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Evaluation of Phytochemical and Anti-Microbial Activity of Ethanolic Extract of *Limonia Acidissima* L. Leaves

Neelamadhab Panda[1], V. Jagannath Patro[2], Basanta Kumar Jena[1*], P.K. Panda[3]

1. Seemanta Institute of Pharmaceutical Sciences, Jharpokharia, Mayurbhanj, Odisha, India. [E-mail: pharma_jena@rediffmail.com]
2. Rolland Institute of Pharmaceutical Sciences, Beherampur, Odisha, India.
3. Utkal University, Bhubaneswar, Odisha, India.

Herbal medicines as the major remedy in traditional system of medicine have been used in medical practices since antiquity. In addition to its ancient historical uses, wood apple is used in several systems of medicine for a variety of ailments. The objective of the present study was to investigate the presence of phytochemicals from the ethanolic extract of *Limonia acidissima* L. leaves and their anti-bacterial action against various Gram-positive and Gram-negative bacteria. The potential antibacterial activity against bacteria was examined by minimum inhibitory concentration and zone of inhibition analysis. Minimum inhibitory concentration values compared with control and zone of inhibition values compared with standard ciprofloxacin. The results revealed that, the ethanolic extract is potent in inhibiting bacterial growth of both gram negative and gram-positive bacteria and comparable with the standard (ciprofloxacin). The 200 µg/ml of ethanolic extract showed the best antibacterial activity as compared to the other concentrations. Hence, this plant can be further subjected to isolation of the therapeutic antibacterials and further pharmacological evaluation.

Keyword: Antibacterial Activity, Ethanolic Extract, MIC, ZOI.

1. Introduction

Plants with medicinal properties have been known for thousands of years and have been used as traditional medicine by the people to treat diseases. Due to many side effects of drugs of medical science and their high cost, the traditional medicines are being used all over the world. Botanically derived medicines have played a major role in human society throughout history and prehistory[1].

Limonia acidissima L. (wood apple) is a member of the family, Rutaceae and is a religious tree planted in temples and gardens. It is an important indigenous tree of India known for its medicinal and processing properties which is a moderate-sized deciduous tree grown throughout India. It is a large tree growing to 9 metres (30 ft) tall, with rough, spiny bark. The leaves are pinnate, with 5-7 leaflets, each leaflet 25–35 mm long and 10–20 mm broad, with a citrus-scent when crushed. The fruit is a berry 5– 9 cm diameter, and may be sweet or sour. It has a very hard rind which can be difficult to crack open, and contains sticky brown pulp and small white seeds[2]. The fruits are woody, rough and used as a substitute for bael in diarrheoa and dysentery[3]. The bark and leaves of the plant are used for vitiated conditions of vata and pita while the fruits are used for tumours,

asthma, wounds, cardiac debility and hepatitis [4] and the leaves were reported to possess hepatoprotective activity [5]. The fruit contains flavanoids, glycosides, saponins and tannins [6]. Some coumarins [7] and tyramine derivatives [8] have also been isolated from the fruits of *Limonia*. Leaves contain stigmasterol, psoralen, bergapten, orientin, vitedin, saponarin, tannins and an essential oil [9], while the fruit shells contain antifungal compounds, namely, psoralene, xanthotoxin, 2, 6-dimethoxybenzoquinone and osthenol [7]. The stem bark of the plant has yielded (-)- (2S)-5,3'-dihydroxy-4'-methoxy-6",6"dimethyl chromeno-(7,8,2",3")-flavanone along with several known compounds, including an alkaloid, five coumarins, a flavanone, a lignan, three sterols and a triterpene, which were found to possess antimicrobial activity [10]. Traditionally the leaves have been used in the treatment of diarrhoea, wound healing and boils, which gives as an idea for its antimicrobial activity. The present investigation was done to find out the antimicrobial potential of the ethanol extract of leaves against some Gram-positive and Gram-negative bacteria, which gives a scientifically proof for its traditional uses.

2. Material and Methods

2.1. Plant Materials

The leaves of the selected plant were collected from the forest of Simlipal Biosphere Reserve, Mayurbhanj, Odisha, India in August 2008. The plant material was identified and authenticated taxonomically at the Central National Herbarium, Botanical Survey of India, Botanical Garden, Howrah-711103, West Bengal, India (Ref. no. CNH/I(60)/2008/Tech-II, dated 27-10-2008). A voucher specimen of the collected sample was deposited in the institutional herbarium for future reference.

2.2. Preparation of Extracts

The leaves of the plant were cleaned, dried under shade and powdered by a mechanical grinder. Hundred grams of the powdered leaves was extracted with the solvent, petroleum ether, chloroform, and ethanol in increasing polarity successively in a Soxhlet apparatus. Petroleum ether was used in initial step of extraction for defatting the plant materials followed by chloroform and ethanol. The successive extracts were separately filtered and concentrated at reduced temperature on rotary evaporator [11]. Then, the percentage yield of extracts were calculated (**Table 1**) and stored in a desiccator for further Phytochemical and pharmacological screening.

