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Anti-biofilm Activity of Extracts of *Caryota no* in *Drosophila melanogaster* Infected with *Pseudomonas aeruginosa*

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Authors' contributions

This work was carried out in collaboration among all authors. Author CAM designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors SSG and SO provided revisions, supervised and approved every step plus additional aspects and details for the subject that were not present in the initial manuscript, along with contributions and article selection for design. All authors read and approved the final manuscript.

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ABSTRACT

Objectives: To investigate the anti-biofilm activity of the n-hexane and methanol extracts of *Caryota no* (CN) seeds against *Pseudomonas aeruginosa* (PA) infection in *D. melanogaster*.
Study Design: Experimental design.
Place and Duration: Sample: African Centre of Excellence for Phytomedicine Research and Development, University of Jos, Jos Plateau State Nigeria between June 2018 and February 2019.
Methods: 20 flies (1-3 days old) were pricked in the dorsolateral aspect of the abdomen with a modified fly needle dipped in biofilm forming strain of *P. aeruginosa* inoculum and left to recover and then transferred into the appropriately labeled vials which include different doses of the extracts or those in various control conditions. This process was done in 2 batches of flies treated

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with extracts prior to infection and flies treated with extracts after infection. The flies were monitored for survival for 72 hours. Mortality records were taken 6 hourly during this period, and the percentage of death determined in each group. The statistical difference among test groups was presumed at $P < .05$.

Results: The result of n-hexane *Caryota no* extract showed significantly ($P < .05$) improved survival (or decreased mortality) when used prophylactically and in curative assays compared to the control. The 400 mg of the n-hexane extract seemed to be the optimal concentration in both prophylactic and curative assays since it showed the least percentage deaths (50% and 43%) respectively. The methanolic extract exhibited a nonsignificant ($P > .05$) decrease in percentage death prophylactically but direct significant ($P < .05$) dose-dependent reduction in percentage deaths than the control in the curative studies with the 400 mg dose also showing the least deaths (47%).

Conclusion: The results suggest that both methanolic and n-hexane extracts of *Caryota no* have potentials as antibiofilm agents.

Keywords: Biofilm; adhesin; Caryota no; Pseudomonas aeruginosa; Drosophila melanogaster.

1. INTRODUCTION

Resistance arises when the two components (the antimicrobial drug, which inhibits the susceptible organism, selects the resistant organism and the genetic resistance determinant in the bacteria selected by the drug) come together in an environment or in a host [1]. This problem has reached historic and global proportions requiring the attention of the world health organization [2] and it has been found that biofilms are central to the problem of resistance.

Biofilms are gelatinous masses of microorganisms encased in exopolysaccharides capable of attaching to virtually any surface or buried firmly in an extracellular matrix as aggregates [3]. They contribute to nearly 80% of all bacterial infections [4]; are tolerant to harsh conditions and are inherently resistant to antibiotics [5]. Biofilms prevent wounds from healing, and keep bladder infections recurring. They may also be an explanation for lingering lyme disease. They cause recurrent bacterial pneumonia, tooth decay, gum disease, sinusitis, ear infections, Legionnaires' disease and mortality in cystic fibrosis and burn patients. These glom onto medical devices where they are deadly, or difficult to eradicate, plague hospitals, and contribute greatly to health care burden [6].

Bacterial colonization of a host tissue begins with the attachment of bacteria to receptors expressed by cells forming the lining of the mucosa [3]. Pathogens colonize different sites in the human body because they express multiple adhesins which are usually proteins that recognize specific receptors expressed at various sites of the host. Adhesins are key mediators of the adhesion stage of biofilm formation [7]. Bacteria also produce different types of polysaccharides that are specifically designed to form the structural components of the biofilm [7].

Bacterial lectins are the most common type of adhesins among both gram positive and negative organisms [8]. These are currently being studied in various centres as it has been observed that inhibiting the attachment of microbes to these lectins can abort infection *ab initio*. The agents used to achieve these inhibitions called adhesin inhibitors or anti-adhesins abound in nature as illustrated by reduction of incidence of urinary tract infections by daily ingestion of about 300 ml of cranberry juice (*Vaccinium macrocarpon*) by patients in a clinical trial [9]. Anti-adhesion therapy and anti-adhesin immunity are meant to reduce contact between host tissues and pathogens, either by prevention (anti-adhesins) or reversal of adhesion (quorum-sensing inhibitors) of the infectious agent.

The history of drug discovery has been a story of serendipity or a result of man's quest to conquer new grounds in scientific development and to satisfy his curiosity. Drug discovery has contributed more to the progress of medicine in the past century than any other scientific factor and *Drosophila melanogaster* serves as a useful model for evaluating biological actions of therapeutic agents against several human diseases [10,11].

A number of phyto-constituents and plant extracts have been proven both clinically and from laboratory tests to possess potent anti-adhesin effects. *Caryota no* is native to Borneo

rainforests. CN belongs to the family, Arecaceae (Palmae) and the genus Caryota consist of 27 species widely distributed throughout Asia [12]. This is truly a tropical palm. It is considered endangered due to deforestation and harvest of edible palm hearts from which is made a sugary drink or wine called jaggery. The closest members of this group *C. urens* and *C. mitis* have been reported to have antimicrobial properties and high amounts of potent antioxidants [13] and have been used traditionally for various ailments. Very little has been reported about this plant, CN despite so much information about closely related members of the genus.

Therefore, the aim of this study is to investigate the antibiofilm activity of the extracts of *Caryota no* in *Pseudomonas aeruginosa* infections in *Drosophila melanogaster* model.

