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Study on the Areca Nut for its Antimicrobial Properties

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ABSTRACT

This study evaluates the antibacterial, antifungal, and antiviral properties of the areca nut *in vitro* using isolated organisms. A variety of human and veterinary isolates, both gram positive and gram negative, were tested against areca nut extract by measuring the growth of the organisms using the spectrophotometric method. It was found that both gram negative and gram positive organisms were susceptible to the areca nut extract. The concentration needed for 100% inhibition of growth was found to be 3.3-7 µg/ml for gram negative organisms and 16 µg/ml for gram positive bacteria. The extract was also found to inhibit the growth of *Candida albicans* at a concentration of 16 µg/ml and inhibited aflatoxin production by *Aspergillus flavus*. The extract was also found to inhibit the viral growth of the New Castle Disease Virus (NDV) and Egg Drop Syndrome Virus (EDS) grown in embryo cultures. These results indicate that betel nut chewing may have significant disinfective properties.

Key words: Areca nut, antibacterial activity, antifungal, antiviral activity

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INTRODUCTION

Chewing the areca nut with betel leaves (betel quid) is a traditional habit in India and in many Asian countries. Betel quid chewing has been reported to improve oral hygiene[1] and motility of food and thereby reduce the absorption of food and thus arrest weight gain. It has also been shown to be anti-diabetic[2] and to reduce cholesterol[3] levels. Areca nut is a mild psychoactive material[4] and has been reported to be an aphrodisiac.

Indians have less dental caries when compared with the Western population.[4] In fact, areca nut has been known to reduce dental caries by possibly inhibiting gram positive microorganisms that are responsible for dental caries.[5] Areca nut has also shown significant antiviral activity against the human immunodeficiency virus (HIV).[6] One of the ingredients in areca nut, catechin, is believed to be a chemo preventive agent against several tumors in animals.[7]

Although there are several reports on the adverse effects of areca nut,[8] no systematic research has been conducted to determine its medicinal properties. In this report, we have studied the anti-bacterial, antiviral, and antifungal activities of areca nut extract in isolated micro-organisms. The main constituents of areca nut are polyphenols, fat polysaccharides, fiber, and protein. Besides these, the nuts contain alkaloids arecoline (0.1-0.7%) and others in trace amounts such as arecadine, guvacoline, and guvacine.

MATERIALS AND METHODS

Bacterial cells: both gram positive and gram negative bacteria were used for this study. Gram positive bacterial cultures are:

Streptococcus mutans was procured from Microbial Type Culture Collection Centre, IMTECH, Chandigarh. *Streptococcus viridans* was procured from CMC, Vellore.

Gram negative bacterial cells *Pseudomonas*, *Klebsiella*, *Proteus*, *E. coli* which were human isolates were obtained from the Department of Microbiology, Amala Cancer Hospital.Veterinary isolates *E. coli, Klebsiella pneumoniae* and *Salmonella typhimurium* were obtained from Veterinary College, Mannuthy.

Fungi: *Candida albicans* was procured from CMC, Vellore. Other fungi used were *Mucor sp, Aspergillus niger,* and *Cladosporium*. These were purchased from IMTECH, Chandigarh. *Aspergillus flavus* was also obtained from IMTECH, Chandigarh.

Viruses: The New Castle Disease Virus (Ranikhet Disease Strain, R2B) was initially derived from a live vaccine of Indovax Pvt. Ltd., Haryana. The Egg Drop Syndrome Virus was procured from the Department of Microbiology, College of Veterinary and Animal Sciences, Mannuthy. These viruses were maintained in the harvested allantoic fluid of chick embryonated and duck embryonated eggs in cold storage at -70ºC.

Embryonated Eggs: On their 7th day of embryonation, chick embryonated eggs and duck embryonated eggs were bought from the Department of Poultry, Veterinary College, Mannuthy Thrissur. The eggs were maintained in an incubator at 37ºC and at 61.2% relative humidity.

All the bacterial cultures were maintained in our laboratory on Nutrient Agar. *Streptococcus viridans* was maintained on blood agar. *Streptococcus mutans* was maintained on brain heart infusion agar. All fungal cultures were maintained on Sabouraud's Dextrose.

Chemicals: Nutrient agar was purchased from HI-MEDIA and brain heart infusion broth was purchased from SRL.

Extraction of areca nut

A hot water extract of the areca nut was prepared by boiling the nuts (100 g) in 500 ml of distilled water for 1 hour. This extract was then concentrated by evaporation. The yield of the extract was found to be 6.4%. The extract was then re-suspended in distilled water and diluted to the desired concentration.

