In vitro antibacterial effects of Alkaloid-rich dried extract from Areca nuts (Semen Arecae Catechi)

Nguyen Thi Thu Hang, Nguyen Thi Minh Thu, Nguyen Phuong Dung, Do Thi Bich Tra, Do Thi Thanh Xuan, Vi Van Binh
Vietnam University of Traditional Medicine

ABSTRACT

Objective: *To test in vitro antibacterial effects of Areca nut dried extract rich in alkaloids.*

Subjects and methods: *The test was conducted on three bacterial strains comprising Pseudomonas aeruginosa ATCC 27853, Staphylococcus aureus ATCC 25923 and Escherichia coli ATCC 11775 by the agar well diffusion method.*

Results: *Areca nut extract (ANE) at all tested concentrations exhibited remarkable in vitro antibacterial activity against three strains of P. aeruginosa, S. aureus and E. coli with their largest antibacterial ring diameters at 1.0 g/ml being 17.10 ± 0.707, 26.67 ± 0.661 and 25.00 ± 0.90 mm, respectively. The diameter values decreased linearly with the test sample's concentrations and were lowest at 0.1 g/ml with values of 7.90 ± 0.64, 12.20 ± 1.45 and 15.80 ± 11.33 mm in order. Moreover, ANE had the highest antibacterial activity against S. aureus, significantly higher than E. coli ($p<0.05$) and P. aeruginosa ($p<0.01$) at each of the same test concentrations. Also, ANE's antibacterial effects reached highest when the bacteria were exposed to it for 2 hours at 37°C before being cultured for 24 hours.*

Conclusion: *ANE showed notable in vitro antibacterial activity against three strains of P. aeruginosa, S. aureus and E. coli at various concentrations of 1.0, 0.5, 0.333, 0.25, 0.20, 0.167, 0.143, 0.125, 0.111 and 0.1 g/ml. The best antibacterial effects were achieved when all bacteria were exposed to ANE for 2 hours at 37°C before being cultured.*

Key words: *Areca nut dried extracts, rich in alkaloids, in vitro, antibacterial effects.*

INTRODUCTION

Areca catechu L. is widely grown at altitudes below 700m in a number of Asian and East African countries like India, China, Thailand, Malaysia, Philippines, Vietnam, and Kenya. It is grown for ornamental, medicinal purposes, and cultural uses in some countries.

The most utilised and studied part of the Areca palm is its seeds which contain the main active ingredient arecoline, an alkaloid that functions as an agonist on muscarinic acetylcholine receptors, leading to cholinergic effects in the parasympathetic nervous system [1],[2]. The water and ethanol extracts of the areca nut had antibacterial [3],[4], anti-inflammatory, antioxidant [5], and antiparasitic effects [1],[2],[6]. In addition, ethanol extract and dichloromethane fraction of Areca nut had antidepressant effects in rats [1].

With remarkable biological effects and being widely grown in Vietnam, the development of medicinal preparations from Areca nut is of great scientific and practical significance. Therefore, the Department of Pharmaceutical Chemistry (Vietnam University of Traditional Medicine - VUTM) has prepared a dried extract rich in alkaloids from Areca nut with 60° ethanol as solvent and some other excipients.

Up to now, there has been no study to evaluate this preparation's antibacterial effect. Thus, we conducted an *in vitro* evaluation of its antibacterial effect on some identified bacterial strains to contribute to the development of a dosage form from Areca nut extract.

MATERIALS AND METHODS

Sample: Areca nuts extract (ANE) rich in alkaloids extracted with 60° ethanol was prepared by the Department of Pharmaceutical Chemistry, VUTM and had met its basic standards. Each 64.48 g of Areca nut extract was extracted from a mixture of 100 g of Areca nuts, 1,800 ml of ethanol 60°, 180 ml of 2% HCl solution, 180 ml of 10% $NaHCO_3$ solution and 5 g of lactose. In which, arecoline content must reach > 0.3% of the dried extract weight [7].

Bacterial strains: Three strains of bacteria consisting of *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 11775 were provided by Institute of Biotechnology, Vietnam National University.

