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Research Article

In vitro anti-oxidant and anti-adhesin assays of n-hexane and methanolic extracts of *Caryota no* seeds

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Abstract

To determine the anti-oxidant and anti-adhesin activities of the n-hexane and methanolic extracts of *Caryota no* (CN) seeds. For anti-adhesin properties, the wells of a sterile flat-bottomed tissue culture plate were filled with 200µL of each extract in several concentrations, incubated and washed twice. 200µL of bacterial suspension was added, incubated and washed thrice with PBS. Adherent bacteria were fixed with methanol, plates emptied, left to dry and stained with crystal violet. Excess stain was rinsed under running water, air dried, bound dye resolubilized with glacial acetic acid, and the absorbance measured using a microplate reader to calculate the percentage inhibition. For antioxidant properties, three different concentrations of the DPPH solution were prepared. 2 mg of each extract was weighed out and dissolved in 10 mL of their corresponding solvent. 1 mL of DPPH solution was added to 2 ml solution of the extracts and ascorbic acid. The reaction mixture was vortexed and left in the dark for 30 minutes then absorbance was measured against blank solution to calculate the percentage scavenging radical inhibition. Statistical difference was presumed at $P < 0.05$. The extracts showed significant ($P < 0.05$) adhesin inhibition activities, n-hexane (64%) and methanolic (46%) at 200µg/mL concentrations. The fractions of the extracts also showed various remarkable results. The anti-adhesin activity increased with increasing extract concentration in both extracts. Antioxidant assays revealed that at 200 mg/mL, both extracts were significantly ($P<0.05$) lower than the control. Both the n-hexane and methanolic extracts showed remarkable adhesin inhibition activities at concentration of 200 µg/mL and antioxidant effects, with the methanolic extract demonstrating comparable levels as the standard, ascorbic acid. This implies that both extracts may serve as veritable natural sources of anti-adhesins, antioxidants and thus adjunct anti-infective agents or immune enhancers.

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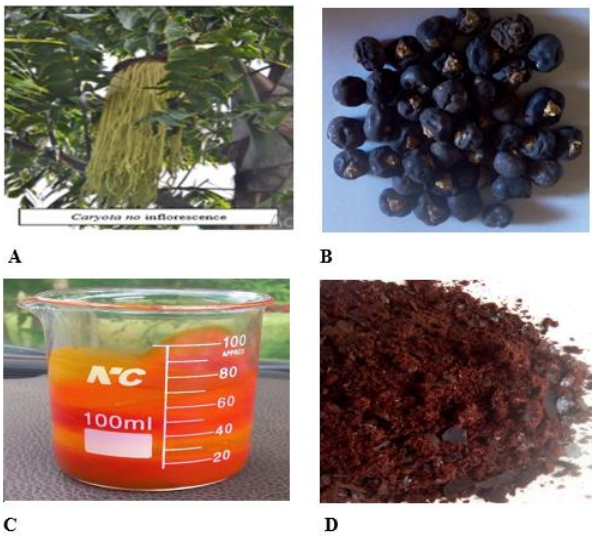
1. Introduction

Colonization of host tissue by bacteria starts with the attachment of bacteria to receptors or structures found on cells forming the lining of the mucosa [1]. This attachment is receptor-ligand specific when it involves living tissues but becomes non-specific when it involves abiotic surfaces [2]. This interaction with receptors on host cell surfaces is mediated by one of the tip proteins, called adhesins [3]. There are fimbrial, non-fimbrial and polysaccharide adhesins which are important mediators of the adhesion stage [4]; are involved in the initiation of infection, biofilm formation and pathogens colonize different sites in

the human body because they express multiple adhesins that can recognize receptors in these sites. Bacteria also produce different types of polysaccharides that are specifically designed to form the structural components of the biofilm [4]. Recently, anti-adhesin studies became topical because of the need to resolve the global pandemic and antimicrobial resistance. These anti-adhesins are useful in the production of vaccines and may serve as adjuncts in the prevention and management of infections since they play a major role in impairing the formation of biofilms.

