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In Vitro Effect of Azadirachtin on Aerobic Bacteria of Rat Intestine

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Neem *(Azadirachta indica* A Juss) based pesticides, containing Azadirachtin (AZ) as primary active ingredient, are being investigated as promising alternatives of synthetic pesticides because they produce less mammalian toxicity and less ecological damage (Sexsena 1989). Although AZ has low acute toxicity with an LD_{50} greater than 5000 mg/kg in the rat (Raizada et al. 2001), the possibility of adverse effects cannot be ruled out. Because of intended use neem pesticide may enter into the environment in considerable amount and most common sites of contamination by overt or covert means are rivers, lakes, reservoirs, soil etc. (Purohit and Dixit 1991). Kreutzweiser (1997) has reported that, based on a proposed application rate of 50g Azadirachtin /hectare, the expected environmental concentration of neem pesticide in water would be 0.35 mg/lit. So an assessment of adverse effects of neem pesticide on non -target organism is warranted.

Reports are available on various pharmacological and medicinal activities of neem compounds (Biswas et al. 2002). Neem products have been shown to posses antimicrobial activity (Sairam et al. 2000; Vanka et al. 2001). Studies conducted by Rao et al. (1986) have shown the *in vitro* antibacterial activity of neem oil. Significant antibacterial activity against Gram-positive and Gram-negative organisms has been shown by an isolated limonoid, along with seven known tetranotriterpenoids from neem fruits (Siddiqui et al. 1992). AZ has been shown to block the development of motile male malarial gamete *in vitro* (Jones et al. 1994). The inhibitory effect of aqueous neem extract on bacterial property in the *in vitro* plaque formation has also been reported (Wolinsky 1996). Das et al. (1999) have reported growth inhibitory property of aquaneem, an emulsified product prepared from neem kernel, against four pathogenic bacteria of fish (i.e. *Aeromonas hydrophila, Pseudomonas fluorescence, Escherichia coli* and *Mycobacterium* sp.) at various concentrations. *In vitro* antibacterial activity of neem oil against pathogenic bacteria has been considered due to the inhibition of cell membrane synthesis in the bacteria (Baswa et al. 2001). There are reports on biological activity of neem extract against various non-target organisms (Kreutzweiser et al. 1999; Rahman et al. 1999). However, no information is available on the effect of AZ on gastrointestinal micro flora of non-target organism, which play important role in the hosts cellular metabolism.

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Possible contamination of water sources could lead to ingestion of AZ by non-target organisms, which could adversely affect gut micro flora. This study deals with the *in vitro* effect of AZ on growth, enzyme activity, lipid peroxidation and membrane constituents of aerobic bacteria isolated from rat large intestine.

MATERIALS AND METHODS

Gut micro floras were isolated from healthy male Wistar albino rats procured from the animal breeding center of Industrial Toxicology Research Centre, Lucknow. The microbes were isolated from large intestine according to the procedure described in Larson et al. (1978), with required modifications. Briefly, laparotomy was performed under light ether anesthesia and aseptically caecum and large intestine was separated. The content of separated segment was suspended in nutrient broth (containing 1.0% peptone, 0.5% yeast extract, 0.5% NaCl, pH 7.0) to give 5% (w/v) suspension. This was allowed to sit for 15 min to settle the food particles and other coarse materials and then centrifuged at 2000 rpm for 5 min at 4^0 C. Various aerobic (facultative) bacteria were purified auxigenically by streaking on agar plates and morphological, physiological and biochemical tests were carried out in accordance with Burgey's manual to identify *Lactobacillus* sp. and *Escherichia coli*.

The effect of Azadirachtin (Technical, 12%; obtained as gift from Southern Petrochemical Industries Corp. Ltd.. Chennai, India) on individual bacteria was determined by growing them separately in the basal salt media (gram / litre- $Na_2HPO_4.H_2O$, 4.0; $KH_2PO_4.3H_2O$, 4.0; NH_4Cl, 2.0; $MgSO_4.6H_2O$, 0.02; $CaCl_2.2H_2O$, 0.01; $FeSO_4.6H_2O$, 0.01; yeast extract, 5.0; 2% glucose and traces of vitamins) containing different concentrations of AZ. Control cultures were devoid of AZ. Overnight nutrient broth grown cells were used as inoculum (2% v/v). Cultures were incubated at 37^0 C on rotary shaker operated at 200±10 r.p.m. Agar 2% (w/v) was added to all broth as and when required. Isolates were maintained either on nutrient agar or nutrient whey agar. Stock solutions of AZ were prepared in DMSO and 2% DMSO, (v/v, final concentration) was maintained in experimental (with AZ) as well as control cultures (without AZ).