Appliances: Equipment and tools used in the study include a Sartorius BP121S analytical balance, a Hirayama HV-110 sterilizer, a Froilabo BC 120 incubator, a Clean Bench microbiological incubator, a refrigerator, a drying oven, McFarland tubes, alcohol lamps, Petri dishes, glass rods, a thermometer, micro pipettes, and Panme calipers with an accuracy of 0.1 mm.

Chemicals: Double distilled water. Mueller Hinton and dry powder nutrient agar media were all pure and manufactured by Himedia (India).

Time and location: This study was conducted between November 2022 and April 2023 at VUTM.

Corresponding author: Nguyen Thi Minh Thu
Phone: (+ 84) 912750167
E-mail: minhthunimpe@gmail.com

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Method

This experiment was carried out by the agar well diffusion method [8],[9].

Microbial culture: Nutrient agar medium was prepared according to routine techniques. Then, all three bacterial strains were transferred separately to Petri dishes containing this nutrient agar medium and stored in a refrigerator at 2-8°C.

Antibacterial effect evaluation of dried Areca nut extract

First, ANE was mixed with distilled water to form a solution with a concentration of 1 g/ml, which was then further diluted with distilled water to form test samples with concentrations of 0.5, 0.333, 0.25, 0.20, 0.167, 0.143, 0.125, 0.111 and 0.1 g/ml.

Next, three standard bacterial strains, obtained from colonies grown in nutrient agar, were mixed with about 2 ml of sterile distilled water to form 3 distinguished suspensions until reaching a turbidity equivalent to the standard turbidity of 0.5 McFarland (10^8 bacteria/ml). These bacteria were transferred to agar petri dishes containing Mueller Hinton medium.

Then, 100 µl of each bacterial suspension was pulled out with a micropipette and poured onto the surface of Mueller-Hinton agar, so that the bacteria were spread evenly on the medium plate. After that, small and high holes on the surface of the agar medium were punched and left at room temperature (25°C) for 30 minutes, then incubated at 37°C. After 24 hours of incubation, the bacterial inhibition zones were measured with Panme calipers with an accuracy of 0.1 mm. The average values of the diameters of the bacterial inhibition zones between 3 tests were considered as the test values to be determined.

Some factors affecting antibacterial effects of Areca nut extract

Exposure time between the test sample and bacterial strains before culturing:

The test samples with 3 concentrations of 0.333, 0.25 and 0.2 g/ml were conducted as above steps, but the exposure time to bacteria were 0.5, 1, 2 and 4 hours before culturing at 37°C for 24 hours. The optimal exposure time was then selected.

Differentiated temperature conditions before culturing:

The test samples with three concentrations of 0.333, 0.25 and 0.2 g/ml were processed as above and exposed to the bacterial strains for the selected optimal time with differentiated temperature conditions of 15, 20, 25, 30 and 35°C before incubation. From this, the optimal exposure temperature was found.

Evaluation criteria

Study indices comprise diameter of antibacterial zones (mm), optimal exposure time between bacterial strains and test sample (hours), optimal exposure temperature (°C) and the best antibacterial concentrations (g/ml).

Data processing

Research data was expressed as $\overline{X} \pm SD$. Data was processed according to medical statistics methods using SPSS 22.0 software. In which, Student T-test was used to compare groups' data. Differences were considered statistically significant with $p < 0.05$.

Research ethics

The study complied with ethical regulations in biomedical research. Bacteria and the test sample were handled properly after the end of the experiment.

RESULTS

The antibacterial properties of ANE are shown in table 1.

