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Studies on the Use of Plant Extracts for the Prevention of Bacterial Biofilms on Urinary Catheters

Running Headline: Prevention of bacterial biofilms

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Abstract

This study was undertaken to evaluate the potentials of six plant extracts to prevent bacterial biofilms on urinary catheters. Uniformly sized sections (9 mm^2) of sterile silicone catheters were impregnated with aqueous extracts of the various plants, dried and sterilized under a UV hood before immersing in fresh urine for 18 – 72 h. Untreated catheter and catheter treated with standard antibiotics (ciprofloxacin and gentamicin), which were similarly immersed in urine, served as controls. Biofilm formation on the catheter sections was evaluated by culture, microscopy and viable cell count procedures. The results showed that bacterial attachment occurred on the catheter sections within 24 h of immersion in urine. The various extracts and standard drugs reduced bacterial attachment on the catheters in the order: ciprofloxacin > *Psidium guajava* > *Aloe vera* > gentamicin > *Gongronema latifolium* > untreated sections. *Carica papaya, Ocimum gratisimum* and *Vernonia amygdalina* potentiated bacterial attachment. Furthermore, the effect of *Psidium guajava* extract was longer-lasting than that of the standard antibiotics. Direct sensitivity tests against the urinary isolates showed that only *P. guajava*, amongst all the plant extracts, was active. This preliminary study highlights the potentials of herbal extracts especially *P. guajava* and *Aloe vera* in preventing biofilm formation on urinary catheters.

Keywords: Biofilms, urinary catheters, plant extracts, *Psidium guajava, Vernonia amygdalina,* ciprofloxacin

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Introduction

Urinary catheters are tubular latex or silicone devices that are inserted through the urethra into the bladder to measure urine output, collect urine during surgery, prevent urinary retention, or control urinary incontinence (Donlan and Costerton, 2002). A catheter once inserted, may readily acquire biofilms on the inner and outer surfaces (Donlan, 2001). Microorganisms gaining access to the surfaces of a catheter may then travel intralumenally to reach the urinary bladder, resulting in infection and the rate may be influenced by the presence of swarming organisms such as *Proteus* spp. (McLean *et al.* 1995; Jones *et al*., 2005).

Urinary catheters are associated with urinary tract infections (UTIs) in individuals requiring catheterization. According to Stickler (1996), 10 – 50% of patients undergoing short-term catheterization (up to 7 days) develop infections, whereas essentially all patients undergoing long-term catheterization (more than 28 days) will develop UTIs. McLean *et al* (1997) further noted that the risk of catheter-associated infection increases by approximately 10% for each day the catheter is in place. Studies have also shown that catheter related UTIs contribute to more than 40% of nosocomial infections (Bronsema *et al*., 1993; Carapeti *et al*., 1996).

Organisms that attach to a catheter and develop the biofilm may be organisms

introduced into the urethra or bladder during insertion of the catheter, organisms from the sheath of exudates surrounding the catheter, or organisms from the collection bag (Donlan and Costerton, 2002). The organisms commonly associated with urinary catheters are *Enterococcus faecalis, E. coli, Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella pneumoniae* and other gram-negative organisms (Donlan, 2002). Several strategies have been attempted to control urinary catheter biofilms, including treatment of catheters with antimicrobial ointments and lubricants, bladder instillation or irrigation, introduction of antimicrobial agents in collection bags, impregnation of the catheter with antimicrobial agents such as silver-oxide, or use of systemic antibiotics (Riley *et* al., 1995; Bologna *et* al., 1999; Donlan, 2001). However, these strategies have been largely ineffective due to the typical recalcitrance of biofilm organisms to antibiotics and other antimicrobial agents; attributed to restricted penetration of the antimicrobials into the biofilm matrix and phenotypic alteration of biofilm organisms. In view of these, intervention strategies targeted at disruption of the initial attachment of organisms remain the best option.

In this study, aqueous extracts of leaves of various plants are evaluated for their effectiveness in inhibiting bacterial attachment and growth on urinary catheters. Plants, because of their often multiple mechanisms of action, minimal side effects, low potential to cause resistance and low cost (Cowan, 1999; Esimone *et al*., 2005) are envisaged as suitable alternatives to conventional antibiotics and antimicrobial agents.