Samples were withdrawn from experimental and control cultures at 1-hr interval up to 24 hr and growth was measured by turbidimetry at 610 nm in a Bausch and Lomb Spectronic-21. Growth measurements were carried out in three replicates and specific growth rate, doubling time and numbers of generations were evaluated according to Espargares and Mariscan (1989). Turbidimetric analysis for microbial growth pattern was further confirmed by measuring total cell proteins at timed intervals. For viability testing serial 10-fold dilutions of sample were prepared in sterile normal saline. Duplicate 100 µl samples were plated on nutrient agar plates, incubated at 37^0 C for 24 hr and then colonies were counted.
In order to prepare cell extract and cell envelope, cells grown in presence or absence of AZ for 10 hr were harvested by centrifugation (8000 rpm, 10 min, 4^0 C). Cells were washed with sterile physiological saline and suspended in suitable buffer for enzyme assays and other measurements following the disruption by four - 15 sec

bursts of Vibronic Ultrasonic Processor. Cell debris was removed by centrifugation at 2000g for 10 min. The resulting supernatant was designated as cell extract. Cell envelope was fractionated by centrifugation of cell extract at 50,000g for 60 min. (Kumar and Upreti. 2000).

Alkaline phosphatase (E.C. 3.1.3.1) and Ca^{2+}-Mg^{2+}-ATPase (EC 3.6.1.3) were estimated in cell extracts according to Weiser (1973) and Hidalgo et al. (1983), respectively. Enzyme units were defined as μ moles of product formed or liberated per min under the assay conditions. Specific activity was expressed as units per mg protein. Protein was determined according to Lowery et al. (1951) using bovine serum albumin as standard. Total lipids were extracted according to Folch et al. (1957). Phospholipids were quantified following digestion with 70% perchloric acid and estimated according to the method of Wagner et al. (1962). Total hexoses were estimated by anthrone reagent method (Roe 1953). Lipid peroxidation was analysed by measuring thiobarbituric acid reactive substance according to Okawa et al. (1980) using malonaldehyde (MDA) as a standard. The results were expressed as mean ± standard deviation (S.D.). Comparisons were made with appropriate control employing Student's *t*- test. Differences were considered significant at $P< 0.05$.

RESULTS AND DISCUSSION

Results on the growth pattern of *E.coli* and *Lactobacillus* sp. revealed more or less similar specific growth rate in presence or absence of AZ up to 100 ppm concentration. Whereas, AZ concentrations more than 200 ppm prompted an appreciable time-dependent inhibition in growth rate vis-à-vis higher doubling time and less numbers of generations in both the bacteria (data not shown). Table 1 shows the specific growth rate values evaluated at the end of 10 hr of growth in absence as well as presence of different concentrations of AZ and Figure 1 depicts the doubling time. It is evident that higher concentrations of AZ significantly

Table 1. Specific growth rate of *E. coli* and *Lactobacillus* sp. for different concentrations of Azadirachtin.

Conc. (in ppm)	Specific growth rate	
	Escherichia coli	*Lactobacillus* sp.
0	0.88 ± 0.09	0.37 ± 0.04
50	0.81 ± 0.09	0.36 ± 0.03
100	0.76 ± 0.04	0.31 ± 0.03
200	0.64 ± 0.06*	0.27 ± 0.02*
500	0.33 ± 0.09*	0.20 ± 0.01*
1000	0.04 ± 0.00*	0.06 ± 0.00*

Samples were withdrawn after 10 hr of growth in presence or absence of AZ. Values shown are mean ± S.D. from three replicate samples. * $P < 0.05$.

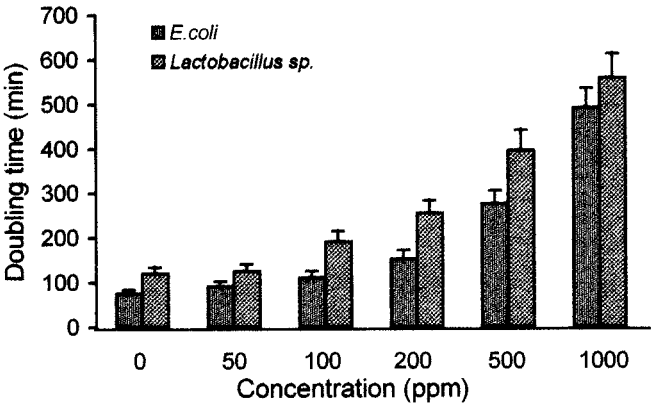

Figure 1. Effect of Azadirachtin on the doubling time of rat intestinal microflora. Samples were withdrawn after 10 hr of growth in presence or absence of AZ. Values are mean ± S. D. from three separate experiments.

inhibited the growth rate. However, the extent of inhibition was more pronounced in *Escherichia coli.*

For quantitative comparison, the number of viable cells in liquid cultures was evaluated by counting colony-forming units on agar plates just after inoculation and at every 1-hr interval (data not shown). Results revealed that cultures with and without AZ had the same number of viable cells in the beginning. No significant change in colony forming units in first 6 hr was evinced with AZ concentrations up to 100 ppm, which was followed by a slight decrease in later time periods. Whereas, with higher concentrations, decrease in cell counts and density was observed as function of time and concentration in comparison to control. It was more pronounced in *E.coli.*

Table 2. *In vitro* effect of Azadirachtin on enzyme activities of rat intestinal microflora.

