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Original Article

Antibacterial activity of ethanol extracts of betel leaf (*Piper betle* L.) and areca (*Areca catechu* L.) nuts against food borne and oral pathogens

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Antimicrobial activity of ethanol extracts of betel leaf (*Piper betle* L.) and areca nut (*Areca catechu*) against six food borne enteric pathogens viz. *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (icddr,b), *Salmonella typhimurium* (AIM-40, icddr,b), *Escherichia coli* (ATCC 25922), *Escherichia coli* O157:H7 (ATCC 12079) and *Bacillus cereus* (ATCC 12079), and four oral pathogens such as isolates 1 & 2 of each of the two bacteria viz. *Staphylococcus* sp. and *Pseudomonas* sp. was investigated. Ethanol extract of betel leaf produced highest zone of inhibition (18.0 ± 1.91 mm) against *Staphylococcus epidermidis* (icddr,b) whereas that of areca nut produced highest zone of inhibition (15.0 ± 0.816 mm) against *Staphylococcus aureus* (ATCC 25923). But no inhibition was found against *Salmonella typhimurium* (ATCC AIM-40, icddr,b), *Escherichia coli* (ATCC 25922), and *Escherichia coli* O157:H7 (ATCC 12079) by the Ethanol extract of betel leaf. The MIC and MBC values of both ethanol extracts indicated that Gram positive organisms were more susceptible than Gram negative organisms. Highest antimicrobial activity of ethanol extracts of betel leaf was found against the isolate 1 of *Staphylococcus* sp. (16.5±0.5) and that of areca nut was recorded against the isolate 1 of *Staphylococcus* sp. (12.0±0.5) and the isolate 2 of *Pseudomonas* sp. (12.0±0.5). Present study reveals the potentials of both the extracts to inhibit food borne enteric and oral pathogens that could be used as food preservatives to prevent the food borne illness as well as for maintaining the oral and gut health.

Keywords: Antimicrobial activity, ethanol extract, betel leaf, areca nut, food borne and oral pathogens

Introduction

The practice of chewing betel leaf (*Piper betel*) and areca nut (*Areca catechu*) is mostly popular as a part of culture in Asian countries[1]. Betel leaf is very popular in Asia particularly in Bangladesh, Burma, China, India, Indonesia, Malaysia, Nepal, Pakistan, Philippines, South Africa, Sri Lanka, and Thailand. Asian emigrants live worldwide used to consume betel leaves. Apart from traditional use, these plants also have many medicinal properties and have been used in folk medicine for many centuries[2,3]. These plant extracts have antiseptic, antibacterial, antifungal and antiviral activity[2]. In oral and gastrointestinal diseases, extracts from betel leaf and areca nut have shown to be effective as antibacterials[4,5].

In recent years, multidrug resistance (MDR) has become a great nuisance due to miss use of antibiotics and the situation is alarming in case of food-borne pathogens[6,7]. So, microbiologists have to look for alternative resources to kill MDR strains of different pathogens. Natural plant extracts can be a great source of antimicrobial substances to fight against drug resistant bacteria. Ethanol extracts of betel leaf and areca nuts have antimicrobial activity against food-borne pathogens[8]. Essential oil from betel leaf was found to inactivate *Vibrio cholerae* in ground chicken meat[9] whereas the ethanol extract of areca nuts possesses antibacterial activity against *Helicobacter pylori*[10].

As these plant extracts have shown potential in medicinal aspect in previous studies, the present study was conducted to observe the antibacterial activity of the ethanol extract of the betel leaf and areca nut against some oral as well as enteric food-borne pathogens.

Materials and Methods

Test organisms

Six organisms such as *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (icddr,b), *Salmonella typhimurium (*AIM-40, icddr,b), *E. coli (*ATCC 25922), *E.coli* O157:H7 (ATCC 12079), and *Bacillus cereus* (ATCC 12079) collected from laboratory of Department of Microbiology and four freshly isolated oral bacteria such as Isolate 1 and 2 of each of the two bacteria such as *Staphylococcus* sp. and *Pseudomonas* sp. in the Food Microbiology Laboratory of the Department of Microbiology, University of Dhaka were used as test organisms.

