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Potent Bactericidal Action of a Flavonoid Fraction Isolated from the Stem Bark of *Butea frondosa*

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Abstract. *The flavonoid fraction isolated from the ethyl acetate fraction (BF-1) of Butea frondosa (L.) stem bark exhibited distinct antimicrobial activity when tested against 129 bacterial strains belonging to 9 different genera of both gram-positive and gram-negative types. Minimum inhibitory concentration (MIC) of the fraction BF-1 was determined following NCCLS guidelines using the agar dilution method. Twenty-four out of 36 strains of Staphylococcus aureus were inhibited by 50-200 mg/l of the fraction. This fraction also inhibited strains of Bacillus spp., Shigella spp., Salmonella spp. and even a few Pseudomonas at concentrations between 50-200 mg/l. Other bacteria including Escherichia coli, Vibrio cholerae and V. parahaemolyticus were moderately sensitive to BF-1. In the in vivo studies, this fraction offered significant protection to Swiss albino mice at a concentration of 80 μg/mouse ($p<0.001$) when they were challenged with 50 median lethal dose of Salmonella enteritidis NCTC 74. A fraction named BF-1 that was isolated from an ethyl acetate fraction of Butea frondosa provided protection against an infection from a Salmonella enteritidis NCTC strain.*

Natural products obtained from different types of plants have made an important impact since ancient times in the recognition and discovery of new therapeutic compounds, for example, quinine obtained from cinchona bark has been used for centuries in different parts of the world to successfully treat malaria. Since 1950, the study of ethnobotany has intensified in many countries, including India. Of 45,000 plant species available in India, medicinal properties have been assigned to several thousand. Hence, it may be useful to look for new drugs from plant sources for diseases where modern drugs are either unavailable or unsatisfactory. Isoflavonoids and phenolic compounds extracted from the plant *Sophora* (Family *Fabaceae*) have been reported to possess antimicrobial property; one of the compounds turned out to be so potent that it could be compared with a sulfonamide (1). Similar compounds were obtained from *Sophora* and *Euchresta* spp. of *Leguminosae* which also proved to be equally powerful antimicrobials (2). The plant *Butea frondosa* is a medium-sized tree growing widely in India and Burma. The leaves, flowers, seeds, barks, roots and stems of this plant have been used for various ailments by many tribes in India. The present study describes the antimicrobial property of a flavonoid fraction obtained from the stem bark of this plant.

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Materials and Methods

Plant materials. The stem barks of *B. frondosa* were collected in September 2005 from the forest region of Orissa, India. The collected bark, along with a complete herbarium of the flowering aerial parts of the plant, was sent for identification and was finally authenticated by the Central National Herbarium, Botanical Survey of India, Howrah, India.

Extraction and isolation. The stem bark was dried in an incubator at 40˚C for 2 days, then crushed in a mechanical grinder to a fine powder of mesh 40. The powder (500 g) was then completely extracted with 2.5 l of 50% ethanol in a Soxhlet apparatus at 65˚C. The resulting extract was filtered and concentrated to half of its volume under vacuum (10 mm of Hg) and at a temperature of 90˚C. The resultant liquid was then extracted with *n*-hexane by shaking gently in a separating funnel (×3). The combined *n*-hexane fractions were evaporated to dryness using a rotary vacuum evaporator. The remaining hydro-alcoholic part was then extracted with ethyl acetate (×3) in a separating funnel. The combined ethyl acetate fraction was evaporated to dryness using a rotary vacuum evaporator. The dried *n*-hexane fraction (BF-1) was yellow in colour while the dried ethyl acetate fraction (BF-2) was sticky reddish-brown in colour. The residual hydro-alcoholic fraction was also evaporated to dryness and labeled as BF-3.

