

Effect of Betel Leaf and Lemon Juice Extracts on *Staphylococcus aureus* *in vitro*

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ABSTRACT

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Staphylococcus aureus is a smart, often drug-refractory pathogen. This necessitates use of alternate compounds as possible therapeutic options. So we tested the efficacy of extracts of betel leaf and lemon on growth, biofilm formation and virulence factors of this pathogen. Both the extracts showed marked inhibition of the pathogen at high concentrations. The extracts were also non-toxic to the host RBC and WBCs. Hence they can be considered as putative therapeutic options against this pathogen.

Introduction

Staphylococcus aureus is an extremely virulent bacterium causing multiple infections in man, like superficial and deep seated infections and also toxin mediated infections (1). This bacterium is usually resistant to multiple antibiotics, like Methicillin and others, which often makes treatment very difficult (2). Treatment can be initiated with Vancomycin in MRSA, but the latter drug is itself very toxic (3). Moreover Vancomycin indeterminate *S. aureus* (VISA) and Vancomycin resistant *S. aureus* (VRSA) are also emerging (2). Hence scientists are now focussing on newer and natural alternative compounds to inhibit or kill *S. aureus* from clinical samples. Sometimes even of bacteria are not killed due to refractoriness to some

compounds or antibiotics, their virulence can be mitigated by some natural components.

The effect of ethanolic extract of paan leaf has already been demonstrated on some clinical strains of *Proteus* spp., *P. aeruginosa* and *S. aureus* (4). Lemon peel and juice have also shown some promising action against mostly Gram negative bacteria causing various infections (5).

Keeping these things in mind, our study was planned to test the efficacy of extracts of betel leaf (paan) and lemon juice of this pathogen and its virulence traits, and also to see the synergistic effect of some antibiotics with these extracts, on *S. aureus* isolates.

Materials and Methods

This was a lab based observational study carried out in OPD and department for a period of 5 months from June 2017 to November 2017. Ethical clearance was obtained from the institute before starting the study. Paan leaves and lemon (*Citrus limon*) were sourced from local vendors, each time from a different vendor. Sweet variant of paan leaf was used. Common lemon was used. Isolates of *S. aureus* were selected randomly from laboratory isolates which were identified by Gram staining, anaerobic mannitol fermentation reaction and positive slide and tube coagulase tests. Paan and lemon juice extracts were prepared by weighing 1 gram paan leaf (dried) and lemon juice in 100 ml Peptone water (pH 7). Other concentrations like 2% and 4% (w/v) were also tested. They were autoclaved at 10 lbs/in² pressure. Isolates were emulsified in (a) 2 ml peptone water, and (b) 2 ml of the extracts, and in both a turbidity of 0.5 MacFarland standard was made. After overnight incubation at 37 °C, 1 loopful from each sets of suspension were subcultured on Mueller Hinton agar and Egg yolk agar. Both media were again incubated similarly at the same temperature. Lecithinase, lipase and protease were denoted on Egg yolk agar by the presence of haziness around colonies, clearing around colonies and pearly sheen on surface of colonies, respectively. Mueller Hinton agar was used to see and compare reduction of colonies due to effect of extract. Time kill analysis was also performed wherein liquid broths were subcultured after every 30 minutes, 1 hour, 2 hours and 4 hours on solid media to see the time when growth inhibition starts. Similarly Egg yolk agar was used to see the alterations of the virulence traits due to effect of extract. Then the growths in peptone water and extracts were decanted, washed thrice with sterile physiological saline and washed for 1 minute with 1% aqueous safranin. They were

again washed with sterile saline, inverted and dried, and observed visually for biofilm formation (test tube method). Antibiotic susceptibility of the isolates were done by Kirby-Bauer disk diffusion method, using common antibiotics like Cefoxitin (30 µg), Azithromycin (15 µg), Clindamycin (2 µg), Cotrimoxazole (25 µg) and Amikacin (30 µg) (6). Another set of antibiotic susceptibility was carried out by putting up disks of antibiotics on MHA, and adding 20 µl from each concentration of extract over the paper disks of antibiotics and then incubation. All results were calculated and compared. Then the toxicity of the extracts was also tested by mixing 1 drop of buffy coat from plasma samples received in lab and as left-over from other tests, and observing the mount microscopically for 30 minutes. All tests were done thrice.

