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Oriental Pharmacy and Experimental Medicine 2008 **8(4)**, 339-348
DOI 10.3742/OPEM.2008.8.4.339

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Anti-*Salmonella* activity of a flavonone from *Butea frondosa* bark in mice

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Received for publication July 30, 2007; accepted January 21, 2008

SUMMARY

Butea frondosa has been used traditionally as a topical formulation in the treatment of many diseases and disorders. Two compounds [BF-1 (crystalline flavonol quercetin) and BF-2 (tannin) from ethyl acetate fraction of ethanolic extract] were isolated from the bark of *Butea frondosa*. The stereostructures of the compounds were determined on the basis of chemical and physico-chemical evidence. BF-1 and BF-2 were screened *in vitro* for possible antibacterial property against 112 bacteria comprising 3 genera of Gram-positive and 12 genera of Gram-negative types. It was found that both BF-1 and BF-2 exhibited inhibitory activity against several bacteria. Most of these strains were inhibited by BF-1 at 50 - 200 μg/ml, while BF-2 (MIC_{50} 400 μg/ml) was much less active. The bacteria could be arranged in the decreasing order of sensitivity towards BF-1 in the following manner: *S. aureus*, *Bacillus* spp., *Salmonella* spp., *Vibrio* spp., *Shigella* spp., *E. coli* and *Pseudomonas* spp. The MIC_{50} of the compound was 50 μg/ml while the MIC_{90} was 100 μg/ml. The decreasing order of sensitivity towards BF-2 was *V. cholerae*, *Bacillus* spp., *S. aureus*, *V. parahaemolyticus*, *Salmonella* spp. and *Proteus* spp. BF-1 was bactericidal in action. *In vivo* studies with this extract showed that it could offer statistically significant protection ($p < 0.01$) to mice challenged with a virulent bacterium. The inhibitory activity of *Butea frondosa* against Gram-positive and Gram-negative bacteria indicates its usefulness in the treatment of common bacterial infections. The potentiality of BF-1 as an antibacterial agent may be confirmed further by pharmacological studies.

Keywords: *Butea frondosa*; ethyl acetate fraction; quercetin; antimicrobial activity; *in vitro*; *in vivo*

INTRODUCTION

Chemotherapeutics and antibiotics have offered effective therapies for the prevention and treatment of many diseases caused by several species of microorganisms for the past several decades. These achievements, however, have also posed the inevitable threat of acquired bacterial drug resistance. Over the last few years, both the frequency and spectrum of multi-resistant species of pathogenic bacteria have noticeably increased (Cohen, 1992; Neu, 1992). Even today, there are species of *Streptococcus, Staphylococcus, Pseudomonas, Enterococcus* and *Mycobacterium* that

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have developed resistance to all existing antibiotic therapies (Davies, 1994, 1996; Nikaido, 1994; Spratt, 1994). Unfortunately, few new antimicrobial drugs are reported to be under active development (Travis, 1994). Under such intimidating circumstances, there is an urgent need to be pursued to meet the upcoming challenges that are being caused by the emergence of multidrug resistance in bacteria.

The use of plant-based therapeutics by man is as old as the history of man himself. Even today, many scholars are of the view that traditional medical systems based on plant products continue to lead in many human groups. It is useless to mention that nearly three-fourth of modern medicines have plant constituents in them in some form. In Ayurveda, about 2000 plant species have been considered to possess medicinal value. Ayurveda, or the science of life, is the holistic alternative science from Asia and is believed to be the oldest healing science in existence, forming the foundation of all others. Asia has a rich resource of medicinal plants that are widely used in the traditional system of medicines since inception of human culture.

The plant kingdom, holding many species of plants, is constantly being screened for its pharmacological properties and new plant drugs are continuing to find their way into the medical world.

There are other important reasons to undertake research on medicinal plants. They include documentation and conservation of the deteriorating traditional medical system, as most of these practices are transmitted by word of mouth. Provision of scientific background in support of folk curative practices and identification of a cheap drug of plant origin with least side effect and maximum therapeutic potential are essential. Finally, efforts should be made to elucidate the mechanism of action of plant-based drugs in animal models.