2. MATERIALS AND METHODS

2.1 Reagents

All chemicals used were of analytical grade. Distilled water was purchased from Africa Centre of Excellence in Phytomedicine Research and Development, Jos, Plateau State, Nigeria. Methanol puris and n-hexane were purchased from Medicom, Jos Plateau State.

2.2 Plant Collection and Preparation

The plant material was collected from Games Village, Abuja FCT, Nigeria. The plant was identified by a taxonomist J. J. Azila in the herbarium of the Federal College of Forestry Jos. The seeds were sorted, air-dried for several days and then pulverized to powder using a commercial grinding machine. The soxhlet extractor was used for extraction of the plant compound using analytical grade absolute n-hexane and 80% methanol as solvents following a method described by Virot et al., [14]. A rotary evaporator was employed to recover the different solvents. The extracts were further dried in a water bath regulated at 40°C and further kept in a fume cupboard. The methanolic extract was later lyophilized using a freeze-drier and stored as crystals in an airtight container because it was found to be hygroscopic.

2.3 Fly Strains

D. melanogaster harwich strain was obtained from Africa Center of Excellence in Phytomedicine Research and Development, University of Jos and maintained at constant temperature and humidity (23°C; 60% relative humidity, respectively) under 12 h dark/light cycle. The flies were cultured by feeding them with a standard medium of the following compositions; 1700 ml of water, 16 g agar agar, 20 g of baker's yeast, 100 g of corn flour, and 1 g of methyl paraben dissolved in 5 ml of absolute ethanol, 1700 ml of water [15].

2.4 Bacterial Strains

Pseudomonas aeruginosa mutant biofilm forming strain (BFF) and non-biofilm forming (NBF) strain were obtained from the department of Pharmaceutical microbiology, University of Nigeria, Nsukka, Nigeria. The Pseudomonas aeruginosa identity is a clinical isolate from a chronic lung infection for the BFF and a wild non-biofilm forming strain.

2.5 *In-vivo* Biofilm Inhibition Studies

From the LC_{50} values [16] and the 28-days survival curves [17], appropriate doses (350, 400 and 500 mg/10 g food) were selected for further studies. These extracts were weighed in dried crystalline form for the methanolic extract and as an oily liquid for the nhexane extract and incorporated into fly food at the appropriate doses.

Several pilots were also carried out for this particular section to establish that the nicking of the dorsal thorax was effectively done to improve skill and to establish infection in the fly as a cause of death and not just the injury alone. The flies were also examined for melanization after some hours.

2.5.1 Acute *P. aeruginosa* infection of Drosophila

The bacterial pellets (the biofilm-forming, -BFF and the non-biofilm forming, -NBF strains of P. aeruginosa) were removed from the stock and cultured in nutrient broth to move them from stationery into active phases of growth. The different cultures were then vortexed to form a suspension which was diluted with 5% dextrose at OD (optical density) of 600nm.

Healthy 3 day-old flies were used in the fly nicking assays according to a modified method of Mulcahy et al., [18]. Flies were sorted following anesthesia on a cold tile. The flies were nicked in the dorsal thorax with a modified fly needle,

which was dipped in different media or in bacterial culture normalized to an optical density at 600 nm of 1.0 in nutrient broth. Different groups of flies were pricked and kept for monitoring and include those not pricked or infected and not on extract (C-INF); those pricked with the biofilm forming organism (BFF) but untreated (C+INF); the 3 groups also infected with BFF but treated with different extract doses (350, 400 and 500 mg/10 g food); the group infected with non-biofilm forming strain (NBF); the group pricked with uninfected or sterile fly needle (UNF); and the group pricked with needle dipped in 5% sucrose (SUC) which is the diluent for the bacterial pellets. After nicking, 20 flies from each group were placed into each appropriate vial and maintained at room temperature. Fly survival was monitored 6 hourly and recorded from 8 to 36 h post-inoculation. Each experiment was done for five independent times. From the records of survival, percentage deaths were calculated. C-INF received plain fly food but not pricked at all; C+ INF received plain fly food but infected with BFF; 350 received 350 mg extract/10 g fly food but infected with BFF; 400 received 400 mg extract/10 g fly food but infected with BFF; 500 received 500 mg extract/10 g fly food but infected with BFF; NBF received plain fly food but infected with NBF; UNF received plain fly food but pricked with sterile fly needle; SUC received plain fly food but pricked with fly needle dipped in 5% sucrose.

2.5.2 Establishment of infection in the fly

This was done by adopting a modified method of Khalil et al. [19] in the following three methods.

2.5.2.1 Microscopy

5 flies were taken from each vial and homogenized in a small Eppendorf tube using the tail of a fly brush. A drop of the fluid diluted in Phosphate Buffered Saline (PBS) pH 7.4 was put on a slide and mounted for viewing under the microscope to identify any microorganisms present. It was then smeared, gram-stained and mounted to be viewed under oil immersion microscope.

2.5.2.2 Macroscopy

Five infected live flies for each infection were homogenized using a pellet pestle (Krackeler Scientific Inc.) in 300 µl PBS, serially diluted and plated onto Brain Heart Infusion agar (BHI; Difco) for PA growth and identification. Plates were incubated at 37°C for 24 hours.

2.5.2.3 Biochemical assay – Catalase test

This was done by homogenizing five flies on a sterile white tile. Few drops of H_2O_2 were added. The presence of bubbles confirmed that a catalase positive organism was present in the mixture.

