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Mwihia Stephen
Mt.Kenya University

Mathew Piero Ngugi
Kenyatta University

John Maingi
Kenyatta University

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Screening of Phytochemicals and Antibacterial Activity of Seed Extracts of Kenyan Sugar Apple (*Annona squamosa*)

[1]Stephen K. Mwihia, [2]Mathew P. Ngugi, [3]John M. Maingi, [4]James k. Kamau, [5]Anne W. Muhuha

[1,2,3,4] Kenyatta University, Department of Biochemistry and Biotechnology, P. O. Box 43844-00100 Nairobi, Kenya
[5]Mt Kenya University, Department of Microbiology, P.O. Box 342-01000 Thika, Kenya

***Abstract*: Investigation of plants for phytochemicals and antibacterial effects is essential for discovery of prospective novel complexes for therapeutic use. In the present assay the seed extracts of *Annona squamosa* were screened for phytochemicals present and antibacterial activities against *Salmonella typhi* (ATCC 1408), *Escherichia coli* (ATCC 25922), and *Klebsiella pneumonia* (ATCC700603) and *Shigella flexneri* (ATCC 12022). Extraction of the active components was done by the use of organic solvents; methanol, dichloromethane and methanol: dichloromethane blend in soxhlet extractor and concentrated in a rotary evaporator. Phytochemical analysis was done through standard qualitative screening procedures. The antibacterial activities were evaluated through the paper disc diffusion method. Phytochemical analysis exhibited the occurrence of fixed oils, alkaloids, saponins, flavonoids, glycosides, tannins, phenols, steroids and terpenoids which are indicated in antibacterial activity. The methanolic extract was the most efficacious in terms of inhibitory activities, exhibiting a MIC range of 15-31.17mg/ml followed by Methanol: dichloromethane with a MIC range of 16.4-56.57mg/ml while dichloromethane displayed the least bacterial inhibitory activity with a MIC range of 47.50-61.83mg/ml. The antibacterial data obtained was assayed against that of the Ciprofloxacin (reference). Ciprofloxacin activity was significantly higher than all the extracts (p<0.05), though some concentrations exhibited equal activities (p>0.05). The extracts have shown antibacterial effects at their MIC against the microorganisms in the study indicating that with further researches these extracts can be used for treating enteric diseases.**

***Keywords*: *Annona squamosa,* Phytochemical, Antibacterial, Paper disc diffusion method, methanol, dichloromethane extract, enterobacteria.**

I. INTRODUCTION

Enterobacteria are Gram-positive microorganisms from *Enterobacteriaceae* family that comprises of many symbionts and pathogens bacteria [1]. Specific enteric pathogens cause infections; the most common etiology being *Escherichia coli*, which is responsible for acute and persistent diarrhoea as well as dysentery [2].Contamination of water with fecal matter infested by enteric pathogens is common in many developing countries as a result of inadequate sanitation [3]. Besides, studies have shown that food-borne outbreaks in the USA are as a result of non-typhoidal salmonellosis from environmental sources [4]. Infectious diarrhoea triggered by enteric bacterial infection remains a major source of mortality throughout the globe in children of up to 5 years of age [5]. In Kenyatta National Hospital, the major public referral and teaching hospital in Kenya, have been reports of high prevalence of *Klebsiella* species infections [6].A study on *Campylobacter*, *E. coli*, *Shigella* and *Salmonella* species show that they are opportunistic infectious agents in patients suffering from HIV and AIDS [7].

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Enterobacterial Infections are treated with antibiotics which include beta-lactams, carbapenems, fluoroquinolone, Sulfamethoxazole-Trimethoprim and aminoglycosides [8]. New antibiotics such as tigecycline and polymyxin B have been shown to have activity against enterobacteria *in vitro* [9], [10], [11]. Drawbacks associated with the continual use of antibiotics include the development of adverse effects, bacteria resistance and high costs of treatment [12].The challenges associated with antibiotic have forced many developing countries to rely on complementary and alternative medicine for primary health care [13].

