Asian Journal of Pharmaceutical and Clinical Research

ANTIBACTERIAL ACTIVITY OF STEM BARK AND ROOT OF INDIAN *ZANTHOXYLUM NITIDUM*

SANJIB BHATTACHARYA[*1], M. K. ZAMAN[1], P. K. HALDAR[2]

The *in vitro* antibacterial activity of aqueous and ethanol extracts from the stem bark and root of *Zanthoxylum nitidum* (Roxb.) DC (Rutaceae), growing in north-east India was evaluated against five Gram-positive bacteria including *Staphylococcus aureus, Streptococcus faecalis, Bacillus cereus, Sarcina lutea, Bacillus subtilis* and two Gram-negative bacteria, *Klebsiella pneumoniae,* and *Escherichia coli*; using disk diffusion method followed by determination of minimum inhibitory concentrations (MIC) by broth dilution method, against sensitive bacteria. All extracts, at higher concentrations showed varying degrees of inhibitory activity against all bacteria; except the aqueous extract of the stem bark, which was inactive against *S. lutea* and *B. subtilis*. The highest activity was exhibited by the ethanol extract of root against *B. cereus*, while the lowest activity was exhibited by aqueous extract of stem bark against *K. pneumoniae*; in the highest (40 mg/ml) concentration. The ethanol extract of root was the most active, showing maximum concentration dependent antibacterial effects and the aqueous extract of stem bark was the least active. Root extracts exhibited higher antibacterial effects than stem bark extracts. Gram-positive bacteria were more sensitive to the extracts, except aqueous extract of stem bark.

Keywords : Antibacterial activity, Indian *Zanthoxylum nitidum*, Minimum Inhibitory Concentration (MIC), Root, Stem bark, Zone of inhibition.

INTRODUCTION

The use of higher plants and their preparations to treat infectious diseases is an age-old practice and in the past possibly the only method available. However, the systematic study of higher plants for detecting antimicrobial activity is of comparatively recent origin[1]. These investigations have been triggered by the emergence and spread of antibiotic resistant microorganisms causing the effective life-span of existing antibiotics limited[2]. Hence, the plant kingdom is being screened for newer and effective chemotherapeutic agents. Higher plants can serve both as potential antimicrobial crude drugs as well as a source of new anti-infective agents[3].

Zanthoxylum nitidum (Roxb.) DC (Rutaceae) is a morphologically variable plant species occurring in south-east Asian countries and in Australia[4]. In India it grows as a large prickly shrub particularly in north-east India (Sikkim, Assam and Nagaland). In India the plant is used traditionally for various medicinal purposes. The root is used in toothache, stomachache, fever, rheumatism, paresis, boils and as an insecticide and piscicide. The fruit is used in the treatment of stomachache, cough, colic vomiting, diarrhoea, and paresis and as an aromatic, stimulant and piscicide. The small branches, seeds and stem bark are prescribed in fever, diarrhea and cholera[5-7].

Several plants of the genus *Zanthoxylum* have been studied and different antimicrobial effects have been reported among them[8]. Yao et al., (2005) found that the ethanol extract from root of Chinese *Z. nitidum* had moderate antibacterial activity against oral Gram-positive bacteria[9]. However, no antibacterial study has been performed with the stem bark of this plant and by using Gram-negative bacteria. Previous workers also reported antispasmodic, anti-tumor, antifungal, antioxidant, analgesic and antiinflammatory activity of the root of non-Indian *Z. nitidum* mainly in China, Japan and Taiwan[10-14]. However, there are no reports of biological investigations carried out on *Z. nitidum* of Indian habitat. The present work therefore, attempts to evaluate the broad spectrum antibacterial activity of the aqueous and ethanol extracts from the stem bark and root of *Z. nitidum* growing in India.

MATERIALS AND METHODS

Plant Material

The fully matured entire plants of *Z. nitidum* were collected during the month of November 2006 from the outskirts of Dibrugarh University campus, in Dibrugarh district of Assam, India. The species was identified by Dr. S. J. Phukan, taxonomist, from Botanical Survey of India, Eastern Circle, Shillong, India, and a voucher specimen (No. DUPS-06-003) was deposited in Department of Pharmaceutical Sciences, Dibrugarh University, for future reference. Immediately after collection, the underground root parts were separated from the shoots. All the prickles were removed from the stems and branches carefully by using a sharp knife, without harming the bark. Then the barks were peeled off from the shoots. The plant materials were shade dried at temperature 21-24^{0}C and ground into coarse powder.