Table 1. Antibacterial activities of ANE against 3 tested bacterial strains

Serial No.	Concentrations of ANE (g/ml)	Diffusion diameters of antibacterial zones (mm)		
		Pseudomonas aeruginosa (1)	Staphylococcus aureus (2)	Escherichia coli (3)
1	1,0	17.10 ± 0.707	26.67 ± 0.661	25.00 ± 0.90
2	0,5	17.05 ± 0.289	26.50 ± 0.541	24.75 ± 1.04
3	0.333	16.95 ± 0.2889	26.33 ± 0.567	24.67 ± 1.145
4	0.25	16.67 ± 0.381	26.10 ± 0.332	24.50 ± 1.19
5	0.20	16.20 ± 0.50	25.95 ± 0.457	23.33 ± 0.633
6	0.167	15.32 ± 0.661	25.00 ± 0.577	22.70 ± 0.377
7	0.143	14.90 ± 0.144	24.01 ± 0.50	22.10 ± 0.661
8	0.125	14.71 ± 0.382	23.35 ± 0.52	20.67 ± 0.643
9	0.111	13.65 ± 2.81	22.65 ± 0.49	18.33 ± 1.792
10	0.10	7.90 ± 0.64	15.80 ± 1.33	12.20 ± 1.45

(p_{1-2}, $p_{1-3} < 0.01$, $p_{2-3} < 0.05$ (The values assessing the differences between groups 1 and 2, 1 and 3, and 2 and 3 are shown in the table above, respectively))

ANE, at all tested concentrations, had good antibacterial effects against all three strains and its antibacterial levels decreased in order with *S. aureus, E. coli* and *P. aeruginosa*. At each concentration, antibacterial zones' diameters of different bacterial strains were statistically significant (p values < 0.05 and < 0.01). The antibacterial ability of the test sample changed linearly with its concentrations, meaning that the higher the concentration of the test sample, the higher the antibacterial ability and vice versa.

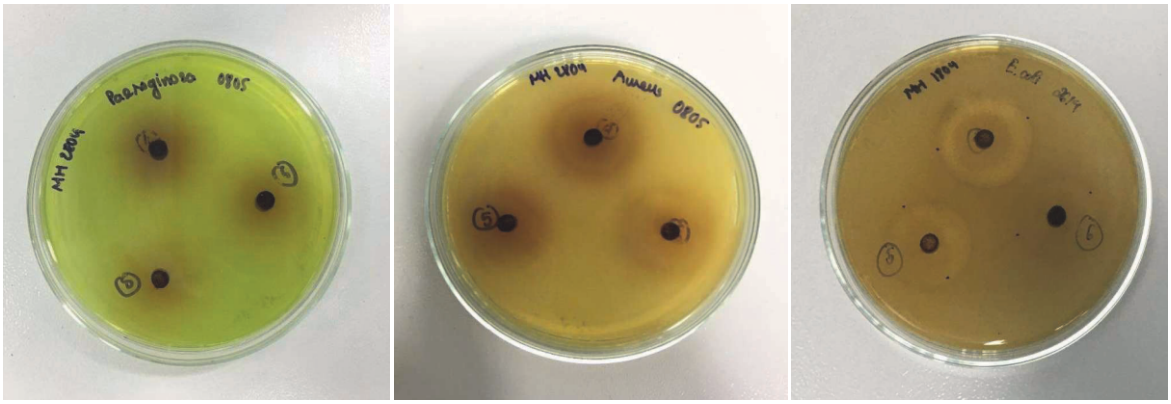

Figure 1. Antibacterial activities of ANE against P. aeruginosa, S. aureus and E. coli (from left to right) at distinguished concentrations of 0.25, 0.20 and 0.167 g/ml

We selected three concentrations of ANE, 0.333, 0.25 and 0.20 g/ml, to evaluate some factors affecting its antibacterial ability. The results are shown in Tables 2 and 3.