Butea frondosa Koenigex Roxb. (Palas), a member of the family Fabaceae/Leguminosae, may be one such example of the indigenous system of medicines. The plant encompasses a wide range of therapeutic activities, some of which have been discovered and put into practice successfully. The various parts used in traditional medicine are gum, seeds, flowers, bark and leaves of the plant. The leaves and flowers of *B. frondosa* are used as astringent, tonic, aphrodisiac, depurative, diuretic and poultice. The seeds of the plant are used as laxative and anthelmintic. The gums of *B. frondosa* are traditionally used for their astringent, antidiarrhoeal, antidysenteric and anthelmintic properties (Kaleysa *et al*., 1962) and also in the treatment of septic sore throat and phthisis (Kirtikar and Basu, 1975; Nadkarni, 1976; Lal *et al*., 1976). *Butea frondosa* is reported to be associated with hepatoprotective (Rane and Grampurohit, 1998), antiestrogenic properties (Kapila *et al*., 1970; Razdan *et al*., 1970; Shah *et al*., 1990; Anonymous, 1998; Bhatwadekar *et al*., 1999). Unfortunately, this particular plant has not been extensively explored. The present study has been undertaken to investigate the antimicrobial activity of the stem bark of this immensely useful plant. The bark of the tree was obtained and dried following standard extraction procedures and tested for antibacterial activity. A detailed chemical characterization was accompanied with an extensive *in vitro* and *in vivo* analysis.

Butea frondosa bark contains many constituents. In this study, we report for the first time the presence of quercetin (flavonone) as one of the constituents. There are reports on the antimicrobial activity of quercetin. However, this study aims to investigate its broad-spectrum efficacy and its ability to protect the mouse from an infection caused by a highly virulent strain of *Salmonella typhimurium*. *S. typhimurium* causes mild gastroenteritis in humans but is lethal in mice. Thus, infection of mice with *S. typhimurium* provides a murine model for typhoid fever as it bears many similarities to human serovar typhi infection. We have already established the characteristics of gastritis caused by *Salmonella typhimurium* (intraperitoneal LD_{50} - 0.95×10^9) in mice and determined the changes of histopathological lesions and bacterial colonization

in mice stomachs with a time course of infection (Dastidar *et al*., 2001, 2004).

MATERIALS AND METHODS

Plant material

The bark of *Butea frondosa* (Family Fabaceae/ Leguminosae) was collected from forest region (Balasore) of Orissa, India. The plant part was authenticated by Central National Herbarium, Botanical Survey of India, Botanical Gardens, Howrah, India. This tree, growing up to 12 - 15 m, is irregularly branched, with a rough grey bark. Leaves are large with petioles 10 - 15 cm long, stipules linear-lanceolate, deciduous, leaflets coriaceous, all obtuse, glabrous above when old, silky and prominently reticulately veined below. Flowers are large, bright orange, in rigid racemes 15 cm long, 3 flowers arising together. Calyx is 13 mm long, dark green; corolla 3 - 3.5 cm long (Tandon and Shivanna, 2003).

Compounds

The bark of *Butea frondosa* was ground by electric grinder and screened through 1.4 mm mesh sieve. The extraction in 98% ethanol was carried out with the extract to solvent ratio 1 : 10 for a period of 20 h with intermittent stirring. The extract was evaporated under vacuum at 50 °C to approximately one-tenth of its original volume, purified by liquid-liquid extraction method with n-hexane, ethyl acetate and distilled water and stored at 4 °C. The color of dried n-hexane and ethyl acetate fractions were yellowish and reddish sticky, respectively.

The physical characteristics (color, nature, solubility, Rf values and melting point or decomposition temperature) of the isolated compounds and their chemical reactions with different reagents were determined with the help of standard methods (Trease and Evans, 1985; Wallis, 1985; Plummer, 1985). The isolation and purification of the compounds were performed by TLC [5 × 20 cm size glass plate, 20% suspension of silica gel G (E-Merck), 0.25 mm layer thickness of spreader, activated at 1100 °C for 30 min, solvent system chloroform : methanol (1 : 1), visualizing agent iodine vapor and UV light (366 nm)].