Table I. *Antibacterial in vitro activity of BF-1.*

Bacteria	Number of strains tested	Minimum inhibitory concentration (mg/l) (Percentage of total strains)				
		50	100	200	400	>400
Staphylococcus aureus	36	2 (5.5%)	12 (33.3%)	10 (27.8%)	10 (27.8%)	2 (5.5%)
Bacillus spp.	6	-	6 (100%)	-	-	-
Escherichia coli	8	-	2 (25%)	-	2 (25%)	4 (50%)
Salmonella spp.	12	1 (8.3%)	4 (33.3%)	4 (33.3%)	3 (25%)	-
Shigella spp.	14	1 (7.1%)	4 (28.6%)	8 (57.1%)	1 (7.1%)	-
Klebsiella spp.	5	-	-	-	-	5 (100%)
Proteus spp.	5	-	-	-	-	5 (100%)
Pseudomonas spp.	10	-	1 (10%)	2 (20%)	2 (20%)	5 (50%)
Vibrio cholerae	25	5 (20%)	9 (36%)	7 (28%)	4 (16%)	-
Vibrio parahaemolyticus	8	-	1 (12.5%)	5 (62.5%)	2 (25%)	-
Total	129	9 (6.98%)	39 (30.2%)	36 (27.9%)	24 (18.6%)	21 (16.3%)

Bacteria. A total of 129 clinical strains of pathogenic bacteria belonging to different gram-positive and gram-negative genera, whose origins are from collections maintained by Professor Dastidar, were employed in this study (Table I). When pertinent, their susceptibility to common antibiotics was determined by Bauer-Kirby test.

Media. Liquid media were prepared from powder: nutrient broth (NB; Oxoid, England, UK); peptone water (PW; 1.0% bacteriological peptone, Oxoid, plus 0.5% Analar NaCl). Solid media were prepared from powder: nutrient agar (NA; Oxoid). The pH of all media was adjusted to 7.2-7.4.

Detection of flavonoids in the BF1-3 fractions. The qualitative method described by Kim *et al*. (3) was strictly followed. To 1 ml aliquot of 1.0% sample solution, 4 ml of double distilled water and 0.3 ml of 5% $NaNO_2$ were added. After 5 minutes, 0.3 ml of 10% $AlCl_3$ was added; after 1 minute 2 ml of 1 M NaOH were added and the reaction mixture was made up to 10 ml with the addition of 2.4 ml of double distilled water. Formation of a pink colour in the mixture within 5 minutes indicated the presence of flavonoids.

Detection of antibacterial activity by in vitro testing. A total of 1600 μg of compound BF-1 was dissolved in 40 ml of nutrient agar maintained at 50˚C, mixed thoroughly and pH adjusted to 7.2-7.4, and two-fold serial dilutions with nutrient agar were made to yield final concentrations of 0 (control), 50, 100, 200, 400 and 800 mg/l and poured onto individual sterile Petri dishes. Gram-positive bacteria were grown in nutrient broth and gram-negative in peptone water at 37˚C for 18 hours. From these cultures, a 2 mm loopful containing approximately 105 colony forming units (CFU) (4) was streaked onto the nutrient agar plates containing different concentrations of BF-1, incubated at 37˚C up to 72 hours for determination of the minimum inhibitory concentration (MIC) of BF-1.

Bactericidal/Bacteriostatic action of BF-1. The highly sensitive bacteria *Shigella dysenteriae* (7) was grown overnight in nutrient broth and an aliquot of 2 ml was added to 4 ml of fresh medium and incubated up to 2 hours to attain the logarithmic growth phase of the culture. From this culture, a 2 mm loopful was streaked onto plates containing different concentrations of BF-1 that equaled or exceeded the previously determined MIC for this fraction. At zero time and intervals of 2 and 18 hours, aliquots were removed from each culture and processed for CFU counting.

Evaluation of the effect of BF-1 on Salmonella-infected mice. Two hundred Swiss white male mice from colony maintained by Professor Dastidar weighing 18-20 g were used for the animal experiments. Animals were maintained at 21±1˚C and 50-60% relative humidity with a photoperiod of 14:10 hours of light:darkness. Water and a dry pellet diet were supplied *ad libitum*. Mortality experiments in these mice with or without BF-1 were carried out by challenging them with 50 median lethal dose (MLD) of a virulent strain of *Salmonella enteritidis* NCTC 74, corresponding to 0.95×10^9 CFU in 0.5 ml nutrient broth (5). Reproducibility of the challenge dose was ensured by determining the optical density in a Klett Summerson colorimeter at 640 nanometers.

