Original Article

Antibacterial Effect of Aqueous and Methanolic extract of Indian bay leaves *(Cinnamomum tamala)* against *Pseudomonas aeruginosa*

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Abstract

Objective: To investigate the antibacterial activities of Indian bay leaf extracts against Pseudomonas aeruginosa.

Methods: This Experimental study was carried out during the period of July 2019 to June 2020 in the Department of Pharmacology and Therapeutics with the collaboration of Department of Microbiology, Mymensingh Medical College, Mymensingh, Bangladesh. The antibacterial activity was tested at different concentrations (20, 10, 5,2.5, 1.25 & 0.625 mg/ml) of both extracts of spice by using disc diffusion & broth dilution method. The extracts were prepared by using solvents aqueous & methanol. The test microorganisms were also tested for their activity against a standard antibiotic Gentamicin (80 mg) by broth dilution method and the result was compared with that of Aqueous and Methanolic extracts.

Results: Among different concentrations of the ALE, 15mg/ml & above concentration showed inhibitory effect against *Pseudomonas aeruginosa.* In case of MLE, 15mg/ml & above concentration showed inhibitory effect against aforesaid bacteria. In disc diffusion method, Zone of inhibition (ZOI) in case of ALE ranged between 6-25mm at different concentration of extracts. Minimum inhibitory concentrations (MIC) of ALE were 15 mg/ml and MLE were 7.5 mg/ml against *Pseudomonas aeruginosa*. This result was also compared against a standard antibiotic Gentamicin where the MICs of Gentamicin were lower in comparison to MICs of ALE & MLE. The present study showed that aqueous and methanolic extracts of leaves demonstrated antibacterial effects against *Pseudomonas aeruginosa*.

Conclusion: From the study it is clearly observed that there is definite antibacterial effect of both the aqueous and methanolic extract of leaves of Cinnamomum tamala against *Pseudomonas aeruginosa.* Further studies are required to detect and isolate the biologically active ingredients present in the Cinnamomum tamala which are responsible for this antibacterial effect. Hopefully, that would lead to the discovery of new and more potent antimicrobial agents isolated from Cinnamomum tamala.

Key words: Antibacterial activity, Cinamomum tamala, *Pseudomonas aeruginosa*, Zone of Inhibition, Minimum

Introduction

Infectious diseases are the leading cause of premature death and kill about 50000 people every day in the world. In recent years, drug resistant pathogenic bacteria have been commonly reported from around the world due to indiscriminate use of antibiotics and anxious situation encourages the development of potent antimicrobial agents.[1] Thus, global attention has been shifted to the search for new chemicals, specifically herbals, for the development of new drugs. Many methods have been used to obtain compounds for drug discovery, including isolation from plants and other natural sources, synthetic chemistry, combinatorial chemistry and molecular modeling.[2]

The spice plant Cinnamomum tamala commonly known as tejpata, belongs to the family Lauraceae, which is native to India, Nepal, Bhutan, Bangladesh. The used parts are leaves, bark and essential oil. Bay leaves are a moderate sized evergreen tree with a height of 8m and a girth of 50 cm. Leaves are lanceolate, glabrous, alternately placed, opposite and short stalked.[3] nerved from the base. The genus Cinnamomum has about 250

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tropical tree and shrub species. The etymology is derived from the Greek word Kinnamomon (meaning spice). The Greeks borrowed the word from the Phoenicians, indicating that they traded with the east from early times. The specific epithet 'tamala' is after a local name of the plant in india.[3,4,5] Bay laurel leaves are short and light to medium green in color, with one large vein below the length of the leaf, while the leaves of the indian bay leaf are about twice as long and broad, usually olive green in color, and with three veins down the length of the leaf.[6] Dried leaves of Cinnamomum tamala are used to flavor a variety of foods. Plant bark and leaves are good source of aromatic essential oil which possess phenolic compounds which show multiple therapeutic effects against Alzheimer'sdisease, diabetes, arthritis and arteriosclerosis.[7] The leaves of the tree are rich in manganese, iron, dietary fiber and calcium. Dried leaves and bark are prescribed for fever, anemia and body odor. People chew dried leaves to get rid of bad breath. Cinnamomum tamala leaves have a strong effect on the biological systems such as immune system, gastro intestinal tract and liver.[8] C.tamala show strong antimicrobial activity against gram-positive bacteria, gram-negative bacteria, and fungi. Oil and its ingredients show strong antimicrobial activity against methicillin-resistant *Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis*, *Helicobacter pylori, Aspergillus fumigatus*. Essential oil showed growth inhibitory effects against *S.aureus* and *Mycobacterium tuberculosis*, *Escherichia coli*.[9]

