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Research article

A flavone compound isolated from the stem bark of *Butea frondosa*: A broad spectrum bactericide especially active on Bacillus

Jiten Mishra[*1], Digambar Bisoi[2], Nityashree Mohapatra[3]

[1]*Roland Institute of Pharmaceutical Sciences, Berhampur, Orissa-760010, India.*
[2]*Sivananda College of Pharmacy, Berhampur, Orissa-760010, India.*

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***Corresponding Author: Jiten Mishra,** Assistant Professor, D. Pharm HOD, Roland Institute of Pharmaceutical Sciences, Berhampur (Gm), Orissa-760010, India.
Email id: hoddpharm@rips.ac.in / jitenmishra000@gmail.com

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Abstract

The Quinoline compound isolated from the ethyl acetate fraction of *B. Frondosa* stem bark showed some significant antimicrobial activity when tested against 106 different bacterial strains belonging to 9 different genera of both Gram-positive and Gram- negative. Minimuim inhibitory concentration of the drug were measured using an agar dilution technique. Twenty four of 36 strains of *Staphylococcus aureus* were inhibited by 100-200 mg/l of drug. This drug also inhibited strains of *Bacillus spp*, *Shigella spp, Salmonella spp* and *Pseudomonas spp* at a concentration of 100-400 mg/l. Other bacteria including *Escherichia* coli, *Vibrio cholerae* and *V. parahaemolyticus* were moderately sensitive to drug. In the *in vivo* studies this compound offered significant protection to swiss albino mice at a concentration of 80 µg /mouse ($P<0.001$) when challenged with 50 median lethal dose of *Salmonella typhimurium* NCTC 74.

Introduction

Systematic search among various pharmacological categories of drugs has shown that several drugs such as bromodiphenhydramine [1], methdilazine [2], diclofenac [3] and trifluoperazine [4] possess moderate to powerful antibacterial action in addition to their pharmacological properties. Such drugs are together designated as non-antibiotic [5, 6].

Naturally occuring products from plants have played an important role in discovery of new therapeutic agents since ancient times, e.g., quinine obtained from *Cinchona* has been successfully used to treat malaria [7]. Isoflavonoid phenolic compounds of the *sophora* genus (leguminosae of pea plant family) have been extracted and studied for pharmacological activity [8-11]. In the present investigation the plant *Butea frondosa* (Fabaceae) is a medium sized tree native of the mountainous regions of India and Burma and now grow wild throughout India [12]. The bark is reported to possess antitumour and antiulcer properties [12]. While stem bark possess antifungal activity [13, 14]. The root bark is used as an aphrodiac, analgesic and anthelmintic [12]. Where as the leaves possess antimicrobial property [15]. The flowers are used for the treatment of liver disorders [16]. Nitrogenous compounds from the seeds [17, 18] have been reported earlier. The purpose of this paper was to look for any antimicrobial activity of flavonoid compound isolated from the stem bark of *Butea frondosa*. The isolated flavonoid was studied and designated as Biological Fraction-1 (BF-1).

Materials and methods

Plant material

The stem barks of *Butea frondosa* were collected in the month of September, 2019 from the forest region (Nilagiri) of Orissa, India. The collected bark along with a complete herbarium of the flowering aerial parts of plant was sent for identification and finally was

authenticated by the Central National Herbarium, Botanical Survey of India, Howrah, West Bengal, India. (Authentication Number-5678/ 2019).

Extraction and isolation

Dried and powered stems (2.5 kg) were extracted with double distilled ethanol (by trial and error method & conclusion got from literature survey) and in several batches and the combined extract concentrated to 500ml. After the addition of water (500ml) the extract was fractionated with n-hexane (5x200ml. 7.20g) and Ethyl acetate (EtOAc) (5x200ml, 12.65g.). A portion (5.2g) of the EtOAc fraction was chromatographed over silica gel (300g), eluting with $CHCl_3$ – MeOH (1:1) to provide compound BF-1 (32mg). The fractions collected were 100ml and monitored by Thin Layer Chromatography (TLC).