Materials and methods

Plants and antibiotics used in the study: Six plants were used in this study and are: Vernonia amygdalina (common name, bitterleaf), Gongronema latifolium (utazi), Ocimum gratissimum (scent leaf or nchuanwu), Carica papaya (paw-paw), Psidium guajava (guava) and Aloe vera. Two antibiotics were used: ciprofloxacin and gentamicin. The plants were collected from different residential and non-residential locations in Nsukka town or purchased from the Nsukka market. All the plants were authenticated by a taxonomist in the Department of Botany, University of Nigeria, Nsukka. The standard antibiotics were purchased from a reputable pharmaceutical shop.

Preparation of extracts and antibiotics: A quantity (500 g) of leaves of each plant was washed in clean tap water and dried briefly under room temperature. The dried leaves were then marcerated and extracted by hand in 1 L of cold distilled water. Cold water extracts were used because many herbal potions are specified to be prepared as cold infusions as hot extraction may lead to loss of some active components (Sofowora, 2006). The crude preparations were filtered through Number 1 Whatman filters and the filtrates (extracts) collected into sterile bottles. For Aloe vera, the gel was extracted by slicing open the leaves and squeezing out the gel. The gel obtained from 500 g of the leaves of the plant was diluted to a solution with 1 L of water and stored in sterile bottles. To prepare a 100 mg ml^{-1} solution of ciprofloxacin, 500 mg of the drug was dissolved in 5 ml of distilled water in a sterile bottle. For gentamicin, the injection form (140 mg ml^{-1}) was used.

Preparation of catheter sections: Sterile silicone catheters were purchased from the market. The catheters were cut into uniformly sized sections first transversely, then longitudinally to expose the lumenal surfaces. Each section measured about 9 mm^2. The sections were then dispensed into Bijou bottles containing different plant extracts and antibiotics and kept at 4^oC for about 18 h to facilitate impregnation of the catheter surfaces with the extracts and antibiotics and prevent growth of contaminants. Following impregnation, the catheter pieces were removed from the extracts and placed in sterile Petri dishes before drying and sterilizing under a UV hood.

Production of catheter biofilm: In order to produce biofilms on the catheter, the treated catheter sections were immersed in fresh normal urine (urine from asymptomatic individual) in separate sterile bottles. The bottles were allowed to stand for 18 – 72 h at room temperature before examining the catheter sections for bacterial attachment. Untreated catheter sections also immersed in urine served as control.

Assessment of biofilms: Biofilm formation on the catheter sections was evaluated by culture, microscopy and viable cell count procedures. For culture, swabs taken from both outer and lumenal surfaces of different treated and untreated catheter

sections were inoculated onto the surfaces of MacConkey and blood agar plates. The plates were then incubated at 37^{o}C for 18 – 24 h. Following incubation, total colony counts were taken and the organisms presumptively identified to at least genus level. Microscopic evaluation was carried out by direct Gram staining of catheter sections removed from the urine. Different treated catheter sections were compared for the degree of attachment of organisms on them.

To determine the actual extent of attachment of cells to the variously treated and untreated catheters, viable counts of attached cells were taken for up to 72 h. A sterile wire loop was used to scrape off cells from the lumenal surfaces of each catheter section after several rinses with water. The cells were scraped into 1 ml of normal saline and a serial dilution made before plating on nutrient agar plates and incubating at 37^{o}C.

Sensitivity of isolates to extracts: The different extracts and antibiotics were evaluated for their activity against the urine organisms by disk diffusion method.

Phytochemical analysis of extracts: For the purpose of phytochemistry, leaves prepared as previously described were extracted with 1 L of methanol, instead of distilled water and filtered through Whatman No. 1 filters. The filtrates were then evaluated phytochemically for the presence of saponin, flavonoids, steroids, alkaloids, tannins, triterpene and glycosides according to the methods of Trease and Evans (1996).

*Analysis of data***:** The data on viable counts were analysed statistically using one-way analysis of variance (ANOVA) and significant means were separated using least significant difference (LSD) at 95% confidence limit.

Results

Production of catheter biofilms: The results showed that bacterial cells were attached (not removed by gentle but thorough rinsing) to the catheter sections within 24 h of immersion in urine. Cultures of swabs taken from catheter sections after 18 h incubation in urine yielded significant growth (Figure 1). Direct microscopic examination of Gram-stained sections also showed presence of attached cells to the surfaces of the catheter (Figure 2).

