

Laboratory Evaluation of Crude Extracts of *Cinnamomum tamala* for Potential Antibacterial Activity

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Abstract

Emergence of microbial resistance is the one of the major problem nowadays; thus there have been tremendous efforts towards finding new chemicals, specifically herbals, for the development of new antimicrobial drugs. *Cinnamomum tamala* (Buch.-Ham.) Nees & Eberm. is an important traditional medicinal plant, mentioned in various ancient literatures such as Ayurveda.. The plant is selected to evaluate the possibility for novel pharmaceuticals having antibacterial potential.

Stem-bark of the plant was extracted using various solvents. Extracts were evaluated for their antimicrobial potential by agar well diffusion assay against a total of six bacterial strains. Active extracts thus obtained were subjected to determine their minimum inhibitory concentration(s) (MICs) followed by their phytochemical analysis.

The current study indicated that the pattern of inhibition depends largely upon the extraction procedure, the plant part used for extraction, state of plant part (fresh or dry), solvent used for extraction and the microorganism tested. Almost all the extracts evaluated showed variable degree of inhibition zones against different bacterial species except hexane extract which was found completely inactive. Other organic extracts viz., ethanol, methanol and ethyl acetate were found to have significant activity against all test bacteria except *Escherichia coli*, which was observed completely resistant to all the extracts. Methanol extract was found comparatively more effective. Organic extracts provided more potent antibacterial activity as compared to aqueous extracts. Gram-positive bacteria were found more sensitive than Gram-negative bacteria.

The study promises an interesting future for designing potentially active antibacterial agents from *Cinnamomum tamala*.

Keywords: Agar well diffusion assay; antibacterial activity; Ayurveda; *Cinnamomum tamala*; MIC.

1. Introduction

Antibiotic chemotherapy has been one of the most important medical achievements of the twentieth century. This therapy is widely practiced for the treatment of various microbiological infections; however, Fleming warned that the misuse of antibiotics could lead to the emergence of resistant forms of bacteria. These drug-resistant strains of microorganisms pose a greater threat to the global public health (Russell, 2002; Goyal *et al.*, 2008; Kaushik *et al.*, 2008). Unfortunately, as we enter the new millennium many of our existing antibacterial agents are under threat due to widespread emergence of bacterial resistance.

A feasible way to combat the problem of microbial resistance is the development of new antibacterial agents for substitution with ineffective ones (Leggadrio, 1995). Thus, global attention has been shifted towards finding new chemicals, specifically herbals, for the development of new drugs. The study indicated that natural products are important sources for new drugs and are also good lead compounds suitable for further modification during drug development. Numerous methods have been utilized to acquire compounds for drug discovery, including isolation from plants and other natural sources, synthetic chemistry, combinatorial chemistry and molecular modeling (Geysen *et al.*, 2003; Lombardino and Lowe, 2004; Kaushik *et al.*, 2009). Plant-based medicines initially dispensed in the form of crude drugs such as tinctures, teas, poultices, powders, and other herbal formulations (Samuelsson, 2004; Kaushik and Goyal, 2008), now serve as the basis of novel drug discovery. A number of medicinal plants have been evaluated for their antibacterial potential (Kaushik and Dhiman, 2000; Kaushik, 1988; Goyal *et al.*, 2009; Kaushik *et al.*, 2009). *Cinnamomum tamala* (Buch.-Ham.) Nees & Eberm. (syn. *Cinnamomum tejpata* Hort.; *Laurus tamala* Buch.-Ham.) belongs to family Lauraceae. The specific epithet '*tamala*' is after a local name of the plant in India. Essential oil extracted from the leaves contains monoterpenoides

including phellandrene, eugenol, linalool and some traces of α -pinene, p -cymene, β -pinene and limonene, phenylpropanoids (Agaoglu *et al.*, 2007). This plant is frequently mentioned in various Ayurvedic literatures for its various medicinal values. The current study refers to the evaluation of the stem-bark extracts of this plant for potential antibacterial activity against some clinically significant bacterial strains as mentioned below.

