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 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 drucei 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 10^8 cfu/ml for agar dilution susceptibility test, 10^7 cfu/ml for well diffusion method and 5 x 10^6 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 10^7 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 10^6 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 x 10^7 cfu/ml according to the protocol mentioned earlier [16]. The fixed concentration of the extracts used was 500 μg/ml (1x MIC) for each, and the viable cell counts were received no scientific research on antibacterial activity of extracts of Bacopa monniera to explore therapeutic effects but which
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 × 10⁵ cfu/ml) in fresh Mueller-Hinton broth for 24 h at 35°C. Bactericidal activity was defined as a ≥3 log₁₀ decrease in the inoculum after 24 h of incubation [17].

The χ² 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 log₁₀ cfu/ml and 6.11 log₁₀ 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 log₁₀ cfu/ml and 1.716 log₁₀ 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 × 10⁵ cfu/ml (5.698 log₁₀ cfu/ml) to 0.00011 × 10⁵ cfu/ml (1.041 log₁₀ cfu/ml) and 0.00048 × 10⁵ cfu/ml (1.68 log₁₀ 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 0.75875 × 10⁵ cfu/ml (4.88 log₁₀ cfu/ml) and 1.62181 × 10⁵ cfu/ml (5.21 log₁₀ 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 × 10⁵ cfu/ml (2.28 log₁₀ cfu/ml) to 0.00048 × 10⁵ cfu/ml (1.68 log₁₀ 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
Neem extract of different arid zone plants against G. coli. Gehlot and Bohra reported the antibacterial potential of two extracts were observed in different time periods. 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 (Bhattatak) 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 12.5-1000 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 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 *P. 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, 1041 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.

**References**