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Evaluation of Phytochemical and Antibacterial properties of leaf extract of *Cinnamomum tamala* oil

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ABSTRACT

Cinnamomum tamala, commonly known as tej patta is widely used as a spice in Indian cuisine for its aroma and flavoring property as the leaves contain essential oil. The essential oil is extracted with the help of the Clevenger apparatus using dry leaves. This oil is mainly used as medicine for releasing gas as well as a carminative agent and diuretic agent. It also improves the digestive system and helps in increasing appetite. This study aimed to determine the phytochemical properties and antibacterial potential of different extracts (aqueous, methanol, and acetone) and oil of *C. tamala* leaves. The phytochemical evaluation shows the presence and absence of alkaloids, flavonoids, phenolic compounds, steroids, tannins, glycosides, terpenoids, saponins, proteins, and carbohydrates in the aqueous, methanol, and acetone extracts. The efficacy of antibacterial properties of prepared extracts was examined against *E. coli* and *Salmonella typhi* (gram-negative bacteria) and *Staphylococcus aureus* and *Bacillus subtilis* (gram-positive bacteria). These bacterial cultures were obtained from IMTech Chandigarh. From the results of the antibacterial study it has become evident that among three extracts, the maximum zone of inhibition was obtained in the aqueous extract which was followed by methanolic and acetone extract. With the help of a Clevenger apparatus, Bay leaf oil was extracted to establish antibacterial properties. Henceforth, to analyze the antibacterial potential of the oil sample, the test was performed against the mentioned bacterial species (*E. coli, Salmonella typhi, Staphylococcus aureus,* and *Bacillus subtilis*) and reported significant antibacterial activities. From the outcome of this study, it has become clear that Bay leaf oil has potent antibacterial properties against selected bacterial species.

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1 Introduction

Cinnamomum tamala is commonly known as Tejpatta. It is a medium-sized evergreen tree acquiring 8 m in height and a girth of 150 cm. The genus *Cinnamomum* consists of 270 species out of which only 20 species are cultivated commercially in India (Mall et al. 2018). The bay leaf trees are commercially cultivated throughout the country from tropical regions to sub-tropical regions of the Himalayas. Stems are rough, having a brown color. The flowers have a whitish type of color and mostly occur in the last week of March or in the April first week. The fruits are drupe and ripens fruits are dark purple. Seed maturity takes place in approximately one year (Sharma et al. 2009).

The leaves of *C. tamala* are used widely in north India as a spice known as Tejpat. These are used as a replacement for paan (betel leaves) in Kashmir (Kirtikar and Basu 1981). Bay leaf is extensively applied as spices in Indian cuisine for its fragrance and flavoring property as leaves containing essential oil. The leaves' essential oil is extracted by the steam distillation process of dry leaves. The chief element of cinnamon oil includes eugenol, sabinene (4.8%), β-caryophyllene (6.6%), curcumenol (2.3%), and germacrene D (4.6%). Its bark produces cinnamaldehyde which is primarily accountable for its fragrance but other components give the unique flavor and odor. Medicinally the oil is mainly used as an anti-flatulent, diuretic and carminative (Showkat et al. 2004). Traditionally, the leaves are utilized in colic and diarrhoeal preparations (Nadkarni 1976; Duke and Fulton 2002). Further, the essential oil of *C. tamala* revealed fungi toxic activity (Dubey et al. 1998).

Bay leaves or Tejpatta are used daily by the Indians in cooking. Since the ancient era, dry bay leaves are mainly used as a spice and green leaves are used for perfumery purposes. It also shows good medicinal properties. As it stimulates the different digestive enzymes which enhance digestion. Bay leaf is an important constituent of several kinds of drugs in the pharmaceuticals industries. It is beneficial in the treatment of muscle and joints complications and helps to relieve arthritis, inflamed joints, muscular pains, rheumatism, and sprains. It also improves the digestive system and helps in increasing appetite. It also helps in controlling infections also (Shu 1998). It also showed antibacterial properties against potent gram-positive and gram-negative bacteria. The present study was focused on the phytochemical analysis of bay leaf along with its antibacterial activity. Moreover, its essential oil was extracted and undergoes antibacterial analysis against potentially pathogenic bacteria.