Its various activities have a very promising role. Therefore, the aim of the present study is to evaluate the antibacterial effects of Aqueous and Methanolic extract of leaves of Indian bay leaf against *Pseudomonas aeruginosa.* This study may be helpful in emphasizing the importance of Indian bay leaf as natural product for controlling drug resistant bacteria which is a major threat to human health and also to compare its sensitivity with commercial antibiotics against bacterial species.

Methods

This Experimental study was carried out in the Department of Pharmacology & Therapeutics in collaboration with the Department of Microbiology, Mymensingh Medical College, Mymensingh, Bangladesh from July 2019 to June 2020.

Tested bacterial strains: Bacterial strains, *Pseudomonas aeruginosa* (ATCC 27853) were used in study. Pure cultures of these were obtained from the Department of Microbiology, Mymensingh Medical College, Mymensingh.

Maintenance of bacterial culture and inoculum preparation: Pure cultures were refreshed and maintained on nutrient agar slants and plates on regular basis. The cultures were streaked on sterile nutrient agar plates and kept in incubator for 24 hours at 37°C and stored at 4 °C. Bacterial cultures were refreshed after every 1 to 2 weeks to avoid contamination. Inoculum was prepared by growing the pure bacterial culture in nutrient broth over night at 37°C.

Plant material: The fresh tender leaves were purchased from rural area of Mymensingh.

Preparation of aqueous leaves extract:
The leaves were washed and dried in shade under room temperature for six to seven days. Finaly dried materials were pulverized into fine powdered substance by a grinder. 50 gm of powder of indian bay leaves were weighed with the electric balance and transferred into one conical flask. Then 500 ml distilled water in the flask was added. The solution was kept at room temperature for at least 24 hr. The aqueous extract was then filtered by using muslin cloth. The filtrate was again filtered using whatman no.1 filter paper under strict aseptic conditions. The resulting filtrate were collected in previously tared sterilized petriplates and dried in rotary flash evaporator at 45°C for proper dehydration. After the complete removal of the solvent, the petriplates were weighed and then the net weight of dried extract was determined and used. 1gm dried extract was then dissolved in 50ml sterilized distilled water.

For preparation of aqueous stock solution, 1gm of ALE was dissolved in 50 ml of D/W to get a concentration of .02 gm/ml i.e 20mg/ml which as labeled as stock solution. From above stock solution different concentration such as 10mg/ml, 5mg/ml, 2.5mg/ml, 1.25mg/ml, 0.625mg/ml were prepared with appropriate volumes of D/W.

Table I: *Preparation of the ALE solutions of diVerent concentration*

Sl. No.	Amount of solution (ml) taken from Stock Solution	Amount of distilled water (ml)	Concentration (milligram /ml)
1	1	31	0.625
2	1	15	1.25
3	1	7	2.5
4	1	3	5
5	1	1	10
6	1	0	20

Preparation of methanolic leaves extract:
The leaves were washed and dried in shade under room temperature for six to seven days. Finaly dried materials were pulverized into fine powdered substance by a grinder. 50 gm of powder of indian bay leaves were weighed with the electric balance and transferred into one conical flask. Then 500 ml 100% Methanol in the flask was added. The solution was kept at room temperature for at least 24 hr. The methanolic extract was then filtered by using muslin cloth. The filtrate again filtered using whatman no.1 filter paper under strict aseptic conditions. The resulting filtrate were collected in previously tared sterilized petriplates and dried in rotary flash evaporator at 45°C for proper dehydration. After the complete removal of the solvent, the petriplates were weighed and then the net weight of dried extract was determined and used. 1gm dried extract was then dissolved in 50ml sterilized distilled water.

For preparation of methanolic stock solution, 1gm of MLE was dissolved in 50 ml of D/W to get a concentration of .02 gm/ml i.e 20mg/ml which as labeled as stock solution. From above stock solution different concentration such as 10mg/ml, 5mg/ml, 2.5mg/ml, 1.25mg/ml, 0.625mg/ml were prepared with appropriate volumes methanol.