Column chromatography was done [absorbent, silica gel for column (60 - 120 mesh), grade (E-Merck), mobile phase; chloroform: methanol (1 : 1), quantity of each fraction collected - 20 ml; glass column size -60 × 30 mm, number of column elutes from columns 1 to 23 (ethyl acetate fraction) and 1 to 8 (n-hexane fraction)]. It was further subjected to chemical tests and confirmation of Rf values, UV-visible spectra [Systronics double beam spectrophotometer-2101, Absorption maxima (λ_{max} nm) 257 to 356], IR spectrum and H-NMR spectrum. Elemental analysis and mixed melting point determination were also performed (Markham, 1982; Breitmaier, 1993). All compounds were stored at 4 °C.

Quercetin (BF-1) and Tannin (BF-2)

Ethyl acetate fraction 10 - 12 yielded a deep yellow sticky mass, which was washed successively with excess amount of petroleum ether to remove the sticky matter and re-crystallized with the help of dilute alcohol to obtain a light yellow crystalline powder. This product was named as BF-1. Ethyl acetate fraction 16 - 19 yielded a reddish brown sticky mass, which was successively washed in petroleum ether to remove the sticky matter and re-crystallized with acetone (irregular fragments). This product was named as BF-2. Melting point of BF-1 was 309 °C to 311 °C, yield was 0.07%, Rf value was found to be 0.97 (chloroform : methanol = 1 : 1). Elemental analysis calculated for $C_{15}H_{10}O_7$ was C 59.51; H 3.30, found were C 59.60; H 3.32. Melting point of BF-2 was 118 °C to 120 °C (decomposed). Yield was 0.29% and Rf value was 0.72 (chloroform : methanol = 1 : 1). The UV-visible spectra, H-NMR and IR spectra of the isolated compounds were determined (data not presented). From all the furnished experimental evidences on mixed melting point determination with the authentic sample of quercetin, it was concluded that the new isolated crystalline

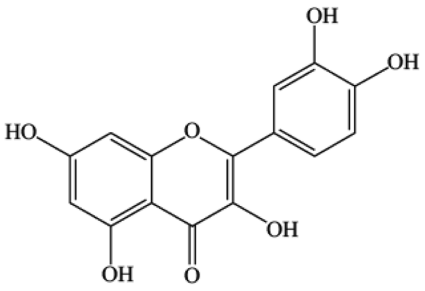

Fig. 1. Structure of BF-1.

compound BF-1 is a flavonoid compound quercetin. BF-2 is found to be tannin. The structure of BF-1 has been described in Fig. 1.

Bacteria

A total of 112 strains of bacteria belonging to 15 different genera comprising 3 Gram-positive and 12 Gram-negative types were tested. These were clinical isolates of human origin, identified as described by Barrow and Feltham (1993) and preserved in freeze-dried state.

Media for antibacterial screening studies

Liquid media used were peptone water [PW, bacteriological peptone (Oxoid) 1% (w/v) plus NaCl (Analar) 0.5% (w/v)] and nutrient broth (NB, Oxoid). Solid media were Mueller–Hinton agar (MHA, Oxoid), nutrient agar (NA, Oxoid) and desoxycholate citrate agar (DCA, Oxoid). MHA was supplemented with 5% defibrinated sheep blood where necessary. The pH was maintained at 7.2 - 7.4 for all the media. NA was used for tests with Gram-positive bacteria and DCA was used for the rest of the bacteria, particularly *Proteus* spp. *Salmonella* strains were grown in Brain Heart infusion (BHI, Difco).

