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A Preliminary Scientific Assessment on *Holarrhena pubescens* Wall

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Abstract

Holarrhena pubescens Wall. (Apocyanaceae) plant is used in traditional systems (Ayurvedic) of medicine, to treat various diseases. The preliminary phytochemical screening of this plant showed positive results for protein, alkaloid, flavonoid and phenolic compounds (ethanol and aqueous extracts of leaf and bark). In both the samples (leaf and bark), carbohydrates and tannin are present in the aqueous extract. Antibacterial activity of the ethanolic leaf extract showed notable activity than the aqueous extract against both tested organisms (*Bacillus cereus* and *Escherichia coli*) at 100µl concentration.

Keywords: Apocyanaceae**,** *B. cereus, E. coli, Holarrhena pubescens,* Leaf and Bark Extract.

1. Introduction

India has been referred to as the medicinal garden of the world. The knowledge of medicinal plants is ancient. In Vedas (Rig Veda and in Atharva Veda) medicinal properties of plants are described and medicinal plants have been known as a rich source of therapeutic agents. WHO estimated that in developing countries, 80% of the population depends on the traditional system of medicine. In recent times, people have become aware of the side effects of synthetic drugs and hence move to plant-based remedies[1].

Holarrhena pubescens Wall. (Synonym: *H. antidysenterica* L.) belongs to the family Apocynaceae. It is a shrub with sub-sessile, opposite, ovate-oblong leaves, and corymbose cyme inflorescence with white flowers. Since Apocynaceae plants contain milky latex, rich in alkaloids, they have immense medicinal significance. Worldwide, the plants of Apocynaceae have been used for their medicinal applications[2]. This plant has been used in the treatment of dysentery, diarrhoea, intestinal worms and diabetes.

2. Materials and Methods

2.1 Plant Collection and Identification

The fresh leaves, and dried bark of *Holarrhena pubescens*, were collected from Chennimalai, Erode in January 2021. It was identified and authenticated taxonomically, with the help of Flora of the Presidency of Madras[3]. The plant parts were shade-dried and mechanically ground to coarse powder. The powdered samples were stored, in an air-tight container for further use.

2.2 Preparation of Herbarium

Following the method of Jain and Rao[4], the collected plant parts were pressed properly, poisoned with 0.1% $HgCl_2$ dissolved in absolute alcohol and mounted with glue on a standard (42 x 28 cm) herbarium sheet. The herbaria were deposited in PG and Research Department of Botany, Vellalar College for Women, Erode, Tamil Nadu.

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2.3 Taxonomic Account of *Holarrhena pubescens* Wall.

Division	:	Tracheobionta
Class	:	Magnoliophyta
Order	:	Gentianales
Family	:	Apocyanaceae
Genus	:	*Holarrhena*
Species	:	*pubescens*
Synonym	:	*Holarrhena antidysentrica* Wall.

Common name:

Tamil	:	Vapali
Hindi	:	Karchi
Telugu	:	Pala Kodaga
Malayalam	:	Kodagapala

2.4 Morphological Characters of *Holarrhena pubescens* Wall.

Stem	:	Woody, white with brown thick bark
Leaves	:	Opposite, ovate, oblong pubescent leaves with pointed tip
Inflorescence	:	Corymbosecymes
Flower	:	White, scentless flowers
Calyx	:	5 narrow-lobed, glandular at the base
Corolla	:	Salver-shaped, slender tube, lobes oblong
Stamen	:	5, included, slightly dilated opposite to petals, Antherlanceolate
Carpel	:	2, free, style-short, stigma-fusiform with bifid tip
Fruit	:	2 terete elongate follicular mericarp
Seed	:	Many linear seeds

3. Phytochemical Screening

As per the methods adopted by Harborne[5], Kokate *et al.*[6] and Prabhakaran[7], phytochemical screening of successive solvent extract was followed. *Holarrhen pubescens* leaf and bark powder (50g) was extracted with ethanol (78°C) using a Soxhlet extractor for 8-10 hours. Finally, the material was macerated using hot water (99.98°C) with occasional stirring for 16 hours and the water extract was filtered. The solvent extract was filtered, evaporated to dryness using the rotary evaporator, concentrated and subjected to further analysis.