***Antibacterial sensitivity testing using disc diVusion method*:** Antibacterial sensitivity test was performed by Kirby-Bauer disc diffusion technique. Filter paper disc of 6mm diameter using Whatman No.1 filter paper was prepared and sterilized. After matching with 0.5 McFarland standards for each isolate, a sterile cotton swab was dipped into bacterial suspension and streaked in three directions on the surface of Mueller Hinton Agar plates and then left for 5-10 minutes in room temperature. The blank discs were aseptically placed over the Mueller Hinton agar plates seeded with the test microorganisms. Then with the help of micropipette 10µl 20mg/ml, 10mg/ml, 5mg/ml, 2.5mg/ml, 1.25mg/ml and 0.625mg/ml concentrations of Aqueous & methanolic leaf Extracts were transferred to different disc aseptically. while 10µL of distilled water & 100% methanol were added in sterile filter paper disc as negative control in both extracts. Plates were incubated at 37°C for 24 hours. After 24 hours the results were recorded. The antibacterial activity results were expressed in term of

Table II: Preparation of the MLE solutions of different concentration

SI. No	Amount of solution (ml) taken from stock solution	Amount of distilled water (ml)	Concentration (milligram /ml)
1	1	31	0.625
2	1	15	1.25
3	1	7	2.5
4	1	3	5
5	1	1	10
6	1	00	20

the diameter of zone of inhibition and <9mm zone was considered as inactive; 9-12mm as partially active; while 13-18mm as active and >18mm as very active as described in Gupta et al[10].

Determination of minimum inhibitory concentration (MIC) of Cinnamomum tamala leaves extract against test bacteria by broth dilution method.

Preparation of ALE stock & working solutions: As described before, 1gm Aqueous extracts powder was dissolve in 50 ml D/W in which 1 ml of solution contained .02 gm or 20 mg of ALE powder and it was stock solution used to prepare ALE working solutions. **Sets-I, II, III, IV, V, VI and VII** respectively were made in different test tubes by mixing measured amount of ALE stock solution with measured amount of nutrient broth medium. The concentrations of these sets were 15mg/ml, 10mg/ml, 7.5mg/ml, 5mg/ml, 2.5mg/ml, 1.25mg/ml and 0.625mg/ml ALE respectively. **Set-VIII (Control-1)** was made with ALE stock solution. **Set-IX (Control-2)** was made with nutrient broth medium. **Set-X (Control-3)** was made with nutrient broth medium in test tubes.

Inoculation of bacterial suspension to working solutions of ALE & MLE in test tubes

After matching the turbidity of bacterial suspension with 0.5 McFarland standards, 20µl of bacterial suspension of *P. aeruginosa* were separately added to each concentration of working solutions of ALE & MLE in separate test tubes. These inoculums were also added to the Control-1 & 2, but not to Control-3. These were Incubated at 37°C for 18-24 hours.

Table III: Composition and different concentrations of working ALE solutions and the controls

No. of Sets	ALE Solution (ml)	Nutrient broth medium (ml)	Total (ml)	Concentration of ALE (mg/ml)	Test Organism (µl)
Set- I	7.5	2.5	10	15	20
Set- II	5	5	10	10	20
Set- III	3.75	6.25	10	7.5	20
Set- IV	2.5	7.5	10	5	20
Set- V	1.25	8.75	10	2.5	20
Set- VI	.625	9.375	10	1.25	20
Set- VII	.3125	9.6875	10	0.625	20
Set- VIII C-1	10	0	10	20	20
Set-IX C-2	-	10	10	-	20
Set-X C-3	-	10	10	-	-

Preparation of MLE stock & working solutions: As described before, stock solution 20 mg/ml. Different sets of working solutions & controls were prepared as described before.