***Corresponding author:** ***[1] Department of Pharmaceutical Sciences, Dibrugarh University, Dibrugarh 786004, Assam, India.**
[2] Department of Pharmaceutical Technology, Jadavpur University, Kolkata 700032, West Bengal, India.
e-mail: sakkwai@yahoo.com.

Preparation of extracts

The air-dried stem bark and root of *Z. nitidum* were extracted with water (doubled distilled water from all glass still) and ethanol (90% v/v) by cold maceration. Powdered plant materials (70g) were macerated with 400 ml of solvent at 21-24^{0}C temperature for 2 days with frequent shaking. After 2 days, the extracts were filtered by using Whatman filter paper (No. 1) and to the marc part 300 ml of the solvent was added and allowed to stand for next two days at same temperature for second time maceration (re-maceration) and after two days, again filtered similarly. The combined filtrates (extracts) were evaporated to dryness in a vacuum oven and stored in a desiccator for future use. The extracts were denoted as RE (Root Ethanol), RA (Root Aqueous), BE (Bark Ethanol) and BA (Bark Aqueous). The dry extracts were dissolved in 25% v/v aqueous dimethyl sulfoxide (DMSO) for use in the study.

Antibacterial activity Disk diffusion method

The antibacterial activities of the aqueous and ethanol extracts of stem bark and root were evaluated by disk diffusion method. The turbidity of bacterial cultures in broth media was adjusted with sterile saline (0.9% w/v) according to 0.5 McFarland turbidity standard, for preparation of the inoculum. Mueller-Hinton agar medium previously prepared and sterilized was cooled down to approximately 45-50^o C. 20-25 ml of this media were poured into 9 cm sterile glass Petri dishes previously marked suitably at the bottom surface, to a depth of approximately 4 mm. The inoculum was added to the molten agar media in the Petri dishes and the plates were swirled gently to disperse the microorganisms homogeneously. The plates were then allowed to solidify. Whatman no. 1 filter paper disks (0.5 cm), previously sterilized were impregnated with each test extracts at four different concentrations (40, 20, 10, and 5 mg/ml) and placed on the solidified surface of the media seeded with respective microorganisms. Similarly, filter paper disks impregnated with the vehicle (25% DMSO) were used as control. No standard antimicrobial agent was employed. Then the Petri dishes were incubated in inverted position at 37^{o}C for 24 h. After incubation the zones of inhibition around the disks were measured by means of a transparent ruler in mm.[15-16]

TABLE- 1 In Vitro Antibacterial Activity of Z. Nitidum extracts by disk diffusion method.

Extracts	Conc. (mg/ml)	Zone of Inhibition (mm)						
		Sa	*Sl*	*Sf*	*Bc*	*Bs*	*Kp*	*Ec*
RE	40	16.0	10.0	14.0	17.0	15.0	8.5	11.0
	20	11.0	7.0	10.0	12.5	9.5	-	7.0
	10	8.5	-	6.0	9.5	6.5	-	-
	5	6.0	-	-	5.5	-	-	-
RA	40	10.0	7.0	9.0	12.0	16.0	9.0	6.0
	20	6.0	-	-	7.5	9.0	5.5	-
	10	-	-	-	5.5	6.0	-	-
	5	-	-	-	-	-	-	-
BE	40	13.0	9.0	7.0	12.5	10.0	6.0	10
	20	8.0	-	-	7.0	6.0	-	5.5
	10	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
BA	40	9.0	-	6.0	7.0	-	6.0	8.0
	20	5.5	-	-	-	-	-	-
	10	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-

RE = Root Ethanol, RA = Root Aqueous, BE = Bark Ethanol, BA = Bark Aqueous

***Sa = Staphylococcus aureus, Sl = Sarcina lutea, Sf = Streptococcus faecalis, Bc= Bacillus cereus, Bs = Bacillus subtilis, Kp= Klebsiella pneumoniae, Ec= Escherichia coli,* - = No Zone.**

Bacteria

The bacteria used in the study included five Gram-positive bacteria, *Staphylococcus aureus, Streptococcus faecalis, Bacillus cereus, Sarcina lutea, Bacillus subtilis* and two Gram-negative bacteria, *Klebsiella pneumoniae* and *Escherichia coli.* All bacterial cultures were obtained from the Department of Microbiology, Assam Medical College, Dibrugarh, Assam, India and maintained in usual laboratory conditions.

