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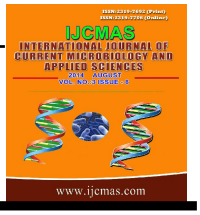

Original Research Article

Enhanced antibacterial potential of ethanolic extracts of neem leaf (*Azadiracta indica* A. Juss.) upon combination with bacteriocin

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Azadiracta indica: Bacteriocin; Antibacterial potential; Synergism

A B S T R A C T

Ethanolic extracts of neem (*Azadirachta indica*) leaf produced significant antibacterial potential against Gram positive *Staphylococcus aureus, Bacillus subtilis and Listeria monocytogenes,* and Gram negative *Pseudomonas aeruginosa* and *Escherichia coli* between 10-20 mg/ml concentration but failed to kill *Salmonella typhimurium* during *in vitro* study. A positive synergism was observed in terms of antibacterial potential of the extract upon combination with bacteriocin from a food grade lactic acid bacterium *Pediococcus acidilactici* LAB001. The combination killed *Bacillus subtilis* and *Pseudomonas aeruginosa* with a cidal mode of action.

Introduction

From ancient times, plants have been used in Ayurvedic medicines. The extracts from different parts of different plants have shown potent antimicrobial effect. It is estimated that there are 250,000 to 500,000 species of plants on Earth (Borris, 1996). Only a very small percentage (1 to 10%) of these is used by humans and other animal species as foods, whereas more are used for medicinal purposes (Moerman, 1996). Recently, much work has been done on extraction of chemicals responsible for the antimicrobial effect from these plant species to know the chemical nature of the products. Plants have an almost limitless ability to synthesize aromatic substances and secondary metabolites. Most of which are phenols or their oxygen-substituted derivatives (Geissman, 1963).

At least 12,000 of secondary metabolites have been isolated, which is less than 10% of the total (Schultes, 1978). Scientific analysis of plant components follows a logical pathway. Plants are collected either randomly or by following leads supplied by local people who has used the plant parts for healing from any particular region where the plants are found (Martin, 1995). Initial screenings of plants for possible antimicrobial activities typically begin by using crude aqueous or alcohol extractions and then followed by various organic extraction methods. Since nearly all of the identified components from plants active against microorganisms are aromatic or saturated organic compounds, they are most often obtained through initial ethanol or methanol extraction (Cowan, 1999).

Neem is one of the most useful traditional medicinal plants in India. It has been described as *Azadirachta indica* as early as 1830 by De Jussieu. Common people use the paste of neem leaves at itching site without knowing any scientific reason. The biological activities of neem leaf extracts have been reviewed beautifully by Biswas et al. (2002). Clinical studies with the dried neem leaf extract showed its effectiveness to cure ringworm, eczema and scabies. Lotion derived from neem leaf, when locally applied, can cure these dermatological dieases within 3-4 days in acute stage or a fortnight in chronic case.

A paste prepared with neem and turmeric was found to be effective in the treatment of scabies within 3-15 days of treatment without any adverse effect. It is proved that extracts of neem leaf contain certain compounds like nimbidine, cyclic trisulphide, cyclic tetrasulphide with a wide spectrum of antibacterial action against Gram- positive and Gram-negetive bacteria. Recently the potential of neem leaves against human pathogenic and drug resistant strains of bacteria have been evaluated by Sarmiento *et al.* (2011) and Margathavalli *et al* (2012).

Recently there are several reports of using herbal derivatives (in both crude and extracted active form) with bacteriocin for increase the antimicrobial potency or probiotic efficiency or to be used as food preservatives.

Synergism is one such way where an interaction arising between two or more factors or agents that produce an effect greater than the sum of their individual effects. In the present study combined effect of bacteriocin and extracts of neem leaf has been tested. This will help to make a formulation, during microbial infection on skin and mixing the extract with bacteriocin.

