

Phytochemical and biological evaluation of stem bark of *Holarrhena pubescens* against oxidative and inflammatory disorders

Sakshi Dhiman

Shoolini University

Rohit Kumar Nadda

Shoolini University

Ruhi Thakur

Shoolini University

Rohit Goyal

Shoolini University

Purna Bhardwaj (✉ premabhardwaj1355@gmail.com)

Shoolini University

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Abstract

Background

Holarrhena has been traditionally used to treat inflammatory disorders and oxidative stress. The purpose of our study was to examine the biological potential of phytoconstituents found in the stem bark of *H. pubescens* using antioxidant and anti-inflammatory *in-vitro* methods.

Method

Dried stem bark of this plant was firstly extracted with n-hexane for defatting and then extracted with hydro-alcohol (90% ethanol). The hydro-alcoholic extract was further fractionated by different solvents (chloroform, ethyl acetate and butanol) and the antioxidant activity was assessed using reducing power assay, free radical scavenging methods for 1,1-diphenyl-2-picrylhydrazyl (DPPH), and hydrogen peroxide radicals. Anti-inflammatory activities were carried out by using albumin denaturation and nitric oxide (NO) assays.

Results

The qualitative phytochemical analysis of n-hexane and hydro-alcoholic extract revealed the presence of alkaloids, saponins, flavonoids, phenols, carbohydrates, proteins and tannins. In quantitative phytochemical analysis of hydro-alcoholic extract showed a higher percentage of phytochemicals than n-hexane extract. Hydro-alcoholic extract and fractions showed significant antioxidant activities against DPPH, H₂O₂, and reducing power assay. Similarly, in anti-inflammatory studies, butanol fraction showed higher protection against albumin denaturation which was 55.81 ± 2.05 whereas in NO assay ethyl acetate fraction was 30.39 ± 1.37 .

Conclusion

Our research concluded that the hydro-alcoholic extract has highly phytochemical properties than n-hexane extract whereas ethyl acetate and butanol fraction showed superior anti-oxidant and anti-inflammatory properties as compared to other fractions. This research supports the plant's traditional use as a medicine.

1. Introduction

Oxidative stress is referred as imbalance between the formation of free radicals and the antioxidant defence system. Reactive oxygen species (ROS) are several types of activated oxygen that induce lipid peroxidation, which may lead to the development of inflammation (1). Free radicals are well

acknowledged to be crucial in the emergence of tissue damage and pathogenic processes. Hemorrhoids, malaria, and diabetes are chronic pathological conditions that are characterized by an excess of free radicals, that is an imbalance between the generation of reactive oxygen species and the antioxidant defence system, and oxidative damage to biomolecules like lipids, proteins, and DNA (2). The natural substances extracted from plants are generally safe and can exhibit overwhelming biological activities, depending on their nature, structure, and interactions with other molecules in the assemblage.

Holarrhena pubescens (family-Apocynaceae) is primarily found in colder climates at higher altitudes. It grows in the Chittagong and Sylhet region of Bangladesh while it is available in the tropical Himalayan region of India (3). The bark has historically been used to treat amoebic dysentery, bleeding piles, diarrhoea, and other gastrointestinal disorders due to its astringent, anthelmintic, stomachic, febrifugal, and tonic characteristics. In traditional Ayurveda, it is used as an herbal remedy for the treatment of Jwaratisara (secondary diarrhoea), Pravahika (amebiasis), Kustha (skin disorder), Atisara (diarrhoea), Asra (blood or blood-related illnesses), and Trisna (thirst) (4). Methanol extract from the entire plant and ethanol extract from the seed have both been shown to have anti-diabetic effectiveness (5, 6). The methanolic and aqueous bark extract of this plant possesses hypoglycemic and hyperlipidemic effects, as well as gut-stimulating, inflammatory bowel diseases and relaxing properties (7–9).

In previous studies, we have found that a very few studies have been conducted on stem bark of *Holarrhena pubescens* for phytochemical analysis, anti-oxidant and anti-inflammatory activities. To support the assertion that traditional healers utilize this plant to cure inflammation and oxidative stress, there is still more research to be done on the detailed phytochemical and biological analyses of this plant. Therefore, in the present study, hydro-alcoholic extract and different fractions of stem bark of *H. pubescens* were analyzed for their phytochemical, antioxidant, and anti-inflammatory activities using the *in-vitro* method.