2 Materials and Methods

2.1 Collection of *C. tamala* leaves

The plant material for the study was purchased from the local markets of Mathura. Cleaning was done two times with running tap water and with distilled water so that all the dust and impurities runoff. At room temperature, leaves were air-dried and then dried in a hot air oven for a few hours. The weight of dry leaves was taken and recorded. It is then crushed by the mixer to get a fine powdered form and the weight of the powder was also taken and recorded (Figure 1). Extraction was carried out using the Soxhlet apparatus. About 30g of leaves powder was taken for each aqueous, acetone, and methanol solvent. Powdered plant material (30g) was uniformly packed in the respective thimbles and covered with cotton wool, the thimble having sample was then placed in the extraction jacket and the continuous extraction was carried out by using respective solvents, i.e., double distilled water (700 ml), acetone (500 ml) and methanol (500 ml). The extraction was carried out till the color of the solvent in the siphon tube become colorless. The temperature of Soxhlet was maintained to approximately 60-70°C for aqueous extraction, 30-40°C for

Figure 1 Preparation of *Cinnamomum tamala* (Bay leaf) (A) Leaf collection (B) Crushed

acetone, and 50°C for methanol extraction. When the extraction is complete, the thimble is removed and again switched on the Soxhlet, and the purified solvent was extracted out from the extract which was used for the further study. Then the extract was poured on a 200mm Petri plate and put overnight in an incubator to incubate it at 50°C till the solvent evaporate, and at last, the extract was collected by scratching it from the plate and then weight the obtained extract and storing it in a sterile vial or sample container.

2.2 Preliminary phytochemical screening of *C. tamala*

2.2.1 Alkaloids

Hager's test has been used for screening the presence of alkaloids in the extract. A saturated solution of picric acid (TNP) was made and then mixed with 2 ml of the respective extracts. The appearance of yellow ppt. indicates the presence of alkaloids.

2.2.2 Cardiac glycosides

For this, 2 ml of glacial acetic acid was collected, and add a drop of $FeCl_3$ solution into it. In this solution, 2 ml of plant extract and 1 ml of concentrated H_2SO_4 were also added. The formation of a brown ring at the interface specifies the presence of de-oxy sugar i.e. the characteristics of cardenolide.

2.2.3 Saponins

2ml of water was added to the dry extract and shake vigorously. The appearance of foam showed the presence of saponins.

2.2.4 Tannins

2 ml of extract was mixed with a few drops of 1% $FeCl_3$, and the appearance of blue-black precipitate in the solution confirms the presence of tannins.

2.2.5 Steroids

The presence of steroids was screened by Salkowski Test. For this, chloroform was added into the crude extract and this was followed by the addition of a few drops of concentrate H_2SO_4, shaking well mix and allowed to stand for some time. The presence of steroids was confirmed by the appearance of red color at the lower layer while the presence of tri-terpenoids was confirmed by yellow color layer formation.

2.2.6 Carbohydrates

For carbohydrate screening, Molisch's Test was carried out, in this, 2 ml of sample was taken in a test tube and a small amount of Molisch's reagent (∝-naphthol dissolved in ethanol) was mixed. This was followed by the addition of concentrate H_2SO_4 slowly through the wall of the test tube, so a ring is formed at the bottom layer, formation of a bluish-violet ring at the junction confirms its carbohydrates.

2.2.7 Proteins

2 ml of extract was taken in a test tube and boiled with Ninhydrin, development of a violet color confirmed the presence of proteins.

2.2.8 Phenolic compound

2 ml of extract was mixed with the Bromine water and the presence of yellow precipitate confirms the presence of phenolic compounds.

2.2.9 Terpenoids

Take 2 ml of chloroform and carefully add concentrated H_2SO_4 to form a layer. Add 2 ml of extracts to it. The appearance of reddish-brown color at the interface authenticates the presence of terpenoids.