The protective effect of BF-1 was determined as follows: 0.1 ml of BF-1 was injected intraperitoneally at a dose of 0.0, 40 and 80 μg per mouse (body weight 18 to 20 grams) 3 hours before a challenge with the determined lethal dose of *S. enteritidis* NCTC 74. The number of mice of the control and BF-1-treated groups was 60, and 20 and 20 respectively. All the mice were sacrificed after 18 hours and their livers and spleens removed aseptically, homogenized in 5 ml of sterile phosphate-buffered saline in a glass tissue homogenizer and aliquots removed and plated for CFU counts. Serum was prepared from blood contained in the heart and processed for CFU counts. This serum was also used for the bioactivity of BF-1 as follows: Millipore filter paper discs of 6 mm diameter, 3 mm thickness, absorbing 0.03 ml were soaked in diluted serum (1/10) and placed on top of agar that was streaked with a 2 mm loopful of an 18-hour broth culture of *S. enteritidis* NCTC 74. The concentration of BF-1 in the sera was deduced by referring to a standard calibration curve prepared with known concentrations of BF-1 and their effects on *S. enteritidis* NCTC determined by CFU counting.

Table II. *Protection of BF-1 afforded to mice receiving a challenge dose of 0.95×10^9 CFU of Salmonella enteritidis NCTC 74 in 0.5 ml of NB.*

	Group	Agent injected per mouse	Number of dead mice after 100 h
Non-treated group	Control (N=60)	0.1 ml sterile saline solution	49
Groups treated with BF-1	Group I (N=20)	40 μg BF-1	8*
	Group II (N=20)	80 μg BF-1	0**

Note. The results presented were obtained from a single experiment involving 100 mice. Other mouse infection studies were conducted during the standardization of the infection and determination of effective BF-1 dose.*$p<0.05$, **$p<0.001$ (χ^2 test).

Table III. *In vitro activity of serum obtained from blood and homogenates of liver and spleen of mice treated for Salmonella enteritidis NCTC 74.*

Group[a]	Treatment	CFU/ml[b]		
		Serum	Homogenate	
		Heart blood	Liver	Spleen
Control	0.1 ml of a sterile saline solution	2.0×10^8 to 6.3×10^8	5.8×10^7 to 9.5×10^8	1.5×10^7 to 9.2×10^8
II	80 μg of BF-1	1.5×10^4 to 7.8×10^5	1.6×10^3 to 3.5×10^4	2.2×10^3 to 5.0×10^4

[a]Each group consisted of 5 mice. [b]CFU counts between the two groups significant; $p<0.01$ in 18-hour samples (Student's *t*-test).

Results

Detection of flavonoids in BF-1. The presence of flavonoids was confirmed only in BF-1 following the procedures described in the Material and Methods.

Determination of MIC of BF-1. The *in vitro* inhibitory activity of BF-1 against a large number of species and strains is summarized in Table I. Briefly, BF-1 had activity against 20% of the strains of *Vibrio cholerae* at a concentration as low as 50 mg/l. At a concentration of 100 mg/l, activity against 100% of *Bacillus* spp. and 33% of *Staphylococcus aureus* strains was evident. With respect to *S. enteritidis* NCTC 74, the infective agent employed in this study, the activity of BF-1 against strains of this bacterium varied (MIC for the bulk of the strains is 200 to 400 mg/l). However, it should be noted that BF-1 had an MIC of 100 mg/l against the strain used for infecting mice (data not shown). Unlike most of the bacterial species shown in Table I, the activity of BF-1 against *Klebsiella* spp. and *Proteus* spp. took place with a minimum concentration of 400 mg/l and hence these species are relatively resistant to this fraction.