Culture Media used

Nutrient agar medium and nutrient broth medium, Mueller Hinton Broth and Mueller Hinton Agar media and different media for biochemical tests such as Phenol red carbohydrate broth, Nitrate broth, Peptone water, MR-VP broth, Simmons' citrate agar, MIU agar, KIA agar were used in this experiment.

Plant Selection and Collection

Fresh betel leaf and areca nut (Figure 1) were collected from local markets of Dhaka, Bangladesh.

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Figure-1. *Betel leaf and areca nuts*

Extraction of ethanol soluble compound from betel leaf for antibacterial activity

The betel leaves were cut into small pieces; washed in distilled water; and dried at 40ºC for 48 hours in an oven. The dried leaves were grounded using a grinder into fine powder. Twenty grams of betel leaf powder were soaked in 80ml of 95% ethanol in sterilized bottle and kept in fume hood chamber for overnight. The ethanol fraction was separated using sterilized cheesecloth and the solvent was evaporated in oven and extract was concentrated. Then the dried extracts were transferred into small McCartney bottles and vials and stored in a refrigerator at 4°C until use for antibacterial bioassay.

Extraction of ethanol soluble compound from areca nut for antibacterial activity

The collected nuts of *Areca catechu* were washed with drinking tap water and air dried to remove the tap water of the surface of nuts. The washed dried nuts were chopped and were crushed by grinder and 25 grams of crushed nuts were dipped into 75 ml ethanol separately into conical flasks stoppard with rubber corks and left for 1 day in shaking incubator. After 24 hours incubation in a shaking incubator these were filtered off using muslin cloth as filter into sterile petri plates and areca nut extract was refrigerated at 4°C, where the solvent was evaporated and extracts were concentrated. About one week was required to dry the extract[10]. The dried extracts were transferred to small McCartney bottles and vials and stored in a refrigerator at 4°C until use for antibacterial assay.

Preparation of inoculum

The stock cultures were transferred to sterile Nutrient Broth and were incubated overnight at 37°C. The overnight growing cultures were sub cultured by streak plate on Nutrient Agar plate and the plates were incubated for 24 hours at 37°C. Then pure colony from each plate was transferred into Nutrient Broth in tube and incubated at 37°C for about 4 hours. Thus the bacterial inocula were prepared for the antibacterial activity against test organisms.

Preparation of extract solution

Four hundred miligram of each extract was dissolved in 2 ml of ethanol in a vial and shaken well. Thus 200mg/ml extract solution was prepared. All of the extract were used for preparing solution for further study. The concentration of the extracts was 200mg/ml.

Preparation of Paper Discs

Six mm filter paper discs were prepared using the Whatman No 1 filter paper. These were autoclaved in petri plate and dried in oven.

Antibacterial Assay

Antibacterial activities of different samples were individually assayed against the test bacteria. *In vitro* antibacterial assay was carried out by disc diffusion method using sterile cotton bud, suspension of tested bacteria (about 10^8cfu/ml) was spread on Mueller-Hinton Agar plates (Seeded plates)[11]. The discs (6 mm in diameter) were impregnated with 50µl of each ethanol extract of betel leaf solutions which was previously prepared at the concentration of 200mg/ml. Each disc was impregnated with 10,000µg extract. Control discs were impregnated with same amount of ethanol. Then the discs were allowed to air drying inside the laminar air flow for 20 min to remove the ethanol by evaporation and were placed on seeded Mueller–Hinton Agar plates. After placing the discs, the plates were kept at 4°C for about 30 minutes to diffuse the extracts from the discs before starting the active growth of the bacteria which could result in overcome of the bacteria and false negative activity of the extracts. Then the plates were incubated at 37°C for 18 to 24 hours. Antimicrobial activity was evaluated by measuring the zones of inhibition against the tested bacteria. Each assay was carried out in triplicate.