5,7,3′,4′ -Tetra hydroxy flavonol (BF-1)

Fractions 10-15 of $CHCl_3$ – MeOH (1:1) eluates yielded a yellow coloured residue, mp 309-311^{o}C, identified by compared the TLC, UV, IR, ^{1}H NMR data of BF-1 with standard flavonol compound, Quercetin [19, 20]. Rf : 0.97 ($CHCl_3$- MeOH; 1:1); UV: λ max (EtOH): 257,356 nm, IR: Vmax cm^{-1}: 3397, 2919, 1621, 1462,1378; ^{1}H-NMR ($CDCl_3$) : H-6′. –H-6′. Ortho-coupling (8.4Hz) at 7.5ppm and 6.9ppm, H-6′. –H-2′ meta coupling (2Hz) at 7.5ppm and 7.5ppm, meta coupling between H-6 and H-8 (1.9Hz) at 6.2ppm and 6.4ppm respectively. These all data were identical in every respect with the authentic sample of quercetin. The isolated pure compound BF-1 was stored at 4^{0}C for further study (Figure 1).

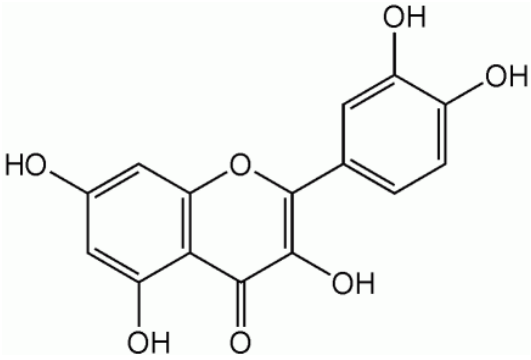

Figure 1. Structure of Quercetin (BF-1), (5,7,3′,4′-tetrahydroxy flavonol).

Bacteria

A total of 10 strains belonging to different genera, comprising both Gram-positive and Gram-negative types were used in this study. All the strains were clinical isolates from human beings. The strains were identified using to Barrow and Feltham's method [21] by Gram Reaction Test, Oxidase Test and Motility test.

Media

The liquid media used were nutrient broth (NB; Oxoid brand) and peptone water (PW; Oxoid bacteriological peptone 1.0% plus Analar NaCl 0.5%). The solid medium was nutrient agar (NA, Oxoid).

Detection of antibacterial activity by in vitro tests

BF-1 was dissolved in dimethyl sulfoxide (DMSO). The solution was then individually added at final concentrations of 0 (control), 100, 200 and 400 mg/l to molten NA, mixed thoroughly and the pH was adjusted to 7.2-7.4 before pouring in to sterile Petri dishes. Gram-positive bacteria were grown in NB and Gram-negative in PW for 18h. The cultures were diluted to contain 10^5 colony forming units (CFUs) in a 2mm 9 internal diameter) loop, spot inoculated on the NA plates and incubated at 37^{0}C up to 72 h to determination the minimum inhibitory concentration (MIC) of BF-1. Dilution Culture is suitable to use for cultivation of bacteria .This was done by 2 fold steps from the original samples to nearly extinction in sterile water depending on source. The dilution made in 96- well plates & spotted in Agar plate.

Bactericidal/bacteriostatic activities of BF-1

Highly sensitive bacterial strain *Shigella dysenteriae* 7 was grown in NB over night, 2ml of which were added to 4ml of fresh NB and incubated for 2h to obtain a logarithmic phase culture. At this stage, the colony forming unit counts of the culture was determined and BF-1 was added into different broths at higher concentrations than the MIC. The CFU counts were determined again after 2, 4, 6 and 18 h.

In vivo tests

A Swiss strain of albino male mice (250/IAEC/RIPS/11/2019) maintained in our animal house was given commercial feed and water ad libitum. Mortality experiments in these mice (18-20g each) with or without BF-1 was carried out by challenging them with 50 median lethal dose (MLD) of a mouse-passaged, virulent strain of *Salmonella Typhimurium* NCTC 74 corresponding to 0.95x 10^9 CFU suspended in 0.5 ml NB [22]. Reproducibility of this challenge dose was ensured by choosing a fixed value of its optical density at 640 nm in a Klett- Summerson Colorimeter. BF-1 was administration at doses of 40 and 80µg/mouse by injecting intraperitoneally (i.p.) 0.1 ml sterile stock solution of the drug containing 400 and 800 mg/l, respectively, or 0.1ml sterile saline (control) 3h before the challenge. The protective capacity of BF-1 was assessed on the basis of the following: (i) When both the infective challenge, as well as, the antibacterial BF-1 was administered; (ii) when BF-1 was administered alone; (iii) when the bacterial challenge plus 0.1ml saline (instead of BF-1) was used. In a similar experiment, 80 mice were

divided into three groups of each 20 mice, receiving the challenge dose, groups I and II received BF-1 40µg/mouse and 80µg/mouse. While group III received only saline 3h before the challenge.