Oxidative stress, which represents extreme amounts of free oxygen radicals in biological fluids in the human body can cause many diseases [5]. Endogenous sources of radicals include mitochondrial electron transport chain, endoplasmic reticulum, peroxisomes, membrane-bound NADPH oxidase, dual oxidases, lipooxygenases and cyclooxygenases which generate reactive oxygen species (ROS) by the release of -H2O2, -ROO, -NO2, -ONOO radicals [6]. Exogenous sources of oxidative stress are cigarette smoking, ionizing and ultraviolet radiations, heavy metals (iron, nickel, cadmium, copper, arsenic), ozone and air pollution and these produce ROS by releasing -ROOH, -RO, -OH, -O2 radicals [7]. ROS play an important role in cell signaling and in regulating the expression of antioxidant genes especially in maintaining oxidative/antioxidant intracellular homeostasis [8], a disruption of which can lead to the depletion of endogenous antioxidant agents. Antioxidants are biochemical substances that interact with free radicals, catalytic metals or chelating agents to reduce or erase the imbalance [9]. Antioxidant defenses could be non-enzymatic from vitamins (A, C, E, carotenoids, ubiquinones, Uric acid, taurine etc) and glutathione or enzymatic systems like catalase, superoxide dismutase, glutathione peroxidase, acetylcholines-terase, glutathione-s-transferases and the thiol redox system [10]. However, when there is impairment or inadequate neutralization of these radicals, pathogenesis ensues causing the living cells and tissues to undergo oxidative damage by free radicals that oxidize biological matter leading to cancers, diabetes mellitus, cardiovascular, neurodegenerative and other chronic disorders [11]. The antioxidant effect, and the beneficial impact on human health and disease prevention after cranberry consumption are well documented [12].

Caryota no is the giant fishtail palm [9] and from this plant, a sugary drink called jaggery was made by the natives. The plant, CN pacifies vitiated pitta, hyperdipsia, arthritis and burning sensation. *Caryota no* is native to Borneo rainforests which is located 1° north of the equator thus making this a truly tropical palm. It is endangered due to deforestation and harvest of edible palm hearts [10]. The physical features of this palm are shown in Fig 1. and the phytochemical analysis of CN showed several bioactive substances hence it would be worth the effort to evaluate the seed extracts of this plant for anti-adhesin and anti-oxidant effects since it is a source of sweet refreshing drink (which are known to commonly possess these pharmacological effects being sought) and since no published research data in these areas on CN are available.



A = *Caryota no* Palms (Source: www.rarepalmseeds.com)
B = Dried ripe seeds of CN
C = A sample of freshly prepared n-hexane extract of CN
D = A sample of freeze-dried methanolic extract still in crystalline form

Figure 1. Physical characteristics of the Palm, seeds and crude extracts of *Caryota no*

2. Materials and methods

2.1 Materials

The mutant strain *Pseudomonas aeruginosa* mutant

biofilm forming strain was 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 nonbiofilm forming strain.

2.2 Methods

2.2.1 Plant preparation

The plant material was collected from Games Village (9.0166 ^{0}N, 7.4475 ^{0}E), Abuja, Nigeria. The plant was identified by a taxonomist at 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.

2.2.2 Preparation of extracts

The soxhlet extractor was used for the extraction of the plant compound using analytical grade 80 % methanol and absolute n-hexane as solvents. A rotary evaporator was employed to recover the different solvents and the extracts were further dried in a water bath regulated at 40 ^{0}C. The methanolic extract was finally lyophilized using freeze dryer and stored as crystals in an airtight container because it was found to be hygroscopic. The percentage yield of each extract was determined by calculation according to equation 1

$$\% \text{ Yield} = \left[\frac{\text{weight of dried extract}}{\text{weight of dried powder before extraction}}\right] \times 100$$

2.2.3 *In vitro* anti-adhesins assay

The anti-adhesive activity of the n-hexane, and methanol extracts of *Caryota no.* against biofilm-forming *Pseudomonas aeruginosa* strain was quantified according to the procedure described by [11]. Briefly, the wells of a sterile 96-well flat-bottomed plastic tissue culture plate (Greiner Bio-One GmbH) were filled with 200 µL of each extract. Extract concentrations tested ranged from 12.5 to 200 mg/mL while control wells contained PBS buffer only. Incubation was for 18 hours at 4 ^{0}C before washing twice with PBS. 200 µL of a washed bacterial suspension (108 CFU mL^{-1}) was added and incubated again for 4 hours at 4 ^{0}C. Unattached micro-organisms were removed by washing the wells three times with PBS and adherent bacteria were fixed with 200 µL of methanol (99 % purity) for 15 minutes. The plates were emptied, dried and stained for 5 minutes with 200 µL of 2 % crystal violet. Excess stain was rinsed out under running tap water, air dried, the dye bound to the adherent micro-organisms was resolubilized with 200 µL of 33 % (v/v) glacial acetic and the absorbance was measured at 630 nm using a microplate reader (model 680, Bio-Rad). The microbial inhibition percentages at different extract concentrations for each microorganism were calculated according to equation 2:

$$\% \text{ Microbial inhibition} = \left[1 - \left(\frac{A_c}{A_0}\right)\right] \times 100$$

A_c = the absorbance of the well with extract concentration, c

A_0 = the absorbance of the control well, 0

2.2.4 *In-vitro* Antioxidant Activity

The antioxidant properties of the n-hexane and methanolic extracts were measured *in vitro* by the inhibition of generated stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical as described by [12]. Three different concentrations (50, 100 and 200 µg/mL) of the DPPH solution were freshly prepared in the same manner as the ascorbic acid. 2 mg of each extract was dissolved in 10 mL of their corresponding solvent 1 mL of DPPH solution was added to 2 mL solution of the extracts and ascorbic acid. The reaction mixture was vortexed and left in the dark at room temperature for 30 minutes. Absorbance was measured using spectrophotometer at 517 nm against the corresponding blank solution. The percentage scavenging activities were calculated according to the following equation 3:

$$\text{DPPH radical scavenging activity (\%)} = \left[\frac{\text{Abscontrol} - \text{Abssample}}{\text{Abscontrol}}\right] \times 100$$

$Abs_{control}$ = the absorbance of DPPH radical and n-hexane/methanol

Abs_{sample} = the absorbance of DPPH radical and sample/standard

2.2.5 Statistical analysis

The data were expressed as mean ± SD (standard deviation), and the statistical analysis was carried out

using one-way analysis of variance (ANOVA) followed by Turkey's post-hoc test followed by Turkey's post-hoc test with the software. Graphpad Prism version 7.0 (GraphPad Software, San Diego, CA, USA). The results were considered statistically significant at $P<0.05$.

3. Results

3.1 Results of Anti-Adhesin Assays

The n-hexane extract showed a remarkable adhesin inhibition activity- 64 % at extract concentrations of 200 µg/mL (Table 1). The antiadhesin activity of the n-hexane fraction was found to be directly concentration-dependent. For the n-hexane extract, the anti-adhesin activity further increased with increasing extract concentrations with the anti-adhesin effect appearing at the concentration of 50 µg/mL. The one-way ANOVA summary of the n-hexane extract revealed a P value of < 0.0001 - **** which was several fold from the standard already set. On further analysis with Turkey's multiple comparisons, it was detected that differences were found to be between the different concentrations of treatment groups.

The methanolic extract showed about 46 % inhibition at extract concentrations of 200 µg/mL (Table 1). For the methanolic extract, the anti-adhesin activity also increased with increasing extract concentration with the anti-adhesin effect appearing at the concentration of 100 µg/mL. The one-way ANOVA summary of the methanolic extract (Table 1) revealed a P value of < 0.0001 - ****. On further analysis with Turkey's multiple comparisons, it was detected that differences were found to be between the different concentrations of the treatment group. Both extracts demonstrated biphasic effects, facilitating variable degrees of bacteria adhesion at very low concentrations until different peaks from which they begin to play anti-adhesin roles. It was observed that some fractions of the extract gave promising adhesin inhibition values like n-hexane, butanol and aqueous fractions (Fig. 2).

3.2 In vitro Anti-oxidant Studies

For the n-hexane extract, the anti-oxidant results increased with increasing concentrations of the extract but the values were significantly ($P < 0.05$) decreased compared to the control (Fig. 3).