TABLE- 2 THE MIC VALUES OF *Z. NITIDUM* EXTRACTS BY BROTH DILUTION METHOD.

Extracts	Minimum inhibitory concentrations (MIC) (mg/ml)						
	Sa	Sl	Sf	Bc	Bs	Kp	Ec
RE	0.1563	0.625	0.3125	0.0781	0.1563	1.25	0.625
RA	0.625	1.25	1.25	0.3125	0.1563	1.25	2.5
BE	0.3125	0.625	1.25	0.3125	0.625	2.5	0.625
BA	1.25	ND	2.5	2.5	ND	5.0	1.25

RE = Root Ethanol, RA = Root Aqueous, BE = Bark Ethanol, BA = Bark Aqueous.
Sa = Staphylococcus aureus, Sl = Sarcina lutea, Sf = Streptococcus faecalis, Bc= Bacillus cereus, Bs = Bacillus subtilis, Kp = Klebsiella pneumoniae, Ec= Escherichia coli.
ND = Not determined, because the extract was inactive in the preliminary screening.

Broth dilution method

The minimum inhibitory concentrations (MIC) of the extracts were determined for the sensitive bacteria (in disk diffusion test) by broth dilution method. All test extracts were serially (two fold) diluted from 40 mg/ml to 0.0781 mg/ml (10 dilutions). To 9 ml of sterile Mueller-Hinton broth in test tubes, 1 ml of varying concentrations of the extracts were added and then a loopful (approximately 0.01 ml) of the bacterial suspensions previously adjusted with sterile saline (0.9% w/v) according to 0.5 McFarland turbidity standard, were introduced to the tubes. Streptomycin sulphate was used as the standard antimicrobial agent, which was also serially (two fold) diluted from 32 mg/ml to 1 mg/ml (6 dilutions) and tested similarly. In each test set, tube containing only medium and inoculum was used as control. Tubes were then incubated at 37^0C for 24 h. After incubation the lowest concentration at which no visible growth was observed (i.e. no turbidity) was regarded as minimum inhibitory concentration.[17-18]

RESULTS AND DISCUSSION

The antibacterial activity of ethanol and aqueous extract from *Z. nitidum* stem bark and root against seven bacterial strains was initially assessed by disk diffusion method. The results are shown in Table 1.

The extracts showed antibacterial activity against most of the tested bacteria mainly at higher concentrations. The ethanol extract of root exhibited moderate to feeble inhibition against all test bacteria with maximum against *B. cereus* (17 mm) and minimum against *K. pneumoniae* (8.5 mm) at highest concentration (40 mg/ml). The activities decreased with decrease in concentration. This extract was found to possess maximum concentration dependent antibacterial effects showing least inhibition even at the lowest concentration of 5 mg/ml against *S. aureus* (6 mm) and *B. cereus* (5.5 mm).

The aqueous extract of root showed comparatively weak inhibitory activity against all test bacteria with maximum against *B. subtilis* (16 mm) and minimum against *E. coli* (6 mm) at 40 mg/ml (highest) concentration. Here also activities decreased with concentration but were not detected up to the lowest concentration employed.

The ethanol extract of stem bark also showed weak activity in comparison with the ethanol extract of root, against all bacteria tested with maximum inhibition (13 mm) against *S. aureus* and minimum against *K. pneumoniae* (6 mm). The effects were observed in the highest and next lower concentration (20 mg/ml) of extract against some bacteria.

The aqueous extract of stem bark exhibited no inhibitory activity against *S. lutea* and *B. subtilis,* and it showed the weakest activity as compared to other three extracts against other five bacteria mostly in the highest (40 mg/ml) concentration.

The results of broth dilution test for MIC of extracts and streptomycin sulfate are shown in Table 2 and 3 respectively. This method was performed only on bacteria that were found to be sensitive to the extracts in the disk diffusion method. Hence, the aqueous extract of stem bark was not tested against *S. lutea* and *B. subtilis* for MIC. The ethanol extract of root had the lowest MIC (0.0781 mg/ml) against *B. cereus*, while the highest MIC was shown by the aqueous extract of stem bark against *K. pneumoniae*; indicating the highest and lowest inhibitions respectively. The results of broth dilution test i.e. MIC values were roughly in agreement with the results of disk diffusion, thereby confirming differential antibacterial effects of the extracts.

