



Research Article

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ANTIMICROBIAL PERFORMANCE OF ETHANOLIC EXTRACT OF *ARECA CATECHU* L SEEDS AGAINST MIXED-ORAL FLORA FROM TOOTH SCUM AND GRAM NEGATIVE LABORATORY ISOLATES

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ABSTRACT

Areca catechu L. nut is a component of betel quid, the chewing of which is a popular or a cultural tradition in some countries in South East Asia. Areca nut contains bioactive components like alkaloids and tannins which have been demonstrated to elicit inhibitory effects in selected microorganisms. Varying concentrations of *Areca catechu* L. nut ethanol extract were tested for antimicrobial activity against 0.5 McFarland of mixed-oral flora and eight gram-negative clinical isolates (*E. coli*, *K. pneumonia*, *P. vulgaris*, *P. aeruginosa*, *S. non-typhi*, *S. typhi*, *S. flexneri* and *V. cholera*) by agar well diffusion method. All concentrations were shown to inhibit growth in all mixed-oral flora models with zones of inhibition ranging from 7 mm to 18 mm. Susceptible patterns were also seen in all gram-negative clinical isolates with the smallest mean of zones of inhibition seen in *Escherichia coli* which is 8 mm at 30 % concentration and *Klebsiella pneumoniae* with no zone of inhibition in both 30 % and 50 % concentration. Highest antimicrobial activities were seen against *Proteus vulgaris* and *Vibrio cholerae* with a mean zone of inhibition of 18 mm at 70 % concentration and a mean zone of inhibition of 16 mm at 50 % concentration respectively. These results were comparable to intermediate susceptible pattern in Ciprofloxacin which is 16 mm to 20 mm and indicates that there are bioactive components present in areca nut that are worthy enough of further study and exploitation for its inherent antimicrobial activity.

Keywords: *Areca catechu* L., Agar-well diffusion, Betel nut, ethanol extract, Gram-negative bacteria, oral flora

INTRODUCTION

A common practice among Asians, most especially the elders is the chewing of betel quid commonly known as “nganga”, a combination of betel nut, betel leaves with lime or tobacco. Chewing betel quid is considered a past time and is a cultural tradition in different countries like India, Indonesia, Arabian Peninsula, some of the Pacific islands such as Micronesia, Fiji, Solomon Islands and the Philippines¹. *Areca catechu* L. is a straight solitary tree that has annular leaf scars. The leaves have leaflets that measure up to 4 cm long. The ovoid fruit turns red or orange when ripe and has a fleshy pericarp and a fibrous mesocarp² seeds are mistakenly called as nuts are used for mastication³. The husks are used as an alternative to toothbrush⁴ and it is also used as a vermifuge in some parts of China⁵. The antimicrobial activity of the betel quid is known due to the discovery that even the betel leaf alone can contribute effectively since it contains alkaloids. The four major alkaloids found in *Areca catechu* L. are arecoline, arecaidine, guvacoline, and guvacine. Arecoline is an oily-liquid that is soluble in water, alcohols and ether; this lipophilic nature can have an entry to the brain and intracellular spaces to fulfil its stimulatory effects⁶. Periodontal diseases are infections caused by bacteria that affect the supporting structures of the teeth such as the gingiva, cementum, periodontal membrane and alveolar bone. Gingivitis, a condition where the gums are inflamed, is associated with accumulations of bacterial plaque. Consequently,

evidences support that normal flora of the oral cavity can partake in occurrence of various systemic diseases. The antimicrobial effect of ethanol extract of *Areca catechu* L. seeds against mixed-oral flora and certain-gram negative clinical isolates were tested using agar-well diffusion method as in the work of Cyriac, *et al*, (2012)⁷. Their work demonstrated positive antimicrobial properties of *Areca catechu* L. nut husk against *Candida albicans* but not against gram-negative isolates. Thus, we conducted a study on the antimicrobial effects of ethanol extract of *Areca catechu* L. nuts/seeds against mixed-oral flora and gram-negative clinical isolates.