Determination of Minimum Inhibitory Concentration (MIC) of the compounds BF-1 and BF-2

The MIC of the compounds with respect to different test bacteria was accurately determined by agar dilution method. For this, the compounds were dissolved in dimethyl sulfoxide (DMSO), and added at concentrations of 0 (control), 10, 25, 50, 100 and 200 μg/ml in molten NA/DCA/MHA and poured in For *Salmonella* strains, broth dilution method was employed. Final inocula of 5×10 Petridishes, according to NCCLS guidelines (NCCLS, 1993; Koneman *et al*., 1997). The organisms were grown in respective liquid media, and the overnight cultures were spot-inoculated on the Petridishes, such that each inoculum contained 2×10^5 CFU (colony forming units). The plates were incubated at 37 °C, examined after 24 h and incubated further for 72 h, where necessary. The lowest concentration of the compound in a plate that failed to show any visible macroscopic growth was considered as its MIC. The MIC determination was performed in triplicate for each organism, and the experiment was repeated where necessary. For *Salmonella* strains, broth dilution method was employed. Final inocula of 5×10^5 to 1×10^6 CFU/ml were prepared by diluting overnight cultures in fresh broth. MIC was determined by spectrophotometric method after 24 h of incubation at 37 °C (Washington and Sutter, 1980). The antimicrobial efficacy of BF1 was compared with that of ampicillin.

Determination of the mode of action of BF-1 on *Salmonella typhimurium* NCTC 74

Salmonella typhimurium NCTC 74 was grown in BHI overnight at 37 °C. From this culture, 2 ml was added to 4 ml of fresh BHI and incubated for 2 h so that the culture could attain the logarithmic growth phase. The number of viable cells (CFU) was then determined and BF-1 was added at a concentration higher than its MIC value (50 μg/ml) with respect to the test bacterium. The CFU counts were determined up to 6 h at intervals of 2 h and then after 18 h. (Krogstad and Moellering, 1990).

***In vivo* tests**

In vivo experiments were performed following our institutional guidelines for the care and use of laboratory animals.

Swiss strain of male white mice, each weighing

20 g, was used for the *in vivo* studies. Animals were maintained at standard conditions at 21 ± 1 °C and 50 - 60% relative humidity, with a photoperiod of 14 : 10 h of light-darkness. Water and a dry pellet diet were given *ad libitum*. The virulence of the test strain *Salmonella typhimurium* NCTC 74 was exalted by repeated mouse passage and the median lethal dose (MLD or LD_{50}) of the passaged strain (corresponding to 0.95×10^9 CFU/mouse suspended in 0.5 ml BHI) served as the challenge dose for all animals (Reed and Muench, 1938). Reproducibility of the challenge dose was ensured by standardization of its optical density in a Klett-Summerson colorimeter at 640 nm.

To determine the toxicity of BF-1, 40 mice were taken, 20 of which were injected 60 μg of the compound and the rest 20 received 30 μg of BF-1. They were kept under observation up to 100 h. Subsequently, two separate groups of mice, 20 animals per group, were kept in different cages. Each animal in Group I was intraperitoneally administered 30 μg BF-1 (0.1 ml from 300 μg/ml solution of BF-1) and Group II was given 60 μg of BF-1 per mouse (0.1 ml from 600 μg/ml solution of BF-1). After 3 h, each group was challenged with 50 MLD of *S. typhimurium* NCTC 74. A control group of 40 mice was also injected similarly with the same bacterial strain, and 0.1 ml sterile saline instead of BF-1. The protective capacity of BF-1 was determined by recording the mortality of the mice in different groups up to 100 h of the treatment, and statistically by χ^2 test.

In another experiment, 4 groups of mice, 5 animals per group, were taken. Groups 1 and 3 were administered 60 μg of BF-1, while groups 2 and 4 were given 0.1 ml sterile saline. After 3 h, all the groups were given a 50 MLD challenge of *S. typhimurium* NCTC 74. After 2 h, groups 1 and 2 were sacrificed. Their heart blood was collected aseptically; their livers and spleens were removed and homogenized in tissue homogenizers. CFU counts of the individual organs were determined separately. The same procedure was applied on groups 3 and 4, 18 h after the challenge. Statistical analysis of the *in vivo* data was done by Student's *t*-test.

