ANTIMICROBIAL ACTIVITY OF VARIOUS EXTRACTS FROM THE LEAVES OF *TAXUS BACCATA* LINN. (TAXACEAE)

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Summary

The present study was aimed to investigate antimicrobial activity of various extracts from the *Taxus baccata* Linn. leaves (TBL). Various extracts viz. n-hexane (HX), dichloromethane (DCM), ethyl acetate (EA), ethanol (ET) and water (WT) prepared from TBL were tested on Gram-negative (9 species) and Gram-positive (4 species) bacteria. The agar disc diffusion test was used to determine the sensitivity of the tested samples while the well micro-dilution was used to determine the minimum inhibitory concentration (MIC). The result of the disc diffusion assay showed that ET prevents the growth of all the 13 tested microbial species while other extracts showed selective activity. The inhibitory activity of the most active extracts viz. EA and WT was noted on 12(92.3%) and 11(84.6%) respectively. The lowest MIC value of 39.06µg/ml was observed with ET on five and WT only one microorganism, which appeared as both these extracts are the most active extracts. This lowest MIC (39.06µg/ml) is about 16-fold greater than that of reference antibiotic indicating very powerful antimicrobial potential.

Key words: *Taxus baccata* (taxaceae), leaves, extracts, antimicrobial activity.

Introduction

Nature has been a source of medicinal agents for thousands of year and a remarkable number of modern drugs have been isolated from natural sources, many of them based on their use in traditional medicine. A rich heritage of knowledge on preventive and curative medicines was available in ancient scholastic work included in Athrva Veda, Charka Sushruta etc. An estimate suggests that about 13,000 plant species worldwide known to have use as drugs. The trends of using natural products have increased and the active plant extracts are frequently screened for new drug discoveries and for the presence of antimicrobials (1). The development of bacterial resistance to presently available antibiotics has necessitated the search of new antibacterial agents. Moreover, the relatively lower incidence of adverse reactions to plant preparations in comparison to modern conventional pharmaceuticals, coupled with their reduced cost, is encouraging both the

consuming public and national health care institutions to consider plant medicines as alternatives to synthetic drugs (2). *Taxus baccata* Linn., (Taxaceae) is an evergreen tree, usually 6 m in height and 1.5-1.8 m in growth, found in the temperate Himalayas at an altitude between 1800 - 3300 m and in the hills of Meghalya and Manipur at an altitude of 1500 m (3). *Taxus baccata* Linn. Leaves (TBL) are reported to be used in traditional medicine as abortifacient, antimalarial, antirheumatic, for bronchitis and as antimicrobial (4-7) and dried leaves and barks are used against asthma (8). Anti-cancer, anti-inflammatory and antinociceptive, anti-fungal, anti-mycobacterial activity of *T. baccata* has been reported (9-12). A large number of phytochemicals like taxoids viz. taxusin, baccatin VI, baccatin III etc, lignans, flavanoids, steroids and sugar derivatives have been isolated from the various species of Taxus including *Taxus baccata* (13,14). Lignans are known to possess various biological activities including antibacterial, antifungal, and antiviral, antioxidant, anticancer and anti-inflammatory effects (15). Present investigation was carried out to report antimicrobial activity of the various extracts of *Taxus* baccata leaves (TBL) against some microorganisms that cause infectious diseases.

Materials and Methods

Plant material

TBL were collected from commercial supplier of Mumbai (India). The plant was identified by Dr. Minoo Parabia, Head of the Department of Bioscience, Veer Narmad South Gujarat University, Surat, Gujarat, India where a voucher specimen has been deposited under the number of TBPK/0404/2007.

Extracts and preliminary phytochemical investigation

Powder of TBL was extracted successively i.e same material (marc) was extracted with new menstruum (solvent) viz. *n*-hexane (HX), dichloromethane (DCM), ethyl acetate (EA), ethanol (ET) and water (WT). Evaporation of the solvent under reduce pressure (60 ºC) provided dry extracts. A preliminary phytochemical screening investigated the presence of alkaloids, saponin, flavonoids, lignan, terpenoids and tannins according to standard methods (16).