MATERIALS AND METHODS

Areca catechu L. nuts were harvested and verified by the National Museum, Botany Division, Manila, Philippines. Ethanolic extract was prepared by authorized personnel from the Chemicals and Energy Division, Department of Science and Technology. 430 g of *Areca catechu* L. nuts were pulverized using Wiley mill and soaked in 1.4 L of 95 % ethyl alcohol for 48 hours. The filtrate was concentrated using rotary evaporator at 60°C under vacuum for 2 hours. Concentrated extract was further evaporated using water bath at 60°C to obtain a semi-solid extract (Crude extraction of 430 g, areca nut produced 1.2 L ethanol extract and concentration of filtrate yielded 65.0 g of semi-solid extract) and diluted to 30 %, 50%, and 70 % concentrations which were then autoclaved prior to use. Mixed-oral flora inoculums were prepared by

harvesting tooth scum and inoculating it in Tryptic soy broth (TSB). Gram-negative laboratory isolates were also inoculated in TSB. Both inoculums were incubated at 35°C for 24 hours and adjusted to 0.5 McFarland prior to seeding in MHA plates bored with wells of 6 mm in diameter and 4 mm depth where varying dilutions (30 %, 50 % and 70 %) of *Areca catechu* L. nuts ethanol extract were subjected for agar well diffusion antibiotic susceptibility testing. MHA test plates were incubated at 35°C for 24 hours and read for zones of inhibition using a Vernier calliper. Control antibiotics of Cefepime, Clindamycin, Erythromycin, and Tetracycline were used as reference antibiotics for mixed-oral flora test inoculums and Ciprofloxacin was used as a reference antibiotic for gram-negative laboratory isolates. All tests were done in duplicate and the mean computed.

RESULTS

Cefepime was most effective against all mixed-oral flora compared to other reference antibiotics with zones of inhibition ranging from 21 mm to 32 mm with a mean zone of inhibition of 28 mm. Clindamycin on the other hand was least effective in inhibiting mixed-oral flora inoculums. There were no zones of inhibition (0 mm) observed in mixed-oral flora inoculums A and B against this reference antibiotic. Ciprofoxacin, used as the reference antibiotic for eight gram negative isolates in the study and demonstrated a mean zone of inhibition of 28 mm with susceptible patterns on all gram-negative isolates except for *E. coli* and *P. vulgaris* which demonstrated 8 mm and 15 mm zone of inhibition respectively. Table 1 showed that as the concentration of *Areca catechu* L. nut ethanol extract increases, zones of inhibition also increased. Identical or smaller zones of inhibition observed in higher concentrations can be attributed to the high viscosity of the ethanol extract which hinders the efficient diffusion of the active component throughout the agar matrix. This is the primary reason why 100 % or pure ethanol extract of *Areca catechu* L. nut was not used in the study; it was very viscous and does not evenly diffuse throughout the agar. Although it exhibited larger zones of inhibition at times compared to 70 % ethanol extract, there is great difficulty in measuring its zone of inhibition owing to the irregular pattern of diffusion of the highly viscous extract in the agar. Susceptibility patterns from mixed-oral flora A, B, C, and D indicates that there were organisms in the mixed microbial community in the oral cavity isolated from tooth scum that were all susceptible to Cefepime, Clindamycin, Erythromycin, and Tetracycline; there were however, organisms that were also resistant to the same antibiotics as demonstrated in mixed-oral flora A which were resistant to Clindamycin, Erythromycin, and Tetracycline and mixed-oral flora B which were resistant to Clindamycin. For both cases of mixed-oral flora A and B organisms which demonstrated resistant patterns against Clindamycin, their microscopic observation shows that these were yeast cells and for the case of mixed-oral flora A, organisms which were found to be resistant to Erythromycin and Tetracycline, microscopic examination shows that these were gram-negative bacilli. All