Table 2. Effects of bacterial exposure time to the sample before being cultured

Serial No.	Exposure time (hour)	Diameters of the antibacterial zones (mm) at different concentrations		
		0.333 g/ml	0.25 g/ml	0.20 g/ml
1	*P. aeruginosa*			
	0	8.5 ± 1.414	9.4 ± 2.142	5.2 ± 2.151
	0.5	12.3 ± 1.932	11.75 ± 3.666	9.6 ± 2.631
	1.0	16.95 ± 2.505	16.67 ± 7.258	16.20 ± 6.584
	2.0	17.2 ± 2.512	17.45 ± 4.283	16.5 ± 6.185
	4.0	17.05 ± 1.944	17.1 ± 4.328	16.1 ± 5.732
2	*S. aureus*			
	0	23.5 ± 0.361	23.1 ± 0.265	22.8 ± 0.404
	0.5	24.1 ± 0.153	24 ± 0.306	23.67 ± 0.324
	1.0	26.3 ± 0.264	26.1 ± 0.251	25.95 ± 0.208
	2.0	26.5 ± 0.208	26.3 ± 0.378	26.15 ± 0.201
	4.0	26.51 ± 0.379	26.25 ± 0.776	26.02 ± 0.703
3	*E. coli*			
	0	20.33 ± 1.256	19.5 ± 1.457	19.1 ± 0.152
	0.5	22.5 ± 0.613	22.1 ± 1.258	21.95 ± 0.305
	1.0	24.67 ± 0.502	24.5 ± 0.173	24.33 ± 0.405
	2.0	25.1 ± 1.263	24.7 ± 1.452	24.4 ± 0.472
	4.0	25.05 ± 1.436	24.67 ± 0.173	24.2 ± 0.205

($p_{2\text{-}1,\ 4hrs} > 0.05$, $p_{2\text{-}0,\ 0.5hrs} < 0.05$)

Table 2 reveals that ANE at all three concentrations of 0.333, 0.25 and 0.2 g/ml showed the best antibacterial effect after 2 hours of exposures to three tested bacterial strains with remarkably higher antibacterial zone diameters compared to those with exposures for only 0.5 and 0 hours ($p<0.05$) but insignificantly different from those with exposures for 1 or 4 hours ($p>0.05$).

Table 3. Effects of temperature when exposing bacteria to the sample on its antibacterial effects

Serial No.	Temperature conditions (°C)	Diameters of the antibacterial zones (mm) at different concentrations		
		0.333 g/ml	0.25 g/ml	0.20 g/ml
1	*P. aeruginosa*			
	15°C	15.67 ± 0.265	15.2 ± 0.125	14.3 ± 0.472
	20°C	16.2 ± 0.324	15.8 ± 0.404	15.5 ± 0.569
	25°C	16.7 ± 0.416	16.45 ± 1.056	16.1 ± 0.258
	30°C	16.95 ± 1.257	16.5 ± 1.235	16.33 ± 1.235
	37°C	17.1 ± 1.569	16.9 ± 1.113	16.8 ± 1.586
2	*S. aureus*			
	15°C	25.6 ± 0.568	25.33 ± 1.263	24.8 ± 0.823
	20°C	25.95 ± 0.982	25.7 ± 0.986	25.2 ± 0.765
	25°C	26.1 ± 1.258	25.8 ± 0.857	25.5 ± 0.586
	30°C	26.5 ± 1.125	26.15 ± 0.756	25.6 ± 1.123
	37°C	26.67 ± 1.569	26.5 ± 0. 306	26.2 ± 1.458
3	*E. coli*			
	15°C	23.67 ± 0.125	23.5 ± 1.415	23.33 ± 1.458
	20°C	24.1 ± 0.358	23.8 ± 1.256	23.65 ± 1.695
	25°C	24.5 ± 0.758	24.2 ± 1.258	23.95 ± 1.269
	30°C	24.67 ± 0.985	24.33 ± 1.352	24.15 ± 1.478
	37°C	24.90 ± 1.225	24.5 ± 1.258	24.20 ± 1.985

($p_{37\text{-}30,\,25,\,20^\circ C} > 0.05$, $p_{37\text{-}15^\circ C} < 0.05$ (The values show the differences in antibacterial levels at 37°C compared to 30, 25, 20 and 15°C))

ANE at all three concentrations of 0.333, 0.25 and 0.20 g/ml exhibited the best activity against *P. aeruginosa, S. aureus* and *E. coli* when exposed to these bacteria for 2 hours at 37°C before culturing for 24hrs likened to other conditions. This was demonstrated by the highest inhibition zone diameters at 37°C among the tested temperature conditions, which were not significantly different from those at 30, 25, and 20°C ($p > 0.05$) but were notably higher than that at 15°C ($p<0.05$).

