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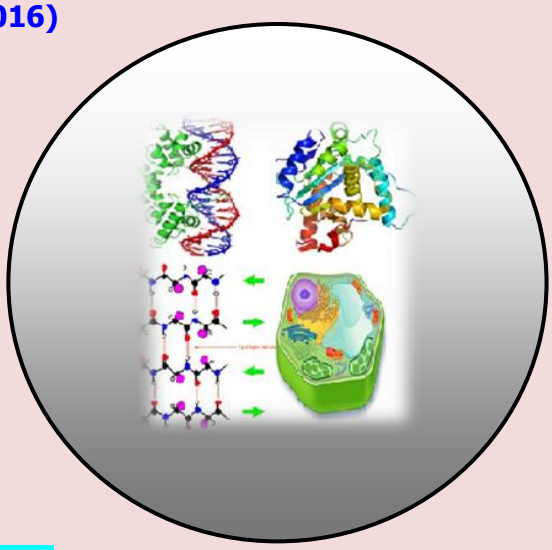

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RESEARCH PAPER

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Bactericidal Effect of Various Sterilizing Sources on Bacteria Isolated From Contaminated Areca Leaf Plates in Tamilnadu

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ABSTRACT

Areca leaf plates are widely used for food handling purpose in various occassions for its biodegradable, compostable and ecofriendly properties. They serve as an perfect alternate to plastics, polymer and paper based plates. There are chances for the presence of contaminants on plates may play a key role to cause the human infection. A total of 60 Arecanut leaf plates samples were collected and identified the pathogenic bacteria by biochemical testing as per the Bergey's manual of determinative bacteriology. The Staphylococcus sp. Bacillus sp. Pseudomonas sp. Proteus sp. and E. coli were the predominant isolates. The bactericidal effect of various sterilizing sources were studied by determining the minimum inhibitory concentration for salt (sodium chloride), Herbal extracts (Neem oil, Neem leaf extract and Tulsi extract) and minimum lethal dose for UV rays and ozone gas. The salt concentration needed for bactericidal activity against Staphylococcus sp. Pseudomonas sp. Proteus sp. and E. coli was found to be 4% and 5% for Bacillus sp.. In herbal extract, 20 µl to 125 µl concentration of Neem oil and Neem leaf extract was showed bactericidal activity against all bacteria. The UV treatment showed that 5 sec needed to kill the Pseudomonas sp. Proteus sp. and E. coli and 10 sec for Staphylococcus sp. and Bacillus sp.. In ozone gas, 5 mins for Pseudomonas sp. Proteus sp. and E. coli and 15 mins for Staphylococcus sp. and Bacillus sp.. The study concluded that the risk of transmission of pathogens in areca leaf plates is high and it can be treated with different sterilizing sources may prevent the harmful infection in human beings in near future.
Keywords: Areca Leaf Plates, Bacteria and Minimum Inhibitory Concentration.

INTRODUCTION

The *Areca catechu L*, belongs to the family *Arecaceae* is commonly called as Betel nut, grows in much of the tropical pacific, Asia and parts of East Asia. It is extensively cultivated in South India as a cash crop, supplies a

pliable material that is amenable to shaping. This part is obtained from the plant leaf part, which in course of its biological life cycle dries, falls and regenerates. This naturally shed leaf sheath is strong, semiflexible yet not brittle, odourless, water and heat resistant [1]. The leaf sheath has its own natural grains and characteristic texture. The leaves/sheath of the betel nut tree is a renewable resource. Hence no trees are cut for manufacturing these products. When compared to bamboo products, *Areca catechu* plant part is used as a raw material for the manufacture of a variety of items of day to day use [2]. The manufacturing process of these plates involves collection of the fresh fallen leaves and stored properly. Then these leaves are cleaned with fresh water. No chemicals or additives is used in any stage of the process [3]**.** Fig 1 shows the picture of Areca leaves and Fig 2 shows the food handling plate manufactured from Areca leaf.