All animals of groups I - III were autopsied 18h after the challenge. The livers and spleens were removed aseptically, homogenized in a glass homogenizer (by using phosphate buffer with Potassium chloride salt and Magnesium chloride salt, then mixture was homogenized) and preserved at -20^{0}C for the subsequent determination of CFU/ml counts; 0.2 – 0.4 ml of heart blood samples were also collected aseptically from these animals at the same time, allowed to clot and analysed for the size of bacteriamia (by clot culture) and concentration of BF-1 in the sera. The latter was also determined at 0h in mice in a separate experiment. The concentration of BF-1 in mouse blood was assayed by measuring the diameter of the inhibition zones by serum-soaked filter paper discs (6 mm diameter, 3mm thick, Millipore, absorbing 0.03 ml) on a lawn seeded with 10^6 bacteria from 18h broth culture of *S. Typhimurium* 74 . BF-l concentrations in the sera were deduced by referring these values to a standard calibration curve prepared with known concentrations of the agent.

Conditions for animal study

Light: - A 14 hour Light/10 hour dark cycle or 12 light/12 dark cycle was maintained.

Temperature: - 65 to 75^{0}F or 18 to 23^{0}C.

Humidity:- 40 to 60%.

Results

Determination of minimum inhibitory concentration (MIC) of BF-1

BF-1 was firstly screened using 14 known sensitive bacteria. The antibacterial potency of BF-1 was further assessed in detail on a total of 106 bacteria both Gram-positive and Gram- negative strains (Table 1). Out of 36 strains of *S. aureus* tested against BF-1, 34 were inhibited at less than 400mg/l. All the six strains of *Bacillus* Spp and two strains of E. coli were inhibited at 100 mg/l.

Five out of 10 *Shigella* Spp were inhibited at <400mg/l of BF-1. Four strains of *Salmonella* Spp were inhibited up to the concentration of 400mg/l. *Pseudomonas* Spp, *V. cholerae* and *V. parahaemolyticus* were also fairly sensitive to BF-1. *Klebsiella* and *Proteus* Spp were not sensitive to BF-1 up to the concentration of 400 mg/l.

Bactericidal action of BF-1

The MIC of BF-1 against *Shigella dysenteriae* 7 was 100 mg/l. At the logarithmic growth phase of the culture, when the CFU counts of the strain was $1.3x10^8$, 200mg/l of BF-1 was added to each and viable count of the culture determined. The CFU count was $1.3x10^5$ after 2h and 10^4 after 4h, while at the end of 6h the CFU count was zero (Figure 2).

In vivo tests

Table 2 shows that in the control group, 32 out of 40 animals died within 100h of the challenge and but there were no deaths in those groups of mice that received different doses of BF-1. There was a significant protection in the drug-treated groups by the compound. Table 3 shows that BF-1 significantly reduced the number of viable bacteria in heart blood, liver and spleen of mice at 18h after challenge compared with the control (saline treated) mice. Statistical analysis showed $P<0.01$.

Table 1. Antibacterial spectra of BF-1.

Bacterial strain	No. Tested	Minimum Inhibitory Concentration (mg/l)			Total Number Inhibited
		100	200	400	
Staphylococcus aureus	36	12	12	10	34
Bacillus Spp	6	6	-	-	6
Escherichia coli	5	2	-	-	2
Salmonella Spp	8	2	1	1	4
Shigella Spp	11	4	1		5
Klebsiella Spp	5	-	-	-	0
Proteus Spp	5	-	-	-	0
Pseudomonas Spp	10	1	2	2	5
V. Cholerae	15	2	1	2	5
V. Parahaemolyticus	5	-	2	-	2