Antimicrobial activity

Microbial strains

The microorganisms used in the antimicrobial tests were Gram-positive (*Staphylococus aureus* ATCC 25925, *Bacillus subtilis* ATCC 6633, *Staphylococcus epidermis* ATCC 12228, *Micrococcus luteus* ATCC 10240) and Gram-negative (*Escherichia coli* ATCC 25922, *Enterobacter aerogenes* ATCC 13048, *Salmonella typhi* ATCC 51812, *Shigella dysentriae* ATCC 25931, *Pseudomonas aeruginosa* ATCC 9027, *Pseudomonas testosteroni* NCIM 5098, *Proteus morganii* NCIM 2040, *Klebsiella pneumonae* NCIM 2719, *Listeria monocytogenes* ATCC 19112) bacteria. All the strains were obtained from C. G. Bhakta Institute of Biotechnology, Bardoli, Gujarat, India.

Culture media

Nutrient agar (NA) (Himedia Laboratories Ltd. Mumbai, India) containing Bromocresol purple was used for the activation of *Bacillus* species (17,18) while NA was used for the other bacteria. The Mueller Hinton Agar (MHA) (Himedia Laboratories Ltd. Mumbai, India) was used in sensitivity assay. Nutrient broth containing 0.05% phenol red and supplemented with 10% glucose (NBGP) was used for MIC and MMC.

Chemicals

Gentamycin (Alkem Laboretories Ltd. Mumbai, India) was used as reference antibiotic (RA) used against bacteria and dimethylsulphoxide (DMSO) (Himedia, Mumbai, India) was used as solvent for tested samples. Other chemicals were used of analytical grade of S.D. Fine Chemicals, Mumbai, India.

Sensitivity test: agar disc diffusion assay

Preparation of discs: Whatmann filter paper (No.1) discs of 6 mm diameter were impregnated with 10 µl of the solution of various extracts (at 20 mg/ml). The discs were evaporated at 37˚C for 24 h. The RA discs (gentamycin for bacteria) were prepared as described above using the appropriate concentrations to obtain discs containing 50 µg of drug. Three discs were prepared for each sample.

Disc diffusion test: The antimicrobial diffusion test was carried out as described by Berghe and Vlietinck (19) using a cell suspension of about 1.5×10^6CFU/ml obtained from a McFarland turbidity standard No. 0.5. The suspension was standardized by adjusting the optical density to 0.1 at 600 nm (SHIMADZU UV-1700 pharmaspec) (20). This was used to inoculate by flooding the surface of MHA plates. Excess liquid was air-dried under a sterile hood and the impregnated discs applied at equidistant points on top of the agar medium. A disc prepared with only the corresponding volume of DMSO was used as negative control. The plates were incubated at 37˚C for 24 h. Antimicrobial activity was evaluated by measuring the diameter of the inhibition zone (IZ) around the disc. The assay was repeated trice and the results expressed using the following symbols: (−) for no activity and (+) for samples with IZ >6 mm.

Determination of minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC):

The MICs of test samples and RA were determined as follows: the test sample was, first, dissolved in DMSO. The solution obtained was added to the NBGP to a final concentration of 312.5 µg/ml for the various extracts and 39.06 µg/ml for the RA. This was serially diluted 2-fold to obtain concentration ranges of 1.2-312.5 µg/ml for the extracts and 0.08–39.06 µg/ml for the RA. One hundred micro litres of each concentration were added in a well (96-wells micro plate) containing 95µl of NBGP and 5µl of the standard inoculums. The final concentration of DMSO in the well was less than 1% (preliminary analyses with 1% (v/v) DMSO/NBGP affected neither the growth of the test organisms nor the change of color due to this growth). The negative control well consisted of 195µl of NBGP and 5 µl of the standard inoculum (21,22). The plates were covered with a sterile plate sealer, then agitated to mix the content of the wells using a plate shaker and incubated at 37˚C for 24 h. The assay was repeated trice. Microbial growth was determined by observing the change of color in the wells (red when there are no growth and yellow when there is growth). The lowest concentration showing no color change was considered as the MIC.

For the determination of the MMC, a portion of liquid (5 µl) from each well that showed no change in color was plated on MHA and incubated at 37˚C for 24 h. The lowest concentration that yielded no growth after this sub-culturing was taken as the MMC.

Results and discussion

Extracts and preliminary phytochemical screening

200 gm of TBL were powdered and sequentially extracted (soxhelation) by 1000 ml of *n*-hexane (yield, 0.8 %), dichloromethane (yield, 1.8 %), ethyl acetate (yield, 1.6 %), ethanol (9.1 %) and water (yield, 8.3 %). The preliminary phytochemical investigation reveals the presence of steroids (HX and DCM), flavonoids (HX, ET and WT), lignan (EA and ET), alkaloid (ET) and saponins (WT).