Histopathological examination

Liver and spleen samples were fixed in 10% neutral formalin for 24 h. Formalin-fixed tissues were dehydrated in an alcohol-xylene series and embedded in paraffin wax. From each block, sections 2 μm thick were prepared and stained with haematoxylin and eosin (HE) for histopathological examination. Changes in the experimental histopathologic parameters for liver and spleen tissues were graded as follows: (-) showing no changes, and (+), (++) and (+++) indicating minimum, moderate and maximum changes, respectively. These values were considered to be non-parametric, and therefore the data was statistically analyzed in order to determine the effects of all groups on each of the experimental parameters, and compare the means of each parameter between the groups. A *p*-value of < 0.05 was considered significant.

RESULTS

Bacterial inhibitory spectra of BF-1 and BF-2

The isolated components of *B. frondosa* were active against both Gram-positive and Gram-negative bacteria. BF-1 was found to be inhibitory against all the bacteria screened, at different concentrations. Out of 112 bacteria, the MIC of BF-1 was 10 - 50 μg/ml for 86. Strains of *Bacillus* spp., *Staphylococcus aureus*, *Salmonella* spp., *Klebsiella* spp. and *Vibrio* spp. were inhibited by BF-1 within 100 μg/ml, while those of *Proteus* spp., *Pseudomonas* spp. and *Enterobacter cloaca* within 200 μg/ml (Fig. 2). The MIC_{50} of the compound was 50 μg/ml, while the MIC_{90} was 100 μg/ml (Table 1).

BF-2 was active at higher concentrations (MIC range from 200 - 1000 μg/ml, MIC_{50} was 400 μg/ml).

Ampicillin was used against 8 strains of Salmonella. The MIC_{50} and MIC_{90} were 8 and -16 μg/ml respectively.

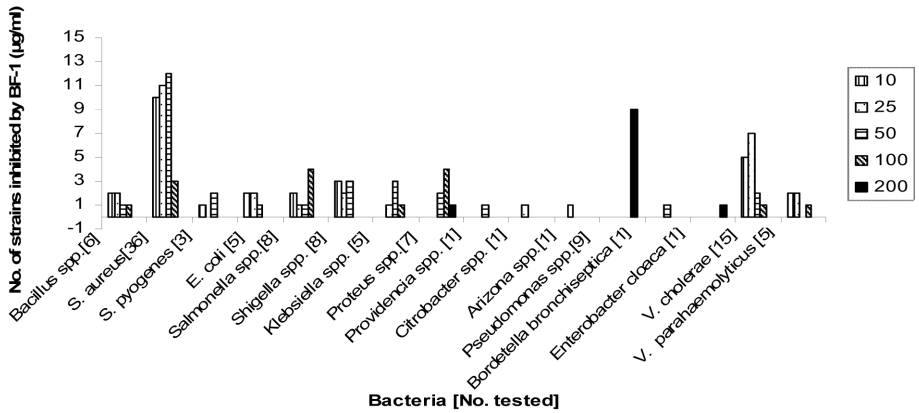

Fig. 2. Antibacterial spectrum of BF-1 in a dose dependent manner.

Table 1. *In vitro* activity of BF-1 tested against a varity of Gram positive and Gram negative bacteria

Bacteria [No of tested]	MIC (μg/ml)		
	Range	MIC_{50}	MIC_{90}
Bacillus spp.[6]	10-100	25	100
Staphylococcus aureus[36]	10-100	25	50
Streptococcus pyogenes [3]	10-100	50	50
Escherichia coli [5]	10-100	25	50
Salmonella spp.[8]	10-100	50	100
Shigella spp. [8]	10-100	25	50
Klebsiella spp. [5]	10-100	50	100
Proteus spp.[7]	50-200	100	200
Providencia spp. [1]	50		
Citrobacter spp. [1]	25		
Arizona spp.[1]	25		
Pseudomonas spp.[9]		200	200
Bordetella bronchiseptica [1]	50		
Enterobacter cloaca [1]	200		
Vibrio cholerae [15]	10-100	25	100
Vibrio parahaemolyticus [5]	10-100	25	100
Total [112]	10-200	25	100