4. Phytochemical Analysis

4.1 Test for Carbohydrates

To the plant extract (1ml), Barfoed's reagent (1ml) was added and heated for 2 mins in a boiling water bath. Red precipitate indicates the presence of carbohydrates.

4.2 Test for Protein

To the plant extract (2ml), Biuret reagent (2ml) was added. The formation of violet colour indicates the presence of proteins.

4.3 Test for Amino acid

To the plant extract (1ml), 5% Ninhydrin solution (3 drops) was added. The mixture was heated in the boiling water bath for 10 minutes. The formation of a characteristic purple colour indicates the presence of amino acids.

4.4 Test for Alkaloids

To the plant extract (1ml), Wagner's reagent (1ml-Iodine in potassium iodide solution) was added. The formation of a reddish-brown precipitate indicates the presence of alkaloids.

4.5 Test for Flavonoids

The plant extract (1ml), was treated with ammonium hydroxide solution. The appearance of yellow fluorescence indicates the presence of flavonoids.

4.6 Test for Phenols

To the plant extract (1ml), distilled water (3ml) was mixed. To this mixture, 3 ml of lead acetate solution (10%) was added. The formation of a white precipitate indicates the presence of phenols.

4.7 Test for Tannin

To the plant extract (1ml), a few drops of ferric chloride solution were added. If hydrolysable tannins are present, blue appears; if condensed tannins are present, green appears.

4.8 Test for Glycosides

The plant extract was treated with chloroform and now the chloroform layer was separated. To this, a diluted

ammonia solution was added. The formation of pink colour indicates the presence of glycosides.

4.9 Test for Terpenoids

To the plant extract (1ml), Chloroform (5ml) and a few drops of conc. H_2SO_4 were added. The formation of a reddish-brown colour at the interface indicates the presence of terpenoids.

4.10 Test for Saponin

To the plant extract (2ml), distilled water (1ml) was added and was shaken in a graduated cylinder for 15 mins. The development of a 1cm foam layer indicates the presence of saponin.

4.11 Test for Coumarin

A small quantity of plant samples was taken in a test tube and covered with filter paper. The test tube was placed in a water bath for several minutes, and the paper was removed and exposed to Ultraviolet (UV) light. If the paper showed green-coloured fluorescence, it confirms the presence of coumarin.

4.12 Test for Quinone

To the plant extract (1ml), conc. H_2SO_4 (2ml) was added. The formation of red colour indicates the presence of quinone.

4.13 Test for Anthroquinone

To the plant extract (0.5ml), a few drops of HCl were added, and the mixture was shaken well. The appearance of pink, violet or red colour in the lower phase indicates the presence of anthroquinone.

4.14 Test for Fixed Oil and Fat

Press the plant extract between two filter papers. Oil stain on the filter paper indicates the presence of fixed oil and fats.

4.15 Test for Gums and Mucilage

To the plant extract (5ml), distilled water (5ml) was mixed. To this mixture, on constant stirring, absolute alcohol (25ml) was added. The appearance of a white or cloudy precipitate indicates the presence of gums and mucilage.

5. Antimicrobial Activity Microorganisms

The microorganisms used in the present study are *Escherichia coli* and *Bacillus cereus*.

6. Inoculum Preparation

Stock cultures were maintained on slopes of nutrient agar, at 4°C. Active cultures for experiments were prepared by transferring a loop full of cells from the stock cultures to, test tubes of nutrient agar medium. These test tubes were incubated without agitation, for 24 hrs at 37°C. The cultures were diluted with fresh nutrient agar broth, to achieve optical densities corresponding to 2 – 10[6] Colony Forming Units (CFU/ml) for bacteria.

7. Media preparation

Composition of Nutrient Agar Medium for bacteria: Beef extract-1.5g, Yeast extract-1.5g, Pepton-5g, Sodium chloride-5g, Agar-15g, pH-7.0, 28.0g of Nutrient agar media, were mixed in distilled water (1000ml). The media was mixed well and sterilized for further use.