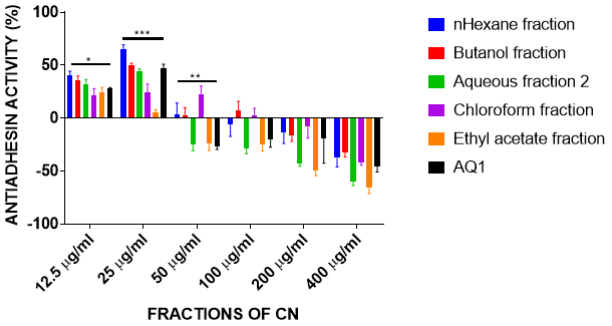


Figure 2. Anti-adhesin activity of fractions of CN seeds extract (n = 5 *$P < 0.05$; **$P < 0.01$; ***P =0.0001– 0.001)

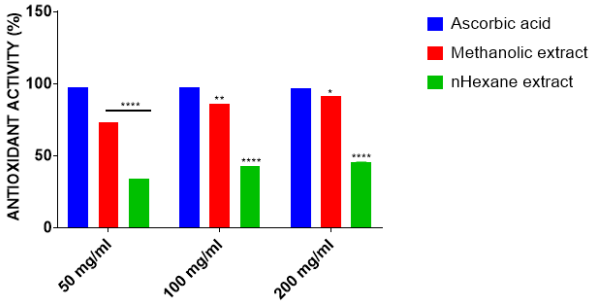


Figure 3. *In-vitro* anti-oxidant activity of extracts of CN seeds (n = 5 *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$)

The antioxidant activity of the n-hexane extract was found to be concentration-dependent and is directly proportional to the extract concentration of the treatment giving a value of 45.64 % at 200 mg/mL.

The methanolic extract of CN at different concentrations also revealed significantly ($P < 0.05$) decreased antioxidant activity compared to ascorbic acid (Fig. 3). The results increased in a direct concentration-dependent manner and were comparable to those of the clinical standard. The one-way ANOVA summary revealed $P < 0.05$ - ****. On further analysis with Turkey's multiple comparisons, it was detected that differences were found to be between the different concentrations of the control and all the different concentrations of treatment group. The methanolic extract however had comparable values with the control (91.67 % and 96.83 % respectively) at the highest test concentration (200 mg/mL) unlike what was seen with the n-hexane extract.

4. Discussion

The antiadhesin activity of the n-hexane fraction was found to be directly concentration–dependent (Table

1). It can be observed that some fractions of the extract gave promising adhesin inhibition values like n-hexane, butanol and aqueous fractions. The acute *In-vivo* antibiofilm assay of these extracts using *Drosophila melanogaster* revealed that the n-hexane extract possesses very significant antiadhesin and quorum sensing inhibitory effects probably because of the presence of an abundance of fatty acids many of which have antibiofilm functions [16, 17] while the methanolic extract exhibited majorly quorum-sensing inhibitory effects [16].

Table 1. *In-vitro* anti-adhesin activity of the extracts of CN seeds

Concentrations (µg/mL)	Percentage Inhibition of Cell Adhesion (%)	
	n-Hexane Fraction	Methanol Fraction
12.50	-95.60±0.25 *	-88.48±0.09 *
25.00	-62.60±0.28 *	-77.58±0.31 *
50.00	55.19±0.16	-330.0±0.44 *
100.00	58.72±0.06	33.03±0.13
200.00	64.46±0.09	45.76±0.06

n = 5 $P < 0.05$, * = statistically significant

Plant extracts may be major sources of anti-adhesins because of their abundant possession of lectins which may interact with surface saccharides on animal cells to block adhesion mediated by lectin-carrying bacteria and may improve clearance of bacteria from the host [18]. Some reviewers reported that dietary constituents containing bioactive compounds like polyphenols and proanthocyanidins (PACs) demonstrate proven antiadhesin activities in variable degrees [19]. Sialylated fat globules which are glycoconjugates in milk inhibited adhesion of fimbriated *E. coli* to buccal epithelial cells [20].

High concentrations of cranberry juice, usually consumed as a cocktail have been shown to inhibit the *Escherichia coli* type 1 fimbrial lectin because proanthocyanidins (condensed tannins) found in cranberry blocked adhesion to and impaired biofilm formation on target tissues of the urothelium by *E. coli* bacteria preventing infection [21]. Some other researchers [22] found that tannins from *Terminalia catappa* leaves significantly inhibited the maturation of biofilms of *P. aeruginosa.* Polyphenols have demonstrated antimicrobial properties [23] while triterpenoids, terpenoids and carotenoids also exhibit antibacterial, antiviral, anti-carcinogenic, and anti-inflammatory activities [23, 5]. Quercetin, a flavonoid inhibits alginate production in a concentration dependent manner leading to decline in adherence during biofilm formation and also reduces exopolysaccharide production required for the initial attachment of *S. aureus* bacteria [24]. Several works have demonstrated *in vitro* inhibition of the growth and adhesion of human uropathogens by the sugar, chitosan [25]; demonstrated *in vivo* in murine model that the chimeric protein, PilQ/PilA antigen of *P. aeruginosa* (vaccine) caused anti-adhesion of *P. aeruginosa* by an anti-pili mechanism [26] and also demonstrated *in vivo* that the adhesin, multivalent adhesion molecule (MAM7) coupled to polystyrene microbeads blocked pilus assembly or function in *P. aeruginosa* in rat model thus, preventing infection [27].