Bactericidal activity of BF-1. The bactericidal activity of BF-1 against the species presented in Table I was close to that of the respective MIC (data not shown).

Animal studies. The lethality of an infection with *S. enteritidis* NCTC 74 is shown in Table II. In the control group (non-treated animals), 49 of the 60 mice died within 100 hours after being infected. In contrast to this lethality, 12 out of the 20 mice that had been pre-treated with 40 μg of BF-1 survived infection and 100% survived when they were pre-treated with 80 μg of BF-1.

Demonstration of the presence of BF-1 in the serum of mice treated with 80 μg of BF-1. The antimicrobial activity of serum obtained from the blood that was removed from the hearts of control and pre-treated BF-1 mice, as well as that of their liver and spleen homogenates, was deduced from a standard curve that plotted the effects of different dilutions of BF-1 against the number of CFU of *S. enteritidis* NCTC 74 as well as by the direct effects of the serum and homogenates on the bacterium. Briefly, as evident from Table III, serum and organ homogenates obtained after 18 hours from mice receiving BF-1 reduced the number of CFU of *S. enteritidis* NCTC 74 by at least 3 to 4 logs as opposed to that produced by controls that received the saline blank. The calculation of the concentration of BF-1 in serum and homogenates slightly exceeded 80 mg/l (data not shown).

Discussion

New antibiotics are steadily being synthesized in the pharmaceutical industry and also in academic institutions, but the problem of drug resistance has remained unchanged among bacterial pathogens. The search for antimicrobials has now been extended to a class of compounds named "non-antibiotics" which are employed for the therapy of non-infectious pathology and which demonstrate significant antimicrobial activity against

some of the most pathogenic infectious agents such as vancomycin resistant/methicillin resistant *S. aureus* (12), multi-drug resistant *Mycobacterium tuberculosis* (13, 14), *etc*. (15). These and many other studies (16) have shown that among such antimicrobials are phenothiazines and many other compounds that possess at least 2 benzene rings (6-11). Earlier studies on the antimicrobial action of flavonoids also indicated that prenylflavonones and isoflavones possessing 2 benzene rings exhibited powerful antibacterial activity (1-2). The flavonoid fraction BF-1 was seen to possess powerful antibacterial activity *in vitro* as well as *in vivo*. While sensitive bacterial strains occurred among *S. aureus*, *Bacillus* spp. and *Vibrio* spp., against *Escherichia coli*, *Shigella*, *Salmonella* and *Pseudomonas*, BF-1 was only moderately active. *Klebsiella* spp. and *Proteus* spp. were found to be resistant.

The results of our study show that a single dose of 80 mg/l of BF-1 protected the mouse from a lethal infection by *S. enteritidis* NCTC 74. Although this dose corresponded well with that deduced from blood and homogenates that significantly reduced the number of CFU of *S. enteritidis* NCTC 74, the actual concentration of BF-1 in the mouse, assuming that the weight of the mouse, roughly 20 grams, is equal to a 20 ml volume of liquid, would be approximately 4 mg/l and hence far lower than the MIC of BF-1 (100 mg/l). How did this low concentration result in the elimination of the *Salmonella* from the infected animal inasmuch as this concentration is 25 times lower than the MIC? Because our previous studies demonstrated that macrophages concentrate phenothiazines to a level that kills intracellular bacteria (12), it appears reasonable to suspect that BF-1 is concentrated by neutrophils, the main phagocytic killing cells of the mouse, to levels that equal its bactericidal activity. However, because some of our previous studies demonstrated that extracts from plants induce T-cells to secrete lymphokines that promote recruitment of killer T-cells (17), the BF-1 fraction may provide similar properties. Nevertheless, further studies are in progress to completely identify compound(s) present in the flavonoid fraction as well as to determine any synergism with antibiotics, antimicrobials and non-antibiotics.

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References

1 Dastidar SG, Mahapatra SK, Ganguli K, Chakrabarty AN, Shirataki Y and Motohashi N: Antimicrobial activity of prenylflavonones. In Vivo *15*: 519-524, 2001.