2.2.10 Flavonoids

2 ml of 2% NaOH was added to the extract which produces yellow color which get disappeared when 2-3 drops of dilute acid are added, this is the best test for the screening of the presence of flavonoids.

2.3 Antibacterial activity of different bay leaf extracts

2.3.1 Test microorganisms

Escherichia coli (MTCC 294) and *Salmonella typhi* (MTCC 660) are the two Gram-negative bacterial strains while *Staphylococcus aureus* (MTCC 3160) and *Bacillus subtilis* (MTCC 2057) are the two Gram-positive bacterial strains used for the analysis of antimicrobial activity.

2.3.2 Preparation of different concentrations of bay leaf extracts

Prepare different extracts (acetone, methanol, and aqueous) as per the method given in section 2.1. The dried extracts were diluted with appropriate solvents to make different final concentrations (25 mg/ml, 50 mg/ml, 75 mg/ml and 100mg/ml) respectively.

2.3.3 Preparation of bacterial inoculum

A loopful culture of each bacterium was inoculated into 4-5 ml peptone water and incubated at 37^0C for 24 hours. Now match this bacterial growth with that of 0.5 Mc Farland standards that are formulated by adding 99.5 ml of 1% (v/v) H_2SO_4 in 0.5 ml of 1.75 % (w/v) $BaCl_2.2H_2O$. If the bacterial growth is dense then dilute it by adding more peptone water to match exactly with the Mc-Farland standard. This concentration is equivalent to 1-2 X 10^8CFU/ml approximately (Baker et al. 1983).

2.3.4 Screening of antibacterial activity of bay leaf extract in different solvents

Disc diffusion method was used to find out the antibacterial activity. Firstly the Nutrient agar media (NAM) was poured into pre-sterilized Petri plates and when it becomes solidified, 0.1 ml of bacterial inoculum (size $1x10^8$) was loaded on the Petri plates and spread using an L-shaped spreader. Now, plates were incubated at 37^0C for one hour. Sterile plain discs were impregnated with different concentrations of extracts (25 mg/ml, 50 mg/ml, 75 mg/ml, and 100 mg/ml) were placed over the Nutrient agar media surface at a specific distance. All the 4 different concentrations of the 3 extracts and 4 discs were impregnated with 15 µL of all respectively and placed on the separate bacterial plates.

Ciprofloxacin, streptomycin, erythromycin, and chloramphenicol antibiotic discs were taken as a positive control for the different bacteria respectively. Disc impregnated with 15 µL of autoclaved distilled water was taken as a negative control for aqueous extract and disc impregnated with 15 µL of a mixture of DMSO and distilled water (20%) was taken as a negative control for acetone and methanol extract. Now plates were incubated at 37^0C for 24 hours for the development of the zone of inhibition. Results were compared with the standard antibiotics discs which serve as positive control and water/DMSO (depending on the solvent used) as a negative control. Each test was carried out three times and the average inhibition zone was documented for all the three extracts and compared it with standard antibiotics i.e. control.

2.4 Screening of antibacterial activity of bay leaf oil

2.4.1 Extraction of bay leaf oil

About 40 g of the powdered leaves is weighed and 600 ml of double-distilled water was taken. Then the Clevenger apparatus was set up and leaves were subjected to hydro-distillation for about 6-7 hrs at a temperature between 30-40^{o}C. After that the apparatus was left undisturbed for some time so that the temperature of the apparatus will decrease, then the essential oil extracted from the leaves was collected and stored at 4^{o}C until used.

2.4.2 Preparation of different concentrations of bay leaf oil

The oil was diluted with ethanol to obtain different concentrations of 50% and 100% (v/v). Furthermore, preparation of bacterial inoculums and screening of antibacterial activity of bay leaf oil was carried out as explained in sections 2.3.3 and 2.3.4.

2.5 Statistical analysis

Results (in terms of zone of inhibition) undergo statistical analysis that is performed by one-way analysis (ANOVA) through SPSS ver. 20.0 software and Duncan's multiple tests (DMRT) at $p < 0.05$ and $p < 0.01$ by determining the significant variation in mean values between the experimental and control values. These values were defined as mean ± S.E.M (standard error mean).