Materials and Methods

Collection of plant material

Fresh leaves of the healthy neem plants (*Azadiracta indica,* L.) were collected from the local area.Its identification was confirmed by Dr. S.Mondal, Angiosperm Taxonomist of the department of Botany, Visva-Bharati. A voucher specimen is kept in the departmental Herbarium.

Preparation of Crude Extracts

Collected leaves were washed properly, air dried, coarsely powdered and were subjected to extraction. Powdered plant materials were kept in proper container and labelled properly. 5 gram of the air dried and coarsely powered plant materials were extracted for overnight with 50 ml ethanol solution under shaking condition. The concentrated extract was then centrifuged at 10,000 rpm for 10 min. The liquid extract was subjected to evaporation for drying and dried extracts were kept at 4^{o}C.

Test micro-organisms

Three Gram positive (*Bacillus subtilis* MTCC 121, *Listeria monocytogenes* MTCC 657, *Staphylococcus aureus* MTCC 96) and three Gram negative bacteria (*Salmonella typhimurium* MTCC 98, *Pseudomonas aeruginosa* MTCC 741 and *Escherichia. Coli* MTCC 1667) used in this study were procured from Institute of Microbial Technology, Chandigarh. All the pathogenic bacteria were grown and maintained on Nutrient broth or agar medium at 37 ^{o}C with regular transfer.

Source of Bacteriocin

Bacteriocin produced by a strain of lactic acid bacterium *Pediococcus acidilactici* LAB001 was purified from 24 h grown culture in MRS broth at 30^{o}C following the

method of Yang *et al.* (1992). Identification of the bacterium was confirmed by morphological, biochemical and molecular characters (Das, 2014).The isolation procedure and detail growth conditions of the strain was described earlier. The bacteriocin produced was a 13.7 KD protein (Das, 2014).

Antimicrobial study

The sensitivity of the selected bacteria to the plant extract was evaluated by agar well diffusion method (Bauer *et al.* 1966). A solution of extract with a concentration of 100 mg/ml in DMSO was prepared. Overnight broth cultures of pathogenic bacteria were spread over the prepared nutrient agar plates by sterile cotton swab. 0.04 ml of different concentrations of the extract (0 to 100 mg/ml) were poured into the wells on the nutrient agar plates and were incubated at 37^{o} C for 24 hours. At the end of incubation diameter of inhibition zones around the wells were measured against each concentration.

Assessment of antimicrobial combination

Effect of antibacterial potential of neem leaf extract, bacteriocin and a combination of both was assessed by counting the numbers of colony forming units by treating the test bacteria with bacteriocin (2000AU) alone, neem leaf extract at its MIC, and a combined mixture of neem leaf extract (MIC amount) and bacteriocin (2000AU) (Ray *et al.* 1999). Equal volume of neem leaf extract and bacteriocin (2000AU) were mixed properly in an eppendroff tube and the mixture was introduced at suitable concentrations into the actively growing (12 h) cultures of *Bacillus subtilis* and *Pseudomonas aeruginosa*. Aliquots were taken out at 0 (control), 6, 12, and 24 h interval after addition of the test compounds and plated onto nutrient agar plates after suitable dilution. Plates were incubated for 24 hours at 37^{o} C. As dried ethanolic extract of neem leaf was dissolved in non-toxic organic solvent DMSO, its effect was also studied in a similar way.