A survey conducted by the WHO, 80% indicated that majority of the people in the third world nations depend on traditional medicine almost exclusively for their main healthcare requirements [14]. Nevertheless, of the likely four million plant spices, only 6% have been evaluated for bioactivity and approximately 15% have been explored for phytochemicals availability [15]. Significant bioactive components of plant origin include alkaloids, tannins, flavonoids and phenolic compounds [16]. Around 14-8% of plants are utilised therapeutically and 74% of plant-derived that have pharmacologically active components are discovered after analysis of folkloric use of the medicinal plants [17].

Annona squamosa also known as sugar apple is *a* plant in the family of *Annonaceae* which a common habitat in tropical forests and cultivated in the wild in various parts of the tropics [18]. Literature of various studies demonstrate that each part of the plant have therapeutic property [19], [20], [21], [22]. In aforementioned study phytochemical analysis on the *Annona squamosa* demonstrated that it carries a varied complexes including acetogenins which are accountable for anti-feedant, anti-malarial, cytotoxic and the immunosuppressive activities. Diterpenes isolated from the *Annona squamosa* possess the anti-HIV principle and the anti-platelet aggregation activity [23]. On the other hand flavonoids are shown to be responsible for activities against bacteria and pests. Number of alkaloids that are reported from this plant belongs to diverse classes such as aporphine and benzoquinazoline. These alkaloids have been associated with the plants' medicinal value [24]. The anti-bacterial activities of the plant compounds such as squamocin, cholesteryl glucopyranoside annotemoyin-1 and annotemoyin-2 have shown that *Annona squamosa* extracts are active against enterobacterial species of *Salmonella typhi ,E. coli, S. shiga, Klebsiella spp, S. flexneriae, S.sonnei, , P. aeruginosa* and *S. dysenteriae,* [25].

This study focused on the phytochemicals present in *Annona squamosa* seed extracts and their use of in the treatment of enterobacterial infections. The screening of this bioactive complexes may afford us the basis for advancement of leads for novel antimicrobial agents. Due to distinctiveness of the seeds property in curing of different ailments, this part was selected for the study .This research describes the presence of some phytochemicals in *Annona squamosa* seeds which are accountable for antibacterial activity particularly against the enteric pathogens.

II. MATERIALS AND METHOD

Collection and Extraction of Plant Materials:

The ripe fruits of *Anonna squamosa* were collected from local farms in Kirinyaga County of Kenya. The seeds were separated from the pulp and dried up then milled into a fine powder using an electric grinder. For each sample, 150 mg of the powdered material was leached separately, in 300 ml of the solvent that included methanol, dichloromethane and blend of methanol and dichloromethane in a ratio of 1:1 for 48 hours to allow the bioactive compounds to dissolve. The mixture was allowed to settle then filtered using Whatman's filter paper (185 mm) followed by steeping of the residual in the solvent for another 48 hours. The procedure was repeated twice and then concentrated at low pressure using a rotary evaporator. Finally, the extracts were preserved in sterile conditions in tightly sealed bottles at 5ºC for future use in the antibacterial assay.

Screening of Phytochemicals:

The seed extracts were evaluated for the presence of quinones, steroids, glycosides, saponins, tannins, flavonoids, saponins, alkaloids, phenol and fixed oils through the methods as described by Raman [26].

Antibacterial Testing Disc Diffusion Method:

Cylindrical discs of equal size were punched out of sterile blotting paper. A Sterile cotton capped stick was dipped in the inoculate broth and spread evenly over the surface of the media in the four different plates for each bacterial species. The sensitivity discs that had been prepared were soaked in the extract dilution and later removed to air dry and to let off the fluid and then placed on plates containing the organisms using sterile forceps and a wire loop to ensure that they adhere properly. The petri dishes were covered and placed in the incubator at 37ºC for 24 hours in an inverted position so that any condensate would not fall on the inoculated media. Zones of inhibition were indicative of the antibacterial effect on *S. typhi*, *Shigella*, *Klebsiella*, and *E. coli*. The zones of inhibitions were measured using a vernier caliper. The negative control used Was Dimethyl sulfoxide (DMSO) while ciprofloxacin was the standard. To guarantee the reproducibility of the results, individual experiments were performed in triplicates. The dilutions that exhibited maximum antibacterial effect were selected for the quantitative assay through a modified agar microdilution technique [27] and used in the determination of the minimum inhibitory concentration (MIC).