2.3. Phytochemical Screenings of the Crude Extracts

The Phytochemical screenings of the crude extracts were carried out by employing standard procedures [12] to identify the chemical constituents like alkaloids, glycosides, tannins, flavonoids, terpenoids, saponins, sterols, carbohydrates, oil and fats, proteins and amino acids etc. present in the extracts shown in **Table 2**.

2.4. Antibacterial Activity of Ethanolic Extract

The ethanol extract of *Limonia acidissima* L. leaves was obtained and tested for the antimicrobial activity against different Gram-positive *(Staphylococcus aureus* ML-59 *Salmonella typhimurium* NCTC 74, *Staphylococcus aureus 29737, Bacillus lycheniformis* 10341), and Gram-negative bacterial strains (*Escherichia coli* K-12 ROW, *Shigella sonnei* 2, *Shigella boydii* 8, *Vibrio cholera* 811, *Vibrio cholera* 854, *Vibrio alginolyteus*). These bacterial strains were obtained from Jadavpur University, Kolkata-32, India. All subculture microbes used were pure culture preserved as slant agar culture at 4°C. The molten nutrient agar

medium containing various concentrations of the extracts (0, 5, 10, 25, 50 and 100) were poured and solidified on to sterile 100 mm petridishes to give sterile nutrient agar plates with varying dilutions of the extract. Then these plates were kept in a refrigerator (4 °C) for 24 h for uniform diffusion of the extract in the nutrient agar media. The plates were then dried at 37 °C for 2 h before spot inoculation[13]. One loop full (diameter 3 mm) of an overnight grown peptone water culture of each test organism was placed in petridish marked by checkerboard technique [14]. The spot inoculated plates were incubated at 37°C for 24 h and the minimum inhibitory concentration values were obtained. Ciprofloxacin was taken as a standard compound for comparing the results obtained. Two sets of two dilutions (100 and 200 µg/ml) each of ethanol extract of *Limonia acidissima* L. leaves and standard ciprofloxacin (solvent: sterile distilled water) were prepared in sterile McCartney bottles. Sterile nutrient agar plates were prepared and incubated at 37 °C for 24 h to check for any sort of contamination. Two sterile filter paper discs (Whatman® filter paper No. 1) of 6 mm diameter were soaked in two different dilutions of the crude extract and placed in appropriate position of the surface of the flooded plate, marked as quadrants at the back of the petridishes. The petridishes were incubated at 37°C for 24 h and the diameter of zones of inhibition were measured in mm. Similar procedure was adopted for the pure ciprofloxacin and the corresponding zone diameters were compared accordingly[15].

3. Result and Discussion

After drying the ethanolic extract of *Limonia acidissima* L. leaves, physical appearance were observed and presented in **Table 1**.

The percent yield (w/w) of these obtained extracts were also measured (**Table 1**), and it was that the percent yield of ethanolic extract (7.89 %) was maximum in comparison with other extracts (chloroform and petroleum ether).

Table 1: Various Extracts of *Limonia acidissima* L. leaves and their Physical Appearances

Sl. No	Name of Extract	Consistency	Color	Odor	Taste	Extractive Value (% w/w)
1	Pet. ether	Sticky Semisolid	Dark-green	Characteristics	Slightly bitter	1.280
2	Chloroform	Sticky Semisolid	Green	Characteristics	Slightly bitter	2.49
3	Ethanol	Sticky Semisolid	Green	Characteristics	Slightly bitter	7.89

The qualitative phytochemical analysis of ethanolic extract of of *Limonia acidissima* L. leaves was performed and the result of this study is presented in (**Table 2**).

Table 2: Qualitative Phytochemical Analysis of Ethanolic Extract of *Limonia acidissima* L. leaves.

Constituents and their respective test	Ethanolic Extract of *L. acidissima* L.
Alkaloids	+
Sterols	+
Flavonoids	+
Tannins	+

Triterpenoids	-
Saponins	-
Glycosides	+
Carbohydrates	+
Proteins and Amino acids	-
Gum and mucillage	-
Fixed Oils and Fats	-

'+' Present, '-' Absent

In ethanolic extract indicates the presence of alkaloids, sterols, flavonoids, tannins, glycosides and carbohydrates. The ethanol extract was taken for antimicrobial activity due to higher yield value and based on the presence of phytoconstituents.