8. Agar Well Diffusion Method

The leaf and bark extracts (ethanol and aqueous) of *Holarrhena pubescens,* were analyzed for antibacterial activity by agar well diffusion method. Nutrient agar media and the petriplates were sterilized by autoclaving at 121°C at 15 lbs pressure, for about 30 minutes. Under the Laminar airflow chamber, about 20 ml of the agar medium was dispensed into each petriplate, to yield a uniform depth (4mm). After solidification of the media (24 hrs) culture of Gram-positive microorganisms-*Bacillus cereus,* and Gram-negative microorganisms - *E. coli* were swabbed on the surface of the agar plates. Using a cork borer well was prepared and loaded with 50µl and 100µl of each sample. 30µg of Kanamycin as positive control, and 50µl of distilled water as negative control was used. To observe the zone of inhibition, the loaded sample plates were incubated at 37 C for 24 hours[8,9].

9. Results

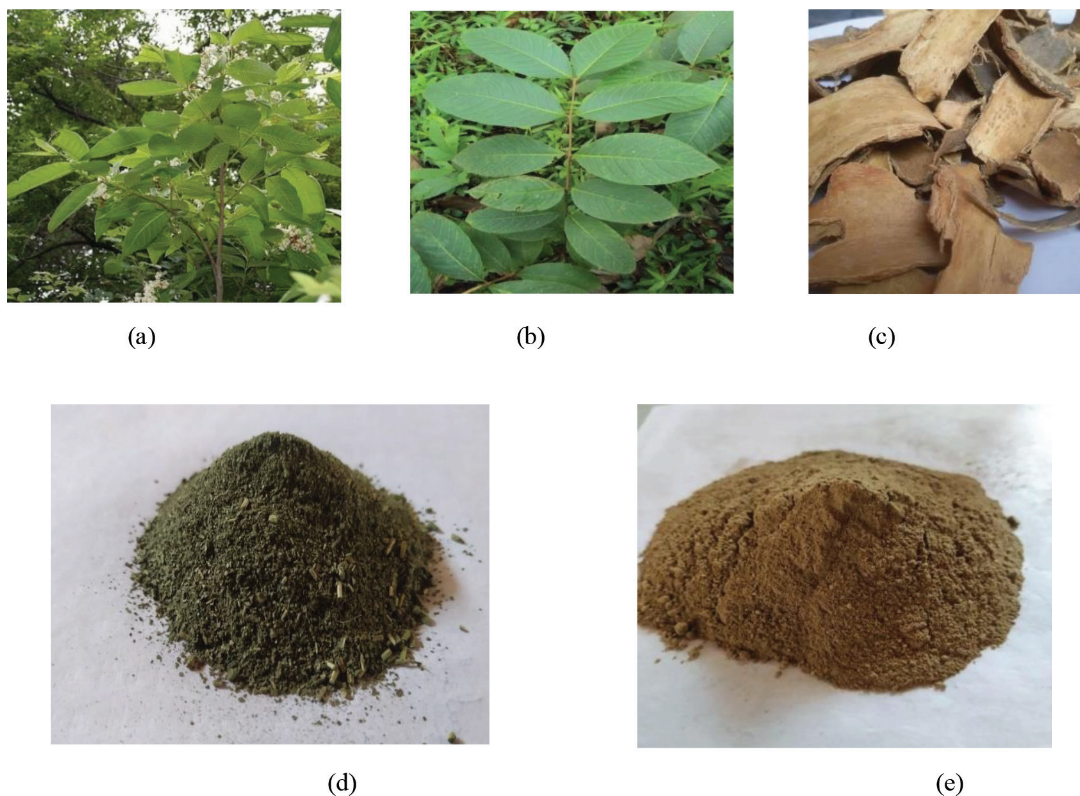

Figure 1. Study plant *Holarrhena pubescens* Wall. (a) Twig (b) Leaves (c) Bark (d) Leaf powder (e) Bark powder.

10. Qualitative Phytochemical Screening of *Holarrhena pubescens* Wall.

The preliminary phytochemical analysis of *Holarrhena pubescens* leaves and bark extracts (ethanol and aqueous) showed the presence of various phytoconstituents and their presence and absence was expressed in (+) and (-) symbols respectively. The result is shown in Table 1.