Data Management and Statistical Analysis:

Raw data on zones of inhibition and the MIC were tabulated on Ms Excel spread sheets where it was organized for statistical analysis. The data was then exported to Minitab statistical software version 17.0 (Minitab Inc., Pennsylvania) for analysis. The data was subjected to descriptive statistics and the results expressed as mean ± standard deviation of the mean. One-way analysis of variance (ANOVA) was used to determine whether there were significant differences between the means of different groups. This was followed by Tukey's post hoc tests for pairwise separation and comparison of means. The values of $p \leq 0.05$ were considered significant. The data was presented in tables and figures.

III. RESULTS

Table I, below shows the qualitative investigation of secondary metabolites of organic seed extracts of *A. squamosa* exhibited the presence of alkaloids fixed oils on dichloromethane extracts while methanolic extracts showed presence of saponins, tannins, glycosides, quinones, terpenoids, flavonoids, steroids and phenolic. Methanolic extracts exhibited more positive results for tannins, alkaloids, flavonoids, steroids and phenols.

Table I: Qualitative Phytochemical composition of Methanolic, Dichloromethanolic and Dichloromethanolic: Methanolic blend of *A. squamosa*

Phytochemical	MeOH extract	DCM extract	DCM: MEOH
Saponins	++	-	+
Tannins	+++	-	++
Glycosides	+	-	+
Quinones	+	-	+
Terpenoids	+	-	-
Alkaloid	+++	+	++
Flavonoids	+++	-	+
Steroids	+++	-	++
Fixed Oils	+	+	+
Phenol	+++	-	+

Key: +++ strongly present; ++ moderately present; + slightly present; - absent

Susceptibility of bacterial isolates on methanolic, dichloromethanolic and dichloromethanolic: methanolic blend seed extracts of *A. squamosal:*

In the current study, four bacterial species were tested against three extracts of *A. squamosa* seeds. The MeOH, DCM and MeOH: DCM blend extracts of *A. squamosa* demonstrated significant antimicrobial activities against *K. pneumoniae* (ATCC700603), *S. flexneri* (ATCC 12022), *S. typhi* (ATCC 1408) and *E. coli* (ATCC 25922). The tested bacterial species showed different patterns of zones of inhibition (Figure I, II and III). The inhibitory effects of the reference drug (Ciprofloxacin) against *K. pneumoniae* and *E. coli* were significantly higher compared from extracts of *A. squamosa* in all concentrations tested ($p<0.05$; Figure I, II and III).

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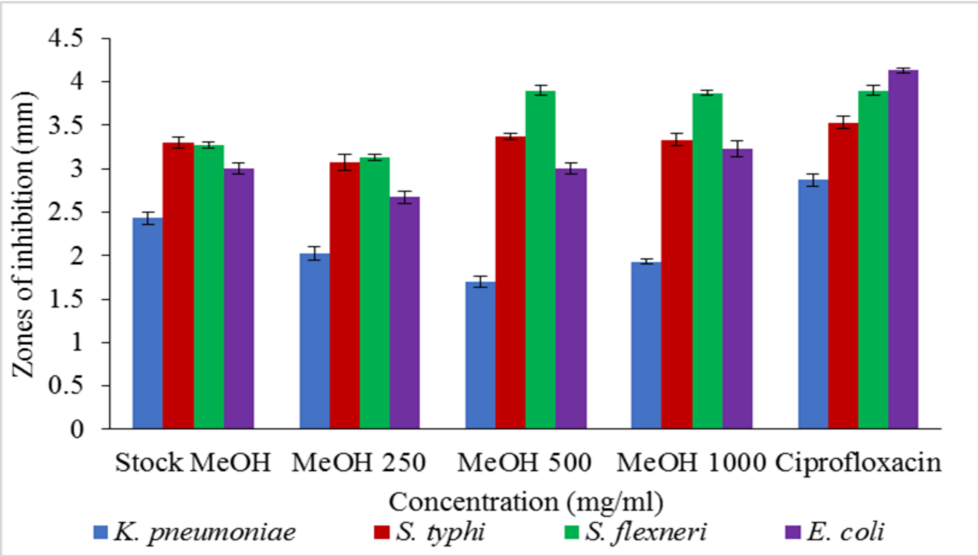