Determination of MIC and MBC

MIC was determined by broth macro dilution method according to Sengul *et al*. (2005). Overnight Mueller-Hinton broth cultures of selected bacteria at 37ºC were prepared[12]. The culture was adjusted to obtain turbidity comparable to that of the turbidity about 0.1 at OD_{600}. The inoculums thus prepared expected to obtain 10^5 to 10^6 cfu/mL. Two fold diluted extract solutions were prepared which ranged from 200mg/mL to 1.56 mg/mL. After incubation at 37ºC for 24 hours, the vials were examined for growth and determination of MIC values of tested extraction, which is bacteriostatic for the test organism. As the extract solution was opaque, further streaking was done from each vial to determine the MIC value. For determination of MBC, the concentration which was bactericidal was then found by subculturing the contents of vials into a series of Mueller-Hinton agar by streaking. Minimum concentration showing no visible growth on agar plate was taken as MBC.

Isolation and identification of oral isolates and antimicrobial activity of ethanol extracts of areca nut and betel leaf against the isolates

Collection of samples

To isolate and presumptive identification of oral bacteria, swabs were taken from oral cavity of volunteers and streaked on Nutrient Agar plates. Pure colonies were isolated by sub culturing from mixed culture. Further biochemical tests were done to

presumptively identify the isolates.

Gram staining for microscopic study

Following isolation, selected isolates was undergone Gram staining for microscopic identification. Gram staining was performed as per procedures described by other workers to determine the size, shape and arrangement of bacteria[13].

Biochemical tests for presumptive identification of the isolates

Subsequently, after Gram staining a series of biochemical tests such as Catalase test, Oxidase test, Citrate test, Kligler Iron Agar test, Methyl Red (MR), Voges Proskauer (VP) test, Urease Test, Indole Test, Nitrate Reduction Test, Lactose/Sucrose/Dextrose Fermentation Test were done described by Quinn (2002) and Bergey's Manual of Determinative Bacteriology,1939[14,15].

Preservation of the isolates

Each pure isolate was cultured in nutrient broth and stored with 20% glycerol into sterile micro centrifuge tube and kept into -20°C. Duplicate sets of micro centrifuge tubes were prepared for each isolate and maintained at -80 °C.

Results

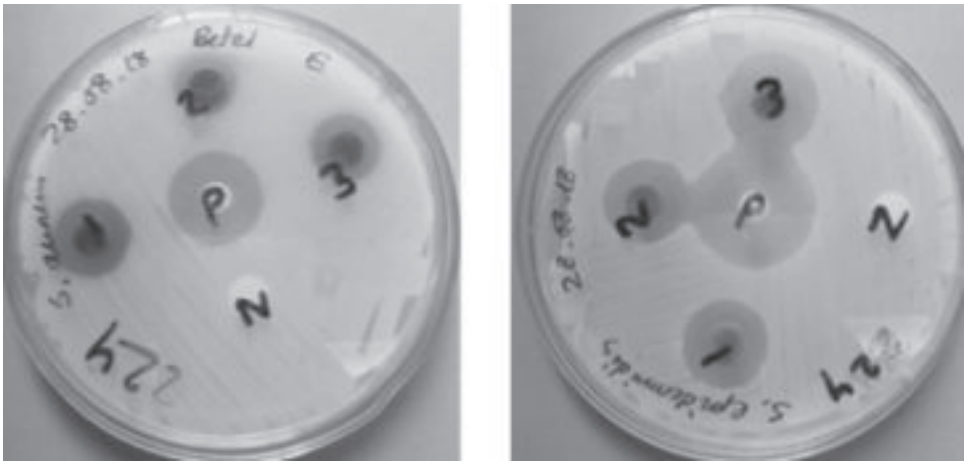
Antibacterial activity of ethanol extracts of betel leaf and areca nut was determined by disc diffusion method against six food borne bacteria such as *S. aureus* (ATCC 25923), *S. epidermidis* (icddr,b), *Salmonella typhimurium (*AIM-40, icddr,b)*, Escherichia coli (*ATCC 25922)*, Escherichia coli* O157:H7 (ATCC 12079)*, and Bacillus cereus* (ATCC 12079) and four oral isolates which were presumptively identified using biochemical tests as *Staphylococcus* sp. and *Pseudomonas* sp. Kanamycin was used as positive control and ethanol was used as negative control. In case of ethanol extract of betel leaf, the highest zone of inhibition was found in *S. epidermidis* (18.0 ± 1.91 mm) and the lowest zone of inhibition was found in *E. coli* O 157:H7 (9.67 ± 0.471 mm). In case of ethanol extract of areca nut, the highest zone of inhibition was found in *S. aureus* (15.0 ± 0.816 mm) and the lowest zone of inhibition was found in *Bacillus cereus* (10.33±0.47 mm). Ethanol extract of areca nut showed no activity against *S. typhimurium*, *E. coli* O157:H7, and *E. coli*. Zones of inhibition of both extracts against food borne pathogens are presented in Table 1. The representative plates for zone of inhibitions of food borne pathogens by ethanol extracts of betel leaf and areca nut were shown in Figure 2.