The results indicated that the root extracts were more effective than the stem bark extracts. This may be due to the fact that the root had more antibacterial constituents, which were less or absent in stem bark; and the stem bark had some green pigments which might interfere with activity. Out of the solvents employed for extraction the ethanol extracts exhibited higher activity against the test organisms. Different solvents have the capacity to extract different antimicrobial constituents from plants. Initial screenings of plants for possible antimicrobial activities can typically begin by using crude aqueous or alcohol extracts. Since nearly all of the identified components from plants active against microorganisms are aromatic or saturated organic compounds, they are most often obtained through initial ethanol or methanol extraction [2, 19].

The extracts were effective against most Gram-positive and Gram-negative bacteria tested, thereby indicating a broad spectrum of activity. However, the results revealed that the Gram-positive bacteria were in general, more sensitive to the extracts, except the aqueous extract of stem bark.

In this investigation the agar disk diffusion method was employed to serve as initial antibacterial screening procedure. The diameter of zone of inhibition is a function of initial concentration (in the disk), solubility, and diffusion rate of the antibacterial compound(s) present in the extract through the agar media and thus not a true measure of effectiveness. [20, 21] Many physical and chemical factors unrelated to antibacterial activity affect the rate of diffusion of compound(s) through bacteria seeded solid media[22]. Therefore, it is not possible to measure the antibacterial activity of a new compound or plant extract in terms of another antibiotic as standard by comparing the diameters of zone of inhibition produced by disk diffusion. Hence, in the present study no antibiotic standard was employed while disk diffusion method was used to assess antibacterial activity.

More sensitive, quantitative and confirmatory results were obtained with broth dilution test. Here the method employed can be termed as broth two-fold macro dilution where the extracts were diluted serially (two-fold) in a sequence of decreasing concentration in broth by using test tubes, and inoculated with test bacteria. The smallest concentration of the extract that prevented visible growth (turbidity) was called the minimum inhibitory concentration (MIC). Here, a reference broad spectrum antibiotic streptomycin sulphate was employed as standard which was also tested for MIC by serial dilution similarly. It was found that the MIC values of streptomycin sulphate were very much lower (2-16 mg/ml) than that of extracts (0.0781-5 mg/ml) from *Z. nitidum*. Furthermore, some MIC values obtained for streptomycin sulfate were roughly in agreement with literature values [16,23]. Therefore, the standard antibiotic was employed not only for comparison but also to ensure the bacteria used in the study and the experimental conditions were appropriate and acceptable.

Yao et al. (2005) reported antibacterial efficacy of ethanol extract from root of *Z. nitidum* growing in China against three oral pathogenic bacteria (*Streptococcus mutans*, *Actinomyces naeslundii* and *Actinobacillus actinomycete micomtans*)[9]. Apart from this study, there are no reports of antibacterial studies on *Z. nitidum*. Present investigation is the first experimental demonstration of any biological activity as well as broad spectrum antibacterial efficacy of *Z. nitidum* growing in India. It is also first report of antibacterial potential of stem bark of *Z. nitidum*. The antibacterial effects of Indian *Z. nitidum* can substantiate its traditional uses in north-east India. The stem bark and root of Indian Z. *nitidum* need further investigations.

TABLE- 3 The Mic Values of streptomycin sulphate by borth dilution method.

Bacteria	MIC (µg/ml)
Staphylococcus aureus	16
Sarcina lutea	8
Streptococcus faecalis	16
Bacillus cereus	4
Bacillus subtilis	16
Klebsiella pneumoniae	2
Escherichia coli	4

MIC = Minimum inhibitory concentration

ACKNOWLEDGEMENT

The authors would like to thank Dr. A. Bhattacharya, Reader and Head, Department of Pharmaceutical Sciences, Dibrugarh University, Dibrugarh, Assam-786004, India, for his kind help and inspiration for the current work.

REFERENCES

1. Skinner FA. Antibiotics. In: Paech K, Tracey MV, editors. Modern Methods of Plant Analysis. Berlin, Gottingen, Heidelberg: Springer-Verlag; 1955. p. 626-654.
2. Cowan MM. Plant products as antimicrobial agents. Clin Microbiol Rev 1999; 12: 564-582.
3. Rios JL, Reico MC. Medicinal plants and antimicrobial activity. J Ethnopharmacol 2005; 100: 80-84.

4. Hu J, Zhang WD, Shen YH, Zhang C, Xu L, Liu, RH, Wang B, Xu XK. Alkaloids from *Zanthoxylum nitidum* (Roxb.) DC. Biochem Syst Ecol 2007; 35: 114-117.

