Antibacterial potential of *Azadirachta indica* seed and *Bacopa monniera* leaf extracts against multidrug resistant *Salmonella enterica* serovar Typhi isolates

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Abstract

Introduction: Neem (*Azadirachta indica*) and brahmi (*Bacopa monniera*) are well known medicinal plants, but their antibacterial activity against the typhoidal pathogen *Salmonella enterica* serovar Typhi has not been studied.
Material and methods: *A. indica* seed and *B. monniera* leaf extracts were assayed for antibacterial activity by agar well diffusion and agar dilution methods in order to determine the zone diameter of inhibition and minimum inhibitory concentration (MIC) values, respectively. Killing efficacy of the extracts was determined at various concentrations in Mueller-Hinton broth. Time-dependent killing was achieved using 500 µg/ml (1× MIC) of the extract for *S. enterica* serovar Typhi strain.
Results: The *A. indica* seed and *B. monniera* leaf extracts showed excellent antibacterial activity against the isolates having zone diameter of inhibition 9-19 mm and 9-18 mm. The MICs for the isolates were in the range 50-500 µg/ml (*A. indica* extract) and 50-600 µg/ml (*B. monniera* extract). The *A. indica* and *B. monniera* extracts were bactericidal against *S. enterica* serovar Typhi at concentrations of 400 µg/ml and 450 µg/ml, respectively. *A. indica* and *B. monniera* against the test microorganisms displayed significant antibacterial (at concentrations ≥250 and ≥300 µg/ml, respectively) as well as time killing (at 500 µg/ml) activities ($p<0.005$).
Conclusions: The results might explain the ethnobotanical use of the studied plants for the treatment of *S. enterica* serovar Typhi infection. This is the first evaluation of *A. indica* and *B. monniera* against *S. enterica* serovar Typhi isolates associated with enteric fever in and around Kolkata (India) in the past one and half decade.

Key words: bactericidal, plant extracts, time kill, typhoidal bacteria.

Introduction

Several antibiotics are currently in use to treat a variety of infectious human diseases. Many of them have, however, a limited antimicrobial spectrum due to the emergence of multi drug-resistant (MDR) bacterial strains; *Salmonella enterica* serovar Typhi, a causal organism of typhoid fever, is such a bacterium that showed resistance to a number of well known antityphoid antibiotics such as ampicillin, chloramphenicol, cotrimoxazole and ciprofloxacin [1, 2].

The persistent increase in MDR *S. enterica* serovar Typhi has led to the introduction of more potent synthetic antibiotics such as the 3rd generation cephalosporins [3]. These antibiotics are scarce, costly and not affordable, particularly in a developing country like India, and therefore make

compliance difficult. Thus there is a need for a continuous search for new effective and affordable antimicrobial drugs. The efforts of scientists in establishing plants with promising antimicrobial property is yielding fruitful results as a number of plants with high antimicrobial property have been elucidated [4-10].

Gehlot and Bohra reported the inhibitory effect of a large number of plant part extracts against *S. enterica* serovar Typhi, excluding neem (*Azadirachta indica*) and brahmi (*Bacopa monniera*) [11]. Atata et al. showed the potential use of extracts of *Enantia chlorantha* for use in the control of several medically important bacterial strains including *S. enterica* serovar Typhi [12]. Ravikumar et al. reported inhibitory activity of *B. monniera* against the human bacterial pathogen *Escherichia coli* [13]. The most active antibacterial plants against both gram-positive and gram-negative bacteria excluding *S. enterica* serovar Typhi were *Thymus vulgaris* and *Thymus origanium* as reported by Essawi and Srour [14]. Okemo et al. reported the antibacterial activity of *A. indica* against bacterial isolates such as *Staphylococcus aureus*, *E. coli* and *Pseudomonas aeruginosa* [15]. Thus, among plants studied to explore therapeutic effects but which receive no scientific research on antibacterial activity against *S. enterica* serovar Typhi are *A. indica* and *B. monniera*. The present communication reports the antibacterial activity of *A. indica* seed and *B. monniera* leaf extracts against MDR *S. enterica* serovar Typhi isolates causing enteric fever in and around Kolkata during 1991-2003.

Material and methods

A total of 60, among blood culture isolates of *S. enterica* serovar Typhi (1991-2003) having various antibiotic resistance pattern, as reported previously by Mandal et al. [1] and Mandal et al. [2], were randomly selected and subjected to the present study.