Figure I: Zones of inhibition of methanolic seed extracts *Annona squamosa*

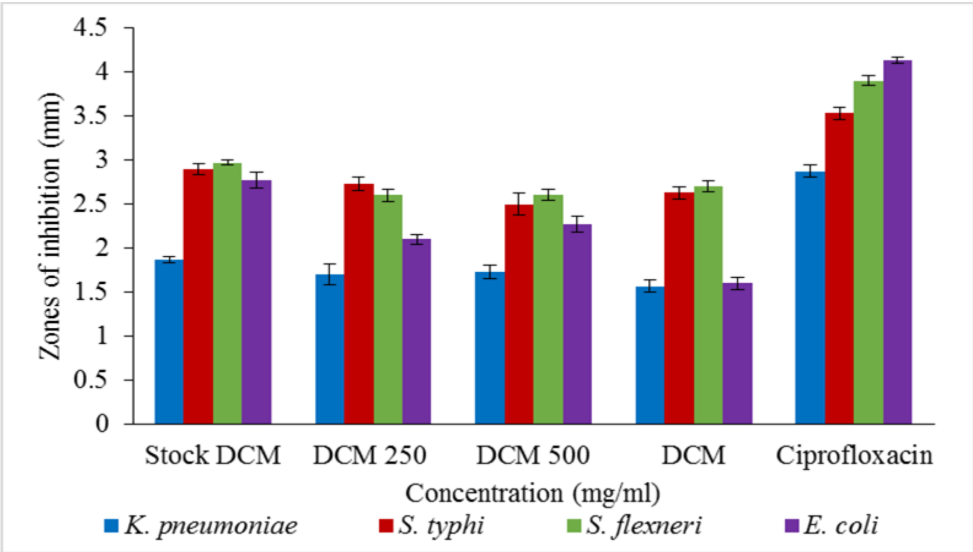

Figure II: Zones of inhibition of dichloromethane seed extracts *Annona squamosa*

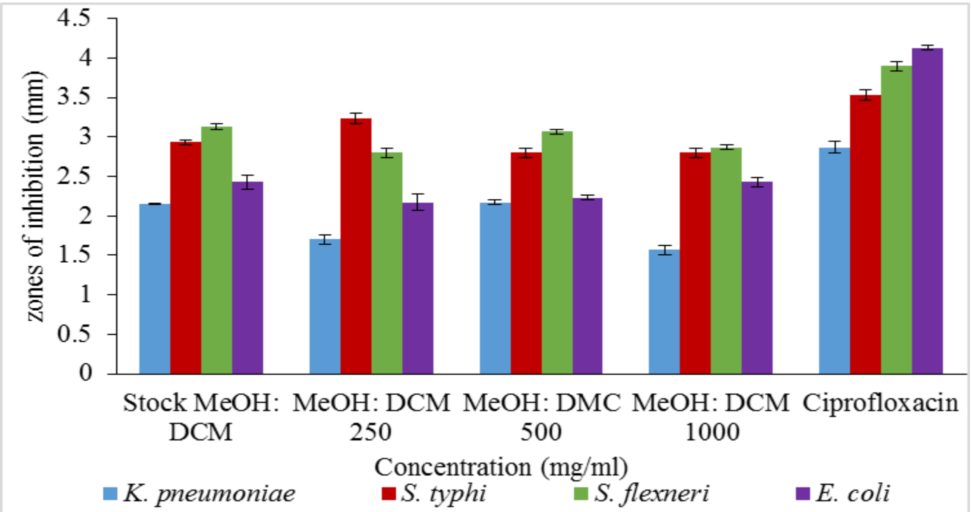

Figure III: Zones of inhibition of dichloromethane: methanolic blend seed extracts *Annona squamosa*