Results and Discussion

Both paan and lemon showed inhibitory action against *S. aureus*, more with lemon juice. Inhibition was more marked with 4% extracts as compared to other concentrations. pH of lemon juice was 5.5 while paan extract was about 6.5. Lemon juice contained some proteins and essential amino acids like Leucine. The extracts were non-toxic to human WBCs and RBCs. Chemical analysis of the extracts showed no ketones in extracts, protein concentration of 6 µg/ml in both extracts at all concentrations, sugar level of 12.8 µg/ml in 2% Paan and 2% lemon extracts, and 0.8 µg/ml and 1.2 µg/ml in 2% and 4% lemon extracts, respectively.

The lemon extracts showed qualitatively large amounts of detectable Leucine by commercial dipstick methods. Tables 1–5 further illustrate the effects. Zone size increase by synergistic effect was only shown by 4% paan extract and has been shown below.

Table.1 Effect of 1% paan and 1% lemon

	Total number of exp.	Normal Peptone water	<u>1% PAAN Extract with peptone water</u>		<u>1% Lemon Extract with peptone water</u>	
			Positive	Negative	Positive	Negative
Bio film	55	55	42	13	42	13
Growth	55	55	55	00	55	00
Lipase	55	55	52	03	55	00
Protease	55	55	04	51	04	51
Lecithinase	55	55	00	55	02	53

Positive: Organisms growth have seen in different % of PAAN and Lemon extract

Negative: Organisms growth have inhibited in different % of PAAN and Lemon extract

Table.2 Effect of 2% paan and 2% lemon

	Total number of exp.	Normal Peptone water	<u>2% PAAN Extract with peptone water</u>		<u>2% Lemon Extract with peptone water</u>	
			Positive	Negative	Positive	Negative
Bio film	30	30	25	05	25	05
Growth	30	30	22	08	24	06
Lipase	30	30	20	10	20	10
Protease	30	30	04	26	04	26
Lecithinase	30	30	02	28	02	28

Positive: Organisms growth have seen in different % of PAAN and Lemon extract

Negative: Organisms growth have inhibited in different % of PAAN and Lemon extract

Table.3 Effect of 4% paan and 4% lemon

	Total number of exp.	Normal Peptone water	<u>4% PAAN Extract with peptone water</u>		<u>4% Lemon Extract with peptone water</u>	
			Positive	Negative	Positive	Negative
Bio film	25	25	03	22	20	05
Growth	25	25	03	22	20	05
Lipase	25	25	03	22	20	05
Protease	25	25	00	25	00	25
Lecithinase	25	25	00	25	00	25

Positive: Organisms growth have seen in different % of PAAN and Lemon extract

Negative: Organisms growth have inhibited in different % of PAAN and Lemon extract

Table.4 Time-kill assay for inhibitory effect of lemon

	Total number of experiment	After 30 minutes	After 1 hours	After 2 hours	After 4 hours	After 24 hours
L2(2% lemon)	10	10(+)	10(+)	10(+)	10(+)	03(-); 07(+)
L4 (4% lemon)	10	10(+)	10(+)	10(+)	0(+)	08(-); 02(+)