The plant materials used in this study consisted of seed of neem (*A. indica*) and leaf of brahmi (*B. monniera*). The fresh leaves were collected from the market, and the seeds were harvested from the tree during May-June in 2005, and seed kernels were taken out. The plant materials thus collected were washed 2-3 times with tap water and finally with distilled water followed by ethanolic wash, and allowed to dry under the shed until constant weight was obtained. The fully dried plant materials were ground into fine powder, and stored in a sterile glass bottle at room temperature.

One hundred grams of ground materials of the two plants were soaked in 250 ml of 95% ethanol contained in two separate 500 ml capacity flasks. The flasks were plugged with cotton wool, wrapped in aluminium foil, shaken vigorously and allowed to stand at room temperature for 24 h. The macerates were squeezed through sterile double-layered cheesecloth, and then filtered with filter paper (Whatman No. 1). Ethanol was evaporated to dryness until constant weight was achieved for each extract, and reconstituted in 50% ethanol to obtain a stock solution of 10 mg/ml. The stock solutions were then filter sterilized and stored in screw capped brown bottles at 4°C. Each extract was tested for purity by plating them on Mueller-Hinton agar and incubated for 24 h at 35°C, and subsequently used to assay for antimicrobial activity.

The bacterial isolates were pregrown in Mueller-Hinton broth (Hi-Media, Mumbai, India) and incubated at 35°C for 24 h. Each of the broth cultures was centrifuged at 7000 rpm for 20 min. The cell pellet was washed by centrifugation at 7000 rpm for 20 min using 10 ml Mueller-Hinton broth. Washed cells were resuspended in Mueller-Hinton broth, diluted serially, and inoculum was adjusted, by colony count technique, to approximately 10^4 cfu/spot for agar dilution susceptibility test, 10^8 cfu/ml for well diffusion method and 5×10^5 cfu/ml for time-kill studies.

Antibacterial activity was determined by agar well diffusion method. Sterile Mueller-Hinton agar (Hi-Media, Mumbai, India) plates (20 ml per plate) were prepared. Approximately 10^8 cfu, from Mueller-Hinton broth culture as mentioned above, were used to spread on the surface of a Mueller-Hinton agar plate and allowed to dry. Three wells (each of 6 mm diameter) were bored on the surface of the agar media on each plate. 50 µl of each extract (equivalent to 500 µg of the extract) was dropped into each appropriately labelled well, and into the remaining well methanol was used as the control. The inoculated plates were allowed to stand at room temperature for 45 min to allow the diffusion of the extracts into the agar to proceed before growth of the organism commenced. The plates were incubated at 35°C for 24 h. The assessment of antibacterial activity was based on measurement of the zone diameter of the inhibition (ZDI) formed around the well.

The minimum inhibitory concentration (MIC) values of the extracts for the isolates were determined by agar dilution method using Mueller-Hinton agar containing different concentration of extract ranging from 25 to 600 µg/ml. Each of the plant extract mixed agar plates was divided into 20 equal sectors and inoculated with approximately 10^4 cfu per spot; thus for 60 isolates three such plates were needed for a single concentration of each extract. The plates were then incubated at 35°C for 24 h. The MICs were defined as the lowest concentration of the extract at which no visible growth was found; hazy growth and one or two colonies on the spot were ignored.

Bacterial killing studies were carried out using the initial inoculum of approximately 5×10^5 cfu/ml according to the protocol mentioned earlier [16]. The fixed concentration of the extracts used was 500 µg/ml (1× MIC) for each, and the viable cell counts were

determined at 0, 3, 6 and 24 h. The effect of varied concentration of the extracts (25-500 µg/ml) on bacterial density (cfu/ml) was determined after incubating the bacterial suspension (5×10^5 cfu/ml) in fresh Mueller-Hinton broth for 24 h at 35°C. Bactericidal activity was defined as a ≥3 $\log_{10}$ decrease in the inoculum after 24 h of incubation [17].

The χ^2-test was employed to compare bacterial growth (in terms of cfu/ml) in presence and absence of *A. indica* seed and *B. monniera* leaf extracts, and their antibacterial activities at different concentrations and at different time intervals. A p value of 0.005 was considered significant.

Results

The agar well diffusion test results are represented in Figure 1. The *S. enterica* serovar Typhi isolates were found sensitive to the ethanolic extracts of the plants (*A. indica* and *B. monniera*) tested showing ZDI 10-19 mm and 10-18 mm, respectively around wells filled with *A. indica* seed and *B. monniera* leaf extracts. Most of the isolates (40, 60%) had larger ZDI (>15 mm) against *A. indica* while only 19 (31.67%) isolates had ZDI >15 mm against *B. monniera*. Ethanol only showed no zone of inhibition.