Determination of antibacterial activity

A loopful of bacterial strains were inoculated into a nutrient broth (100 ml) and kept overnight at room temperature for incubation. About 0.1 ml of this overnight culture was incubated in the presence and absence of escalating concentrations of the extract (10-100 µg) for 8 hours in 3 ml of nutrient broth. The absorbance was read at 530 nm immediately after inoculation of bacterial cells and continued for 8 hours at intervals of 1 hour. The experiment was set up in triplicates and the average value was taken. Inhibition of bacterial growth was calculated by comparing the optical densities of the control and experimental tubes [Table 1].

Determination of antifungal activity of extract

Tube method: A loopful of unicellular fungi was inoculated into the Sabouraud's dextrose broth (100 ml) and kept overnight at room temperature for incubation. About 0.1 ml of this overnight culture was incubated in the presence and absence of escalating concentrations of the extract (10-100 µg) for 8 hours in 3 ml of nutrient broth. The absorbance was read at 530 nm, immediately after inoculation of the unicellular fungi, and continued for 8 hours at intervals of 1 hour. The experiment was set up in triplicates [Table 2].

Disk diffusion (Kirby Bauer) method

Sabouraud dextrose broth cultures were prepared in test tubes. Cultures maintained for 72 hours were taken. The surface of the Sabourauds Dextrose agar plates was scrubbed with sterile cotton swabs to prepare lawn cultures. This was done 5 minutes after the agar surface had dried. Wells were dug using cork borer aseptically. These wells were saturated with areca nut extract with

Table 1: Anti-bacterial activity of areca nut extract

Strains	Percent inhibition			
	10 µg	20 µg	50 µg	100 µg
Gram negative				
Veterinary isolates				
E coli	0	50	100	100
Klebsiella pneumoniae	80	100	100	100
Salmonella typhimurium	57	100	100	100
Human isolates				
Pseudomonas	36	100	100	100
Klebsiella	100	100	100	100
Proteus	100	100	100	100
E coli	0	0	100	100
Gram positive				
Human isolates				
Streptococcus mutans MTCC 497	0	74	100	100
Streptococcus viridans (1603)	11	11	100	100

Concentration is expressed per 3 ml of medium.

Table 2: The effect of areca nut extract on *Candida albicans*

Concentration + Strain	Percent inhibition					
	1 h	2h	3h	4h	5h	6h
10 µg + *C.albicans*	0	0	0	0	0	0
20 µg + *C.albicans*	0	0	0	0	0	0
50 µg + *C.albicans*	100	100	100	100	100	100
100 µg + *C.albicans*	100	100	100	100	100	100

Concentration is expressed per 3 ml of medium

concentrations ranging from 100 μg to 1000 μg. The plates were incubated at 37ºC for 24-48 hours. The diameter of zones of the inhibition was measured using a scale to the nearest millimeter.[9]

Determination of the effect of food additives on Aflatoxin production by *Aspergillus flavus*

A loopful of *A flavus* culture, which was originally grown on Sabouraud Dextrose Agar, was uniformly suspended in 5 ml of glucose ammonium nitrate medium containing the mineral supplements that had been incubated at 37 ºC for 6 days. About 100 μl of this suspension was inoculated into test tubes containing 2 ml of glucose ammonium nitrate medium with different concentrations of areca nut extract ranging from 10–250 μg/ml. All the tubes were kept in triplicates incubated at 30ºC for 6 days. Aflatoxin was extracted using the modified Pon's method.[10] The toxin concentration was estimated in correlation with the intensity of fluorescence of standard aflatoxin on thin layer chromatography (TLC) plates.[11]

Determination of antiviral activity of areca nut extract inoculated chick or duck egg

The New Castle Disease Virus, a RNA virus, belongs to the genus Rubulavirus of the family Paramyxoviridae.[12] The Egg Drop Syndrome virus or Duck Adenovirus I is a non-enveloped haemagglutinating DNA virus.[13] Both the viruses showed properties of haemagglutination of red blood cells (RBCs). Six-day old chick or duck embryonated eggs were swabbed clean with alcohol and kept at 37.1ºC at 61.2% relative humidity before the experiment. Fertile embryonated eggs were divided into 8 sets (5 eggs/group).

Group I: Normal; Group II: 0.1 ml NDV alone; Group III: 0.1 ml NDV + 5 μg Extract/Egg; Group IV: 0.1 ml NDV + 20 μg Extract/Egg; Group V: 0.1 ml NDV + 50 μg Extract/Egg; Group VI: 0.1 ml NDV + 100 μg Extract/Egg; Group VII: 0.1 ml NDV + 200 μg Extract/Egg

Group I: Normal; Group II: 0.1 ml EDS alone; Group III: 0.1 ml EDS + 5 μg Extract/Egg; Group IV: 0.1 ml EDS + 20 μg Extract/Egg; Group V: 0.1 ml EDS + 50 μg Extract/Egg; Group VI: 0.1 ml EDS + 100 μg Extract/Egg; Group VII: 0.1 ml EDS + 200 μg Extract/Egg

Areca nut extract was inoculated with a 22 ½ gauge needle syringe into the allantoic cavity. Every day the eggs were candled and the development of the embryo was noted. Fertile eggs that lacked embryonic development and eggs containing dead embryos were checked. After 5 days of virus and areca nut extract inoculation, all the embryos were killed. Chilled, harvested, and haemagglutination titre was assayed as given below. The growth and morphologies of the embryo and of the Chorio Allantoic Membrane were noted.