concentrations of ethanol extract of *Areca catechu* L. nut demonstrated zones of inhibition against mixed-oral flora A, B, C, and D with the smallest diameter measuring 7 mm (30 % in mixed-oral flora B, Figure 1) and the largest measuring 18 mm (50 % and 70 % in mixed-oral flora C, Figure 2 and 3). Minimum inhibitory concentration of *Areca catechu* L. nut ethanol extract effective against mixed-oral flora isolated from tooth scum was found to be 30 % concentration with 9 mm, 7 mm, 16 mm, and 17 mm zones of inhibition for mixed-oral flora A, B, C, and D respectively. Mean diameter of zones of inhibition from duplicate trials of the varying concentrations of *Areca catechu* L. nut ethanol extract demonstrated antimicrobial activity against all gram-negative clinical isolates (*E. coli*, *K. pneumonia*, *P. vulgaris*, *P. aeruginosa*, *S. non-typhi*, *S. typhi*, *S. flexneri* and *V. cholerae*). The smallest mean of zones of inhibition were seen in *E. coli* with 8 mm at 30 % concentration (Figure 4) and *K. pneumoniae* which demonstrated no zone of inhibition in both 30 % and 50 % concentrations indicating that *Areca catechu* L. nut ethanol extract is least effective for these two organisms, particularly *K. pneumoniae* which produces a very thick capsule layer which can be observed in its highly mucoid colonial characteristic. Highest antimicrobial activity were demonstrated against *P. vulgaris* and *V. cholerae* which demonstrated a mean zone of inhibition of 18 mm at 70 % concentration (Figure 5) and a mean zone of inhibition of 16 mm at 50 % concentration (Figure 6) respectively; these results were comparable to intermediate susceptible pattern in Ciprofloxacin which is 16 mm to 20 mm.

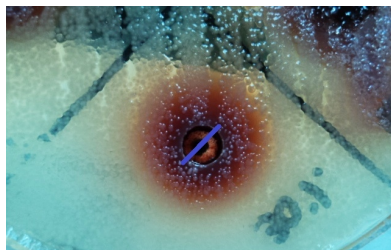
DISCUSSION

This study observed the antimicrobial activity of ethanol extract of *Areca catechu* L. nut in 30 %, 50 %, and 70 % concentrations against mixed-oral flora isolated from tooth scum and eight gram-negative clinical isolates namely: *E. coli*, *K. pneumoniae*, *P. vulgaris*, *P. aeruginosa*, *S. non-typhi*, *S. typhi*, *S. flexneri* and *V. cholerae*. Agar well diffusion method was used to evaluate antimicrobial activity by measuring zones of inhibition⁸; all tests were done in duplicate and demonstrated clear evidences of antimicrobial activity. The use of mixed-oral flora in this study served as an effective simulation of the *in-vivo* microbial community found in the oral cavity in an *in-vitro* test environment. The oral flora which is commonly composed of organisms including bacteria, fungi, mycoplasma, protozoa and viral flora which may persist from time to time although, bacteria are the predominant group⁹ composed mainly of *Streptococcus mutans*, *Streptococcus salivarius*, *Streptococcus anginosus*, *Streptococcus mitis* and *Lactobacillus acidophilus*¹⁰. *Areca catechu* L. nut contains 50-60 % sugars, 15 % lipid (glycerine of lauric, myristic and oleic acid), 15 % condensed tannins (phlobatannin, catechin), polyphenolics (NPPF-86IA, NPF-86IB, NPF-86A and NPF-86B) and 0.2-0.5 % alkaloids (arecoline, arecaidine, guvacine and guvacoline^{11,12}; these alkaloids, tannins and polyphenols possess antihelminthic, antifungal, antibacterial, anti-inflammatory and antioxidant activities¹³.