DISCUSSION

In this study, the antibacterial effects of ANE were evaluated by the agar well diffusion method [8],[9], which is a popular method recently with its advantages of simplicity, low cost, and rapid results. The antibacterial ability of the test samples was exhibited through the diameters of the bacterial inhibition rings. The results from tables 1 - 3 reveal that ANE at concentrations of 1.0, 0.5, 0.333, 0.25, 0.20, 0.167, 0.143, 0.125, 0.111 and 0.1 g/ml all have good antibacterial effects against 3 species of *Pseudomonas aeruginosa* (aerobic Gram-negative bacilli), *Escherichia coli* (rod-shaped Gram-negative bacteria) and *Staphylococcus aureus* (gram-positive cocci). The diameters of their antibacterial rings were largest at the concentration of 1.0 g/ml, respectively 17.10 ± 0.707, 25.00 ± 0.90 and 26.67 ± 0.661 mm. The diameter values decreased linearly with the concentration of the test sample and were lowest at the concentration of 0.1 g/ml with values of 7.90 ± 0.64, 12.20 ± 1.45 and 15.80 ± 11.33 mm in order.

Furthermore, ANE had the best effect on killing *S. aureus*, lower on *E. coli* and lowest on *P. aeruginosa*. These were shown by the diameters of the inhibition zones with *S. aureus* being statistically higher than those with *E. coli* and *P. aeruginosa*, p values < 0.05 and < 0.01 respectively. The diameters of the inhibition zones of ANE with *S. aureus* and *E. coli* were notably higher than those with *P. aeruginosa* at all tested concentrations ($p<0.01$). These prove that ANE has its superb effects on killing both gram-negative and gram-positive bacteria, especially *P. aeruginosa*, a species of bacteria that is quite arduous to

execute [10]. The novelty in our study is that we used ANE in finished form with its ingredients as described in the Sample section.

Apart from that, we evaluated some factors affecting ANE's antibacterial ability including exposure time and temperature to bacteria before culturing at 37°C for 24 hours. We discovered that the exposure time periods of ANE to all 3 bacterial species were best within 2 hours, which were not significantly different from those within 1 and 4 hours ($p>0.05$) but remarkably better than those of 0 and 0.5 hours ($p<0.05$). Similarly, the exposure temperature conditions of all 3 species to ANE were best at 37°C which were not notably different from that at 30, 25 and 20°C ($p>0.05$) but significantly better than that at 15°C ($p<0.05$). These results demonstrate that, before culturing for 24 hours, all three strains need to be exposed to the test sample for 2 hours at 37°C to achieve optimal antibacterial effects.

Our results above are also consistent with some previous studies. Jam et al. also used a similar method to evaluate the effect of ANE using ethanol, methanol and water against strains of *Staphylococcus aureus, Escherichia coli, Salmonella enterica*, and *Enterobacter aerogenes* bacteria. The authors found that all extracts had good antibacterial effects, in which the ethanol extract showed better anti-*E. coli* effect than that of methanol extract at a concentration of 100 mg/ml [3]. M.A. Rahman et al., when studying ANE with n-hexane, ethanol and water, realized that the ethanol extract had good anti-*S. aureus* effect with antibacterial zone diameters from 11-14 mm. These values are slightly lower than our results due to the lower test concentrations of Rahman. Additionally, the ethanol extract of ANE gave an MIC of 0.188 mg/ml against *S. aureus* [4].

In the present study, we did not focus on determining the MIC or MBC values of ANE, but mainly evaluated experimental conditions such as temperature and exposure time to the test sample before culturing to see how they affected the antibacterial activity of ANE. Further studies to determine these values and compare them with standard concentrations of reference antibiotics are essential to provide more information on the antibacterial activity of ANE.

CONCLUSION

ANE demonstrated its impactful *in-vitro* antibacterial activity against three bacterial strains comprising *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 11775 and *Staphylococcus aureus* ATCC 25923 at concentrations of 1.0, 0.5, 0.333, 0.25, 0.20, 0.167, 0.143, 0.125, 0.111 and 0.1 g/ml. With the same tested concentrations, ANE had its highest antibacterial effect against *S. aureus*, lower against *E. coli* ($p<0.05$) and lowest against *P. aeruginosa* ($p<0.01$). In addition, the antibacterial effects of ANE reached highest when the bacteria were exposed to it for 2 hours at 37°C before being cultured for 24 hours.

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