Antimicrobial effects

From the results of the disc diffusion assay (Table 1), it showed that the ET (at 200 µg/disc) prevented the growth of all 13 tested microbial species. Other compound showed selective activity. Inhibitory effects of EA and WT extract noted on 12 (92.3 %) and 11 (84.6 %) respectively while DCM and HX noted on 10 (80%) of the 13 tested microorganisms. EA and ET showed antimicrobial effect against all gram-negative bacteria while only ET showed antimicrobial against all gram-positive bacteria.

The MIC values ranged from 39.06 to 156.25 µg/ml (Table 2) of ET and WT on all the tested microorganisms. The results of Table 2 also confirm the antimicrobial activity of HX and DCM. However, at the tested MIC limit of 312.5 µg/ml, the inhibitory activity of HX and DCM was noted on 10 (80%) of the 13 tested microorganisms while that of EA and WT was observed on 12 (92.3%) and 11 (84.6%) respectively. Regarding the degree of activity of the tested samples, all extracts could mostly be considered as very potent. The lowest MIC values of 39.06 µg/ml for ET, EA and WT is 16 fold greater than the corresponding value (2.4 µg/ml) for gentamycin used as RA. This lowest MIC value (39.06 µg/ml) was observed with ET on five microorganisms namely *Staphylococcus aureus, Micrococcus luteus, Escherichia coli, Shigella dysenteriae* and *Listeria monocytogenes* and with WT only one microorganism namely *Bacilus subtilis*. The lowest MIC value of 78.12 µg/ml observed with HX on *Escherichia coli*, with DCM on *Enterobater aerogens* and with EA on *Bacilus subtilis, Escherichia coli, Shigella dysenteriae* and *Pseudomonas testosteroni* on the tested microorganisms. This data supported the interesting antimicrobial activity of all extracts from the TBL following the disc diffusion test. This can be confirmed by the results of the MMC determination also reported in Table 3.

These MMC determination results showed that values were obtained with ET 13 (100%), EA and WT 10 (80%) and 9 (69.2%) and HX and DCM 7 (54%) of the 13 tested microorganisms. However, the overall results of Table 2 indicated that the obtained MMC values recorded with each extract was not more than 4-fold greater than their MIC. This indicates that the cidal effects of tested samples on many of the tested microorganisms could be expected (23) (Carbonnelle et al., 1987). The results of this study indicate that these plant extracts could possibly use as antimicrobial but more in-vivo experiments are necessary. The antimicrobial activity of the TBL may be due to the presence of antimicrobial compounds. To the best of our knowledge, the antimicrobial activity of the TBL of different extracts is being reported for the first time. However, the antimycobacterial activity of the ET of the TBL was previously demonstrated (24) (Erdemoglu et al., 2003).

The antimicrobial activity of the HX, ET, EA, WT from the TBL may be due to presence of lignans (15) (Cho et al., 2001), flavonoids (24) (Erdemoglu et al., 2003; (25) Cown, 1999) but further studies are required for DCM from, which compounds are responsible for antimicrobial activity.

The present study provides as important basis for the use of various extracts from the TBL for the treatment of infections associated to the studied microorganisms.

Table 1: Antimicrobial activity[#] of various extracts from the leaves of *T. baccata* and reference antibiotic determined by disc diffusion test.

Microorganisms	Tested samples[a]					
	HX	**DCM**	**EA**	**ET**	**WT**	**RA**
Gram positive bacteria						
Staphylococcus aureus ATCC 25925	-	+	+	+	-	+
Bacilus subtilis ATCC 6633	+	-	+	+	+	+
Staphylococcus epidermis ATCC 12228	+	+	-	+	+	+
Micrococcus luteus ATCC 10240	+	+	+	+	+	+
Gram negative bacteria						
Escherichia coli ATCC 25922	+	+	+	+	+	+
Enterobater aerogens ATCC 13048	+	+	+	+	+	+
Salmonella tyhpi ATCC 51812	+	+	+	+	+	+
Shigella dysenteriae ATCC 25931	+	+	+	+	+	+
Pseudomonas aeruginosa ATCC 9027	+	-	+	+	+	+
Pseudomonas testosterone NCIM 5098	-	+	+	+	-	+
Proteus morganii NCIM 2040	+	+	+	+	+	+
Klebsiella pneumonae NCIM 2719	-	-	+	+	+	+
Listeria monocytogenes ATCC 19112	+	+	+	+	+	+

(+): active, (-): not active,

[#] Antimicrobial activity: extracts were tested at 200 µg/disc.