2. Materials and Methods

Plant material

Stem-bark of *Cinnamomum tamala* was collected during the late 2007 from NCR-Delhi, India. The material was taken to the laboratory and was authenticated by Prof. P. Kaushik at Gurukul Kangri University, Haridwar, India.

Extract preparation

Plant material was washed repeatedly under running tap water, followed by sterilized distilled water. Bark was further air-dried on filter paper at room temperature and then powdered with the help of sterilized pestle and mortar under aseptic condition. Plant material was then extracted using the following methodology (Gupta *et al.*, 2009).

Aqueous extraction

10g of powdered bark was mixed well in 100ml distilled water with constant stirring for 30 minutes. The solution was kept at room temperature for at least 24h and then filtered using muslin cloth. The filtrate was centrifuged at 5000rpm for 15 minutes. The supernatant was again filtered using Whatman's Filter No. 1 under strict aseptic conditions. The filtrate was collected in fresh sterilized glass tubes and stored at 4°C until use. Aqueous extract was prepared in final concentration of 100 mg/ml.

Extraction using Organic Solvents

10g of plant part was thoroughly mixed with 100ml organic solvent (*viz.*: ethanol, methanol, ethyl acetate and hexane). The mixture thus obtained was filtered through muslin cloth and then re-filtered by passing through Whatman's filter No. 1. The filtrate was then concentrated by complete evaporation of solvent at room temperature to yield the pure extract. Stock solutions of crude extracts were prepared by mixing well the appropriate amount of dried extracts with appropriate solvent to obtain a final concentration of 100 mg/ml. Each solution was stored at 4°C after collecting in sterilized glass tubes until use.

Bacterial Strains

Six bacterial strains namely; *Escherichia coli* MTCC-739, *Salmonella typhi* MTCC-531, *Bacillus cereus* MTCC-430, *Bacillus subtilis* MTCC-736, *Staphylococcus aureus* MTCC-740 and *Streptococcus pyogenes* MTCC-442 were selected for the present study. All strains were collected from the Microbial Type Culture Collection (MTCC), India. The bacterial cultures were maintained in nutrient agar slants at 37°C. Each of the microorganisms was reactivated prior to susceptibility testing by transferring them into a separate test tube containing nutrient broth and incubated overnight at 37°C.

Antibacterial Susceptibility Assay

Agar well diffusion assay (Perez *et al.*, 1990) was the key process used to evaluate the antibacterial potential of *Cinnamomum tamala* bark extracts. Extracts were first sterilized by sterile membrane syringe filter (pore size 0.45 μ m, manufactured by Pall Life Sciences). Petri dishes (100mm) containing 18ml of Mueller Hinton Agar (MHA) were seeded with approximately 100 μ l inoculum of bacterial strain (inoculum size was adjusted so as to deliver a final inoculum of approximately 10⁸ CFU/ml). Media was allowed to solidify. Wells of 6mm diameter were cut into solidified agar media using a sterilized cup-borer. 100 μ l of each extract was poured in the respective well and the plates were incubated at 37°C overnight. The experiment was performed in triplicate under strict aseptic conditions to ensure consistency of all findings. The antibacterial activity of each extract was expressed in terms of the mean of diameter of zone of inhibition (in mm) produced by each extract at the end of incubation period.

Organic solvents used in preparation of extracts were also used as negative controls during the study. Tetracycline (5 μ g/ml) was used as a standard antibiotic (*i.e.* positive control) in the present study for a comparative analysis with the effectiveness of various plant extracts against selected microflora.

Assessment of Minimum Inhibitory Concentration

MIC (minimum inhibitory concentration) of active extracts thus obtained were further examined by standard two-fold microdilution broth methodology (NCCLS, 1997). A stock solution of each active extract was serially diluted in 96-wells microtiter plate with Mueller Hinton broth to obtain a concentration ranging from 8.0 μ g/ml to 4096 μ g/ml. A standardized inoculum for each bacterial strain was prepared so as to give an inoculum size of approximately 5 x 10⁵ CFU/ml in each well. Microtiter plates were then kept at 37°C for an overnight incubation. Following incubation, the MIC was calculated as the lowest concentration of the extract inhibiting the visible growth of bacterial strain using reflective viewer.