The results of the antioxidant studies (Fig. 3) revealed that the methanolic extract had comparable values with the control at the highest test dose unlike what was seen with the n-hexane extract. The method used compared the extracts to a standard clinically approved antioxidant, ascorbic acid and showed that the methanolic extract was significantly different from the control. The antioxidant activity of the n-hexane extract was found to be dose-dependent and is directly proportional to the concentration of the treatment. These results agree with the GCMS results revealing the presence of ascorbic acid and probably other anti-oxidant molecules [15] and the in vivo anti-oxidant assays of these extracts [29, 30].

Among fifty six tested wild fruits in a research, *Caryota mitis* was one of eight that showed the highest total phenolic contents and highest antioxidant activities, and suggested a potential for development of natural antioxidants and functional foods or drugs [28]. This plant is a close relative of the index plant since they belong to the same genus, Caryota. A review by a group of researchers on different works on *Annona muricata* revealed that it has potent antioxidant properties already established by in vitro free radical scavenging activities of the leaves, seeds, pulp and various parts of the plant [29]. Cranberries contain chemically diverse, secondary metabolites, and is an important source of antioxidants, such as

polyphenols (flavonoids, phenolic acids, anthocyanins, tannins), ascorbic acid, and triterpene compounds. The antioxidant activity in cranberry correlated with the triterpenoids [9]. Flavonoids are important in plant defense and are strong antioxidants [23].

All these observed anti-adhesin and antioxidant effects from both extracts of CN may be due to some bioactive compounds especially since phytochemical assays and GCMS revealed that the n-hexane extract demonstrated the presence of flavonoids, triterpenoids and anthraquinones in small quantities, cardiac glycosides, terpenoids and phenols in moderate quantities and large amounts of steroids. The methanolic extract showed large amounts of ascorbic acid, carbohydrates and tannins, moderate quantities of cardiac glycosides, steroids and anthraquinones while saponins, alkaloids and phenols were present in minute amounts [Unpublished]. Carotenoids, ascorbic acid and terpenoids are present in the n-hexane extract in remarkable amounts, alongside small amounts of triterpenoids which may explain the antioxidant capacity of the n-hexane extract. These results also agree with the in vivo anti-oxidant assays of these extracts [30, 31].

In summary, these extracts have demonstrated anti-adhesin (since anti-adhesin therapy and immunity are meant to reduce contact between host tissues and pathogens) and anti-oxidant effects (which are known to improve the immune system and enhance resistance against infectious microbes). These activities may be explained by the presence of appropriate combination of bioactive compounds already established by phytochemical assay of CN.

5. Conclusions

The presence of both extracts showed anti-adhesin activities which imply that the extract has a modulatory effect on virulence and the infection process and may serve as a potential natural source of anti-adhesins. The antioxidant profile of both extracts was also demonstrated with the methanolic extract showing comparable values to the control in activity and so imply that this extract may be a veritable plant source of anti-oxidants. The combination of these two effects makes the extracts to be potential sources of anti-infective agents or immunity boosters.

Author Contributions

Conceptualization, C.A.M., B.C.U.; Methodology, C.A.M.; Software, C.A.M.; Validation, C.A.M., B.C.U., M.D.; Formal analysis, M.D.; Investigation, C.A.M.; Resources, C.A.M.; Data curation, C.A.M.; Writing – original draft preparation, C.A.M.; Writing – review & editing, B.C.U.; Visualization, M.D.; Supervision, M.D.; Project administration, M.D., C.A.M.; Funding acquisition, C.A.M.

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Conflicts of interest

There is no conflict of interest among the authors

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