2. Material And Methods

2.1 Plant collection and its Authentication

The stem bark of *H. pubescens* (Kutaja) which belongs to the family Apocynaceae was collected from District Kangra (Jawali) H.P, India, at an altitude of 625m, 32°9' 0" North, 76° 1' 0" East. The sample was collected in May and was authenticated from the Botanical Survey of India (BSI), Dehradun with voucher no. 734. The stem bark was washed with water, shade dried (15–20 days) and pulverized to coarse powder up to sieve mesh size 10 and preserved in an air-tight container for further studies.

2.2 Chemicals

For extraction and fractionation, solvents were used like, n-hexane, ethanol, chloroform, n-butanol and ethyl acetate, Silica gel 60 F254 HPTLC aluminum sheets were purchased from Loba Chemie, Mumbai, India. Other chemicals like Aluminium chloride, Sodium hydroxide, Potassium persulphate, Sodium nitrite,

Rutin, Quercetin, Conessine, Gallic acid, and Lupeol were procured from Sigma-Aldrich (St. Louis, MO, USA).

2.3 Statistical analysis

Results were analysed as mean $\pm$ standard deviation (SD). Statistical analysis was performed by one-way ANOVA followed by post hoc Bonferroni and Tukey's multiple comparison test. The software GraphPad Prism (version 8.0) was used and $p < 0.05$ was considered to be statistically significant.

2.4 Preparation of plant extract

300 g of coarse powder of stem bark of *H. pubescens* was firstly extracted with n-hexane for defatting and then extracted with 90% ethanol by using Soxhlet apparatus for 48 hrs. Both the solvents were filtered by a sterilized Whatman No. 1 filter paper and dried under vacuum using a rotary evaporator at 40°C. The dried extracts were stored in an air-tight container in a refrigerator at 4°C for future use.

2.5 Preparation of sub-fractionations

The hydro-alcoholic extract was further fractionated by using different solvents i.e., ethyl acetate, chloroform, and n-butanol. For preparing fractions the 5g of hydro-alcoholic extract was dissolved in distilled water. The aqueous extract was filtered by using filter paper and transferred into a separating funnel, and further fractioned based on polarity using chloroform, ethyl acetate, and n-butanol. Solvents from the fractions were evaporated to dryness under vacuum using a rotary evaporator at 40°C and dried fractions are stored in an air-tight container under refrigeration (3–4°C) for further use (10).

3. Phytochemical Studies

3.1 Preliminary (qualitative) phytochemical screening

n-hexane and hydro-alcoholic extracts were subjected to preliminary phytochemical screening employing the standard procedures to investigate the phytoconstituents like saponins, flavonoids, alkaloids, carbohydrates, tannins, proteins, and phenols (11–13).

3.2 Quantitative phytochemical screening

n-hexane and hydro-alcoholic extracts were used for quantitative screening of various secondary metabolites which are followed by using different systems:

3.2.1 Alkaloids

10 mg of plant extracts were denatured in DMSO with 1 ml HCL (2 N). The prepared solution was shifted to a separating funnel and rinsed three times in 10 mL chloroform. Then the pH of the washed solution was adjusted by adding 0.1 N NaOH. Then 5 ml of bromocresol green solution (69.8 mg bromocresol green along with 3 ml of 2N NaOH in 5 ml distilled water until dissolved completely and then the solution was diluted with distilled water) and 5 ml of phosphate buffer was prepared by adjusting the pH (pH 4.7).

2M sodium phosphate (71.6 gm Na_2HPO_4 in 1 L distilled water) with 0.2 M citric acid (42.02 g citric acid in 1 L distilled water) was mixed to the solution. The mixture obtained was extracted with 1-, 2-, 3-, and 4- ml chloroform by vigorous shaking of the mixture. The reaction mixture was collected in a 10 ml volumetric flask. The complex's absorbance in chloroform was measured at 470 nm in comparison to a blank (14).

3.2.2 Flavonoids

0.1 ml of 10% aluminium chloride (dissolved in 100 ml of distilled water) and then 0.1 ml of 1M sodium acetate were added to the prepared plant mixture. The absorbance was taken at 415 nm using a UV/visible spectrophotometer. The total flavonoid content was calculated from the calibration curve of rutin (standard) and results were expressed as rutin equivalent (mg/g) (15).

3.2.3 Carbohydrates

Total carbohydrate content was estimated by anthrone reagent. 1 ml of sample was taken and 5 ml of HCl (2.5N) was added to it and heated in a water bath for 3 hours at 40°C and then samples were cooled down. The contents were centrifuged after neutralization, and 0.1 ml of the supernatant was used for analysis. 4 mL anthrone reagent was added, which was again heated in the water bath for 8 minutes. Final absorbance was taken at 639 nm using UV/visible spectrophotometer. Diosgenin is used as the standard and the concentration of carbohydrates present in an unknown sample was determined using a standard graph (16).