Isolation and identification of organisms: Three different organisms were isolated from the urine cultures and were presumptively identified based on morphological and biochemical characteristics according to standard microbiological procedures (Cheesbrough, 2004). The organisms were identified as Pseudomonas aeruginosa, Escherichia coli and coagulase-negative Staphylococcus sp.

Effects of extracts and antibiotics on bacterial attachment: The effects of different plant extracts and antibiotics used for the catheter pretreatment were evaluated by considering generally, the presence of organisms on the catheter sections, the type of organisms attaching to the catheters and the number of organisms attached. The attachment of cells to the catheter surfaces was dependent on the catheter pretreatment. Ciprofloxacin-coated sections appeared to have the least number of attached cells, followed by Psidium guajava, followed by Aloe vera and then gentamicin, Gongronema latifolium and the untreated sections. Carica papaya, Ocimum gratissimum and Vernonia amygdalina-treated sections appeared to have more cells attached than the untreated sections. These observations were supported by viable counts determined from scrapings of cells from the catheter sections, which showed the actual number of attached cells per section (Figure 3). The results clearly show that treatment of catheter sections with ciprofloxacin, Psidium guajava, Aloe vera and gentamicin caused significant ($p < 0.05$) reductions in the number of attached cells. Furthermore, the results show that the effect of Psidium guajava extract was longer-lasting than that of the antibiotics and by the end of 72 h, the sections treated with the extract had fewer cells attached than those treated with ciprofloxacin (Figure 4).

Sensitivity of organisms to the extracts: Direct sensitivity tests to determine the activity of the plant extracts and antibiotics against the urine isolates showed that both antibiotics were active against the organisms. However, of all the extracts, only *P. guajava* extract had activity against the organisms (Table 1).

Phytochemistry of plant extracts: The different plant extracts contained various amounts of different photochemical components (tannins, glycoside, saponin, flavonoid, alkaloid and triterpene) as shown in Table 2.

Table 1. Sensitivity of test organisms to antibiotics and various extracts applied to catheter sections.

Organisms	Antibiotics		Extracts					
	CIP	GEN	VA	AV	PG	GL	CP	OG
Pseudomonas aeruginosa	S (55 mm)	S(31mm)	R	R	S(11mm)	R	R	R
Staphylococcus sp.	S(56 mm)	S(42mm)	R	R	S(14mm)	R	R	R
Escherichia coli	S(47 mm)	S(36mm)	R	R	S(15mm)	R	R	R

S = sensitive, R = resistant. Numbers in parenthesis represent inhibition zone diameters.

Table 2. Phytochemical composition of some of the plant extracts

Phytochemical component	Relative concentration in plant extract		
	Vernonia amygdalina	***Psidium guajava***	***Carica papaya***
Tannin	+++	+++	+
Glycoside	++	-	+
Saponin	+++	++	+
Flavonoid	+++	+++	+
Aklaloid	+++	-	+
Triterpene	-	+++	-

Key: - = not detected; + = present in trace concentration; ++ = present in moderate concentration; +++ = present in high concentration

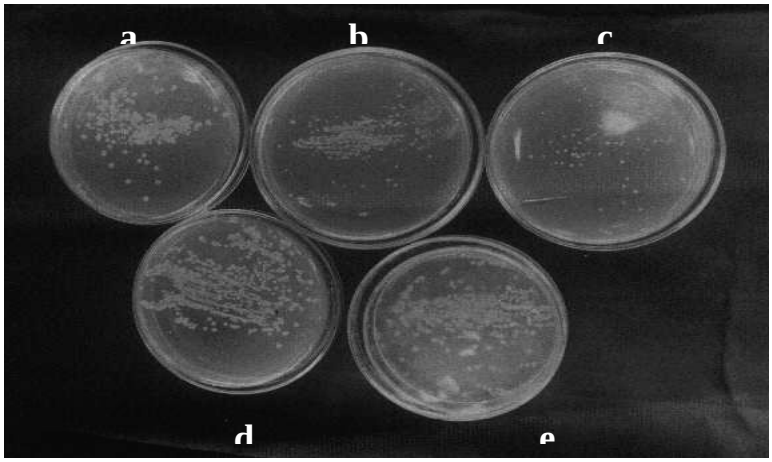

Fig. 1. Culture results of swabs taken from different treated and untreated catheter sections immersed in normal urine for 18 h: a, *Aloe vera*; b, *P. guajava*; c, ciprofloxacin; d, *Vernonia amvadalina*: e. untreated.