Fig. 1. Areca leaves

Fig. 2. Areca leaf plate

Areca plates are ideal for parties, picnics, marriages, religious functions, wedding, tours, sports events, family get together, meetings, restaurants, coffee shops and any indoor and outdoor caterings. They can also be used to handle any type of foods like hot, wet, oily, greasy and cold food items. They are economical and save on transportation, water and cleaning work to the caterers, picnic, tour and event organizers. It is easily disposed off after use and over a period of time decays with the soil. They are 100% biodegradable, compostable, eco friendly, oven safe, water resistant and can hold both hot and cold, freezer safe and more importantly do not react with food or liquid when used. Hence does not affect the taste and flavour of the food items. They are perfect alternate to plastics/polymer based products and also paper based products. Moreover these trees are grown in hilly areas without any assistance of fertilizers and hence there will not be any traces of pesticides in the leaves [4]. The constituents of the leaf sheaths are cellulose 43%, Crude fibre 33%, Ash 5% and moisture. Cellulose is commonly degraded by an enzyme called cellulase. Cellulases are consortia of complex hydrolytic enzymes capable of hydrolyzing these materials to smaller sugar components like glucose units. This enzyme is produced by several microorganisms, mainly by bacteria and fungi [5, 6, 7]. The cellulolytic bacterial species like *Pseudomonas* sp, *Cellulomonas* sp, *Bacillus* sp, *Micrococcus* sp, *Cellovibrio* sp and *Sporosphytophaga* sp. were also reported [8]. The specific cellulolytic bacterial species is found to be depending on the source of occurrence [9]. The cellulose is the highly presented sugar in arecanut plates and act as a major source for cellulolytic microbes such as bacteria and fungi. These microbes are freely floated in air and it can be attached easily on the surface of the plates when it is kept in unhygienic pack and place. So that, the above condition make the way to increase the microbial load on plate surface. In this condition if we use these plates for eating purpose, it will leads to transmit pathogens to the humam beings and cause unwanted health problems. Based on the above aspects the present study was carried out to determine the bactericidal activity of various sterilizing sources on bacterial isolates isolated from contaminated areca leaf plate surface.

MATERIAL AND METHODS

Collection of areca leaf plates

In this study, Sixty Areca leaf plates were collected from various shopping sites by using sterile polyethylene bags and maintained at room temperature in laboratory for microbiological study.

Isolation and identification of bacteria from areca leaf plates

Areca leaf plate samples were aseptically swabbed and the swabs were put in sterile nutrient broth tube and incubated at 37ºC for 24 to 36 hrs. After incubation, the cultured broth was spread on nutrient agar plate for single colony isolation. Single colony culture was stabbed into nutrient agar contained in screw capped vials for further process. Bacterial morphology was studied microscopically and identified by biochemical testing as per the Bergy's manual of determinative bacteriology.

Bactericidal activity of various sterilizing sources on bacteria

The isolated bacteria were inoculated into the nutrient broth tube containing various concentration of sodium chloride (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 and 10%) (gm/100ml) and incubated at 37°C for 24 hours. After incubation period, the minimum inhibitory concentration were observed using the turbidity method.

Herbal treatment

The minimum inhibitory concentration of herbal extracts were determined by agar well diffusion method. Bacterial isolates were swabbed on nutrient agar plates and agar wells were created by using cork borer. The Neem leaf and Tulsi leaf were obtained from agricultural farms and crude extracts were collected by using mortar and pestel. The commercial neem oil was purchased from the open market and sterilized before use. The different concentrations (25µl, 50µl, 75µl, 100µl, 125µl) of crude extracts and Neem oil were added into the agar wells on plates. These plates were incubated at 37°C for 24 hrs. After incubation period the zone diameter of inhibition was measured and recorded.

UV treatment

The isolates were spread on nutrient agar plate and kept it for 10-15 minutes in room temperature. The agar plates were marked into two portions-one portion was ultraviolet light exposed and other portion was covered with sterile glass plate. The agar plates were exposed to UV light at 3, 5, 10, 30 seconds and 1, 2, 5 minutes at 254 nm. After exposure to UV light, the agar plates and control plate were incubated at 37^{o}C for 24 hrs. The control plates were not exposed to UV light. After incubation period, the UV sensitive and resistance isolates were determined.

Ozone treatment

The isolates were swabbed on nutrient agar plates and exposed into ozone gas in different time intervals (5,15, 30, 45, 60 minutes). After exposure, the plates were incubated at 37ºC for 24 to 36 hrs. After incubation period the ozone sensitive and resistance isolates were determined.

RESULTS AND DISCUSSION

Isolation and prevalence of bacteria in areca leaf plates

In this study, the sixty different sizes of areca leaf plates were collected and microbial analysis were carried out using swab method (Fig 3). The highest prevalence was observed in *Staphylococcus* sp. (90%) followed by *Bacillus* sp. (83%), *Pseudomonas* sp. (68%), *Proteus* sp. (60%) and *E. coli* (48%) (Table 1). The present study revealed that areca leaf plates was mostly contaminated with bacteria when compared to other microbes. The contaminants are mainly attached on the plate surface after manufactured process in unit and unhygienic condition in shop etc. The production units are situated in an open air environment, hence there are also possibilities for air borne pathogens. So far there has been no evidence of disease outbreaks in the community caused by food consumption using these plates.