Kinetic studies on the action of BF-1

The MIC of BF-1 against *Salmonella typhimurium* NCTC 74 was found to be 25 μg/ml. At the logarithmic growth phase of the culture, when the CFU count of the strain was 1.3×10^8, 50 μg/ml (2 × MIC) of BF-1 was added. Subsequently, the CFU counts of the culture were determined; they were 1.3×10^5 after 2 h, 1×10^4 after 4 h and 0 after 6 h, as well as at the end of 18 h (Fig. 3).

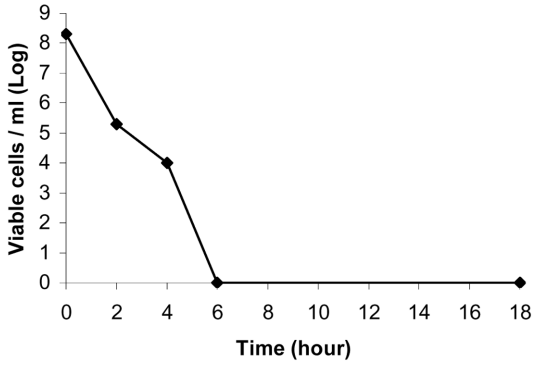

Fig. 3. Mode of action of BF-1 against *Salmonella typhimurium* NCTC 74.

Protective capacity of BF-1 *in vivo*

None of the animals in the two groups of mice (20 in each) receiving 30 μg or 60 μg of BF-1 died, proving thereby that the compound was non-toxic for the animals. Subsequently, two more groups of mice (20 per group) that were given 30 μg or 60 μg of BF-1 were challenged with 50 MLD (Table 2) of *S. typhimurium* NCTC 74 after 3 h. In the first group (30 μg/mouse), 8 out of 20 animals died, while in the other group (60 μg/mouse), none of the animals died. In the control group of 40 mice challenged with the same strain, 32 animals died within 100 h. On the basis of the data obtained, statistical analysis showed $p < 0.01$, according to chi-square test (Table 3). In Fig. 4, it is seen that BF-1 significantly reduced the number of viable bacteria in heart blood, liver and spleen of mice 18

Table 2. Median lethal dose of *Salmonella typhimurium* NCTC 74

Batch	CFU	OD*	Mortality data D/S	Accumulated values from mortality data		MLD	50 MLD
				Mortality ratio** D/D+S	%		
1	2.2×10^8	111	6/6	10/10	100		
2	1.9×10^7	97	2/6	4/8	50		
3	3.5×10^6	88	1/6	2/11	18.1	1.9×10^7 CFU	$50 \times 1.9 \times 10^7 = 0.95 \times 10^9$
4	2.6×10^5	73	1/6	1/15	6		
5	4.0×10^4	66	0/6	0/20	0		

*Reading taken in a Klett-Summerson colorimeter at 640 nm.
**Mortality ratio calculated according to the formula of Reed and Muench, 1938.

Table 3. Effect of BF-1 on survival of challenged mice

Control Group* [Without BF-1]			Test Group* [with BF-1]		
Saline/mouse	Mice died [Out of 40]	% Mortality	Injected/ mouse	Mice died [Out of 20]	% Servility
0.1 ml	32	80	30 mg	8	60
			60 mg**	0	100

*Received a challenge dose of 0.95×10^9 CFU in 0.5 ml BHI of *Salmonella typhimurium* NCTC 74
**$p < 0.01$, using chi-square test.