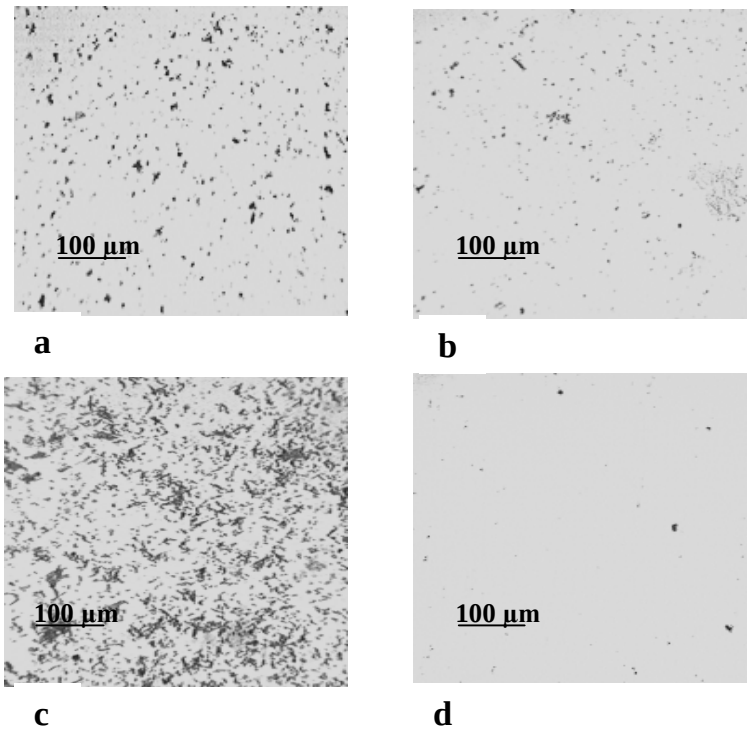

Fig. 2. Composite of the microscopic appearances of the lumenal surfaces of pretreated catheter sections immersed in urine for 24 h: a, untreated catheter; b, *P. guajava*-treated; c, *V. amygdalina*-treated; and d, ciprofloxacin-treated. Cells attached to the catheter sections were Gram-stained *in situ.* Note the reduced number of cells on ciprofloxacin and *P. guajava* treated surfaces compared to the untreated section. Also note the increased number of cells on the *V. amygdalina*-treated section.