2.6 Statistical Analysis

The data was expressed as mean ± SEM (standard error of mean) of five parallel measurements, and the statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Turkey's post-hoc test and two-way ANOVA in cases of comparisons with the software, Graphpad prism version 7.0 (Graph Pad Software, San Diego, CA, USA). The results were considered statistically significant at $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Antibiofilm Studies with n-Hexane Extract of CN

The result (Fig. 1) showed significantly (P = .002; .036) and insignificantly (P = .069) decreased mortality rate in the test groups (400 mg; 500 mg) and (350 mg) respectively compared with the positive control (C+INF). Here, the test groups (350- 60%; 400 -50%; 500 -58%) survived more than the positive control (82.6%). In other words, they caused significant reductions in the number of deaths. The groups on 400 mg/kg recorded the lowest death rate. 400 mg/10 g food of the n-hexane extract of CN appears to be the optimal dose in drosophila antibiofilm study against PA. The C+INF group caused very significant (P < .0001) increase in fly death com*p*ared to the C-INF group. The control which was infected but untreated (C+INF) died en-masse which agrees with the pilot studies (results not shown). The uninfected/pricked (UNF) has lower deaths (48%) than the C-INF (29.4%) showing that injury alone is a compounding variable, the sucrose-treated group has 44% deaths while the non-biofilm infected group (NBF) expectedly has the least percentage deaths (30%) among all infected flies implying lower virulence of that strain of PA but it still recorded insignificantly (P = .999) higher percentage deaths than C-INF. D'Argenio et al. [20] illustrated by their work that certain strains of *P aeruginosa* are more virulent than others in fly killing. The differences between the negative control (C-INF) and the 400 mg extract

group show that the group performed as well as the uninfected and unpricked control (C-INF) since the difference is statistically insignificant (P = .124). This implies that the n-hexane extract of CN has a prophylactic (anti-adhesin) activity against PA biofilm infection in DM.

Exposure to n-hexane extract of CN seeds significantly (P < .05) increased percentage survival in *D. melanogaster* prophylactically. This result (Fig. 2) also reveals significantly (P = .003; .001; .024) improved survival rates between the test groups (350 mg = 47%; 400 mg = 43%; 500 mg = 54% respectively) and the positive control, C+INF (86.6%) but insignificantly (P = .550; .812; .166 respectively) increased percentage death compared to C-INF. All other groups displayed similar survival percentages as in the prophylactic assay above. Once again, the group on 400 mg/10 g food showed the highest protection (least percentage death) for the flies. The NBF also showed insignificantly (P = .999) higher percentage death than C-INF. This implies that the n-hexane extract of CN has a curative (quorum-sensing disrupting) activity against PA biofilm infection in DM.

Exposure to n-hexane extract of CN seeds significantly (P < .05) increased percentage survival in *D. melanogaster* curatively. The groups on 400 mg/kg recorded the lowest death rates among treatment groups for both prophylactic and curative groups (Fig. 3). 400 mg/kg of the n-hexane extract of CN appears to be the optimal dose in drosophila antibiofilm study against PA. There were significantly (P < .05) reduced death rate in treatment-before infection groups and the treatment-after infection as well as groups within each assay when comparing the two results (Fig. 3) by 2-way ANOVA. The differences when comparing before infection and after infection groups were not significant (P = .306) across the rows and also insignificant (P = .657) in the overall interaction. The differences between each column for each of the treatment groups (350 mg; 400 mg; 500 mg) were all insignificant (P = .871; .995; .999) respectively. However all the treatments after infection produced higher survival records than treatments before infection although at insignificant levels. It may be safely deduced that the n-hexane extract of CN has both anti-adhesin (prophylactic) effect and quorum-sensing disrupting (curative effect) against PA biofilm infection in DM.

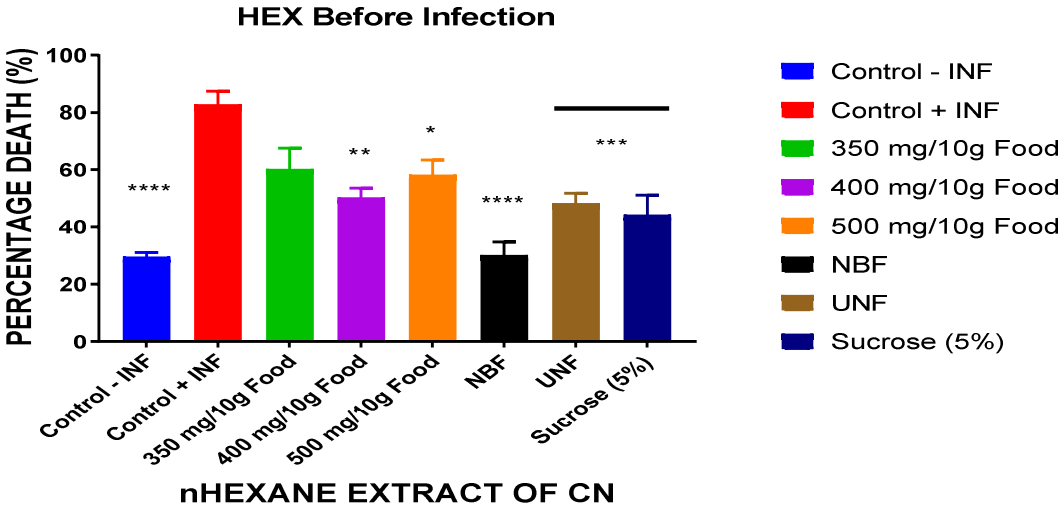

Fig. 1. Antibiofilm activity of n-hexane extract of CN-treated flies before exposure to PA infection