Determination of Haemagglutination titre in embryonated eggs[14]

Harvested allantoic fluid (0.1 ml) was serially diluted in a round bottom 96-welled plate containing 0.1 ml phosphate buffered saline (PBS)/well (pH 7.2). Approximately 0.1 ml of packed chicken/duck RBC was added to each well, mixed gently, and allowed to stand at room temperature for 20 minutes. The dilution of clear agglutination was observed, noted, and tabulated Table 3. Control wells contained no test substance, but had only 0.1 ml of PBS and 0.1 ml of chicken RBC.

Antifungal activity of areca nut extract

The tube method was used against unicellular yeast like fungus *Candida albicans*. The plate method was employed against mycelial forms *Mucor* sp, *Aspergillus niger, Cladospr ium* sp and the unicellular budding fungus *Candida albicans*. Areca nut extract did not inhibit the growth of mycelial fungal forms such as *Mucor, Aspergillus niger,* and *Cladosporium*. However, it inhibited the growth of unicellular fungus *Candida albicans* and the concentration needed for 100% inhibition was found to be 16.67 μg/ml.

Inhibition of aflatoxin production by areca nut extract

Areca nut extract was also tested for its inhibitory activity against aflatoxin production of *Aspergillus flavus*. Areca nut extract was shown to inhibit aflatoxin production by *Aspergillus flavus*. The most effective concentration of areca nut extract for 85% inhibition of the production of aflatoxin by *Aspergillus flavus* is between 100-250 μg/ml.

Table 3: The antiviral activity of areca nut extract

Treatment	Haemagglutination titre
NDV alone	2048
NDV + 1 μg of the extract in 0.1 ml	2048
NDV + 2 μg of the extract in 0.1 ml	2048
NDV + 5 μg of the extract in 0.1 ml	256
NDV + 10 μg of the extract in 0.1 ml	32
NDV + 20 μg of the extract in 0.1 ml	32
EDS alone	1024
EDS + 1 μg of the extract in 0.1 ml	1024
EDS + 2 μg of the extract in 0.1 ml	1024
EDS + 5 μg of the extract in 0.1 ml	1024
EDS + 10 μg of the extract in 0.1 ml	512
EDS+ 20 μg of the extract in 0.1 ml	256

Concentration of the extract is expressed per egg. NDV=New Castle Disease virus; EDS= Egg Drop Syndrome virus.

Antiviral activity of the areca nut against NDV in chicken embryonated eggs and EDS in duck embryonated eggs were also studied. The areca nut extract showed significant antiviral activity against NDV and EDS viruses. In both virus-alone treated groups, the virus significantly inhibited the growth of the embryos. The haemagglutination assay measure of viral propagation was done against chicken RBCs for NDV and duck RBCs for EDS. In the case of NDV, the virus-alone treated group had a titre of 2048. The administration of areca nut extract at a concentration of 20 μg/egg reduced the titre to 32. The group treated with the EDS virus alone had a titre of 1024. The areca nut extract administered at a concentration of 20 μg/egg reduced the titre to 256. The results of haemagglutination titre are listed in Table 3.

DISCUSSION

Our investigations for this study were aimed at assessing the effects of areca nut extract against a battery of gram positive human isolates and gram negative veterinary and human isolates. The aqueous extract of *Areca catechu* L. was found to be effective for inhibiting the growth and propagation of several bacteria. It was found to inhibit the growth and propagation of *Streptococcus mutans*, the bacteria that causes dental caries.

The envisaged use of areca nut extract, as an antiviral agent against avian viruses such as New Castle Disease Virus and Egg Drop Syndrome Virus is also a subject of interest. Determination of haemagglutination titre is the index of the amount of virus present in the allantoic cavity at the end of 5 days. The results indicate the concentration dependent reduction of virus in the allantoic fluid. Further investigations should be carried out to support the application of the aqueous extract of Areca catechu L. as a potent antiviral agent. In fact, areca nut extract has been shown to inhibit replication of HIV.[15]

Antifungal studies showed that areca nut extract is an effective inhibitor of the unicellular fungi. The use of areca nut extract in a dose-dependent manner to prevent the aflatoxin production by *Aspergillus flavus* can be of industrial importance, especially in the chicken feed industry, where afatoxin has been a major poisoning agent, due to the multiplication of the fungal contaminant *Aspergillus flavus*.