Results and Discussion

Ethanolic extracts of shade dried and powdered neem leaves showed antibacterial activity against all the tested bacteria belonging to both Gram positive and Gram negative groups except *Salmonella typhimurium* at different concentrations (Table 1). The inhibition zone was found at 10 mg/ml concentration in case of Gram positive bacteria and 20 mg/ml in case of Gram negative bacteria. To know the combined effect of neem leaf extract and bacteriocin, one Gram positive bacterium *Bacillus subtilis* and one Gram negative bacterium *Pseudomonas aeruginosa* were taken (Table 2). CFU count was made by treating the test bacteria with bacteriocin (2000AU) alone, neem leaf extract at its MIC, and a combined mixture of neem leaf extract (MIC amount) and bacteriocin (2000AU). The MIC value of neem leaf extract against *S. aureus* was 10 mg/ml and the same against *P.aeruginosa* was 20 mg/ml. After 24 hours incubation, the number of viable cells in terms of CFU became 10^4 in case of *B. subtilis* and 10^5 in case of *P. aeruginosa* when treated only with neem leaf extract. In case of only bacteriocin treatment the number of CFU became around 10^5 and 10^6 per ml respectively for the bacteria. This reduction in number with time was gradual and not very fast. It is somewhat comparable with a static mode of action. In general most of the bacteriocin derived from the genus *Pediococcus* spp could kill only Gram positive bacteria (Drider et al., 2006)but the bactericin derived from the present strain

P.acidilactici LAB001 could kill Gram negetive *P.aeruginosa to some extent.* However, during the treatment with mixture of neem leaf extract and bacteriocin the number of viable cells decreases drastically. Neem leaf extract or bacteriocin showed bacteriostatic mode of action but their combined effect exhibited bacteriocidal mode of action.

From the results presented in Table 1 and Table 2 it is found that crude ethanolic extract of neem leaf not only kills surface infecting *S.aureus*, food borne as well as food spoilage pathogen *L.monocytogenes* but also it kills endospore forming *B. subtilis* very effectively. The extract although could kill Gram negative *E. Coli* and *P. Aeruginosa* but failed to kill a strain of *S. typhimurium* MTCC98. Bacteriocin derived from lactic acid bacterium *P. Acidilactici* LAB001 isolated from ready to eat meat product enhanced the potentiality of the extract significantly thus increased the prospect of its application.

Table.1 Antibacterial activity of ethanolic extract of *Azadirachta indica* leaves at different concentrations (mg/ml)

Microorganisms	Zone of Inhibition												
	Concentration of the crude neem leaf extract (mg/ml)												
	0	1	5	10	20	30	40	50	60	70	80	90	100
Bacillus subtilis	-	-	±	+	++	++	++	+++	+++	+++	+++	+++	+++
Listeria monocytogenes	-	-	-	+	+	+	++	++	++	+++	+++	+++	+++
Staphylococcus aureus	-	-	±	+	+	++	++	++	+++	+++	+++	+++	+++
E.coli	-	-	-	-	+	+	+	++	++	++	++	+++	+++
Pseudomonas aeruginosa	-	-	-	-	+	+	+	+	++	++	++	+++	+++
Salmonella typhimurium	-	-	-	-	-	-	-	-	-	-	-	-	-

(-): no inhibition ;(±): very little inhibition; (+): inhibition zone diameter upto 7 mm.; (++): inhibition zone upto 12mm.; (+++): inhibition zone more than 12mm. Results are average of at least three observations.

Table.2 Effect of neem leaf extract, bacteriocin, and a combination of neem leaf extract and bacteriocin on actively growing culture of *Bacillus subtilis* and *Pseudomonas aeruginosa.* Results are average of three observations

Treatment combination	Number of colony forming units per ml							
	Bacillus subtilis				*Pseudomonas aeruginosa*			
	0 hr	6 hr	12 hr	24 hr	0 hr	6 hr	12 hr	24 hr
DMSO	$2x10^8$	$1.7x10^8$	$1.2x10^8$	10^8	7×10^8	6.8×10^8	6.8×10^8	6.6×10^8
Neem leaf extract	$2x10^8$	10^6	$6x10^4$	10^4	10^8	10^7	10^6	10^5
Bacteriocin 2000AU	$2x10^8$	$5x10^6$	10^6	10^5	10^8	$2x10^7$	10^7	10^6
Neem leaf extract + Bacteriocin	$2x10^8$	$3x10^6$	$5x10^3$	10^2	10^8	$2x10^6$	$8x10^4$	10^3

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