Conc. (in ppm)	Specific activity (units / mg protein)			
	Alkaline phosphatase		Ca^{2+}- Mg^{2+}- ATPase	
	E. coli	*Lactobacillus* sp.	*E. coli*	*Lactobacillus* sp.
0	2.49 ± 0.01	1.17 ± 0.02	0.29 ± 0.01	0.18 ± 0.01
50	2.66 ± 0.09	1.22 ± 0.08	0.28 ± 0.03	0.16 ± 0.01
100	2.71 ± 0.04	1.32 ± 0.14	0.24 ± 0.01*	0.14 ± 0.01*
500	2.73 ± 0.11	1.46 ± 0.03*	0.19 ± 0.01*	0.14 ± 0.01*
1000	3.06 ± 0.03*	1.73 ± 0.01*	0.06 ± 0.00*	0.11 ± 0.00*

Cell extract was prepared from cells grown in presence or absence of AZ for 10 hr. Values are mean ± S.D. from three separate experiments. * $P < 0.05$.

Table 3. *In vitro* effect of Azadirachtin on phospholipid content of rat intestinal micro flora cell envelope.

Conc. (in ppm)	μ g / mg protein	
	Escherichia coli	*Lactobacillus* sp.
0	56.2 ± 2.3	38.0 ± 3.4
50	54.5 ± 4.5	37.5 ± 3.6
100	46.3 ± 4.7*	37.4 ± 4.1
500	36.5 ± 3.7*	36.6 ± 3.8
1000	30.6 ± 3.2*	35.6 ± 4.0

Cell envelope was prepared from cells grown in presence or absence of AZ for 10 hr. Values are mean ± S.D. from three separate determinations. *$P < 0.05$.

Alkaline phosphatase and Ca^{2+}-Mg^{2+}-ATPase activities were analysed in cell extracts prepared after 10 hr growth in absence or presence of AZ (Table 2). In the presence of 500 and 1000 ppm of AZ, alkaline phosphatase activities were increased 25% and 48%, respectively, in *Lactobacillus* sp. For *E. coli*, however, alkaline phosphatase activity was increased only 23% by 1000 ppm AZ. Conversely, Ca^{2+} Mg^{2+}-ATPase activities were significantly inhibited in both the bacteria grown in presence of AZ with concentrations higher than 100 ppm. Inhibition of 78% and 37% was evident with 1000 ppm in *E.coli* and *Lactobacillus* sp., respectively. The extent of alterations in these two transport enzymes was found to be genus specific.

Exposure of *E.coli* to concentrations of AZ above 100 ppm produced a concentration-dependent decline in the phospholipid content of the cell envelope (Table 3). No such effect was induced in *Lactobacillus* sp., further suggesting the specificity of deleterious effect of AZ. In addition, AZ exposure did not alter the hexose content of cell extracts, or lipid peroxidation levels in cell extract or cell envelope fractions of either bacterium (data not shown).

The results indicated that the growth of these two common rat intestinal bacteria was inhibited by higher concentrations of AZ. The present study also indicated significant alteration in phospholipid content of *E.coli* cell envelope. No significant alteration in protein contents was noticeable. The specific activities of alkaline phosphatase and Ca^{2+} Mg^{2+}-ATPase were found to be altered. The extent of alteration varied in two intestinal bacteria suggested the genus specificity. The genus-to-genus variation of AZ effect could be due to the biochemical versatility and also due to the nature of cell envelope of these two bacteria (Coosterten et al. 1974). Present study showed no propensity of AZ for lipid peroxidation, which is a common feature of most of the natural products. No significant difference in lipid peroxidation has also been demonstrated in *E.coli* following azzarene treatment (Catallo et al. 1992).

Mallett et al. (1986) demonstrated that *in vitro* culture of mixed bacterial population of large intestine of rat, provides an alternative to animal studies for the investigation of foreign compounds metabolism by flora. An *in vitro* simulation of rat gastrointestinal micro flora has also been used to study the metabolic activity and the effect of xenobiotics (Ames et al. 1999). Moreover, microbes are being used as a biological model for the development of relatively quick toxicity tests based on inhibition of growth and on certain enzymatic alterations (Mamber et al. 1986; Cenci et al. 1987; Kumar and Upreti 2000). The present *in vitro* study indicates that Azadirachtin affects the growth of rat intestinal bacteria and serves to demonstrate possible effects that may occur under *in vivo* conditions on these friendly bacteria. However, *in vivo* effect needs further validation.

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