2 Dastidar SG, Manna A, Asok Kumar A, Mazumdar K, Dutta NK, Chakrabarty AN, Motohashi N and Shirataki Y: Studies on the antibacterial potentiality of isoflavones. Int J Antimicrob Agents *23*: 99-102, 2004.

3 Kim D, Jeong SW and Lee CY: Antioxidant capacity of phenolic phytochemicals from various cultivars of plums. Food Chem *81*: 321-326, 2003.

4 McFarland J: Nephelometer: an instrument for estimating the number of bacteria in suspensions used for calculating the opsonic index and for vaccines. J Am Med Assoc *49*: 176-178, 1997.

5 Reed LJ and Muench H: A simple method of estimating fifty percent end points. Am J Hygiene *27*: 493-497, 1938.

6 Molnar J, Mandi Y and Kiraly J: Antibacterial effect of some phenothiazine compounds and the R-factor elimination by chlorpromazine. Acta Microbiol Acad Sci Hung *23*: 45-54, 1976.

7 Chattopadhyay D, Dastidar SG and Chakraborty AN: Antimicrobial property of methdilazine and its synergism with antibiotics and some chemotherapeutic agents. Arzneim-Forsch/Drug Res (FRG) *38*: 869-872. 1988.

8 Kristiansen JE: The antimicrobial activity of non-antibiotics. Acta Path Microbiol Scand *100*: 45-54, 1992.

9 Dastidar SG, Ganguly K, Chaudhury K and Chakraborty AN: The anti-bacterial action of diclofenac shown by inhibition of DNA synthesis. Int J Antimicrob Agents *14*: 249-251, 2000.

10 Dasgupta A, Jeyaseeli L, Dutta NK, Mazumdar K, Karak P, Dastidar SG, Motohashi N and Shirataki Y: Studies on the antimicrobial potential of the cardiovascular drug lacidipine. In Vivo *21*: 847-850, 2007.

11 Dasgupta A, Dastidar SG, Shirataki Y and Motohashi N: Antibacterial activity of artificial phenothiazines and isoflavones from plants. *In*: Heterocyclic Compounds. N Motohashi (ed.). Berlin, Springer-Verlag, 2008.

12 Martins M, Bleiss W, Marko A, Ordway D, Viveiros M, Leandro C, Pacheco T, Molnar J, Kristiansen JE and Amaral L: Clinical concentrations of thioridazine enhance the killing of intracellular methicillin-resistant *Staphylococcus aureus*: an *in vivo*, *ex vivo* and electron microscopy study. In Vivo *18*: 787-794, 2004.

13 Ordway D, Viveiros M, Leandro C, Bettencourt R, Almeida J, Martins M, Kristiansen JE, Molnar J, and Amaral L: Clinical concentrations of thioridazine kill intracellular multidrug-resistant *Mycobacterium tuberculosis*. Antimicrob Agents Chemother *47*: 917-922, 2003.

14 Amaral L, Martins M, and Viveiros M: Phenothiazines as anti-multi-drug resistant tubercular agents. Infect Disord Drug Targets *7*: 257-265, 2007.

15 Martins, M, Dastidar SG, Fanning S, Kristiansen JE, Molnar J, Pagès JM, Schelz Z, Spengler G, Viveiros M, and Amaral L: Potential role of non-antibiotics (helper compounds) in the treatment of multidrug-resistant Gram-negative infections: mechanisms for their direct and indirect activities. Int J Antimicrob Agents *31*: 198-208, 2008.

16 Kristiansen JE and Amaral L: The potential management of resistant infections with non-antibiotics. J Antimicrob Chemother *40*: 319-327, 1997.

17 Ordway D, Hohmann J, Viveiros M, Viveiros A, Molnar J, Leandro C, Arroz MJ, Gracio MA and Amaral L: *Carpobrotus edulis* methanol extract inhibits the MDR efflux pumps, enhances killing of phagocytosed *S. aureus* and promotes immune modulation. Phytother Res *17*: 512-519, 2003.

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