Table IV: Composition and different concentrations of working MLE solutions and the controls

No. of Sets	MLE stock Solution (ml)	Nutrient broth medium (ml)	Total (ml)	Concentration of MLE mg/ml	Test Organism (µl)
Set- I	7.5	2.5	10	15	20
Set- II	5	5	10	10	20
Set- III	3.75	6.25	10	7.5	20
Set- IV	2.5	7.5	10	5	20
Set- V	1.25	8.75	10	2.5	20
Set- VI	.625	9.375	10	1.25	20
Set- VII	.3125	9.6875	10	0.625	20
Set- VIII C-1	10	0	10	20	20
Set-IX C-2	-	10	10	-	20
Set-X C-3	-	10	10	-	-

Table V: Composition and different concentrations of working Gentamicin solutions and the controls

No. of Sets	Gentamicin stock solution-2 (ml)	Nutrient broth Medium (ml)	Total (ml)	Concentration of of Gentamicin (µg/ml)	Test Organism (µl)
Set-I	2	8	10	2	20
Set-II	1.5	8.5	10	1.5	20
Set-III	1	9	10	1	20
Set-IV	0.75	9.25	10	0.75	20
Set-V	0.5	9.5	10	0.5	20
Set-VI	0.25	9.75	10	0.25	20
Set-VII (C-1)	_	10	10	_	20
Set-VIII (C-2)	_	10	10	_	_

Examination of growth of test organisms in different concentrations of ALE & MLE

After 18 to 24 hours of incubation, the growth of test organisms in different preparations of ALE & MLE were examined and compared against that of controls by matching their turbidity. The clear preparations were considered as "No growth" of bacteria and turbid ones, as "Growth of bacteria". The MIC was reported as lowest concentration of ALE & MLE required to prevent the visible growth of test organisms.

***Testing antimicrobial activity of a standard antibiotic*:**

The test microorganisms *P. aeruginosa* was also tested for their activity against the antibiotic Gentamicin (inj. 80mg) by broth dilution method.

Results

In this study Indian bay leaves were found effective against the test bacterial strains. In disc diffusion method, ALE showed varying degrees of antibacterial activity starting from 15mg/ml. *Pseudomonas aeruginosa* was found to be most susceptible to ALE (25mm) at 20mg/ml concentration. In case MLE 15mg/ml and above concentrations showed inhibitory effect against *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* was found to be most susceptible to MLE (26mm) at 20mg/ml concentration. In Negative control (disc containing only D/W & methanol) showed no activity against any bacteria. The results of leaves extract by broth dilution technique were also compared against a standard antibiotic Gentamicin as shown in figure. By broth dilution technique, the MICs of Aqueous extract of leaves was 15mg/ml, the MIC of Methanolic extract of leaves was 7.5mg/ml. MIC of Gentamicin was 1µg/ml against *P. aeruginosa*. According to CLSI (2016), standard sensitive MICs of Gentamicin for test bacteria were ≤4µg/ml which was consistent with my study results[11].

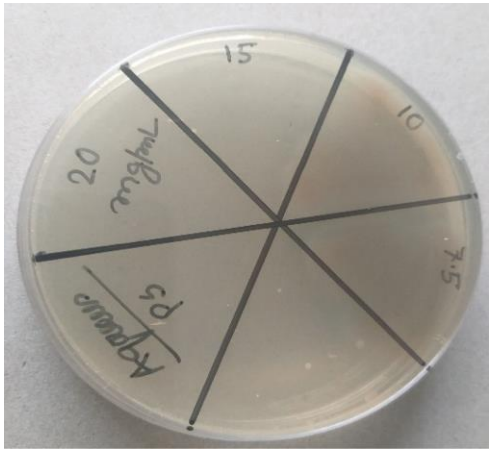

Figure 1: Antibacterial sensitivity testing of ALE against *Pseudomonas aeruginosa*

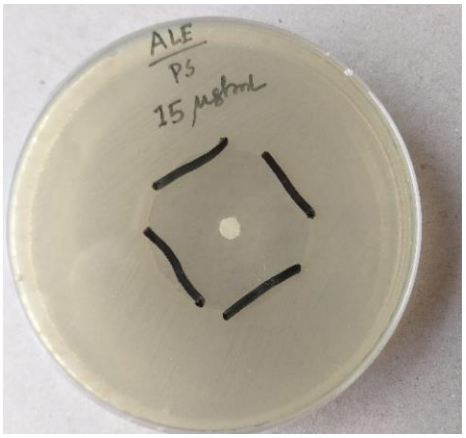

Figure 2: Repeat antibacterial sensitivity testing of ALE against *Pseudomonas aeruginosa.*

Table VI: Antibacterial activity of different concentrations of ALE measured in Zone of Inhibition