The MICs of the extracts of *A. indica* seed and *B. monniera* leaf are represented in Figure 2. The *S. enterica* serovar Typhi isolates showed MICs ranging from 50 µg/ml to 500 µg/ml and 50 µg/ml to 600 µg/ml, respectively, for *A. indica* seed and *B. monniera* leaf extracts. For 8 (13.33%) isolates, the MICs were between 50 and 150 µg/ml of both *A. indica* seed and *B. monniera* leaf extracts. The MICs of *A. indica* seed extract for 41 (68.33%) isolates and those of *B. monniera* leaf extract for 39 (65%) isolates were 200-300 µg/ml and 300-600 µg/ml, respectively.

The activity of varying concentrations of *A. indica* and *B. monniera* extracts against *S. enterica* serovar Typhi is represented in Figure 3. The *S. enterica* serovar Typhi strain did not respond to 25 µg/ml concentration of either the *A. indica* or *B. monniera* extract, and the counts increased from the initial inoculum to 6.04 $\log_{10}$ cfu/ml and 6.11 $\log_{10}$ cfu/ml, respectively. The *A. indica* and *B. monniera* extracts started to show a growth inhibitory effect at concentrations of 50 µg/ml and 100 µg/ml, respectively. Bactericidal activities were found at concentrations of 400 µg/ml of *A. indica* and 450 µg/ml of *B. monniera* extracts, when the initial inoculum was reduced, respectively, to 2.33 $\log_{10}$ cfu/ml and 1.716 $\log_{10}$ cfu/ml, in 24 h. The *A. indica* seed and *B. monniera* leaf extracts at concentrations ≥250 and ≥300 µg/ml, respectively, displayed a significant growth inhibitory effect ($p<0.005$) on *S. enterica* serovar Typhi with respect to the initial inocula used in the experiments. There were no significant differences between antimicrobial activity of *A. indica* seed and *B. monniera* leaf extracts at concentrations >400 and <400 µg/ml; however, a significant difference was observed at concentration 400 µg/ml ($p<0.005$).

Figure 4 represents the killing effect of *A. indica* and *B. monniera* extracts at the concentration of 500 µg/ml on *S. enterica* serovar Typhi; the two extracts reduce the cells from 5×10^5 cfu/ml (5.698 $\log_{10}$ cfu/ml) to 0.00011×10^5 cfu/ml (1.041 $\log_{10}$ cfu/ml) and 0.00048×10^5 cfu/ml (1.68 $\log_{10}$ cfu/ml), respectively, in 24 h of exposure. At the concentration of 500 µg/ml, both *A. indica* and *B. monniera* extracts kill the microorganism very effectively in the first three hours, reducing the population to 0.75857×10^5 cfu/ml (4.88 $\log_{10}$ cfu/ml) and 1.62181×10^5 cfu/ml (5.21 $\log_{10}$ cfu/ml), respectively. At this dose *A. indica* showed bactericidal activity in 6 h and *B. monniera* in 24 h, when the surviving cells were reduced, respectively, to 0.0019×10^5 cfu/ml (2.28 $\log_{10}$ cfu/ml) to 0.00048×10^5 cfu/ml (1.68 $\log_{10}$ cfu/ml). Both the extracts (500 µg/ml) showed significant killing activity ($p<0.005$) on the test microorganisms in different

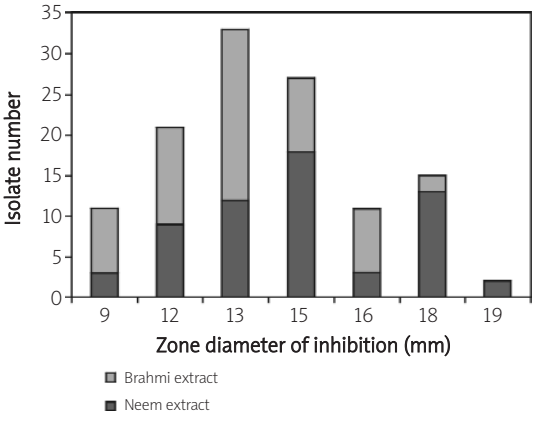

Figure 1. Zone diameter of inhibition of *A. indica* (neem) seed and *B. monniera* (Brahmi) leaf extracts against *S. enterica* serovar Typhi isolates