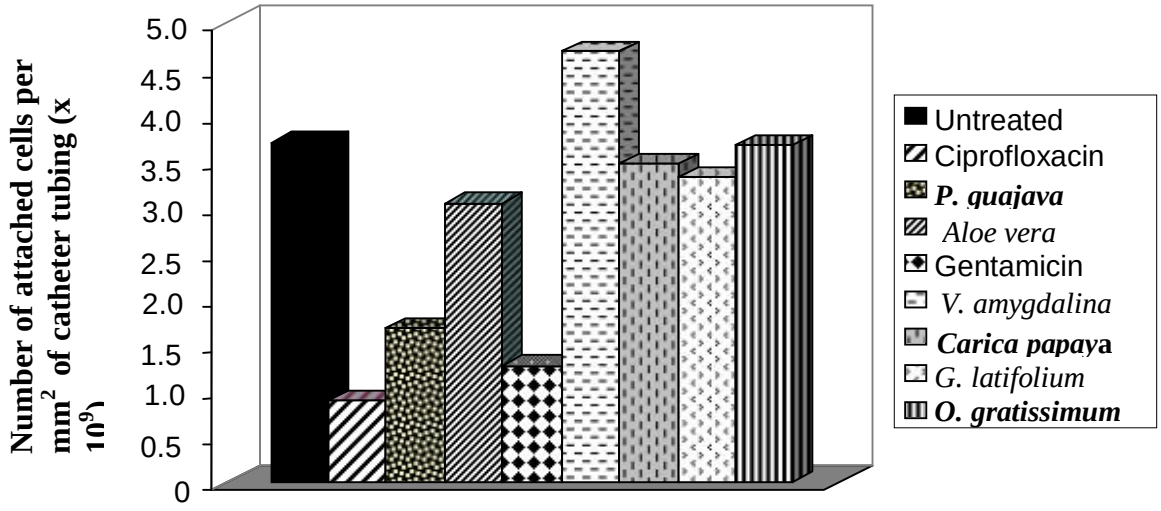

Fig. 3. Attachment of cells, within eighteen-hour period, to silicon cathether surfaces coated with antibiotics and aqueous extracts of various plants. Cell numbers represent mean values of viable counts obtained from scrapings from triplicate catheter sections. Note the significant ($p < 0.05$) reduction in cell numbers following treatment of catheter sections with *Psidium guajava,* Ciprofloxacin, Gentamicin and *Aloe vera.*

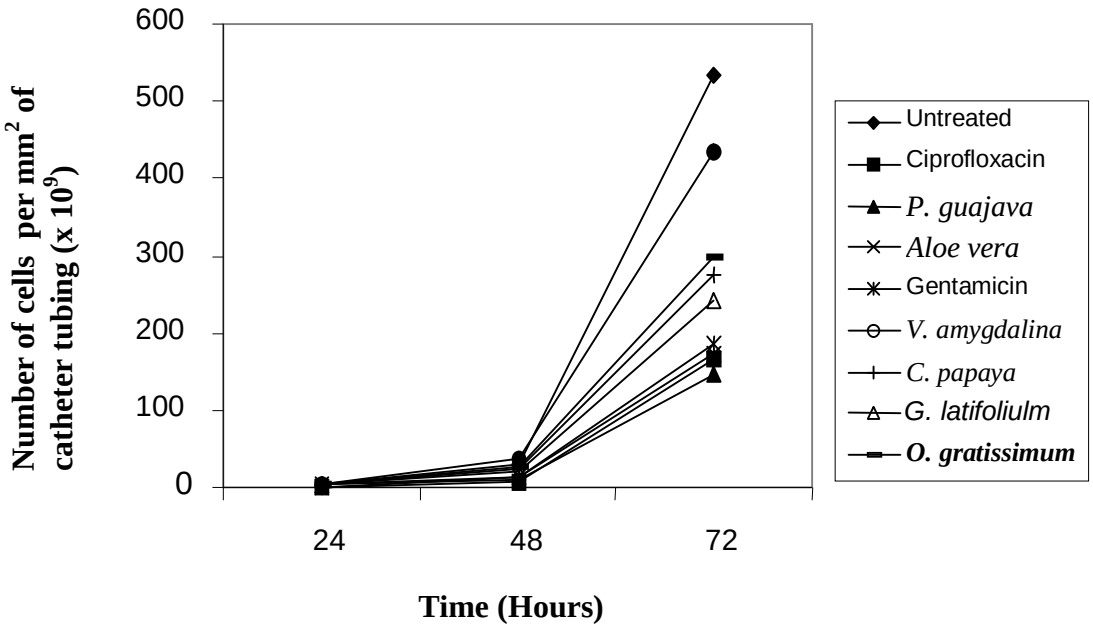

Fig. 4. Attachment and growth of cells on silicon catheter surfaces coated with antibiotics and aqueous extracts of various plants. Cell numbers represent mean values of viable counts obtained from scrapings from triplicate catheter sections. Note the significant ($p < 0.05$) reduction in cell numbers following treatment of catheter sections with *Psidium guajava,* Ciprofloxacin, Gentamicin and *Aloe vera*.

Discussion

Results from this study showed that the aqueous extract of the leaves of *Psidium guajava* significantly ($p < 0.05$) reduced bacterial attachment to the intralumenal surfaces of silicone urinary catheters while the extract of *Vernonia amygdalina* promoted attachment. Macroscopic examination of culture plates of scrapings from uniformly sized, pre-treated catheter sections immersed in urine for 18 h at 37°C, showed that fewer bacterial cells were recovered from *P. guajava* extract-treated catheters than untreated catheters (Figure 1). This observation was further confirmed by microscopic examination of Gram-stained catheter sections and viable counts taken over a period of 72 h (Figures 2 – 4).