5. Kirtikar KR, Basu BD. Indian Medicinal Plants. Vol I. New Delhi: Bishan Singh and Mahendra Pal Singh, International Book Distributors;1933. p. 463-464.

6. Anonymous. Wealth of India, Raw Materials. Vol. XI. New Delhi: Council of Scientific and Industrial Research; 1976. p. 24.

7. Kanjilal UN. The Flora of Assam. Vol. I (Part I). New Delhi: Omsons Publications; 1997. p. 201.

8. da-Silva SL, Figueredo MS, Yano T . Antibacterial and antifungal activities of volatile oils from *Zanthoxylum Rhoifolium* leaves. Pharm Biol 2006; 44: 657-659.

9. Yao S, Min L, Xiang ZDD. Activity of *Zanthoxylum* species and their compounds against oral pathogens. Chin J Nat Med 2005; 3: 248-251.

10. Suffness M, Cordell GA. Antitumor alkaloids. In: Brossi A, editors. The Alkaloids, Chemistry and Pharmacology. Vol. XXV. London: Academic Press Inc. Ltd.; 1985. p. 178-187.

11. Fang SD, Wang LK, Hect SM. Inhibitors of DNA topoisomerase I isolated from the roots of *Zanthoxylum nitidum*. J Org Chem 1993; 58: 5025-5027.

12. Hu K, Dong A, Liu H, Feng H, Sun Q, Yao X. Bioactivity of traditional Chinese herbal medicine against *Pyricularia oryzae*. Pharm Biol 1999; 37: 225-230.

13. Hu J, Zhang WD, Liu RH, Zhang C, Shen YH, Li HL, Liang MJ, Xu XK. Benzophenanthridine alkaloids from *Zanthoxylum nitidum* (Roxb.) DC and their biological activities. Chem Biodivers 2006; 3: 990-995.

14. Shyur LF, Tsung JH, Chen JH, Chin CY, Lo CP. Antioxidant properties of extracts from medicinal plants popularly used in Taiwan. Int J Appl Sci Engg 2005; 3: 195-203.

15. Goren N, Woerdenbag HJ, Johanson CB. Cytotoxic and antibacterial activities of sesquiterpene lactones isolated from *Tanacetum praeteritum* subsp. *praeteritum*. Planta Med 1996; 62: 419-422.

16. Yamac M, Bilgili F. Antimicrobial activities of fruit bodies and/or mycelial cultures of some mushroom isolates. Pharm Biol 2006; 44: 660-667.

17. Pepeljnjak S, Kalodera Z, Zovko M. Investigation of antimicrobial activity of *Pelarogarium radula* (Cav.) L'Herit. Acta Pharm 2005; 55: 409-415.

18. Vimala Y, Elizabeth KM. Antimicrobial activity of *Decalpis hamiltonii* on some microbial isolates of spoiled vegetables and pathogenic microorganisms. Indian J Microbiol 2006; 46: 397-399.

19. Eloff JN. Which extractant should be used for the screening and isolation of antimicrobial compounds from plants? J Ethnopharmacol 1998; 60: 1-8.

20. Seeley HW, VanDemark PJ. Microbes in Action, A Laboratory Manual in Microbiology. Bombay: D.B. Teraporevala Sons & Co. Pvt. Ltd.; 1975. p. 163-164.

21. Prescott LM, Harley JP, Klein DA. Microbiology. Oxford: Wm. C. Brown Publishers; 1993. p. 328-330.

22. Cynthia H, O'Callaghan. Assessment of a new antibiotic. In. Hugo WB, Russel AD, editors. Pharmaceutical Microbiology. 3rd ed. Oxford: Blackwell Scientific Publications; 1983. p. 122-134.

23. Saad HEA, Sharkawy SHE, Shier WT. Biological activities of pyrrolidinoindoline alkaloids from *Calycodendron milnei*. Planta Med 1995; 61: 313-316.

24. Anhalt JP, Washington II JA. Antimicrobial susceptibility tests of aerobic and facultatively anaerobic bacteria. In: Washington JA, editors. Laboratory Procedures in Cilnical Microbiology. New York, Heidelberg, Berlin and Tokyo: Springer-Verlag; 1985. p. 281-305.

25. Reeves DS, White LO. Principles and methods of assaying antibiotics. In: Hugo WB, Russel AD, editors. Pharmaceutical Microbiology. 3rd ed. Oxford: Blackwell Scientific Publications; 1983. p. 140-148.