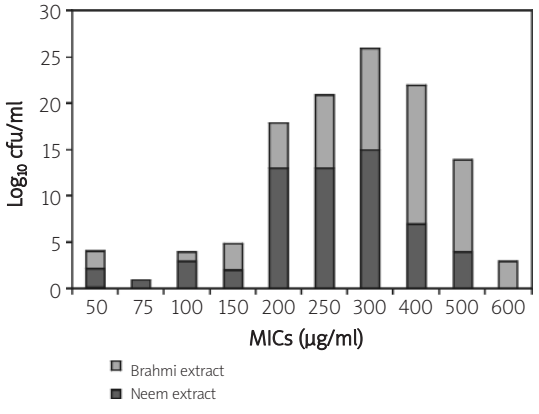

Figure 2. Minimum inhibitory concentration (MIC) values of *A. indica* (neem) seed and *B. monniera* (Brahmi) leaf extracts for *S. enterica* serovar Typhi isolates

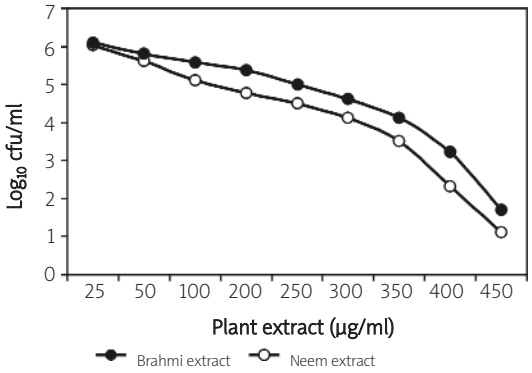

Figure 3. Effect of various concentrations of *A. indica* (neem) seed and *B. monniera* (Brahmi) leaf extracts on growth of *S. enterica* serovar Typhi

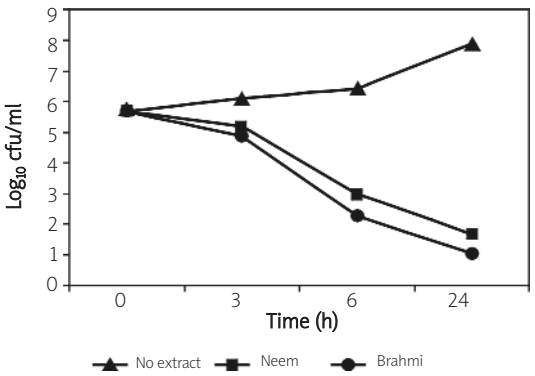

Figure 4. Killing activity of *A. indica* (neem) seed and *B. monniera* (Brahmi) leaf extracts on *S. enterica* serovar Typhi

time periods (3, 6, and 24 h) when compared to the microbial growth (in terms of cfu/ml), in several time changes, in the extract-free broth; however, no significant differences between killing activities of the two extracts were observed in different time periods.

Discussion

For a long period of time, plants have been a valuable source of products to treat a wide range of medical problems, including ailments caused by microbial infection. Numerous studies have been carried out in different parts of the globe to extract plant products for screening antibacterial activity [13, 14, 18-20]. Antibacterial activity of different plant extracts against *S. enterica* serovar Typhi has been reported earlier by several authors who did not include *A. indica* and *B. monniera* in their studies. Gehlot and Bohra reported the antibacterial potential of different arid zone plants against *S. enterica* serovar Typhi [11]. The alcoholic extract of *Semecarpus anacardium* (Bhallatak) showed antibacterial activity in vitro against gram-positive as well as gram-negative strains including *S. enterica* serovar Typhi [21]. The acetone and alcoholic extracts of the leaves of *Cassia alata* showed significant in vitro antibacterial activity against *S. enterica* serovar Typhi [22]. Atata et al. reported antibacterial activity of *E. chlorantha* extract against *S. enterica* serovar Typhi [12].

In the present study, *A. indica* seed and *B. monniera* leaf extracts showed antibacterial activity in vitro against *S. enterica* serovar Typhi, the causative organism of typhoid fever. The ZDI obtained around the wells containing *A. indica* seed extract (9-19 mm) and *B. monniera* (9-18 mm) indicated their more or less similar efficacy against *S. enterica* serovar Typhi isolates. Atata et al. reported 18 mm ZDI using ethanolic extract of *Enantia chlorantha* (20 mg/well) against *S. enterica* serovar Typhi [12]. Zy et al. showed antibacterial activity of sage (*Salvia officinalis*) and parsley (*Petroselinum sativum*) on *S. enterica* serovar Typhi, and they obtained ZDI >10 mm using 1000-4000 µg per well [23]. Such variation in ZDI might be due to variation in active compounds, acting as the antibacterial agents, present in the plant extracts used; the solvent used for the extraction of plant materials, the bacterial species and their source of isolation are the other factors. Banso and Adeymo reported ZDI 8.5-15 mm using *Bacopa* extract at different concentrations (10-30% w/v in ethanol) against clinical isolates of *Ps. aeruginosa*, *Klebsiella pneumoniae* and *E. coli* [24].