The ethanol leaf extract of *Holarrhena pubescens* disclosed the presence of amino acid, protein, carbohydrate, flavonoid, alkaloid, phenol, tannin, anthroquinone, quinone, and mucilage and gums and the absence of terpenoid, glycoside, saponin, coumarin and fixed oils. The leaf (aqueous extract) showed positive results for protein, carbohydrate, flavonoid, alkaloid, phenol, tannin, and coumarin and negative results for amino acid, glycoside, terpenoid, saponin, quinone, anthroquinone, fixed oils and fats and mucilage and gums.

H. pubescens bark (ethanolic extract) showed the presence of amino acid, protein, flavonoid, alkaloid, phenol, coumarin, quinone, anthroquinone, mucilage and gums and the absence of carbohydrate, tannin, glycoside, terpenoid, saponin and fixed oils and fat. The aqueous extract of bark revealed the presence of amino acids, proteins, carbohydrates, flavonoids, alkaloids, quinine, phenol, and the absence of glycoside, tannin, terpenoid, saponin, coumarin, anthroquinone, fixed oils and fat and mucilage and gums.

11. Antibacterial Activity of *Holarrhena pubescens* Wall.

The antibacterial activity of *Holarrhena pubescens* leaf and bark extracts (ethanol and aqueous) at different concentrations like 50µl and 100µl against *Bacillus cereus* and *Escherichia coli* were analyzed, by measuring the zone of inhibition. The results are shown in Tables 2 and 3, Figure 2. The results clearly showed that the plant extracts were specific in their action against both, Gram-positive and Gram-negative bacteria. In *B. cereus*, ethanolic leaf extract exhibited more inhibitory zone at

100µl (2.2cm) concentration when compared to 50µl (1.8cm) concentration. However, in the aqueous extract, the same bacteria exhibited nil zone of inhibition in both concentrations. When compared to aqueous extract, the ethanolic leaf extract exhibited a higher zone of inhibition in *B. cereus*.

In *E. coli*, the ethanolic leaf extract exhibited more inhibitory zone at 100µl (2.2cm) concentration when compared to 50µl (1.6cm) concentration. Aqueous extract did not produce any zone of inhibition in both concentrations. When compared to aqueous extract the ethanolic leaf extract of *H. pubescens* exhibited a higher zone of inhibition against *E. coli*. The ethanolic extract of bark showed a higher zone of inhibition at100µl (2.1cm) concentration against *B. cereus* when compared to 50µl (1.6cm) concentration. In the aqueous extract, the same bacteria showed a maximum zone of inhibition at 100µl (1.5cm) and a minimum zone of inhibition at 50µl (1.2cm) concentration. The ethanolic bark extract of *H. pubescens* exhibited a higher zone of inhibition against *B. cereus*.

In *E. coli*, the ethanolic bark extract showed maximum zone of inhibition (2.6cm) at 100µl concentration when compared to 50µl (2.2cm) concentration. In the aqueous extract, the same bacteria showed maximum zone of inhibition (1.8cm) at 100µl concentration and minimum zone of inhibition (1.5cm) at 50µl concentration. The ethanolic bark extract exhibited a maximum zone of inhibition in *E. coli* when compared to the aqueous extract.

Table 1. Preliminary Phytochemical Screening of *Holarrhena pubescens* Wall.

S. No.	Phyto chemicals	Leaves		Bark	
		Ethanol	Aqueous	Ethanol	Aqueous
1.	Amino acid	+	-	-	-
2.	Protein	+	+	+	+
3.	Carbo hydrate	+	+	-	+
4.	Flavonoids	+	+	+	+
5.	Alkaloids	+	+	+	+
6.	Glycosides	-	-	-	-
7.	Terpenoids	-	-	-	-
8.	Phenols	+	+	+	+
9.	Tannin	+	+	-	+
10.	Saponin	-	-	+	-
11.	Quinone	+	-	+	+
12.	Anthro quinone	+	-	+	-
13.	Coumarin	-	+	+	-
14.	Fixed oil and Fat	-	-	-	-
15.	Gums and Mucilage	+	-	+	-

+ indicates Presence ;- indicates absence.

