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Analysis of phytochemistry and antibacterial property of aquoeus and ethanol extracts of *Dichora febrifuga* leaf from Darjeeling Himalayas

Rupam Mandal[1], Priyankar Sanphui[2*]
[1] Assistant Professor, Department of Botany, Sree Chaitanya College, Habra, North Parganas, West Bengal, India
[2] Assistant Professor, Department of Zoology, Sree Chaitanya College, Habra, North Parganas, West Bengal, India

Abstract
Plants are used as source of medicine from ancient times. The Darjeeling Himalayas is home to a huge number of medicinal plants used by the local people. *Dichora febrifuga* is a plant that grows in this region and is known to have medicinal values among local peoples. The study was conducted to determine the phytochemical properties and antibacterial potential of the aqueous and ethanol extract of *Dichora febrifuga* leaves. Our result indicates presence of different phytochemicals in the aqueous and ethanol extracts of *Dichora febrifuga* leaves. Both the leaf extracts showed antibacterial activity against Gram positive bacteria *Bacillus subtilis* and Gram negative bacteria *Klebsiella pneumoniae*. The maximum antibacterial activity was exhibited by ethanol extract of leaf against *Bacillus subtilis.* The extracts showed better antibacterial potential against Gram positive bacteria *Bacillus subtilis* compared to gram negative bacteria *Klebsiella pneumoniae.*

Keywords: dichora febrifuga, anti-bacterial property, phytochemicals, Darjeeling Himalayas

Introduction
Bacteria were among the first life forms to appear on Earth about 4 million years ago and are present in most of its habitats like soil, water, acidic hot springs, radioactive waste and deep portion of earth's crust constituting a large domain of prokaryotic microorganisms [7]. Though some bacteria are beneficial, some are pathogenic. Diseases like tetanus, typhoid fever, cholera, syphilis, leprosy are caused by bacteria.
The most common fatal bacterial diseases are respiratory infections with tuberculosis alone killing about 2 million people per year mostly in sub-Saharan Africa (2002 WHO mortality data). In developed countries antibiotics are used to treat bacterial infections in human and animals which in turn contribute to the rapid development of antibiotic resistance in the bacterial population. The rise in antibiotic resistance in bacteria is a matter of concern and medical researchers all over the world are struggling to develop multiple drug therapy and screening of new sources of antimicrobial agents.
Natural substances have been used as antimicrobial agents from ancient times. Ancient Egyptians and ancient Greeks used specific plant and molds extracts to treat infections [13].
The use of herbal medicine is mentioned in ancient Indian texts like Rigveda, Atherveda, Charak Samhita and Sushruta Samhita. The eastern Himalayan region includes the district Darjeeling is one of the hotspot of floristic biodiversity. Many systems of herbal medicine is followed in this area.There are a number of plants used in herbal medicine, but proper scientific documentation of their medicinal properties have not been made.
The present work was carried out with a view to documenting the ethno-medicinal knowledge about the plant *Dichora febrifuga*. This plant is used for the oral infection, on cut wounds, and as coagulating agents by the folk practicing jaributy system in Darjeeling hills.

Dichora febrifuga was described by João de Loureiro in 1790. It is a evergreen flowering rare plant in the family Hydrangeaceae. The shrub reaches height to 1-2 meters. The leaves are lanceolate, serrate and petiolate *Dichroa febrifuga* produces panicles of light-blue cup-shaped flowers from April to June. The flowers are hermaphrodite (have both male and female organ).The shrubs produce berries of dark blue colour later in autumn. The shrub can grow in partial sun or shade. It grows in evenly moist well drained soil and is intolerant to drought.
This shrub is found in the Himalayan region extending from northern India to Burma, Thailand, Indo-China, and China, southward to mountainous areas of Malaysia, Indonesia, Philippines and New Guinea [4]. It has also been reported to be found in Darjeeling Himalayas [5].
The plant is commonly used in Chinese herbalism, where it is considered to be one of the 50 fundamental herbs. They are also known as Chinese quinine. The leaves are purgative. They are used in the treatment of the stomach cancer. The juice of the leaves is used in Nepal to treat coughs, colds, and bronchitis. The juice of the root is used in Nepal to treat fevers and indigestion. A decoction of stem bark is used in the treatment of fevers.
Hence based on these previous reports and with respect to the therapeutic usefulness of natural bio-molecules, this study was taken up with an aim to evaluate the phytochemical present and antibacterial potential of aqueous and ethanol extract of leaves of *Dichora febrifuga* from Darjeeling Himalayas.