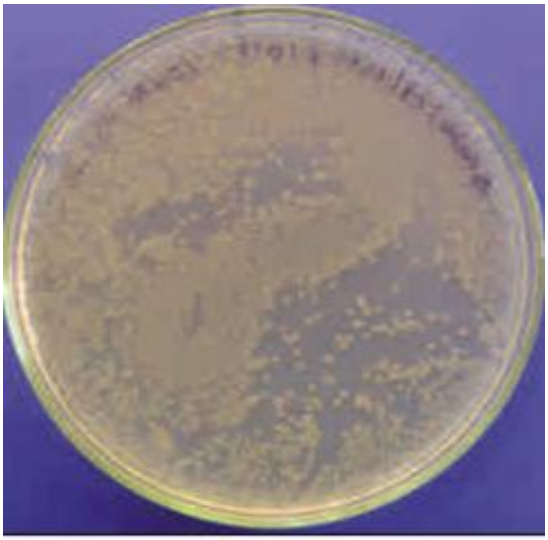

Fig. 3. Isolation of bacteria on nutrient agar

Table 1. Prevalence of bacteria in areca leaf plates

S.No	Prevalent isolates	Bacterial count (CFU/ml)	Percentage
1	*Staphylococcus* sp.	54	90
2	*Bacillus* sp.	50	83
3	*Pseudomonas* sp.	41	68
4	*Proteus* sp.	36	60
5	*Escherichia coli*	20	48

Determination of minimum inhibitory concentration for various sterilizing sources on bacteria

In salt concentration method different concentration of salt containing broth were prepared, *Staphylococcus* sp. *Pseudomonas* sp. *Proteus* sp. and *E. coli* was controlled at 4% salt concentration and *Bacillus* sp. at 5% salt concentration (Table 2). The results revealed that, the bactericidal activity of sodium chloride against *Bacillus* sp. were noted at 5% salt concentration when compared to other bacteria. Because, it have an ability to produce the spore to protect itself from unfavourable condition so more salt needed to kill that. Microbial load has been studied in coins, currency notes, foods and so on. There are no reports regarding the microbial load of areca leaf plates till date.This study is the first report to the microbial diversity and the microbial load of the areca leaf plates.

Table 2. Determination of minimum inhibitory concentration for sodium chloride

S.No	Bacterial isolates	Sodium chloride concentration (gm/100ml)									
		1	1.5	2	2.5	3	3.5	4	4.5	5	10
1	*Staphylococcus* sp.	+	+	+	+	+	+	-	-	-	-
2	*Bacillus* sp.	+	+	+	+	+	+	+	+	-	-
3	*Pseudomonas* sp.	+	+	+	+	+	+	-	-	-	-
4	*Proteus* sp.	+	+	+	+	+	+	-	-	-	-
5	*Escherichia coli*	+	+	+	+	+	+	-	-	-	-

Note: +→ Growth positive (Turbidity), - → **Negative**

Areca leaf plates were treated with different concentration of herbal extracts 20, 50, 75, 100 and 125 µl such as Neem oil, Neem leaf extract and Tulsi extract.

The studies revealed that Tulsi extract was effective at a concentration of 125 µl with the maximum zone diameter of 0.8 mm for *Staphylococcus* sp., *Proteus* sp. and 100 µl for *Bacillus* sp. taken for the study. In neem oil and neem leaf extracts showed that no inhibitory zone against all isolates at all concentrations ranging from 20 µl to 125 µl (Table 3 and Fig 4).

Table 3. Determination of minimum inhibitory concentration for Herbal extracts

S.No	Bacterial isolates	Herbal extracts	Concentration (µl)				
			25	50	75	100	125
			Zone of inhibition(mm)				
1	*Staphylococcus* sp.	Neem Oil	-	-	-	-	-
		Neem Leaf Extract	-	-	-	-	-
		Tulsi Extract	0.4	0.5	0.5	0.6	0.8
2	*Bacillus* sp.	Neem Oil	-	-	-	-	-
		Neem Leaf Extract	-	-	-	-	-
		Tulsi Extract	0.2	0.5	0.6	0.7	0.7
3	*Pseudomonas* sp.	Neem Oil	-	-	-	-	-
		Neem Leaf Extract	-	-	-	-	-
		Tulsi Extract	0.3	0.4	0.5	0.6	0.7
4	*Proteus* sp.	Neem Oil	-	-	-	-	-
		Neem Leaf Extract	-	-	-	-	-
		Tulsi Extract	0.5	0.6	0.7	0.8	0.8
5	*Escherichia coli*	Neem Oil	-	-	-	-	-
		Neem Leaf Extract	-	-	-	-	-
		Tulsi Extract	0.4	0.5	0.6	0.7	0.7