3 Results and Discussion

3.1 Phytochemical analysis

The results of phytochemical evaluation revealed the difference in the presence and absence of alkaloids, flavonoids, phenolic compounds, steroids, tannins, glycosides, terpenoids, saponins, proteins, and carbohydrates in the Aqueous, Methanol, and Acetone extracts (Table 1). Similar types of results were also obtained by Saluja et al. (2010) and Gill et al. (2015). The presence of these phytochemicals provides a source for various medicinal properties against various pathogenic bacteria and lethal diseases. The important studies conducted on *C. tamala* are for its hypolipidemic effects (Dhulasavant et al. 2010), anti-diabetic and antioxidant (Chakraborty and Das 2009), anti-ulcer (Eswaran et al. 2010), anti-inflammatory (Gambhire et al. 2009), anti-diarrhoeal (Rao et al. 2008), immunosuppressive (Chaurasia et al. 2010) and anti-bacterial (Goyal et al. 2009). Phytochemicals like terpenoids and tannins are credited with anti-inflammatory and analgesic properties. Hence, the presence of all these phytoconstituents enables plants enough potent in the treatment of lethal human infections.

Table 1 Phytochemical screening of different Bay leaf extracts

Tests Name	Results		
	Aqueous	Methanol	Acetone
Alkaloids	-	-	+
Cardiac Glycosides	+	+	-
Saponins	+	-	-
Tannins	+	+	+
Steroids	+	-	+
Carbohydrates	+	+	-
Proteins	-	+	-
Phenolic compounds	-	-	-
Terpenoids	-	-	-
Flavanoids	+	+	+

3.2 Antibacterial Activity of bay leaf in different solvents

Antibacterial activities of bay leaf (*C. tamala)* in different solvents (acetone, aqueous, and methanol) were tested against selected pathogenic bacteria and their zones of inhibition were analyzed (Table 2). All the leaf extracts of *C. tamala* showed significant ($p<0.01$) bactericidal potential against both gram-positive (*B. subtilis*

Table 2A Antibacterial activity of aqueous bay leaf extracts against pathogens

S. N.	Name of Bacteria	Leaf Extract Concentrations				Control +VE	Control −VE
		25 mg/ml	50 mg/ml	75 mg/ml	100 mg/ml	Erythromycin	D.W.
		Zone of inhibition (in cm)					
1.	*E. coli*	-	0.7±0.2	1.1±0.1	1.6±0.2	3.0±0.1	-
2.	*Staphylococcus aureus*	-	0.6±0.2	0.9±0.2	1.0±0.1	2.1±0.1	-
3.	*Salmonella typhi*	-	0.5±0.1	0.6±0.2	0.8±0.2	2.2±0.1	-
4.	*Bacillus subtilis*	-	0.4±0.1	0.5±0.1	0.9±0.2	2.6±0.2	-

*Statistical analysis using one way ANOVA/ DMRT revealed results to be significant ($p < 0.01$)

Table 2B Antibacterial activity of methanol bay leaf extracts against pathogens

S. N.	Name of Bacteria	Leaf Extract Concentrations				Control +VE	Control −VE
		25 mg/ml	50 mg/ml	75 mg/ml	100 mg/ml	Erythromycin	Methanol
		Zone of inhibition (in cm)					
1.	*E. coli*	0.5±0.1	0.9±0.2	1.0±0.1	1.1±0.1	1.6±0.2	-
2.	*Staphylococcus aureus*	0.5±0.1	0.6±0.2	0.6±0.2	0.7±0.2	1.6±0.2	-
3.	*Salmonella typhi*	-	0.5±0.1	0.7±0.2	1.0±0.1	3.0±0.1	-
4.	*Bacillus subtilis*	-	0.6±0.2	0.7±0.2	0.9±0.2	2.1±0.1	-

*Statistical analysis using one way ANOVA/ DMRT revealed results to be significant ($p < 0.01$).