Table 1: Zones of Inhibition Observed in Mixed-oral flora and Gram-negative Clinical isolates

Zones of inhibition measured in millimetres (mm)								
Organism	Reference Antibiotics				Test Mean			
	FEP-30	CC-2	E-15	TE-30	30 %	50 %	70 %	
Mixed-oral flora	A	21	0	0	0	9	14	14
	B	30	0	12	16	7	7	8
	C	27	26	19	10	16	18	18
	D	32	11	13	12	17	16	15
		CIP-5						
Gram-negative clinical isolates	<i>E. coli</i>	8				8	11	13
	<i>K. pneumoniae</i>	33				0	0	9
	<i>P. vulgaris</i>	15				12	14	18
	<i>P. aeruginosa</i>	31				8	11	12
	<i>S. non-typhi</i>	33				9	9	11
	<i>S. typhi</i>	34				9	11	10
	<i>S. flexneri</i>	34				13	13	14
	<i>V. cholerae</i>	33				15	16	15

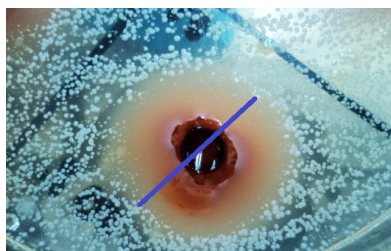
Legend			
Reference antibiotics			
Code:	Name:	Concentration in micrograms (mcg)	Susceptible (S), Intermediate (I), Resistant (R)
FEP-30	Cefepime	30	24 (S), 22-23 (I), 15 (R)
CC-2	Clindamycin	2	19 (S), 16-18 (I), 15 (R)
E-15	Erythromycin	15	21 (S), 16-20 (I), 15 (R)
TE-30	Tetracycline	30	23 (S), 19-22 (I), 18 (R)
CIP-5	Ciprofloxacin	5	21 (S), 16-20 (I), 15 (R)



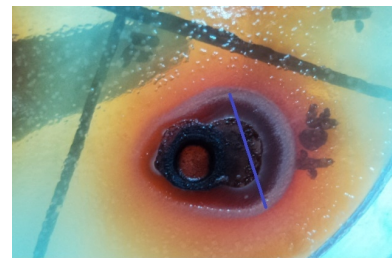
Mixed-oral flora B, 30%, 7 mm
 Figure 1: mixed oral flora B, 30 %, 7 mm



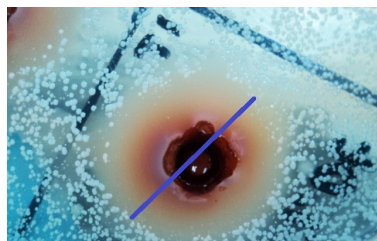
E. coli, 30%, 8 mm
 Figure 4: *E. coli*, 30 %, 8 mm



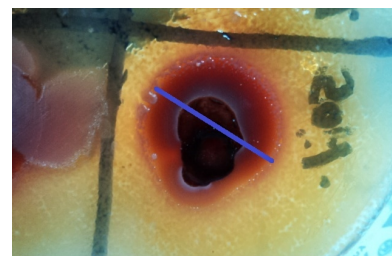
MIXED-ORAL FLORA C, 50%, 18 mm
 Figure 2: mixed-oral flora C, 50 %, 18 mm



P. vulgaris, 70%, 17 mm, Trial 1
 Figure 5: *P. vulgaris*, 70 %, 17 mm (Trial 1)



MIXED-ORAL FLORA C, 70%, 17 mm
 Figure 3: mixed-oral flora C, 70 %, 17 mm



V. cholerae, 50%, 15 mm, Trial 1
 Figure 6: *V. cholerae*, 50 %, 15 mm (Trial 1)

In a study by Anjali and Rao (1995) water and methanol extracts of the seeds in various ages presented higher percentage of tannin and total phenols than other parts of the areca tree extracts¹³ which makes the nut an abundant source of bioactive components with antimicrobial activity. *Areca catechu* L. nut extracts in this experiment proved hard to manage when incorporated in systems with protein constituents like broth media. It produced instant turbidity when applied; which is the primary reason why the researchers used plated media methodology for evaluating antimicrobial activity. Formation of turbidity was attributed to areca extract constituents capable of effectively and instantly precipitating proteins. In line with these it can be suspected that areca extract constituents may have strong effects in inhibiting signal molecules elucidated by microbes by denaturing it. Mixed oral floras from tooth scum were used to simulate, for the first time, the in vitro growth behaviours of microbes in the actual community they were isolated from.