Minimum inhibitory concentrations (MICs) of seed extracts of *A. squamosa* on selected enterobacterial species:

Table II, showed that the minimum inhibitory concentration of (MIC) The minimum inhibitory concentrations values of MeOH, DCM and DCM: MeOH blend extracts of *A. squamosa* were significantly higher than the reference drug (Ciprofloxacin) against all the tested bacterial species ($p<0.05$; Table II). The MeOH extract produced significantly lower MIC values against *K. pneumoniae*, *S. typhi* and *S. flexneri* ($p<0.05$; Table II). The DCM extract produced significantly high MIC values against the bacterial species tested ($p<0.05$; Table II). In addition, the MeOH, DCM and DCM: MeOH blend extracts of *A. squamosa* were not bactericidal against all the bacterial species tested.

Table II: Minimum inhibitory concentration (MIC)

	Minimum inhibition concentration (mg/ml)			
Bacteria / Treatment	***E. coli***	***K. pneumoniae***	***S. flexneri***	***S. typhi***
Ciprofloxacin	0.01 ± 0.01^{d}	0.01 ± 0.01^{d}	0.01 ± 0.01^{d}	0.05 ± 0.02^{d}
MeOH extract	31.17 ± 0.60^{b}	30.83 ± 0.44^{c}	31.17 ± 0.09^{c}	15.63 ± 0.01^{c}
DCM extract	47.50 ± 0.06^{a}	61.83 ± 0.93^{a}	47.60 ± 0.06^{a}	47.80 ± 0.15^{a}
DCM: MeOH extract	16.40 ± 0.06^{c}	56.57 ± 0.03^{b}	32.50 ± 0.29^{b}	25.20 ± 0.20^{b}

Values expressed as the mean ± standard deviation for three replicates. Values with the same superscript letter are not significantly different along the column by one-way ANOVA followed by Tukey's post hoc test ($p>0.05$).

IV. DISCUSSION AND CONCLUSION

Although there is a lot of dedicated efforts in treatment of bacterial infections by means of conventional medicine, exploration for alternative therapeutic agents is required. Antibiotics are used to treat bacterial infections and they are among the most commonly used and abused drugs [27].Moreover, synthetic antibiotics are linked with widespread of undesirable effects [28] which may reflect the toxicological or pharmacological properties of the antibiotic or may involve hypersensitivity [29].This calls for use of plant-derived molecules for the treatment of bacterial infections.

Annona squamosa is a widely used shrub with fruits that are edible and is a used both in industrial and therapeutic products. It is associated with the presence of alkaloids, fixed oils, tannins and phenols [30]. In the current study, the qualitative phytochemical screening of the extracts established that the seed extracts exhibited a host of antibacterial molecules. Also, it was shown that polar solvent extracts were better antibacterial which was in agreement with aforementioned work by Gowdhami *et al*. [31], which indicated that the phytochemical present in seed extracts of *Annona squamosa* are better captured in polar solvents. The results also demonstrated that the interaction between dichloromethane and methanol was additive synergy, which explains why the dichloromethane: methanol blend extract was potent than the dichloromethane extract.

This study pinpoints the potential of *Annona squamosa* seed extracts as a source of antimicrobial agents. The *in vitro* study revealed that traditional medicine can be as efficacious as conventional medicine to fight infectious microbes. The extracts MIC against bacterial species of the study ranged from 15.63-56.57 mg/ml. The MIC for Ciprofloxacin ranged from 0.01 - 0.5 mg/ml. The remarkable variance in the MIC values for Ciprofloxacin and the extracts suggests that Ciprofloxacin is considerably potent than the seed extracts. Nevertheless, since Ciprofloxacin is pure and refined it can be assumed that in refined form, the seed extracts could actually be more effective against the bacterial species. This study suggests that the seed extracts of *Annona squamosa* are a potential candidate for further exploitation in the advancement of pharmacologically active agents due to their abundance of phytochemical content.

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