Table 2C Antibacterial activity of acetone bay leaf extracts against pathogens

S. N.	Name of Bacteria	Leaf Extract Concentrations				Control +VE	Control −VE
		25 mg/ml	50 mg/ml	75 mg/ml	100 mg/ml	Erythromycin	Acetone
		Zone of inhibition (in cm)					
1.	*E. coli*	-	0.4±0.1	0.7±0.2	0.8±0.2	1.0±0.1	-
2.	*Staphylococcus aureus*	0.5±0.1	0.6±0.2	0.6±0.2	0.9±0.2	1.0±0.1	-
3.	*Salmonella typhi*	-	0.5±0.1	0.6±0.2	0.8±0.2	2.1±0.1	-
4.	*Bacillus subtilis*	0.4±0.1	0.5±0.1	0.5±0.1	0.7±0.2	1.9±0.2	-

*Statistical analysis using one way ANOVA/ DMRT revealed results to be significant ($p < 0.01$)

(MTCC 2057), *S. aureus* (MTCC 3160) and gram-negative (*E. coli* (MTCC 294), *S. typhi* (MTCC 660) (Table 2A, 2B and 2C). A dose-dependent bactericidal activity was noticed in all the bay leaf extracts. From the results, it has been clear that the aqueous plant extract shows better inhibition against *E. coli* and *S. aureus*. This result was similar to those of other studies that reported antibacterial activity (Parekh and Chanda 2007; Hassan et al. 2016; Bharadwaj et al. 2020). The methanolic extract shows better inhibition against *E. coli* and *S. typhi*, the similar findings were observed by Parekh and Chanda (2007). Maximum activity was observed against *S. aureus* in acetone extract. These results are in agreement with the findings of Singh et al. (2018) and Dash et al. (2020). The antibacterial activities accessed were compared with that of standard broad-spectrum antibiotics tetracycline as positive control and water and DMSO (according to solvent) as a negative control.

3.3 Antibacterial Activity of bay leaf oil in different solvents

Antibacterial activity of bay leaf oil (*C. tamala*) was performed in different concentrations (50% and 100% v/v) against both gram-positive (*B. subtilis* MTCC 2057, *S. aureus* MTCC 3160) and gram-negative (*E. coli* MTCC 294, *S. typhi* MTCC 660) bacterial strains. All the bay leaf oil extracts showed significant ($p<0.01$) bactericidal potential (Table 3). From the results obtained it has been clear that there is a dose-dependent bactericidal activity. The maximum zone of inhibition was found at 100% (v/v) concentration against *S. aureus* in the volatile oil. These results are in agreement with various previous researchers (Parekh and Chanda 2007; Borkataky and Sood 2014; Hassan et al. 2016; Heer et al. 2017; Manandhar et al. 2019; Dash et al. 2020). The antibacterial activities performed were compared with that of standard broad-spectrum antibiotics as positive control and ethanol as negative control (Table 3).

Table 3 Antibacterial activity of Bay leaf oil against bacterial pathogens

S. N.	Name of Bacteria	Leaf Extract Concentrations		Control +VE	Control −VE
		50 mg/ml	100 mg/ml	Erythromycin	Ethanol
		Zone of inhibition (in cm)			
1.	*E. coli*	0.7±0.2	0.8±0.2	2.0±0.1	-
2.	*Staphylococcus aureus*	0.6±0.2	0.9±0.2	2.1±0.1	-
3.	*Salmonella typhi*	0.6±0.2	0.8±0.2	1.5±0.1	-
4.	*Bacillus subtilis*	0.5±0.1	0.5±0.1	2.0±0.1	-

*Statistical analysis using one way ANOVA/ DMRT revealed results to be significant ($p < 0.01$).

Conclusion

The multiple benefits of *C. tamala* made it a true miracle of nature. From this study, it becomes clear that active phytochemicals were present in the various extracts of bay leaf powder and due to the presence of these phytochemicals, all three extracts showed significant antibacterial activity. The aqueous plant extract shows better inhibition against *E. coli* and *S. aureus* while Methanolic extract showed better inhibition against *E. coli* and *S. typhi*. Maximum activity was observed against *S. aureus* in acetone extract and volatile oil.

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