Materials and Methods
Materials
Ammonium solution, Sulfuric acid, Iron (III) chloride, Nitric acid, Chloroform, Sodium hydroxide, Sodium chloride, Mercury (II) chloride, Potassium chloride, Magnesium acetate tetrahydrate, were purchased from

Merck, Acetic acid glacial was purchased from Glaxo India Limited, LB was purchased from SRL and plastic wares were purchased from Tarsons.

Collection of Plants

Fresh leaves of *Dichora febrifuga* were collected from road side of Darjeeling under Padmaja Naidu Zoological Park area. Sample collection was conducted during the months of August. The leaves were placed in polythene bags and transported to the laboratory.

Preparation of extracts

Cold water extract: The aqueous extract of dried *Dichora febrifuga* leaves was made in distilled water as described earlier [1, 9]. About 5 gm of dried leaf powder was taken and mixed with 50 ml of distilled water. The mixture was taken in a sterile conical flask, plugged with sterile cotton and kept in shaker with 200rpm for 24 hours. After 24 hours the mixture was centrifuged at 5000 rpm for 15 minutes and filtered through muslin cloth.

Ethanol extract: The ethanol extract was also prepared through the same protocol as followed for cold water extract. Only the leaf powder was dissolved in 70% ethanol.

Test for alkaloids

The dry sample (1 gm) was taken in a test tube. Then 3 ml of ammonium solution was added in the test tube. Now the sample solution was allowed to stand for few minutes. After some time 10 ml of chloroform was added followed by addition of 2 ml of Mayer's reagent. If cream coloured precipitation appears at the bottom of the test tube,it indicates presence of alkaloid in the sample.

Test for glycosides

Keller kiliani test was performed to detect the presence of glycosides as described earlier [8]. Briefly, a mixture of 4ml glacial acetic acid and 1 drop of 2% $FeCl_3$ was mixed with 10ml of test sample. 1ml of concentrated sulfuric acid was added. A brown ring is formed between two layers indicating presence of glycosides.

Salkowski's test was also performed to detect presence of glycosides. Concentrated sulfuric acid was added to the test sample. A reddish brown colour formation indicates presence of glycosides [8]

Test for steroids

To determine the presence of steroids, 1 ml of sample solution is mixed with 10 ml of chloroform. Then 1ml of concentrated sulfuric acid was added into the test tube by wall. The colour of upper layer turned red and sulfuric layer show yellow colour with green fluroscence.This indicates presence of steroid [14].

Test for saponin

The extracts were diluted with distilled water in a test tube and then the test tube was shaken for 15 minutes. The formation of foam layer on top indicates the presence of saponin. [8]

Test for tannin

To test whether tannin is present the following procedures were performed

a. 1 ml of ethanol extract was dissolved in 2 ml of distilled water. Then 2-3 drops of diluted ferric chloride solution was added. If the colour changes from green to blue it indicates presence of catechin tannin. On the other hand, if the colour changes from blue to black it indicates presence of gallic tannin.
b. 2 ml of aqueous extract was dissolved in 2 ml of distilled water. Then 1-2 drops of diluted ferric chloride was added. Appearance of dark blue or green colour indicates presence of tannin. [2]

Test for flavonoids

A few drops of sodium hydroxide solution were added to the extract. If an intense yellow colour appears in the extract which becomes colourless on adding few drops of dialuted (50%) sulfuric acid, it indicates presence of flavonoids [15].

Test for quinone

The presence of quinone was detected by adding few drops of concentrated sulfuric acid or aqueous sodium hydroxide solution in the test sample. Appearance of any colour indicates presence of quinine

Test for phenol

Presence of phenol was detected by adding 5% ferric chloride to the sample. Appearance of dark green colour indicates presence of phenol [9].

Test for coumarin

To detect the presence of coumarin, a few drops of the extract was taken in a test tube. A few drops of alcoholic sodium hydroxide solution was added to the extract. Appearance of yellow colour indicates presence of coumarin [11].

Test for Anthraquinone

Few drops of magnesium acetate was added in the test sample and shaked well for few minutes. Appearance of light pink colour indicates presence of anthraquinone.

Test for xanthoprotein

Presence of xanthoprotein was detected by adding ammonia to the extract. If the colour changes from deep orange yellow, the sample contains xanthoprotein.

Evaluation of Anti-Bacterial property

The antibacterial activity was determined by disc diffusion method. In this method small round piece of whatman filter paper was made through the punching machine. The paper discs were dipped in leaf extract and placed on the agar plate containing bacteria. For control, the solvent dipped discs were used. The plates were incubated for 24 hours at 37^0 C. Following incubation, the zone of inhibition was observed around the disc in some plates. The zone of inhibition was measured by vernier caliper. The experiments were repeated three times. The mean value of zone of inhibition against each bacterium is documented here.