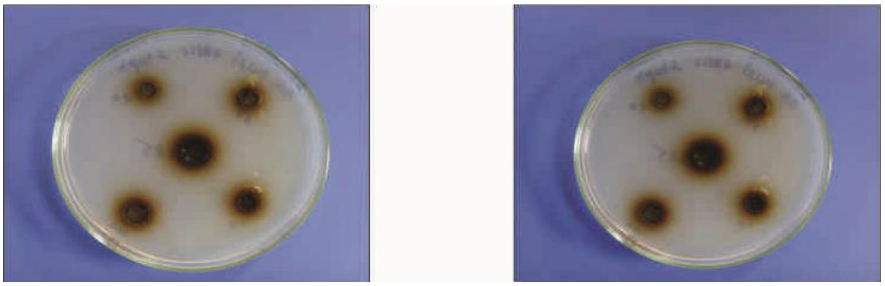

Figure 4. Bactericidal activity of herbal extracts on agar well diffusion method

Table 4. Determination of minimal lethal dose for UV rays

S.No	Bacterial isolates	Time of UVexposure (254 nm)						
		3 sec	5 sec	10 sec	30 sec	1 min	2 min	5 min
1	*Staphylococcus* sp.	+	+	-	-	-	-	-
2	*Bacillus* sp.	+	+	-	-	-	-	-
3	*Pseudomonas* sp.	+	-	-	-	-	-	-
4	*Proteus* sp.	+	-	-	-	-	-	-
5	*Escherichia coli*	+	-	-	-	-	-	-

The isolates were treated with UV rays for different period 3, 5, 10, 30 seconds and 1, 2, 5 minutes. Gram positive rod, *Bacillus* sp. and Gram positive cocci *Staphylococcus* sp. was controlled with 10 seconds exposure time where as *Pseudomonas* sp. *Proteus* sp. and *E. coli* growth was controlled just in 5 seconds of UV exposure (Table 4).

The UV treated one portion of plates showed no growth of isolates and another untreated portion showed that bacterial growth (Fig 5).

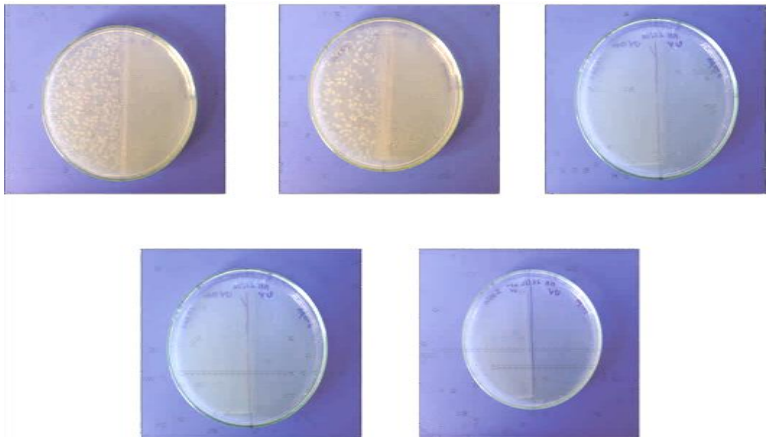

Figure 5. Inactivation of bacteria by UV rays in different time of exposur

The isolates were treated with ozone gas for different period 5, 15, 30, 45 and 60 minutes. Gram positive rod, *Bacillus* sp. and Gram positive cocci *Staphylococcus* sp. was controlled with 15 minutes exposure time where as *Pseudomonas* sp. *Proteus* sp. and *E. coli* growth was controlled just in 5 minutes of ozone treatment (Table 5). Manufacturers should moniter the quality of the areca leaf plates through out the production process and ensure that the final product is of good quality until it reaches the customer.The results of the study revealed that sodium chloride, various herbal extracts like neem oil, neem leaf extract, Tulsi extract, UV radiation and ozone gas are effective in reducing the microbial load of the areca nut plates. These methods are harmless, nontoxic, reliable, safe and cheaper. So the arecanut plate manufacturers are recommended to use above source to prevent the food borne illness.

To date no outbreaks of food borne and other illness have been associated with infection from using such plates. However, evidence for the presence of bacteria on these plates reinforces the need for strict adherence to hygenic practices among food handlers using these plates.

Table 5. Determination of minimal lethal dose for ozone gas

S.No	Bacterial isolates	Time of ozone exposure				
		5 min	15 min	30 min	45 min	60 min
1	*Staphylococcus* sp.	+	-	-	-	-
2	*Bacillus* sp.	+	-	-	-	-
3	*Pseudomonas* sp.	-	-	-	-	
4	*Proteus* sp.	-	-	-	-	-
5	*Escherichia coli*	-	-	-	-	-

CONCLUSION

In recent years, areca leaf plates were used as a food handling purpose in various places of Tamilnadu. The plates were stored at shops in a long time without hygenic condition and the same also maintained by customer until usage. During this time the plates were contaminated by pathogenic microbes.

In another point of view, the plates manufacturing units are not packing in hygenic method at the production point itself and also the products are not subjected to such quality control tests at Tamilnadu, In this study, we recommended that "manufacture shall use suitable sterilization source to destroy microbial load on areca leaf plates and proper packing shall be done with sterile container to keep hygenic until end use.

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