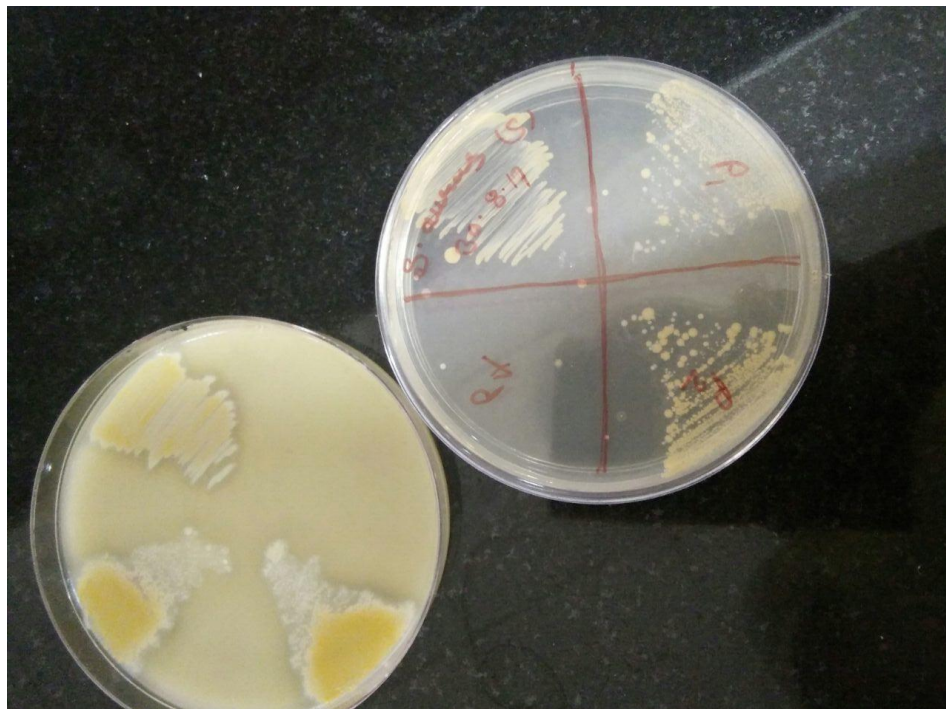
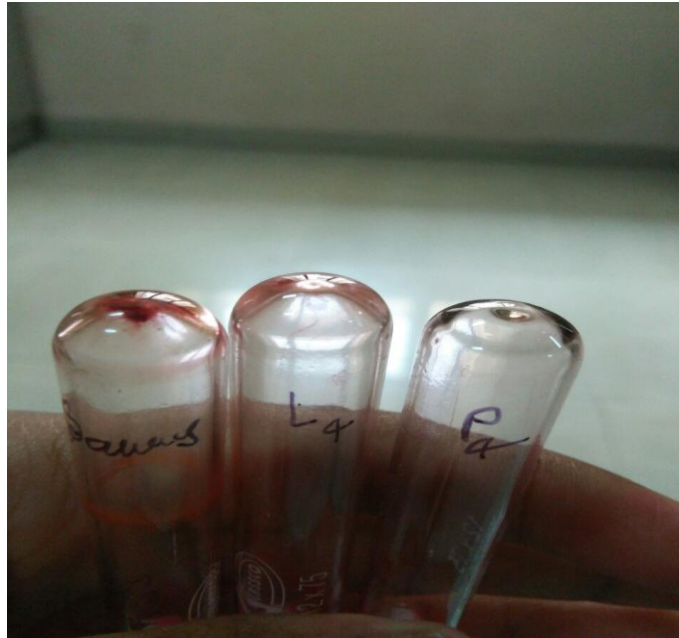
+: positive growth

-: negative growth

Table.5 Sensitivity zone size

AB disc	NET	AZM	CX	COT	CD	FR	LEVO
On MHA media	26mm	Resistant	6mm	22mm	15mm	Resistant	22mm
On MHA AB disc with P4 20µl (4% paan)	32mm	6mm	15mm	22m	28mm	28mm	28mm

MSSA and MRSA pose serious health threats



Both MSSA and MRSA pose serious health threats. They are notorious for causing deadly infections like pneumonia, osteomyelitis and skin infections, both in community and outpatient setting (7). Being notoriously resistant to multiple antibiotics, there is acute and urgent need of natural and alternative compounds to treat infection by *S. aureus*.

Betel leaf is a common ingredient of Indian dishes and also very commonly used as a mouth freshener (4). It is widely available and cultivated in Malaysia, India and Thailand among other South Asian countries. These aspects have not been seen or studied earlier and are quite interesting. Since there was inhibition of virulence factors, this can be used as anti-virulence therapy in this pathogen causing infections.

Also synergistic effects with antibiotics were observed, which means that they can be used in conjunction with antibiotics also and there was no inhibition of effect of antibiotics. The putative inhibitory molecule in lemon extract on *S. aureus* isolates can be Leucine, since *in-vitro* it has shown to be antibacterial, as reported in several studies (8)

More studies are awaited in this regard. Mostly effects started after 4 hours of incubation. Since autoclaved extracts were used, it means it can be used in febrile states also. Extracts were also non-toxic and hence presumably safe for human use.

Paan and lemon juice can be used to treat *S. aureus* infection at least *in-vitro*, and further *in-vivo* studies can confirm these findings.

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