Results

Phytochemical analysis of *Dichora febrifuga* leaf extracts

The presence of different phytochemicals in aqueous and ethanol extract of leaves of *Dichora febrifuga* were tested. Various phytochemicals were detected in the samples. Results indicate presence of alkaloid, coumarin, Xanthoprotein and quinine in both aqueous and ethanol extract of the samples. On the other hand, glycosides,

steroids and anthraquinone are absent in all the samples tested. Saponin and Flavonoids were found in aqueous extract of the sample only. Tannin and phenols were only found in ethanol extract of the sample.

Table 1: phytochemical analysis of leaf extracts of *Dichora febrifuga*

Phytochemicals	Aqueous extract	Ethanol Extract
Alkaloid	+	+
Glycosides	-	-
Saponin	+	-
Tanin	-	+
Flavonoids	+	-
Steroids	-	-
Phenols	-	+
Coumarin	+	+
Quinone	+	+
Anthraquinone	-	-
Xanthoprotein	+	+

Antibacterial property of *Dichora febrifuga* leaf extracts

The antibacterial property of the aqueous and ethanol leaf extracts of *Dichora febrifuga* was studied using disc diffusion method. The antibacterial property was tested on one Gram positive bacteria (*Bacillus subtilis*) and one gram negative bacteria (*Klebsiella pneumoniae*). Our result indicated that both of the leaf extracts exhibited antibacterial property (Table 1). The highest inhibition was shown by ethanol extract (100 mg/ml) against Gram positive bacteria *Bacillus subtilis.* The results are graphically represented in Figure1. Our result also indicate that both the extracts are comparatively more effective on Gram positive bacteria *Bacillus subtilis* than Gram negative bacteria *Klebsiella pneumonia*

Table 2: Zone of inhibiton of *Dichora febrifuga* leaf extracts

	Aqueous extract		Ethanol Extract	
	50 (mg/ml)	100 (mg/ml)	50 (mg/ml)	100 (mg/ml)
Gram positive bacteria *Bacillus subtilis*	11 ± 1	14.1 ± 0.2	13.3 ± 0.8	15.2± 0.6
Gram negative bacteria *Klebsiella pneumoniae*	8.1 ± 0.13	11.2 ± 0.49	9 ± 0.27	13 ± 0.23

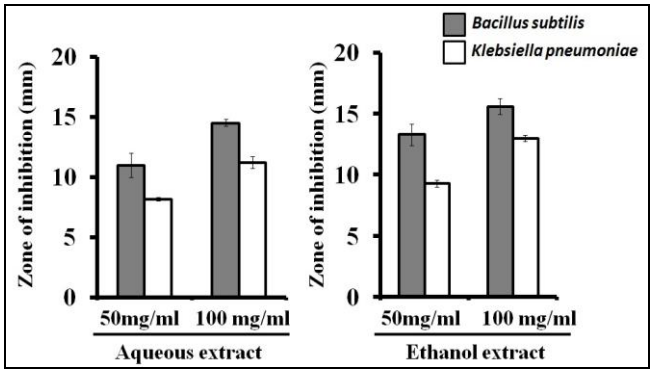

Fig 1: Antibacterial activity of aqueous and Ethanol extract of leaves of *Dichora febrifuga* from Darjeeling

Himalayas. Aqueous and ethanol extract of *Dichora fibrifuga* leaf (50mg/ml and 100 mg/ml) were prepared. Disc diffusion method was used to study the antibacterial activity of the extracts on Gram positive bacteria *Bacillus subtilis and* Gram negative bacteria *Klebsiella pneumoniae*. Zone of inhibition (in mm) are represented graphically. Data represented as mean ± SEM of three independent experiments.

Discussion and Conclusion

Plants have been the source of multiple drugs since ancient times. There is enormous number of medicinally important plants in nature.

Medicinal plants play a vital role in treatment of diseases including microbial infection, fever, and diabetes in traditional medicine. The antimicrobial activity of most of the plants is due to presence of alkaloids and flavonoids. [3,6]

Dichora febrifuga is an important medicinal plant largely used by the local people of Darjeeling as well as Nepal. The present research investigates the phytochemistry and antibacterial efficacy of aqueous and ethanol extract of leaf of Dichora *febrifuga.* The antibacterial property was tested on one gram positive (*Bacillus subtilis)* and one gram negative bacteria (*Klebsiella pneumoniae).* Both the extracts exhibited antibacterial property against both the bacteria used. Ethanol extracts showed more antibacterial activity. Both the extracts were more potent against gram positive bacteria *Bacillus subtilis* compared to gram negative bacteria *Klebsiella pneumoniae*

In conclusion, it can be said that our study indicates antibacterial property of leaf extracts of *Dichora febrifuga*. Further investigation in this direction is required to identify the active components and pave the way for preparation of antimicrobial formulations.

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