Betel nut chewing has been implicated as a cause of cancer.[16] However, the chewing of tobacco along with betel nut may be the major causative agent of oral cancer. Areca nut has also been implicated as a cause of submucous fibrosis, which is probably due to decreased collagen metabolism. Results indicated in the manuscript should be taken into perspective on the possible use of betel nut chewing in oral hygiene and possible inhibition of dental caries and oral candidiasis.

REFERENCES

1. Ling LJ, Hung SL, Tseng SC, Chen YT, Chi LY, *et al*. Association between betel quid chewing, periodontal status and periodontal pathogens. Oral Microbiol Immunol 2001;16:364-9.
2. Chempakam B. Hypoglycaemic activity of arecoline in betel nut Areca catechu L. Indian J Exp Biol 1993;31:474-5.
3. Park YB, Jeon SM, Byun SJ, Kim MS, Choi MS. Absorption of intestinal free cholesterol is lowered by supplementation of *Areca catechu* L. extracts in rats. Life Sci 2002;70:1849-59.
4. Gupta PC, Ray CS. Epidemiology of Betel Quid Usage. Ann Acad Med Singapore 2004;33:31-6.
5. Hung SL, Lin YW, Wang YH, Chen YT, Su CY, Ling LJ. Permeability of *Streptococcus mutans* and *Actinobacillus actinomycetemcomitans* through guided tissue regeneration membranes and their effects on attachment of peridontal ligament cells. J Peridontal 2002;73:69-76.
6. Kusumoto IT, Nakabayashi T, Kida M, Miyashiro H, Hatlori M, Namba T, Shinotohnok. Screening of various plant extract used in Ayurvedic medicine for inhibitory effects on Human Immunodeficiency Virus type-1(HIV) protease. Phytotherapy Res 1995;2:180-4.
7. Jeng JH, Wang YJ, Chiang BL, Leo PH, Chan CP, Ho YS, *et al*. Roles of Keratinocyte inflammation in oral cancer regulating the prostaglandin E2, interleukin-6 and TNF-α production of oral epithelial cells by areca nut extract and arecoline. Carcinogenesis 2003;24:1301-15.
8. Van Wyk, Stander CW, Padayachee A, Grobler Rabie AF. Oral Squamous Carcinoma in South African Indians and areca nut chewing. J Immigr Refug Stud 1980;12-5.
9. Prescott LM, Harley JP, Klein DA. Antimicrobial Chemotherapy. Microbiology 1993;2:328-9.
10. Pons WA, Cuculia AF, Lee LS, Robertson JA, Franz SO, Goldblatt LA. Determination of aflatoxin in products. Use of aqueous acetone for extraction. J Assoc Agri Chem 1966;67:554.
11. Rati ER, Prema V, Shantha TJ. J Food Sci Technol 1987;24:90.
12. Rima BK, Alexander DJ, Billeter MA, Collins PL, Kingsbury DW, Lipkind MA, Nagai YO, Rvell C, Pringle CR, Meulen TV. Paramyxoviridae. In: Murphy FA, Fauquet CM, Bishop DH, Ghabrial SA, Jarvis AW, Martelli GP, Mayo MA, Summers MD, editors. Virus Taxonomy. 6thReport of the International Committee on Taxonomy of Viruses. Vienna and New York: Springer-Verlag; 1995. p. 268-74.
13. Benko M, Marrach B, Russell WC. Family Adenoviridae. In: Van Regenmortel MH, *et al*. editor. Virus Taxonomy, Classification and nomenclature of viruses. Seventh Report of the International Committee on Taxonomy of Viruses San Diego. CA: Academic Press; 2000. p. 227-38.
14. Vijayan P, Raghu C, Ashok G, Dhanaraj SA, Suresh B. Antiviral activity of plants of Nilgiris. Ind J Med Res 2004;120:24-9.
15. Kusumoto IT, Nakabayashi T, Kida H, Miyashiro H, Hattori M, Nasmba T, Shimotohnok. Screening of various plant extract used in Ayurvedic medicine for inhibitory effects on human immunodeficiency virus type -1 (HIV-1) protease. Phytotherapy Res 1995;3:180-4.
16. Chang MC, Ho YS, Lee PH, Chan CP, Lee JJ, Hahn LJ, Wang YJ, Jeng JH. Areca nut extract and Arecoline induced the cell cycle arrest but not apotosis of cultured oral KB epithelial cells: Association of glutathione, reactive oxygen species and mitochondrial membrane potential. Carcinogenesis 2001;1527-35.

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