*C-INF received plain fly food but not pricked at all; C+ INF received plain fly food but infected with BFF; 350 received 350 mg extract/10 g fly food but infected with BFF; 400 received 400 mg extract/10 g fly food but infected with BFF; 500 received 500 mg extract/10g fly food but infected with BFF; NBF received plain fly food but infected with NBF; UNF received plain fly food but pricked with sterile fly needle; SUC received plain fly food but pricked with fly needle dipped in 5% sucrose. Data presented as Mean ± S.E.M = Mean values ± Standard error of means of five independent biological replicates for each extract concentration (n = 20) Extracts: significant from positive control (C+INF), * P < 0.05; ** P < 0.001; *** P = 0.0002 - 0.0004; **** P < 0.0001*

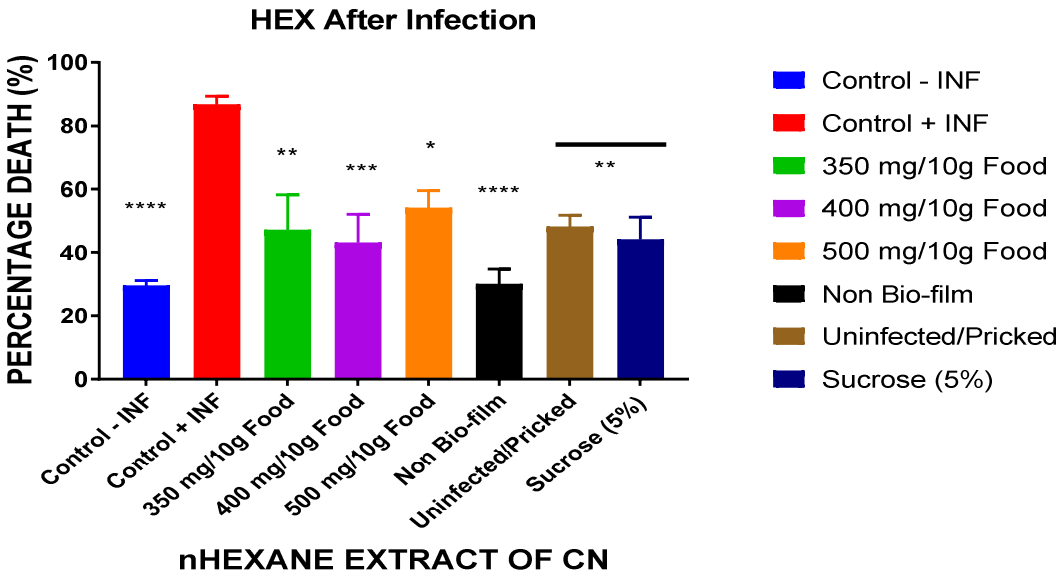

Fig. 2. Antibiofilm activity of n-hexane extract of CN-treated flies after exposure to PA infection

*C-INF received plain fly food but not pricked at all; C+ INF received plain fly food but infected with BFF; 350 received 350 mg extract/10 g fly food but infected with BFF; 400 received 400 mg extract/10 g fly food but infected with BFF; 500 received 500 mg extract/10 g fly food but infected with BFF; NBF received plain fly food but infected with NBF; UNF received plain fly food but pricked with sterile fly needle; SUC received plain fly food but pricked with fly needle dipped in 5% sucrose. Data presented as Mean ± S.E.M = Mean values ± Standard error of means of five independent biological replicates for each extract concentration (n = 20) Extracts: significant from positive control (C+INF), * P < 0.05; ** P < 0.001; *** P = 0.0002 - 0.0004; **** P < 0.0001*

Exposure to n-hexane extract of CN seeds significantly (P < .05) increased percentage survival in *D. melanogaster* prophylactically and curatively. Several Salvia (Sage) species widely used as spices were evaluated for their antimicrobial activities, including their anti-adhesive and anti-biofilm effects. *Salvia triloba* extract demonstrated significant bactericidal activity against methicillin resistant *Staphylococcus aerus* (MRSA). *S. triloba* extract and volatile oil were active against biofilms, demonstrating anti-adhesion and anti-biofilm activities, respectively but the antimicrobial activities of other Salvia species were negligible [21]. Five lipid compounds isolated from soft coral *Eunicea sp*. and three terpenoids together with a mixture of sterols from *Eunicea fusca* collected at the Colombian Caribbean Sea showed different effectiveness against biofilm formation by three marine bacteria associated with immersed fouled surfaces [22], Ochrobactrum pseudogringnonense, *Alteromona macleodii* and *Vibrio harveyi*, and against two known biofilm forming bacteria, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. It was reported that short- and medium-chain fatty acids exhibit antimicrobial activity. Oxylipin synthesis is mainly catalysed by fatty acid dioxygenases and monooxygenases, although they can also be produced by non-enzymatic chemical oxidation of fatty acids. These compounds mediate hormone-like functions and are also involved in defence responses and development [23]. In investigating the reasons for the different results using the feeding or pricking model of infection we hypothesized that this was probably because P. aeruginosa cannot acquire free fatty acids from the intact digestive tract of the flies as it does from the wounded tissues in the pricking model [23]. Formic, capric, and lauric acids are broadly inhibitory for bacteria [24]. Undecylenic acid is another medium chain fatty acid known for its anti-biofilm ability – including the disruption of troubling biofilms of *Candida albicans*. These fatty acid inhibitors contribute to the formation and interaction of species within biofilms [25]. Many of the bioactive compounds already mentioned in these other works were also found present in the n-hexane extract and they include very high quantities of steroids up to 3+ and moderate quantities of cardiac glycosides, phenols and terpenoids up to 2+. The Gas chromatography mass spectrometry (GCMS) assay also reported numerous short and medium