The highest activity of ethanol extract of betel leaf was found against *Staphylococcus* sp., isolate 1 (16.5±0.5 mm) with the lowest activity against *Staphylococcus* sp., isolate 2. The reason behind the significant different antibacterial activities against the *Staphylococcus* spp. (isolates1 and 2) was unknown. On the other hand, the highest

Table 1. *Screening of antibacterial activity of ethanol extracts of areca nut and betel leaf against some food borne pathogens*

Organisms	Betel leaf (20% ethanol extract)	Areca Nut (20% ethanol extract)	Kanamycin (30 µg/disc)
S. aureus	15.33±0.5	15.0±0.816	20.0±0.0
S. epidermidis	18.0±1.91	13.67±0.5	25.0±0.0
B. cereus	13.67±0.46	10.33±0.47	22.0±1.0
S. typhimurium	11.67±0.47	-	17.5±2.5
E. coli O157:H7	9.67±0.471	-	16±0.0
E. coli	11.67±0.48	-	14.0±0.0

Mean diameter of zones of inhibition in mm including the diameter of the disc 6 mm. Mean ± SD, (n=3)



(a) (b)

Fig. 2. *Ethanol extract of (a) betel leaf against Staphylococcus aureus and (b) areca nut against Staphylococcus epidermidis. (1, 2, 3 numbers represent ethanol extract impregnated discs, P, positive control and N, negative control).*

activity of ethanol extract of areca nut was found against oral strains of *Staphylococcus* sp., isolate 1(12.0±0.5), and *Pseudomonas* sp., isolate 2 (12.0±0.5) with the lowest activity against *Staphylococcus* sp., isolate 2 (8.6±0.75). Zones of inhibition of both extracts against oral pathogens are presented in Tables 2.

The representative plates for zone of inhibitions of oral pathogens by ethanol extracts of betel leaf and areca nut were shown in Figure 3.

Determination of MIC and MBC

The MIC and MBC values of ethanol extracts of betel leaf and areca nut were determined. The MIC & MBC values are listed in Table 3. The highest MIC value (0.625 %) of ethanol extract of betel leaf with the highest MBC value (1.25) was found against *S. aureus* and *S. epidermidis*. On the other hand, the highest MIC value (0.625 %) of ethanol extract of areca nut with the highest MBC value (1.25) was found against *S. aureus, S. epidermidis and Bacillus cereus*. In case of oral isolates the highest MIC value (0.625%) of ethanol extracts of betel leaf with the highest MBC value (1.25%) was found against *Staphylococcus* sp. isolate 1, and *Pseudomonas* spp., isolates 1 and 2. On the other hand, the highest MIC value (1.25%) of ethanol extract of areca nut with the highest MBC value of (2.5%) was found against *Staphylococcus* sp., isolate 1.

Table 2. *Screening of antibacterial activity of ethanol extracts of areca nut and betel leaf against oral pathogens*

Organisms	Betel leaf (10% ethanol extract)	Areca Nutt) (10% ethanol extrac	Kanamycin (30 µg/disc)
Staphylococcus sp.Isolate 1	16.5±0.5	12.0±0.5	19.5±0.5
Staphylococcus sp.Isolate 2	6.5±0.5	8.6±0.75	20±0.0
Pseudomonas sp. Isolate 1	14.0±0.5	10.0±0.7	23±0.5
Pseudomonas sp. Isolate 2	10.5±0.69	12.0±0.5	21.5±0.5