Table 3: MIC of ethanolic extract of *Limonia acidissima* L. leaves against different bacteria

Name of bacteria	**Growth in nutrient agar containing different concentrations of ethanolic extract of *L. acidissima* L. leaves in µg/ml**					
Gram-positive bacteria	**0**	**5**	**10**	**25**	**50**	**100**
Staphylococcus aureus ML-59	+	+	+	+	+	-
Bacillus licheniformis 10341	+	+	+	+	-	-
Salmonella typhimurium NCTC 74	+	+	+	+	+	-
Staphylococcus aureus 29737	+	+	+	+	-	-
Gram-negative bacteria						
Escherichia coli K-12 ROW	+	+	+	+	-	-
Shigella sonnei 2	+	+	+	+	-	-
Salmonella typhi 59	+	+	+	+	+	-
Vibrio cholera 811	+	+	+	+	+	-
Vibrio cholera 854	+	+	+	+	+	-
Vibrio alginolyteus	+	+	+	+	+	-
Shigella boydii 8	+	+	+	+	+	-

'+' growth, '-'no growth

The observations of the MIC study has been tabulated in (**Table 3**) and it was found that the minimum inhibitory concentration of the ethanol extract was found to be varying between 5- 100 µg/ml, with respect to most of the test bacteria. The MIC of ethanol extract for bacterial strains like *Bacillus licheniformis* 10341, *Staphylococcus aureus 29737, Escherichia coli* K-12 ROW and *Shigella sonnei* 2 were found to be 50 µg/ml, for *Staphylococcus aureus* ML-59, *Salmonella typhimurium* NCTC 74, *Salmonella typhi* 59, *Vibrio cholera* 811, *Vibrio alginolyteus, Vibrio cholera* 854 and *Shigella boydii* 8 were at 100 µg/ml.

Table 4: Zone of inhibition produced by ethanol extract and ciprofloxacin

Name of bacteria	**Zone of inhibition (mm)[a]**			
	Ethanolic extract of *L. acidissima* L. leaves		**Ciprofloxacin**	
Gram-positive bacteria	**100 µg/ml**	**200 µg/ml**	**100 µg/ml**	**200 µg/ml**
Staphylococcus aureus ML-59	18.2 ± 0.32	22.47 ± 0.80	23.50 ± 0.88	28.33 ± 0.75
Bacillus licheniformis 10341	15.41 ± 0.32	16.83 ± 0.22	17.66 ± 0.72	27.28 ± 0.65
Salmonella typhimurium NCTC 74	10.52 ± 0.82	12.41 ± 0.78	23.50 ± 0.86	29.52 ± 0.76
Staphylococcus aureus 29737	19.82 ± 0.71	22.80 ± 0.61	22.26 ± 0.50	25.22 ± 0.80

Gram-negative bacteria				
Escherichia coli K-12 ROW	13.28 ± 0.62	14.82 ± 0.68	15.56 ± 0.52	19.12 ± 0.89
Shigella sonnei 2	14.78 ± 0.43	16.28 ± 0.41	16.33 ± 0.52	18.25 ± 0.78
Salmonella typhi 59	8.21 ± 0.31	9.32 ± 0.38	27.22 ± 0.76	35.22 ± 0.52
Vibrio cholera 811	13.45 ± 0.82	17.42 ± 0.32	15.48 ± 0.83	20.12 ± 0.71
Vibrio cholera 854	13.31 ± 0.81	16.72 ± 0.42	20.06 ± 0.51	25.14 ± 0.81
Vibrio alginolyteus	12.81± 0.82	15.81± 0.87	22.00 ± 0.75	28.44 ± 0.68
Shigella boydii 8	14.48 ± 0.61	16.23 ± 0.71	26.06 ± 0.83	29.03 ± 0.80

[a]Tests are done in triplicate and values were expressed as mean ± standard deviation; Zone of inhibitions was measured as diameters of inhibited zones in mm; ciprofloxacin was used as positive control.

The result of ZOI of the extracts and its comparison with standards antibiotic, ciprofloxacin (100 µg/ml and 200 µg/ml) was recorded in (**Table 4**). The antibacterial efficacy of the extract of *Limonia acidissima* L. leaves was found to decrease in the following order against different tested bacterial *Staphylococcus aureus* 29737, *Staphylococcus aureus* ML-59, *Bacillus licheniformis* 10341, *Shigella sonnei* 2, *Shigella boydii* 8, *Vibrio cholera* 811, *Vibrio cholera* 854, *Escherichia coli K*-12 ROW, *Vibrio alginolyteus*, *Salmonella typhimurium* NCTC 74 and *Salmonella typhi* 59.

From the results of MIC, ZOI values and their comparison to that of the standard ciprofloxacin, it is evidenced that the ethanol extract was potent against Gram-positive and Gram-negative bacteria. The compounds responsible for this antibacterial activity had not been investigated. However, preliminary phytochemical analysis of the ethanol extract revealed the presence of alkaloids, sterols, flavonoids, tannins, glycosides and carbohydrates. The antibacterial properties of the plant may be attributed to the individual or combined chemical groups. The findings of the present investigation offer a scientific support to the ethno medicinal use of the plant by the traditional healers.

4. Conclusions

From the above results, it is possible to conclude that, the ethanolic extract of *Limonia acidissima* L. leaves possess a broad spectrum of activity against a panel of different types of bacteria responsible for the most common bacterial diseases. The ethanol extract of *Limonia acidissima* L. leaves can potentially be used in the treatment of various infectious diseases caused by various pathogenic bacteria that are showing resistance to currently available antibiotics. These promissory extracts of *L. acidissima* L. leaves open the possibility of finding new clinically effective antibacterial.

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