[a]Tested samples: *n*-hexane extract (HX), dichloromethane extract (DCM), ehtylacetate extract (EA), ethanol extract (ET), water extract (WT), reference antibiotic (RA) gentamycin.

Table 2: Minimum inhibition concentration (μg/ml) of various extracts from the leaves of *T. baccata* and reference antibiotic.

Microorganisms	Minimum inhibition concentration					
	HX	**DCM**	**EA**	**ET**	**WT**	**RA**
Gram positive bacteria						
Staphylococcus aureus ATCC 25925	-	312.5	156.25	39.06	-	9.76
Bacilus subtilis ATCC 6633	156.25	-	78.12	78.12	39.06	4.88
Staphylococcus epidermis ATCC 12228	78.12	156.25	-	78.12	156.25	4.88
Micrococcus luteus ATCC 10240	156.25	156.25	156.25	39.06	78.12	9.76
Gram negative bacteria						
Escherichia coli ATCC 25922	78.12	156.25	78.12	39.06	78.12	4.88
Enterobater aerogens ATCC 13048	156.25	78.12	156.25	78.12	78.12	2.44
Salmonella tyhpi ATCC 51812	156.25	312.5	156.25	156.25	156.25	2.44
Shigella dysenteriae ATCC 25931	312.5	156.25	78.12	39.06	78.12	4.88
Pseudomonas aeruginosa ATCC 9027	156.25	-	156.25	78.12	156.25	4.88
Pseudomonas testosteroni NCIM 5098	-	156.25	78.12	78.12	-	2.44
Proteus morganii NCIM 2040	312.5	312.5	156.25	156.25	156.25	9.76
Klebsiella pneumonae NCIM 2719	-	-	156.25	78.12	312.5	4.88
Listeria monocytogenes ATCC 19112	156.25	156.25	312.50	39.06	156.25	4.88

n-hexane extract (HX), dichloromethane extract (DCM), ehtylacetate extract (EA), ethanol extract (ET), water extract (WT), reference antibiotic (RA) gentamycin.

Table 3: Minimum microbicidal concentration (µg/ml) of various extracts from the leaves of *T. baccata* and reference antibiotic.

Microorganisms	Minimum microbicidal concentration					
	HX	**DCM**	**EA**	**ET**	**WT**	**RA**
Gram positive bacteria						
Staphylococcus aureus ATCC 25925	-	>312.5	312.5	78.12	-	9.76
Bacilus subtilis ATCC 6633	312.50	-	156.25	156.25	156.25	9.76
Staphylococcus epidermis ATCC 12228	156.25	312.50	-	78.12	312.5	9.76
Micrococcus luteus ATCC 10240	312.50	312.50	312.5	78.12	312.5	9.76
Gram negative bacteria						
Escherichia coli ATCC 25922	156.25	312.5	156.25	78.12	312.5	9.76
Enterobater aerogens ATCC 13048	312.5	312.5	>312.5	156.25	312.5	4.88
Salmonella tyhpi ATCC 51812	312.5	>312.5	312.5	78.12	>312.5	9.76
Shigella dysenteriae ATCC 25931	>312.5	156.25	156.25	78.12	156.25	9.76
Pseudomonas aeruginosa ATCC 9027	312.5	-	156.25	156.25	312.5	4.88
Pseudomonas testosteroni NCIM 5098	-	312.5	156.25	156.25	-	4.88
Proteus morganii NCIM 2040	>312.5	>312.5	312.5	312.5	312.5	9.76
Klebsiella pneumonae NCIM 2719	-	-	312.5	78.12	>312.5	4.88
Listeria monocytogenes ATCC 19112	>312.5	156.25	>312.5	78.12	156.25	9.76

n-hexane extract (HX), dichloromethane extract (DCM), ehtylacetate extract (EA), ethanol extract (ET), water extract (WT), reference antibiotic (RA) gentamycin.

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