All the chemical ingredients used in present study were of analytical grade and were purchased from Hi Media, India.

Phytochemical Analysis

Active extract was analyzed for its phytochemistry by using methods as described by Harborne (Harborne, 1973).

3. Results

Stem-bark extracts of *Cinnamomum tamala* were evaluated for *in vitro* antibacterial potential by agar well diffusion assay, the result of which has been mentioned in Table 1. Almost all the extracts evaluated showed variable degree of inhibition zones against different bacterial species except hexane extract which was found completely inactive. Other organic extracts viz., ethanol, methanol and ethyl

acetate were found to have significant activity against all test bacteria except *Escherichia coli*, which was observed completely resistant to all the extracts. Methanol extract was found comparatively more effective with zone sizes ranging from 11.26 mm to 20.77 mm. Ethanol extract showed inhibition in the range of 11.83 mm to 17.90 mm, slightly lower than that of methanol extract. Ethyl acetate extract was shown to have mild activity with average zone size approximately 12 mm and maximum inhibition of *Staphylococcus aureus* with 15 mm inhibition zone. Aqueous extract was found effective only against *Staphylococcus aureus* and *Bacillus cereus* with zone size approximately 11 mm against each bacterium. *Salmonella typhi* and *Streptococcus pyogenes* were shown to have mild susceptibility against ethanol, methanol and ethyl acetate extracts with zone size ranging between 11 to 14 mm only.

Table 1. *In Vitro* Antibacterial Activity of Aqueous and Organic Extracts of *Cinnamomum tamala* Stem-Bark.

Type of Extract	Zone of Inhibition* (in mm diameter)					
	Gram-negative Bacteria			Gram-positive Bacteria		
	<i>Escherichia coli</i>	<i>Salmonella typhi</i>	<i>Streptococcus pyogenes</i>	<i>Staphylococcus aureus</i>	<i>Bacillus subtilis</i>	<i>Bacillus cereus</i>
Ethanol	NI	11.93±0.51	13.87±0.81	17.90±0.17	12.50±0.87	11.83±1.60
Methanol	NI	11.26±0.68	13.53±0.92	20.77±0.64	13.96±0.45	14.30±0.75
Ethyl Acetate	NI	12.17±0.58	12.23±1.08	15.06±0.31	11.67±1.15	12.33±1.26
Hexane	NI	NI	NI	NI	NI	NI
Aqueous Extract	NI	NI	NI	11.76±0.25	NI	11.10±0.66
Tetracycline ⁺	29.50±0.50	25.83±1.61	29.83±1.89	32.50±1.50	34.17±1.76	32.16±1.04

Note: Values of the observed zone of inhibition (in mm diameter) including the diameter of well (6 mm) after 24 hours incubation against different bacterial species when subjected to different extracts in agar well diffusion assay. Assay was performed in triplicate and results are the mean of three values ± Standard Deviation. In each well, the sample size was 100 µl. Inhibition observed in extracts due to solvent were assessed through negative controls. 'NI'-No Inhibition Zone was observed. ⁺Tetracycline (5 µg ml⁻¹) was used as standard antibiotic.

Table 2. Minimum Inhibitory Concentration of Active Crude Extracts of *Cinnamomum tamala* Stem-Bark.