chain fatty acids which may explain these pharmacologic effects (unpublished). The bacterium PA displays a diol synthase activity that transforms several monounsaturated fatty acids into mono- and di-hydroxylated derivatives. A study [23] has shown that oxylipins derived from this activity inhibit flagellum-driven motility and upregulate type IV pilus-dependent twitching motility of P. aeruginosa. Consequently, these oxylipins promote bacterial organization in microcolonies, increasing the ability of P. aeruginosa to form biofilms in vitro and in vivo (in Drosophila flies). It was also demonstrated that oxylipins produced by P. aeruginosa promote virulence in Drosophila flies [23].

3.2 Antibiofilm Studies with Methanolic Extract of CN

The result (Fig. 4) reveals statistically insignificant (P = .969) for 350mg; (P = .889) for 400 mg; (P = .560) for 500 mg decreases in death rate among treated groups compared with the positive control, C+INF (84.8%) but all of them caused very significantly (P < .0001) higher percentage mortality than C-INF (24.8%). This prophylactic assay displays a dose dependent pattern of biofilm inhibition with the highest dose, 500 mg/10 g food having the least percentage deaths (72%) than the other two extract doses (350 mg/10 g food = 78%; 400 mg/10 g food = 76%) as shown graphically (Fig. 4). However, the reduction in incidences of deaths given by the extract-treated groups were insignificant (P > .05) compared to the control. The other three groups (uninfected, non-biofilm infected and sucrose groups) had similar pattern as already seen earlier with n-hexane extract. It can be deduced from this experiment that the methanolic extract of CN has insignificant prophylactic (anti-adhesin) activity against PA biofilm infection in DM.

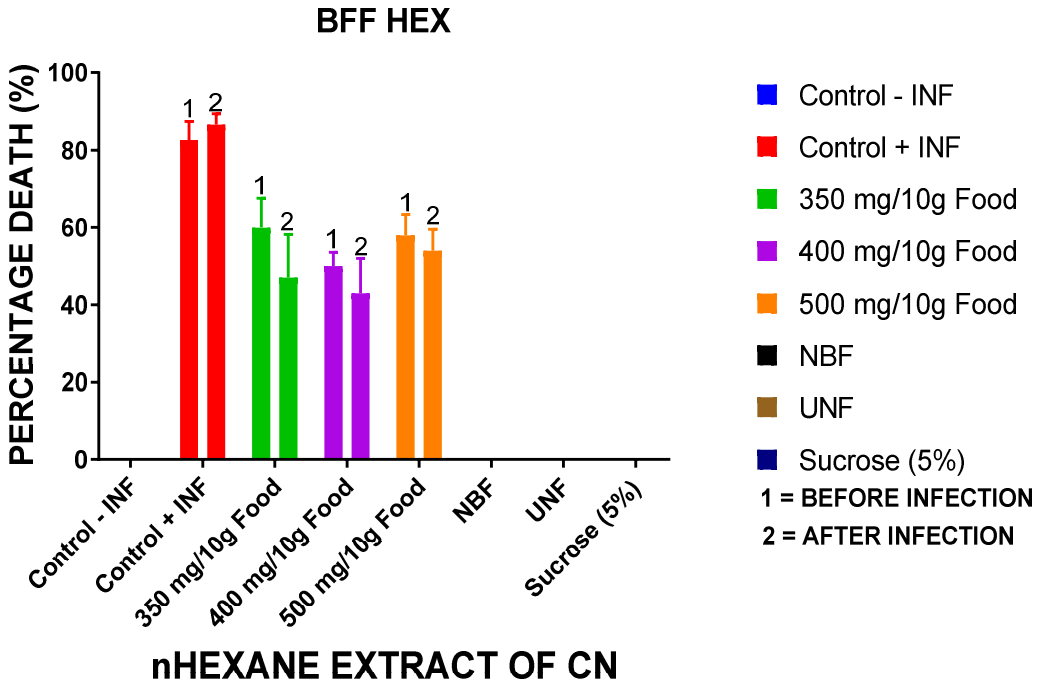

Fig. 3. Antibiofilm activity of n-hexane extract of CN-treated flies before and after exposure to PA infection

*C-INF received plain fly food but not pricked at all; C+ INF received plain fly food but infected with BFF; 350 received 350 mg extract/10 g fly food but infected with BFF; 400 received 400 mg extract/10g fly food but infected with BFF; 500 received 500 mg extract/10g fly food but infected with BFF; NBF received plain fly food but infected with NBF; UNF received plain fly food but pricked with sterile fly needle; SUC received plain fly food but pricked with fly needle dipped in 5% sucrose. Data presented as Mean ± S.E.M = Mean values ± Standard error of means of five independent biological replicates for each extract concentration (n = 20) Extracts: significant from positive control (C+INF), * P < 0.05; ** P < 0.001; *** P = 0.0002 - 0.0004; **** P < 0.0001*