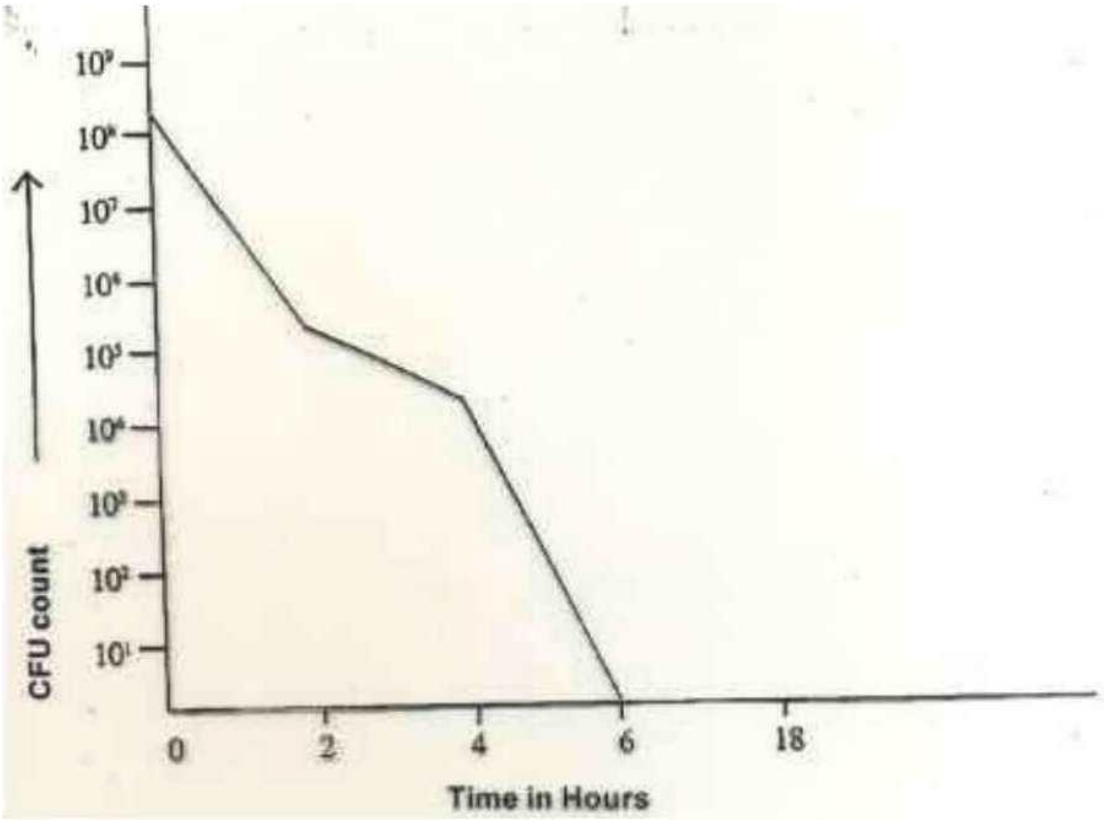

Figure 2. Bactericidal activity of BF-1 on *Shigella dysenteriae* 7 strain.

Table 2. Determination of mouse protective capacity of BF-1 *in vivo.*

Control group[a]		Test group[b]	
Drug Injected / Mouse	**Mice Died (Out of 40)**	**BF-1 (µg) injected/ Mouse**	**Mice Died (Out of 20)**
0.1 ml sterile saline	**32**	**-**	**40**
8 ml sterile saline	**-**	**80**	**-**
0 ml sterile saline	**-**	**-**	**-**

P<0.001, using chi-square test.
[a]Received a challenge dose of 0.95x10[9] CFU in 0.5ml NB of *S. Typhimurium* NCTC 74.
[b]None of the animals died when 40 or 80µg of BF-1 was injected to 2 separate groups of mice (20 mice in each), i.e, BF-1 was found to be non-toxic to mice.

Discussion

New antibiotic are being steadily synthesized by industry and also in academic institutions but the problem of drug resistance is continually increasing. Search for antimicrobials using a different approach has shown many compounds with worthwhile antimicrobial action. Such studies have revealed that several antihistamines [1, 2], neuroleptics [4], antihypertensive [23] and anti-inflammatory drugs [3] may be developed into proper antimicrobial agents.

Conclusion

In the present study, there was a similar approach to detect antimicrobial properties of compound isolated from plant extract. The compound BF-1 showed a significant inhibitory action against bacteria both *in vitro* and *in vivo*. It was most active against *Staphylococcus aureus* and *Bacillus* Spp, and then in decreasing order against *Shigella*, *Pseudomonas*, *Salmonella*, *Vibrio cholerae*, *Escherichia coli* and *Vibrio parahaemolyticus* Spp. The MIC of BF-1 ranged from 100 to 400 mg/l for most organisms. The compound BF-1 showed significant bactericidal activity. It gave significant protection to mice challenged with a virulent *Salmonella* and there was no toxic side effects. Therefore, BF-1 may be suitable for further development into newer antimicrobial in due course.

Author contributions

All the authors have contributed equally in designing, drafting the manuscript as per the journal submission

format. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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