Concentrations of ALE solutions in milligram/ml	Zone of Inhibition (ZOI) in mm Pseudomonas aeruginosa
20	25
10	08
5	07
2.5	06
1.25	06
0.625	06
Control	06

Table VII: *Bacterial zone of inhibition at diVerent concentrations of ALE for repeat experiment*

Concentration of ALE in each disc	Zone of Inhibition (expressed in mm) Pseudomonas aeruginosa
15 mg /ml	20

Table VIII: Antibacterial activity of different concentrations of MLE measured in Zone of Inhibition

Concentrations of MLE solutions in milligram /ml	Zone of Inhibition (ZOI) in mm Pseudomonas aeruginosa
20	26
10	09
05	07
2.5	6.5
1.25	06
0.625	06
Control	06

Table IX: Bacterial zone of inhibition at different concentrations of MLE for repeat experiment

Concentration of MLE in each disc	Zone of Inhibition (expressed in mm) Pseudomonas aeruginosa
15 mg/ml	20

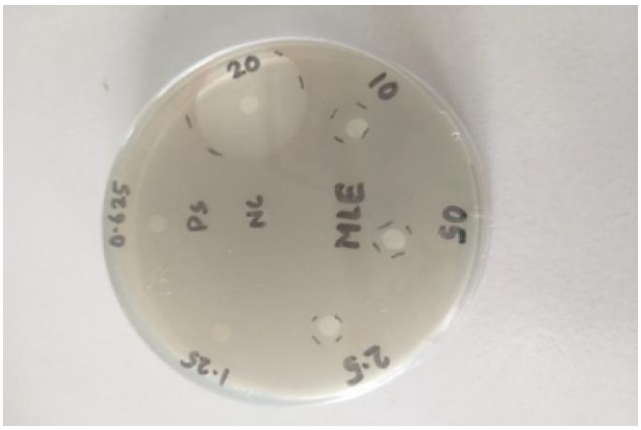

Figure 3: Antibacterial sensitivity testing of MLE against *Pseudomonas aeruginosa*

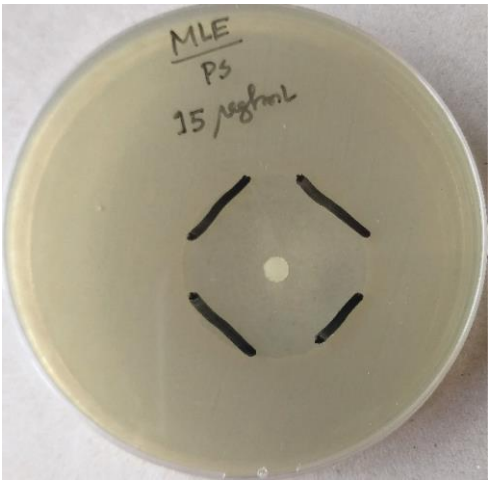

Figure 4: Repeat antibacterial sensitivity testing of MLE against Pseudo*monas aerugi*

Figure 5: *MIC of Methanolic Extract of leaves (MLE)*

Table X: *MIC of ALE against Pseudomonas aeruginosa.*

No. of sets	Concentrations (ALE) mg/ml	*P. aeruginosa*
Set-I	15	No Growth
Set-II	10	Growth
Set-III	7.5	Growth
Set-IV	05	Growth
Set-V	2.5	Growth
Set-VI	1.25	Growth
Set-VII	0.625	Growth
Set-VIII C-1	20	No growth
Set-IX C-2	NB media + Bacteria	Growth
Set-X C-3	NB media+ No Bacteria	No growth

Table XI: *MIC of MLE against Pseudomonas aeruginos*

No. of sets	Concentrations (MLE) mg/ml	*P. aeruginosa*
Set-I	15	No growth
Set-II	10	No growth
Set-III	7.5	No growth
Set-IV	5	Growth
Set-V	2.5	Growth
Set-VI	1.25	Growth
Set-VII	0.625	Growth
Set-VII C-1	20	No growth
Set-IX C-2	NB media + Bacteria	Growth
Set-X C-3	NB media +Bacteria	No growth.