Active Crude Extract	Test Microorganism	Concentration of Extracts* (in µg ml ⁻¹)										MIC (in µg/ml l ⁻¹)
		4096	2048	1024	512	256	128	64	32	16	8	
Ethanol	<i>S. aureus</i>	-	-	-	-	+	+	+	+	+	+	512
Ethanol	<i>S. pyogenes</i>	-	+	+	+	+	+	+	+	+	+	4096
Ethanol	<i>B. subtilis</i>	+	+	+	+	+	+	+	+	+	+	>4096
Methanol	<i>S. aureus</i>	-	-	-	-	-	+	+	+	+	+	256
Methanol	<i>S. pyogenes</i>	-	+	+	+	+	+	+	+	+	+	4096
Methanol	<i>B. subtilis</i>	-	+	+	+	+	+	+	+	+	+	4096
Methanol	<i>B. cereus</i>	-	-	+	+	+	+	+	+	+	+	2048
Ethyl acetate	<i>S. aureus</i>	-	-	+	+	+	+	+	+	+	+	2048

Note: Different concentrations of active crude extracts evaluated in 96-well microtiter plate using Microbroth Dilution Assay as recommended by NCCLS. All values are expressed in µg ml⁻¹; (-) represents 'No Growth Observed'; (+) represents 'Growth Observed'.

Inhibitory concentrations (MICs) of *Cinnamomum tamala* extracts as evaluated by microbroth dilution assay are shown in Table 2. *Staphylococcus aureus* was found most susceptible bacterium as it was significantly inhibited at 256 µg/ml and 512 µg/ml concentrations of methanol and ethanol extracts, respectively. The bacterium was inhibited by ethyl acetate extract at 2048 µg/ml concentration. *Streptococcus pyogenes* was inhibited at 4096 µg/ml of both methanol and ethanol extracts. MIC of methanol extract was found to be 2048 µg/ml and 4096 µg/ml against *Bacillus cereus* and *Bacillus subtilis*, respectively. Ethanol extract was not found satisfactory for *Bacillus subtilis* inhibition in the entire range of concentrations (*i.e.* MIC >4096 µg/ml).

4. Discussion

Results of the present study clearly demonstrated that *Cinnamomum tamala* stem-bark extracts revealed a good antibacterial activity. Some earlier researches carried out on the other species of *Cinnamomum* were also in concordance with our results. The essential oil from the bark of *Cinnamomum zeylanicum* showed *in vitro* antimicrobial activity against several microorganisms (Palmer *et al.*, 1998; Prabuseenivasan *et al.*, 2006). *Cinnamomum cassia* bark extract prepared in alcohol showed good antibacterial activity with 7-29 mm size of inhibition zones against the test organisms. Ethyl acetate and acetone extracts showed no antibacterial activity against one or more bacterial strains (Ates and Erdogrul, 2003). The activity was explained due to the presence of certain volatile oil components such as cinnamic aldehyde and eugenol (Baratta *et al.*, 1998). In our study, qualitative analysis reveals the presence of certain secondary metabolites including terpenoids, tannins, alkaloids and flavonoids.

The present study also confirms the use of organic solvents in the preparation of plant extracts as compared to aqueous extracts. The polarity of antibacterial compounds make them more readily extracted by organic solvents, and using organic solvents does not negatively affect their bioactivity against bacterial species. The data also showed that some antimicrobial substances could only be extracted by organic solvents, suggesting that organic solvents are clearly better solvents of antimicrobial agents (Thongson *et al.*, 2004).

In our study, Gram-positive bacteria were found to have more susceptibility as compared to Gram-negative bacterial species. This was also confirmed by the study of *Cinnamomum verum* bark as evaluated by Nanasombat and Lohasupthawee (Nanasombat and Lohasupthawee, 2005) that

showed mild activity against several *Enterobacteria*. *Cinnamomum zeylanicum* bark showed no activity against *Escherichia coli*, while it was moderately active against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Agaoglu *et al.*, 2007). The hypothesis can be given to the differences in the chemical composition and structure of cell wall of both types of microorganisms.

The current study supports the traditional advantages of the studied plant and suggests that some of the bark extracts possess compounds with good antimicrobial properties that can be used for the therapy of infectious diseases caused by certain pathogens. The most active extracts can be subjected to isolation of the therapeutic antimicrobials and carry out further pharmacological evaluation by several methods such as NMR, Mass Spectrometry, UC-MS, LC-MS etc.

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