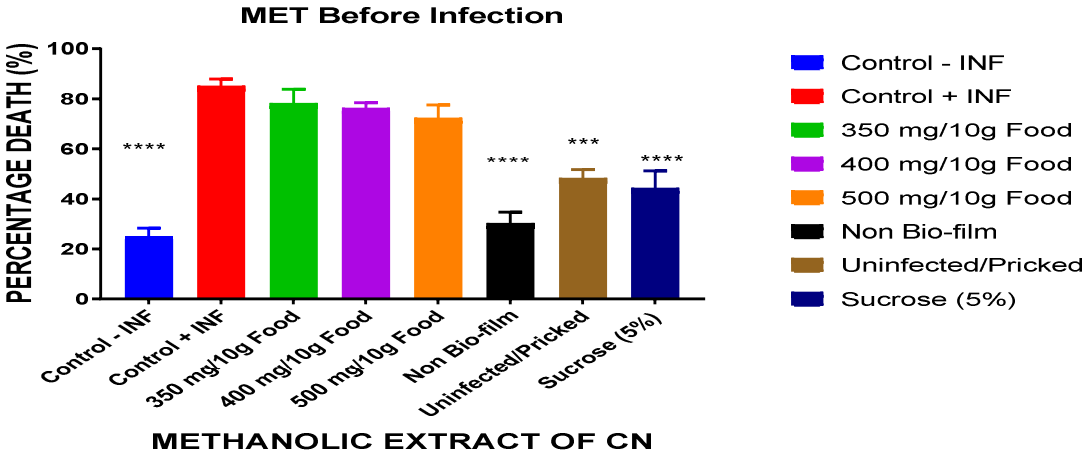

Fig. 4. Antibiofilm activity of methanolic extract of CN-treated flies before exposure to PA infection

*C-INF received plain fly food but not pricked at all; C+ INF received plain fly food but infected with BFF; 350 received 350 mg extract/10 g fly food but infected with BFF; 400 received 400 mg extract/10 g fly food but infected with BFF; 500 received 500 mg extract/10 g fly food but infected with BFF; NBF received plain fly food but infected with NBF; UNF received plain fly food but pricked with sterile fly needle; SUC received plain fly food but pricked with fly needle dipped in 5% sucrose. Data presented as Mean ± S.E.M = Mean values ± Standard error of means of five independent biological replicates for each extract concentration (n = 20) Extracts: significant from positive control (C+INF), * P < 0.05; ** P < 0.001; *** P = 0.0002 - 0.0004; **** P < 0.0001*

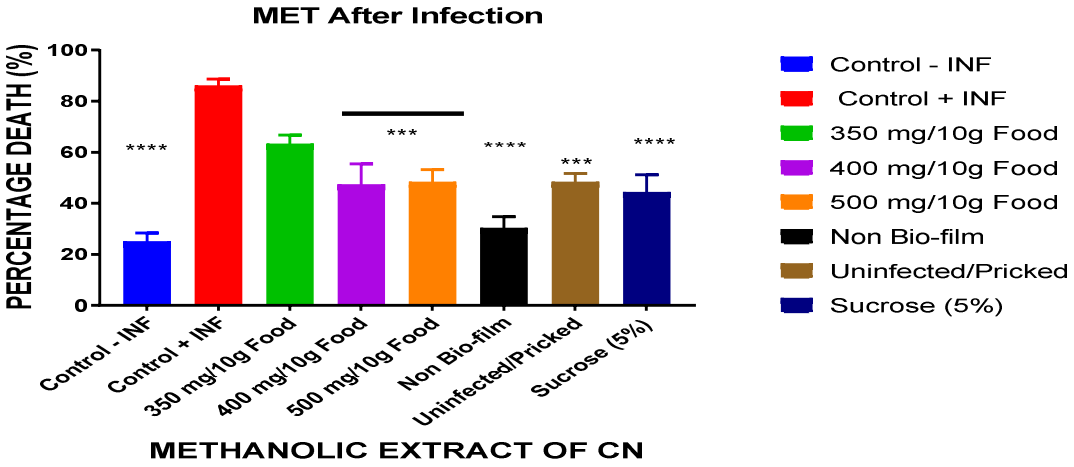

Fig. 5. Antibiofilm activity of methanolic extract of CN-treated flies after exposure to PA infection

*C-INF received plain fly food but not pricked at all; C+ INF received plain fly food but infected with BFF; 350 received 350 mg extract/10 g fly food but infected with BFF; 400 received 400 mg extract/10 g fly food but infected with BFF; 500 received 500 mg extract/10 g fly food but infected with BFF; NBF received plain fly food but infected with NBF; UNF received plain fly food but pricked with sterile fly needle; SUC received plain fly food but pricked with fly needle dipped in 5% sucrose. Data presented as Mean ± S.E.M = Mean values ± Standard error of means of five independent biological replicates for each extract concentration (n = 20) Extracts: significant from positive control (C+INF), * P < 0.05; ** P < 0.001; *** P = 0.0002 - 0.0004; **** P < 0.0001*

Exposure to methanolic extract of CN seeds increased percentage survival in *D. melanogaster* nonsignificantly (P > .05) prophylactically. The result (Fig. 5) reveals statistically significant (P = .0002) for 400 mg; (P = .0004) for 500mg and nonsignificant

(*P* = .073) decreases in death rate among treated groups compared with the C+INF (85.8%) and vice versa with C-INF. Biofilm inhibition significantly increases with increasing extract dose. The higher extract doses (350-63%; 400 -47%; 500 -48%) tended to protect the flies better than lower doses (the 400 mg/10 g food dose once again showed the maximum effect) and all the treatment groups did fare better than C+INF. All groups except the 350mg group were insignificantly different from the C-INF which may suggest that the mechanism of biofilm inhibition with the methanolic extract is curative (disruption of already formed biofilm) rather than prophylactic. It may be safely deduced that the methanolic extract of CN has a quorum-sensing disrupting (curative effect) against PA biofilm infection in DM.