Mean diameter of zones of inhibition in mm including the diameter of the disc 6 mm. Mean ± SD, (n=3)

(a) (b)

Fig. 3. *Ethanol extract of (a) betel leaf against Staphylococcus sp., isolate 1 and (b) areca nut against Pseudomonas sp., isolate 2. (1, 2, 3 numbers represent ethanol extract impregnated discs, P, positive control and N, negative control).*

Table 3. *MIC/MBC values of ethanol extracts of betel leaf and Areca nut*

Test organisms	Antibacterial activity			
	Ethanol extract of betel leaf		Ethanol extract of Areca nut	
Food borne pathogens	MIC (%; w/v)	MBC (%; w/v)	MIC(%; w/v)	MBC(%; w/v)
S. aureus	0.625	1.25	0.625	1.25
S. epidermidis	0.625	1.25	0.625	1.25
B. cereus	5.0	2.5	0.625	1.25
S. typhimurium	5.0	2.5	N/A	N/A
E. coli	5.0	2.5	N/A	N/A
E. coli O157:H7	10.0	20.0	N/A	N/A
Oral pathogens				
Staphylococcus sp., isolate 1	0.625	1.25	1.25	2.5
Staphylococcus sp., isolate 2	10.0	20.0	10.0	20.0
Pseudomonas sp., isolate 1	0.625	1.25	5.0	2.5
Pseudomonas sp., isolate 2	0.625	1.25	5.0	2.5

N/A, Not applicable

Discussion

The importance of finding natural antimicrobials against infectious pathogens of human ailments is increasing day by day. Betel leaf (*Piper betle* L.) and areca nut (*Areca catechu*) are being used for a long time in folk medicine for their antimicrobial, antihaemolytic, antifungal and antioxidative properties[16].

The present study was done to find out the antimicrobial activity of ethanol extracts of betel (*Piper betle* L.) leaf and areca (*Areca catechu*) nut against some food borne enteric pathogens and oral isolates. From the experimental findings it was apparent that ethanol extract of betel leaf showed antibacterial activity against all the test organisms studied, but ethanol extract of areca nut was not found effective against *S. typhimurium, E. coli and E. coli* O 157:H7. The Gram positive organisms were found more susceptible than Gram negative organisms as higher zone of inhibition was found in Gram positive organisms than Gram negative organisms.

Rahman *et al.* (2014) reported that the ethanol extract of areca nut showed no activity against *E. coli and P. aeruginosa*, which did not support the finding of this present study[17]. This may be due to different sources of isolation of *Pseudomonas* sp. Rahman *et al.* (2014) also reported that ethanol extract of areca nut has activity against oral pathogens like *Pseudomonas* sp., *S. aureus* which is similar with the activity found in case of oral isolates in this study[17].

The ethanol extract of betel leaf showed antimicrobial activity against *S. aureus, E. coli,* and *E. coli* O157: H7 which supported the findings of other workers[9]. Unlike the present study, no activity of ethanol extracts of betel leaf against *E. coli* and *S. aureus* was found by other workers[18].

This might be due to strain difference. Datta *et al*, (2011) reported zone of inhibition (12±0.25 mm) of *S. aureus* using 20% ethanol extract of betel leaf[19] which is lower than the zone size (15±0.816 mm) against *S. aureus* found in this study.

From this discussion it can be concluded that a little variable activity was found in comparison to different studies, but most of the data of this study have correlated with the findings of other studies. This deviation of test results might have occurred due to strain difference, sources, assessment methods like disc diffusion or agar well diffusion method, different extraction methods, solvents used in extraction, other environmental parameters during experiment. These factors might interfere with the test result variation.

Conclusion

From the experimental findings in this study, it is evident that areca nut and betel leaf have antimicrobial activity against both Gram positive and Gram negative food borne enteric and oral pathogens. So, these extracts have potential to be used as food preservative, against food borne diseases, to improve oral and gut health, prevention of boils, abscess etc. Further research should be done to find the purified compounds for antimicrobial activity, effect of temperature and pH, molecular mechanism of these extracts antibacterial activity. The side effects of using these extracts should be evaluated too.

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