It was interesting to note that while the *P. guajava* extract seemed less effective than one of the test antibiotics, ciprofloxacin, within the first 24 h, by 72 h, there were fewer cells on the *P. guajava*-treated catheter sections than on the ciprofloxacin-treated sections (Figure 4). This suggests that the inhibitory action of the extract of *P. guajava* leaves was longer-lasting than that of the antibiotic. This could be either because the extract was more stable or because of less resistance of the organisms to the extract. It has been previously suggested that there is limited effectiveness of antibiotics in treating mature biofilms due to restricted penetration of the drugs into the biofilm matrix and antimicrobial resistance of biofilm organisms resulting from physiological changes in the cells (Ganderton *et al*., 1992; Donlan, 2002; Jones *et al*., 2005). The results from this study therefore support the idea of plant extracts as suitable alternatives to antibiotics as suggested by some authors (Cowan, 1999; Esimone *et al*., 2005).

The antibiotics and extracts were evaluated directly for antimicrobial activity against three organisms isolated from urine. The results as seen in Table 1 showed that while both antibiotics were effective against the organisms, only *P. guajava* extract, of all the

plant extracts tested, was active against the test organisms. The zones of inhibition measured were however less than the zones measured for the two antibiotics used in this study. It must be pointed out, however, that the extracts used in this study were not concentrated and this could account for the difference in activity.

Plants such as *Psidium guajava, Vernonia amygdalina* and *Ocimum gratissimum* are known in Nigerian folklore for their uses in the treatment of various ailments, particularly malaria (Irvine, 1961; Iwu, 1993). These plants have been reported to possess antimicrobial activity in addition to their other health benefits, including the lowering of blood glucose, cholesterol and free radicals (Ajali, 2004; Chah *et al*., 2005; Sofowora, 2006; Ehiaghonare, 2007). *Psidium* guajava, in particular, has been shown to have broad-spectrum antibacterial effects (Esimone *et al.,* 2003; Chah *et al*., 2005). Esimone *et al.* (2003) showed that the aqueous leaf extract of the plant had higher *in vitro* activity against a number of test bacteria than six conventional antibiotics. The inhibitory activity observed with the *P. guajava* extract in this study was therefore not surprising. *Psidium guajava* plants are distinctive shrubs with aromatic leaves and berry fruits, belonging to the family Myrtaceae. Ethnopharmacologically, the decoctions of the leaves and unripe fruits are used in the treatment of malaria, skin eruptions, internal haemorrhage, measles and dysentery by oral administration (Iwu, 1983).

While there have been no reports on the bioactive compounds in extracts of the plant, the phytochemical analysis of methanolic leaf extracts in this study indicated the presence of high concentrations of tannins, flavonoids and triterpenes. At least one of these components (flavonoids) is known to contain an agent, flavone, which has been reported to be antibacterial (Oliver-Bever, 1986).

The enhancement of growth rather than inhibition, observed with the extract of *V. amygdalina* was unexpected, since the plant is generally considered to have antimicrobial action. The antimicrobial activity is usually attributed to saponins (Ajali, 2004), which were found to be present in this study. However, there was also a high concentration of glycosides, which may have been used as substrate by the bacteria. It was also noteworthy and surprising that while *Aloe vera* inhibited attachment of cells to the catheter, there was no measurable inhibition zone observed on direct sensitivity studies. We can only speculate here that the thick *Aloe vera* gel did not diffuse through the agar.

The problem of bacterial biofilms is an old one and many intervention strategies have been attempted without success; the most effective so far being the coating of catheters with antibiotics. To date, there have been no reports of attempts to use plant extracts or plant products in the control of urinary catheter biofilms. The only reported studies involving plants were conducted using cranberry juice, which is commonly used and recommended for management of UTIs (Jepson *et al*., 1998; Jepson *et al*., 2001; Morris and Stickler, 2001). In those studies, however, the research evidence did not support the effectiveness of cranberry juice for either prevention or treatment of UTIs. The present study is therefore the first study to investigate the effectiveness of direct application of plant extracts to urinary catheters as an intervention strategy for biofilm problems.

Several important inferences may be drawn from the results of this study: catheterization should ideally be short-term, preferably less than 72 h; if longer periods of catheterization are necessary, such catheters must be periodically (48 to 72 h intervals) removed, flushed out thoroughly before reinsertion; and plant extracts such as that of *P. guajava*, found in this study to be effective, can be used in the treatment of such catheters. The results from this study show that some plant extracts may be developed, perhaps with further purification and concentration of various phytochemical constituents, and applied to catheter surfaces to help in the control of bacterial biofilms.

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