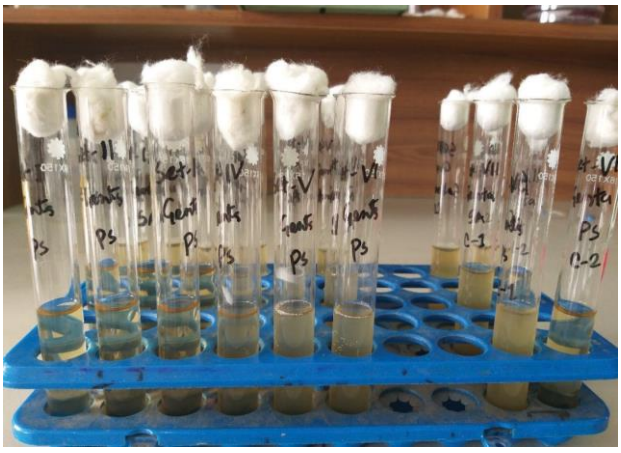

Figure 6: *MIC of Gentamicin*

Discussion

In the present study, in vitro antibacterial activity of ALE and MLE was quantitatively evaluated on the basis of zone of inhibition by disc diffusion method and the MIC was assessed by broth dilution technique. Different concentrations of the extract exhibited varying degrees of inhibitory effect. Several studies have been conducted to evaluate the antibacterial properties of Cinnamomum tamala

A study was carried out by Ajay et al.[12] to assess Antibacterial activity and phytochemical profile of Cinnamomum tamala leaf extracts and oil against different pathogenic bacteria *including S.aureus and P.aeruginosa.* In aqueous extract ZOI of *S.aureus* was 24 mm and *P.aeruginosa* was 25 mm at 5 mg/ml concentration. For *P.aeruginosa*, 25 mm ZOI was seen at 20 mg/ml which is similar with that study. Assessment of Bioactivity of Cinnamomum tamala against pathogenic bacteria including *staphylococcus aureus salmonella typhi, pseudomonas aeruginosa* was tested by Sukumar el at.[13] in agar diffusion method using aqueous and methanolic extract. In aqueous extract highest ZOI for *S.aureus* was 4 mm, for *P.aeruginosa* was 2mm at highest concentration at 5mg/ml. In present study highest ZOI were 18 mm, 25 mm for *P.aeruginosa* respectively at highest concentration at 20 mg/ml. This a bit difference in results may be due to the use of clinical strains of organisms by the researchers.

Anuj et al.[14] investigated the antibacterial activity of Cinnamomum tamala against some bacteria including *S.aureus, P.aeruginosa* and some fungal pathogen by disc diffusion method. In Methanolic leaves extract for *P.aeruginosa* ZOI was 9±0.67, 7±1.23, 6±0.25mm at 200 mg/ml, 100 mg/ml, 50 mg/ml concentration respectively. In present study ZOI for *P.aeruginosa* was 9 mm, 7 mm, 6.5 mm at 10 mg/ml, 05 mg/ml, 2.5 mg/ml concentration respectively. This research result is somewhat similar to this result while considering different concentration of the extract.

A study was carried out by Sukumar et al.[13] to assessment of bioactivity of cinnamomum tamala against *S.aureus, S.typhi, P.aeruginosa* and other bacteria by borth dilution method using aqueous and methanolic extract. In aqueous extract, the MIC for *S.aureus* 10 mg/ml, for *S.typhi* MIC was 13 mg/ml and the MIC for *P.aeruginosa* was 13 mg/ml. This result is somewhat similar with my study. In my study, ZOI for *P.aeruginosa* was 15 mg/ml. Another study was carried out by Sukumar et al.[15] to assess the antibacterial activities of

Cinnamomum tamala against *S.aureus, S.typhi, P.aeruginosa* and other bacteria by borth dilution method using aqueous and methanolic extract. In methanolic extract, the MIC for *S.aureus* 2.25 mg/ml, for *S.typhi* MIC was 9 mg/ml and the MIC for *P.aeruginosa* was 9 mg/ml. In present study the MIC for *P.aeruginosa* was 7.5 mg/ml. This finding of present study is somewhat similar with that study.

Conclusion

It is clear that extracts of indian bay leaf have a potential to be developed as a therapeutic agent in preventing bacteria related diseases. Further studies are required to detect and isolate the biologically active ingredients present in the indian bay leaves which are responsible for this antibacterial effect. The practice of using medicinal plants like Indian bay leaf as supplementary or alternative medicine in developing countries will reduce not only the clinical burden of drug resistance development but also the side effects and cost of the treatment as compared to synthetic compounds.

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