This way the researchers demonstrated the antimicrobial activity of *Areca catechu* L. nut ethanol extract on mixed microbial community without eliminating the synergistic or antagonistic effect that each microbe exert on each other. Inhibitory effects were demonstrated in this study set up by zones of inhibition which can be attributed to the alkaloids in areca-nut¹⁴ and was clear evidence that the ethanol extract does exert an antimicrobial effect on some microbes found in the oral cavity even if they are incorporated in mixed biofilm communities. Furthermore, similar effects of inhibition were observed in varying degrees against the eight gram-negative clinical isolates. Also, betel quid extracts are mutagenic against bacteria; similar studies of aqueous extracts of areca nut produced gene conversion in yeast and arecoline and other areca-nut alkaloids gave positive responses in most bacterial mutagenicity assays¹⁵. A study by Ghanwate and Thakare (2012) demonstrated that all ingredients of betel quid showed reduction in microbial population of oral cavity individually and in different combinations, betel nut decreased oral flora greatly; and showed highest antimicrobial activity against enteric pathogens¹⁶ which confirmed the results of this study as similar inhibitory effects were demonstrated against *S. typhi*, *S. non-typhi*, *S. flexneri* and *V. cholerae*. Studies by De Miranda *et al*, (1996) reported that areca nut extract exerts a direct antimicrobial effect against oral bacteria, including *S. mutans*, *S. salivarius*, *C. albicans*, and *F. nucleatum*¹⁷ which also correlates with the results of this study against mixed-oral flora. In betel quid chewing, auto-oxidation of polyphenols in areca nut generates the superoxide anion (O_2^-) especially at the high pH of slaked lime. The superoxide anion is converted to H_2O_2 which reacts in the presence of copper and iron to generate hydroxyl radicals which are toxic to some bacteria and fungi^{18,19}. Conversely, results in other studies demonstrate that decrease in oxidation-reduction potential of the gingival region and in the pH pose restrictions in antimicrobial action of bioactive components of areca nut extract as shown in the study by Kenney *et al*, (1975) where there were no inhibition for gram-negative anaerobic bacteria which causes dental caries²⁰. Regarding the antifungal property of *Areca catechu* L. extract, a study by Cyriac *et*

al, (2012) demonstrated inhibitory effects against *Candida albicans* with zones of inhibition measuring 6 mm in diameter in MHA agar using 50 ul extract of the nut husk alone⁷; it tested its antimicrobial activity against oral pathogens namely: *C. albicans*, *S. mutans*, *S. salivarius*, *S. mitis*, *L. acidophilus*, and *P. intermedia* and demonstrated positive result only for *C. albicans* and no effect against the other oral pathogens. This can be explained by the results of our study; resistant organisms in mixed-oral flora in this study were found to be yeast cells which may or may not be *C. albicans*; suffice to say, there may be bioactive components present in the husk that is specifically effective against *C. albicans* but not against other oral pathogens. In addition, bioactive components against bacteria may be present in the husk but in very low concentrations if not at all absent which is inadequate to demonstrate antimicrobial activity towards other oral pathogens. Conversely, there are bioactive components present in the *Areca catechu* L. nut ethanol extract which explains the antimicrobial effect exerted against mixed-oral flora organisms from tooth scum and clinical isolates in this study. The former rationale is evidence that there may not be any other constituents exerting antagonistic or negating effects to inhibit the positive action of the bioactive agents present in the ethanol extract of *Areca catechu* L. nut²¹. In summary, ethanol extract of *Areca catechu* L. nut possess antimicrobial properties when applied in aerobic environments owing to the dependency of the bioactive agents in oxidation-reduction reactions¹⁸⁻²⁰. Unidentified bioactive components elicit precipitation of proteins which may explain its antimicrobial potency of inhibiting proliferation of mixed bacterial community enclosed in bio films as exhibited by its inhibitory effect against several models of mixed-oral flora. It is highly recommended that the bioactive agents responsible for precipitating proteins be identified and quantified for applications in disrupting the functions of signalling molecules as well as in other laboratory methodologies having for its principle protein precipitation.

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