12. Discussion

12.1 Qualitative Phytochemical Screening of *Holarrhena pubescens* Wall.

Preliminary phytochemical analysis of *H. pubescens* leaf disclosed the presence of carbohydrates, protein, amino acid, alkaloids, flavonoids, phenol, tannin, quinone, anthroquinone and mucilage and gums in ethanol extract. This is by, an earlier investigation[10]. Pawankumar[11] revealed the presence of protein, carbohydrate, alkaloid, tannin, saponin, and glycoside in the aqueous extract of *H. pubescens* leaf and this coincides with the current study. The qualitative phytochemical analysis of the bark of *H. pubescens* showed the presence of protein, amino acid, alkaloid, phenol, flavonoid, coumarin, quinone, anthroquinone, mucilage and gums in the ethanol extract and carbohydrate, protein, amino acid, alkaloids, phenol, flavonoid, quinone in the aqueous extract. Ganapathy *et*

Table 2. Anti-bacterial activity of *Holarrhena pubescens* Leaves (Agar well diffusion method)

S. No.	Microorganism	Solvent	Zone of Inhibition (cm)			
			Control (50µl)	Leaves		Standard Antibiotic (Kanamycin) 30mcg/disc
				50µl	100µl	
1.	*Bacillus cereus*	Ethanol	-	1.8	2.2	2.3
		Aqueous	-	-	-	2.4
2.	*Escherichia coli*	Ethanol	-	1.6	2.2	2.5
		Aqueous	-	-	-	2.5

Table 3. Antibacterial activity of *Holarrhena pubescens* Bark (Agar well diffusion method)

S. No.	Microorganism	Solvent	Zone of Inhibition(cm)			
			Control (50µl)	Bark		Standard Antibiotic (Kanamycin) 30mcg/disc
				50µl	100µl	
1.	*Bacillus cereus*	Ethanol	-	1.6	2.1	2.3
		Aqueous	-	1.2	1.5	2.4
2.	*Escherichia coli*	Ethanol	-	2.2	2.6	2.5
		Aqueous	-	1.5	1.8	2.5