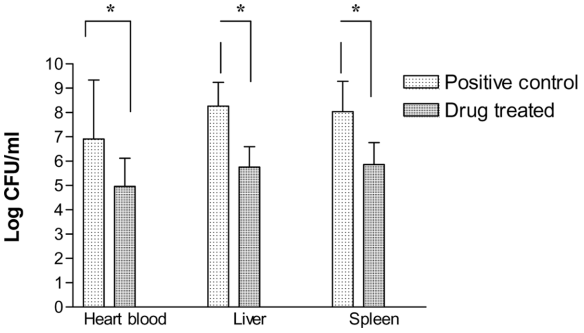

Fig. 4. Effects of BF-1 on CFU counts of *Salmonella typhimurium* NCTC 74 in organs of acute infected mice. *Viable counts between two groups significant; $p < 0.01$.

h after challenge, compared with the control (saline treated) mice. Statistical analysis showed $p < 0.01$ for 18 h samples.

The histopathological sections of liver revealed a considerable 10 fold decrease ($p < 0.05$) in number of severe infiltration with granular leucocytes and numerous micro-abscesses in infected mice treated with BF-1 as compared to the untreated ones.

DISCUSSION

The physico-chemical properties of the isolated flavonoid compound BF-1 were found to be identical with those of an authentic sample of a standard flavonone compound, quercetin (Sigma, USA) (Harborne, 1983). From previous reports, it is known that the *B. frondosa* flowers contain flavonoids. Along with the chalcone-free butein and butin, the other significant constituent is butrin. Isobutrin, which gets converted to butrin on storage, is also present. The plant gives a red gum, called Bengal gum or Butea gum.

Seeds yield a yellow oil, proteolytic and lipolytic enzymes palasonin, h-heneico-sanon and lactone, alpha-amyrin, b-sitosterol, b-D-glucoside, sugars and mono-spermin. Lectins are also found [www.tattvasherbs.com].

Naturally occurring products from plants have played a major role in the discovery of active therapeutic agents since ancient times. One of the foremost examples is quinine that was obtained from the *Cinchona* bark. *B. frondosa* has a strong anthelmintic effect. Extracts of different parts of the plant has been studied. It is used in Ayurveda as a rasayana and anthelmintic. Palasonin, an active principle in seeds, works effectively against *Ascaris lumbricoides in vitro* and *in vivo* on *Toxocara canis*, at

levels similar to piperazine. In mice, the LD_{50} was 7.5 g/kg. Chronic oral exposure to substantial doses of the seeds of the plant leads to nephrotoxicity and anaemia in rats (Ramachandran *et al*., 2004), dogs and rabbits (Mengi and Deshpande, 1995; Kawase *et al*., 2000). Hepato- and splenomegaly, with congestion, gross dilatation of the stomach and gastric inflammation were also seen. The seed oil showed cardiac effects with inhibition of ventricular movement, reduction in the amplitude of contraction of isolated frog heart and duodenal muscle relaxation in the rabbit (Kawase *et al*., 2000). This study, along with earlier studies (Shirataki *et al*., 2001; Dastidar *et al*., 2001, 2004; Mazumder *et al*., 2003) has proved that various plants are still some of the rich sources of products with efficient medicinal values. Flavones are phenolic structures containing one carbonyl group. Flavonoids are also hydroxylated phenolic substances but occur as a C_6-C_3 unit linked to an aromatic ring (Fig. 1). Since they are known to be synthesized by plants in response to microbial infection (Dixon *et al*., 1983; Soman *et al*., 2004), it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and also with bacterial cell walls. Their beneficial effects on the immune systems may be brought about by their ability to increase innate immune defense mechanisms (Gubarev *et al*., 1998). Quercetin, the flavonoid compound possessing two six-membered fused rings as chromanone (present in quercetin), has often been reported to possess antimicrobial activity. However, the presence of this compound in stem bark of *Butea frondosa* is yet unreported. Moreover, although several parts of this plant have been investigated and have yielded products of clinical importance, its stem bark has not been explored for antimicrobial properties. This study reports the existence of quercetin in the stem bark of *Butea frondosa* and presents its antimicrobial spectrum *in vitro* and *in vivo*. It further suggests that quercetin, isolated from stem bark of *Butea frondosa,* has potential for being developed into an antimicrobial agent that is expected to assist in the general battle against antibiotic-resistant bacteria.

ACKNOWLEDGEMENTS

This work was supported by grants provided by the Department of Science and technology, West Bengal, India.

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