Similar to our study, the earlier authors determined the MICs of extracts of different medicinal plants. The extracts from jambolan (*Syzygium cumini*) and clove (*Syzygium aromaticum*) showed activities against several bacterial strains at concentrations ranging from 50 to 500 µg/ml, and from 20 to 250 µg/ml, respectively [15]. Saxena et al. documented MICs of 12.5-1.000 µg/ml when testing *Rhus glaba* extracts on both gram-negative and gram-positive bacteria [25]. MIC of *E. chlorantha* ethanolic and methanolic extracts on *S. enterica* serovar Typhi was 50 mg/ml [12]. Banso and Adeymo recorded ethanolic *Bacopa* extract MICs as 20, 10 and 15% (w/v), respectively for *Ps. aeruginosa*, *K. pneumonia* and *E. coli* [24]. The MICs of the extracts, 50-500 µg/ml for *A. indica* and 50-600 µg/ml for *B. monniera*, as reported in this communication, indicated their strong antibacterial activity on *S. enterica* serovar Typhi. However, it is important and interesting to note that most of the isolates (n=41, 68.33%) showed *A. indica* seed extract MICs of 200-300 µg/ml, while the MICs of *B. monniera* leaf extract for the maximum number of isolates (n=39, 65%) were 300-600 µg/ml.

Banso and Adeymo [24] reported that an increase in the concentration of *Bacopa* extracts showed higher antibacterial activity against gram-negative bacteria (*Ps. aeruginosa*, *K. pneumonia* and *E. coli*), and stated the possibility of the fact that organisms need higher concentration of extracts to inhibit growth or kill them depending on their cell wall

components, and thus antimicrobial substances in the extracts might affect synthesis of the peptidoglycan layer of the cell wall. Okemo et al. reported concentration-dependent killing of *Ps. aeruginosa* and *E. coli* with *A. indica* extracts, and mentioned that the mode of action of *A. indica* extracts is strongly cell wall related [15]. It is interesting to note that for *S. enterica* serovar Typhi, in the present study, the killing was both dosage and time dependent. Higher concentration of *A. indica* seed (400 µg/ml) and *B. monniera* leaf (450 µg/ml) extracts showed bactericidal activity, while in the presence of very low concentration (25 µg/ml) of the *A. indica* and *B. monniera* extracts *S. enterica* serovar Typhi showed luxuriant growth, and the population increased up to 6.04 $\log_{10}$ cfu/ml and 6.11 $\log_{10}$ cfu/ml, respectively. The killing efficacy of *A. indica* extract at concentration 500 µg/ml (1× MIC) was very high in the first 3 h, while the rate of killing with 500 µg/ml (1× MIC) *B. monniera* extract was found high up to 6 h, and respectively after 3 h and 6 h, the activity of the extracts was possibly reduced. *S. enterica* serovar Typhi was tested at MIC (500 µg/ml) of *A. indica* and *B. monniera* extracts and found to act slowly, after showing a high killing rate in 3 and 6 h, on the bacterial population, leaving viable cell counts, 1.041 $\log_{10}$ cfu/ml and 1.68 $\log_{10}$ cfu/ml, respectively, in the presence of *A. indica* and *B. monniera* extracts at 24 h, and thus extracts exhibiting bactericidal activity for *S. enterica* serovar Typhi. Thus data presented in this communication support the view put forward by Banso and Adeymo [24] and Okemo et al. [15] regarding the mode of action of *A. indica* and *B. monniera* extracts.

Conclusions

Enteric fever due to the infection of MDR *S. enterica* serovar Typhi is most difficult to treat with conventional antibiotics, especially in a developing country like India. In our study, the ethanolic *A. indica* seed and *B. monniera* leaf extracts displayed strong anti-*S. enterica* serovar Typhi activity, suggesting the extracts could be potential sources of chemotherapeutic agents for inclusion in anti-*S. enterica* serovar Typhi regimens. However, further investigation to distinguish the components of the extracts and their individual antimicrobial effect is required in order to obtain potential non-antibiotic drugs.

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