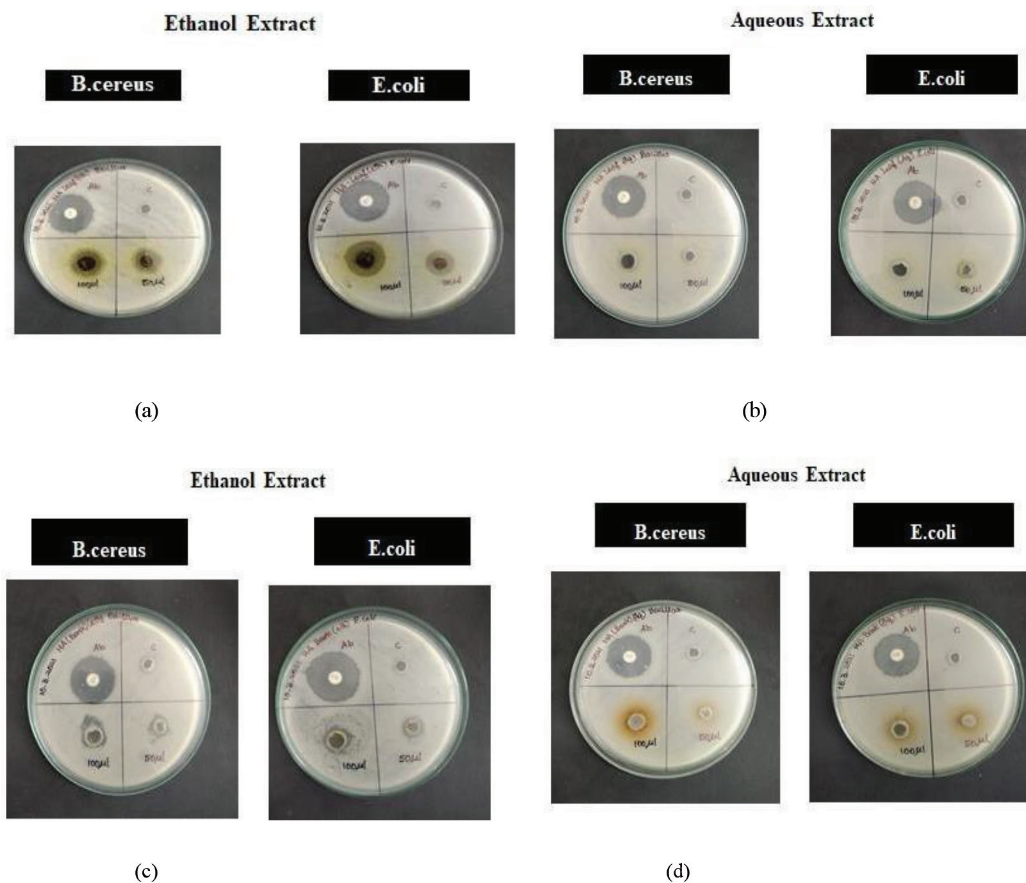

Figure 2. **(a & b)** Antibacterial activity of *Holarrhena pubescens* Bark by Agar well diffusion method, **(c&d)** Antibacterial activity of *Holarrhena pubescens* Leaves by Agar well diffusion method.

al.[12] revealed the presence of alkaloid, flavonoid, quinone and sterolin bark extract (ethanol) and this agrees with the present study.

12.2 Antibacterial Activity of *Holarrhena pubescens* Wall.

Ethanol extract of *H. pubescens* leaf, was significantly active against *Bacillus cereus* (2.2cm) and *Escherichia coli* (2.2cm) at 100 µl concentration. Likewise, the ethanol extract of bark, at 100 µl concentrations was active against *E. coli* (2.6cm) and *B. cereus* (2.1cm).

Pawankumar *et al.*[11] reported that the ethanolic leaf extract of *H. pubescens* was active against *E. coli, Pseudomonas aeruginosa, Staphylococcus aureus, and Salmonella spp*.

The present study coincides with this finding. Suchitra *et al.*[13] reported that the crude methanolic bark, seed and callus extract obtained from *Holarrhena antidysenterica* showed maximum zone of inhibition against bacteria like

S. aureus, Salmonella typhimurium and *E. coli* at 100 µl concentration. This result was by the present investigation.

According to Raman *et al.*[14], the bark extract of *H. pubescens,* at 100µg/ml concentration inhibited all the tested organisms - *B. subtilis, B. cereus, B. megaterium, E. coli, INAVA ET (Vibrio), Shigella dysenteriae, S. sonnei, Salmonella typhi, Staphylococcus aureus, Pseudomonas*. Similarly, the ethanolic bark extract of *H. pubescens*, inhibited *Bacillus cereus* and *Escherichia coli* at 100 µl concentration in the current study.

13. Conclusion

Holarrhena pubescens Wall. is used to treat rheumatism, skin diseases, diarrhoea, gastrointestinal infections, cough and cold, typhoid fever, malaria and spleen infection. The preliminary phytochemical screening of *H. pubescens* Leaf and bark (ethanol and aqueous extracts) showed the presence of protein, alkaloid, flavonoid and phenolic compounds in both extracts. Glycoside, terpenoid and fixed oils were absent in both, tested extracts. Carbohydrate and tannin are present only in the aqueous extract of both samples. Quinone, anthroquinone and gums and mucilage showed positive results in ethanolic extract of tested samples. The antibacterial activity of *H. pubescens* leaf (ethanol extract) showed notable activity than the aqueous extract against both the tested organisms (*B. cereus* and *E. coli*) at 100µl concentration. The ethanolic bark extracts exhibit significant activity against both the tested organisms at 100µl concentration than the aqueous bark extract.

Based on the results, it is very clear that *Holarrhena pubescens* is a potential medicinal plant. The experimental findings of the current study are a substantial step to control the bacterial strains and, it further requires a long-term study to evaluate the therapeutic efficacy of plant parts to establish as a drug.

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