Exposure to methanolic extract of CN seeds increased percentage survival in *D. melanogaster* significantly (*P* < .05) curatively. In these particular experiments, (Fig. 6), extremely great differences were observed both between the two major variables (before infection and after infection), and also between the different treatment groups within each experiment when they were compared using 2-way ANOVA. There is interaction whereby the effect of treatment in relation to control are in opposite effects. The differences when comparing before infection and after infection groups were significant (*P* < .0001) across the rows and (*P* = .028) in the overall interaction. The differences between each column for each of the treatment groups were insignificant (*P* = .427) for 350 mg but significant (*P* = .006) for 400 mg and (*P* = .036) for 500 mg. However two of the treatments (400 mg; 500 mg) after infection produced higher survival records than treatments before infection at significant levels. It may be safely deduced that the methanolic extract of CN has only a quorum-sensing disrupting (curative effect) against PA biofilm infection in DM.

Exposure to methanolic extract of CN seeds increased percentage survival in *D. melanogaster* nonsignificantly (*P* > .05) prophylactically but significantly (*P* < .05) curatively. Qualitative phytochemical analysis of the methanolic extract of CN showed that it contains large quantities of carbohydrates and tannins up to 3+, but moderate quantities of cardiac glycosides and steroids up to 2+. These constituents are also validated by GCMS results of this sample which elucidated some sugars (unpublished). Studies by other researchers have also gone a long way to validate that various types of sugars can block the initial binding between *P. aeruginosa* and carbohydrate

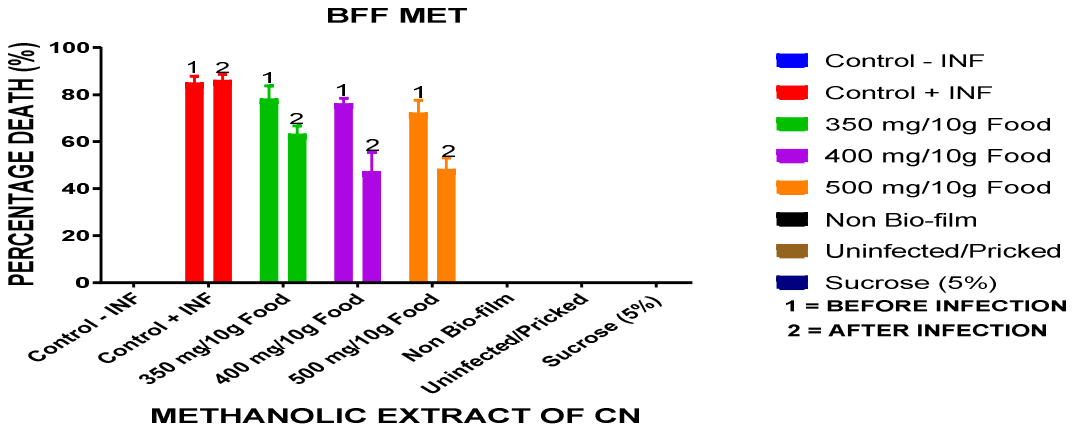

Fig. 6. Antibiofilm activity of methanolic extract of CN-treated flies before and after exposure to PA infection

*C-INF received plain fly food but not pricked at all; C+ INF received plain fly food but infected with BFF; 350 received 350 mg extract/10 g fly food but infected with BFF; 400 received 400 mg extract/10 g fly food but infected with BFF; 500 received 500 mg extract/10 g fly food but infected with BFF; NBF received plain fly food but infected with NBF; UNF received plain fly food but pricked with sterile fly needle; SUC received plain fly food but pricked with fly needle dipped in 5% sucrose. Data presented as Mean ± S.E.M = Mean values ± Standard error of means of five independent biological replicates of for each extract concentration (n = 50).I Extracts: significant from positive control (C+INF), * P < 0.05; ** P < 0.001; *** P = 0.0002 - 0.0004; **** P < 0.0001*

chains of host cell surface glycoproteins and mucins [26] which is an early and crucial step in the establishment of *P. aeruginosa* infection. Bacterial binding to the carbohydrate chains can therefore be competitively blocked by treatment with exogenous sugars as shown by the works of Chabre et al. [27]. In 2012, Hartmann et al. [28] showed that mannosides are effective inhibitors of the uropathogenic *Escherichia coli* lectin FimH and have shown therapeutic promise in murine models for the treatment of *E. coli* urinary tract infections [29]. Several groups have also shown that inhalation of the dry powder form of mannitol has shown promise in the treatment of bronchiectasis and CF [30]. Administration of various lectin inhibitors markedly reduces *P. aeruginosa*-induced lung injury and mortality *in vivo* [31], inhibits biofilm formation, and enhances dispersion of established biofilms [32]. Xylitol is a low-carb sweetener found in toothpaste and diet sodas. When bacteria incorporate xylitol into the biofilm, it makes for a flimsy structure [24].

The study by Bucior et al. [33] proves that administration of a sugar mixture that consists of the three sugars present in *N*- and *O*-glycans, Man, Fuc, and Gal, is an effective adjunctive therapy when used in combination with conventional antibiotics for the treatment of pulmonary infections caused by both non-mucoid and mucoid strains of *P. aeruginosa*. The sugar mixture inhibits bacterial adhesion in vitro and functions synergistically when administered with conventional antibiotics in a murine model of acute pneumonia. *In vitro*, the sugar mixture induces formation of bacterial clusters that are more susceptible to antibiotic killing, providing an additional explanation for their efficacy *in vivo*. These studies introduce a simple, inexpensive, and effective novel form of adjunctive therapy that could aid existing antibiotic treatment of acute and chronic *P. aeruginosa* infections.

The leaf extract of *Pongamia pinnata* showed significant antibiofilm activity [24]. The antimicrobial activity of the plant extract is attributed to the presence of phenolic compounds, such as alkaloids, flavonoids, terpenoids and polyacetylenes [34].

3.3 Confirmation of Infection in Drosophila Flies

It was established that the flies died from the infection and not just from the wound or chance event. This was done using macroscopic, microscopic and biochemical methods.

3.3.1 Macroscopy

It was observed from macroscopy that there were numerous PA organisms from the plates for biofilm-forming infected dead (Plate 1A) and live (Plate 1B) flies and non-biofilm forming infected dead flies (Plate 1C). The non-biofilm forming PA (Plate 1D) with lower virulence was harvested scantily from the live flies because it is probable that the immune system of the flies were able to extinguish the infection. A study [18] also demonstrated that infection with a less virulent *P. aeruginosa* strain resulted in immune potentiation. The uninfected flies (Plate 1E) could not demonstrate visible growth of PA while the cultures from both stocks (Plate 1F) could only produce scanty growth probably because they were each in their stationery phases.

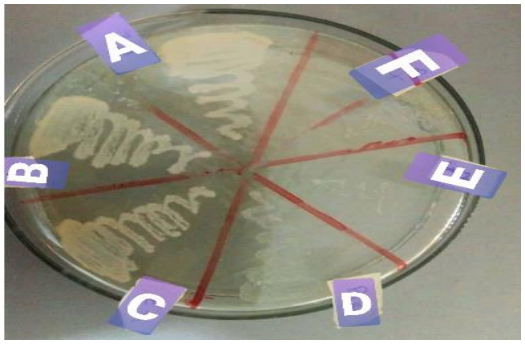

Plate 1. Macrosopic confirmation of fly infection after exposure of flies to PA under different conditions

A = biofilm forming infected dead flies. B = biofilm forming infected live flies. C = non biofilm forming infected dead flies. D = non biofilm forming live flies. E = uninfected control flies. F = biofilm and non-biofilm controls from stock

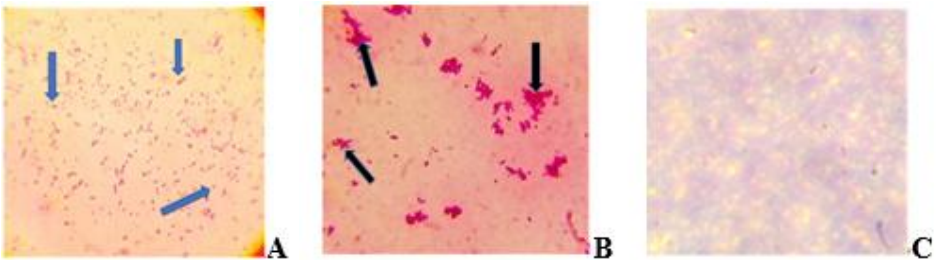

Plate 2. Microscopic confirmation of fly infection after exposure of flies to PA under different conditions

A = Infected flies with bacteria appearing singly (BLUE ARROWS). B = Bacteria in cluster from stock (BLACK ARROWS). C = Uninfected flies. Scale bars in indicate 100 mM. At least three infected flies were examined from two separate infections and representative images are shown. Magnification X100

3.3.2 Microscopy

The microscopy illustrated that the infected flies (Plate 2A) with numerous bacteria shown in blue arrows appearing singly while the uninfected flies (Plate 2C) showed no PA organisms under microscopy. The bacteria from the stock solution of biofilm forming PA (Plate 2B) appear in clusters shown by black arrows.

2A shows flies with disseminated systemic acute infection in very large numbers and when compared with 2C, it can be seen that the uninfected flies expectedly show no PA microorganisms. The microorganisms from 2B appear in clusters since they are of the biofilm stock.

3.3.3 Biochemistry -- catalase test

There was a persistent production of gas when a drop of hydrogen peroxide (H_2O_2) was added to a drop of homogenate from an infected treated fly and this is not an absolute proof of the presence of PA. It is rather a non-specific test for PA but could be used for other catalase positive organisms like *Listeria monocytogenes*, *Aspergillus spp*, *Escherichia coli*, *Shigella spp*, *Salmonella spp* and enterobacter.

4. CONCLUSION

The present work clearly demonstrates the antibiofilm activity of both n-hexane and methanolic extracts of *Caryota no* seeds. The acute antibiofilm assay revealed that the n-hexane extract possesses very significant antiadhesin and quorum sensing inhibitory effects while the methanolic extract of the extract exhibited only quorum- sensing inhibitory effects. These effects may be due to some bioactive compounds such as steroids, cardiac glycosides, phenols, carbohydrates and tannins and further buttressed by their presence from GCMS. It can therefore be safely concluded that both n-hexane and methanolic extracts of CN possess antibiofilm properties both as prophylactic (antiadhesin) and curative (quorum sensing inhibiting effects). However, isolation of the individual bioactive constituent and subjecting it to this biological activity and possible anti-COVID 19 activity is in progress.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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