3.2.4 Phenols

1 ml of this prepared solution is transferred into a test tube then 0.5 ml (2N) Folin Ciocalteu reagent and 1.5 ml (20%) sodium carbonate (Na_2CO_3) solution was added to it. The total volume was made up to 8 ml by adding distilled water which is further followed by vigorous shaking and the final solution was allowed to stand for 2 hours. The final absorbance of the reacted solution was taken at 765 nm using UV/visible spectrophotometer. The total phenolic content was estimated by using a standard calibration curve from various diluted concentrations of gallic acid (standard) and was expressed as mg of GAE/g of the extract (17).

3.2.5 Saponins

Vanillin solution is prepared by adding 1 g of vanillin to 70 ml of ethanol. 2ml of the prepared solution is taken and, in that solution, 2 ml of 72% of the sulfuric acid solution is added. The prepared solution was mixed properly and heated using a water bath at 100°C (10 min.) and further cool down for 10 min. Final absorbance was measured at 544 nm against a blank solution using UV/visible spectrophotometer and diosgenin was used as standard (18).

3.2.6 Proteins

The protein content was evaluated using Lowry's method. 1 ml of the sample prepared in phosphate buffer was combined with 0.5 ml of 0.1 N NaOH and 5 ml of alkaline copper reagent, and the mixture was

incubated for 30 minutes at room temperature. 0.5 ml of Folin–Ciocalteu reagent was added to the mixture and incubated for 10 minutes at room temperature. The absorbance was measured against blank at 660 nm. The analysis was done in triplicates, and the results were expressed in mg/g of sample and bovine serum albumin (BSA) was used as standard (19).

3.2.7 Tannins

1 ml of prepared solution was transferred into the test tube and the volume was made up to 3 ml by using distilled water. 0.5 ml of Folin-Ciocalteu Phenol reagent was added to the solution. After that, in the next step 1 ml of (35%) sodium carbonate (Na_2CO_3) solution is added and further diluted to 10 ml using distilled water. The mixture was properly shaken and allowed kept at room temperature for 30 minutes. Tannic acid was used as a standard reference solution. The final absorbance of standard solutions was measured against blank at 725 nm using a UV/visible spectrophotometer. Total tannin content was expressed as mg of TA/g of the extract (20).

4. Evaluation Of Anti-oxidant Activity

4.1 DPPH (1–1 diphenyl-2-picrylhydrazyl radical scavenging) assay

DPPH radical scavenging activity was performed by the method of (21). Various concentrations (20 $\mu\text{g/ml}$, 40 $\mu\text{g/ml}$, 80 $\mu\text{g/ml}$, 160 $\mu\text{g/ml}$, 200 $\mu\text{g/ml}$) of hydro-alcoholic extract and its fractions i.e., chloroform fraction, ethyl-acetate fraction, n-butanol fraction and water fraction were mixed with 1.0 ml of methanolic solution of DPPH radicals, resulting in the final concentration of DPPH that is 0.2mM. The mixture was shaken vigorously and left it stands for 30 min dark covered with aluminium foil and the absorbance was measured at 517nm. Ascorbic acid was used as standard. Each sample was measured in triplicate. Mean and Standard deviation ($n = 3$) were calculated (22). The percentage inhibition of DPPH of the sample was calculated according to the equation given below:

$$\% \text{Inhibition} = \frac{\text{Abs. of control} - \text{Abs. of sample}}{\text{Abs. of control}} \times 100$$

Results have also been reported as IC 50, which is the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%. The graph was plotted with the average of the three determinations.

4.2 Reducing power assay

The reducing power of compounds was assessed by the method of (23). Different concentrations of extract and fractions were mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% of potassium ferrocyanide in each test tube. 2.5 ml of 10% TCA was added to the mixture after it had been incubated at 50°C for 20 min. The mixture was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution was separated and (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of FeCl_3 (0.1%), and the absorbance at 700 nm was measured. An increase in absorbance of the reaction mixture

indicates increased reducing power. Values from the experiment were taken in triplicates and expressed as equivalents of ascorbic acid in µg/mg of the samples.

4.3 H₂O₂ (Hydrogen peroxide scavenging) assay

Hydrogen peroxide (20mM) solution was prepared in phosphate-buffered saline (PBS, pH 7.4) in various concentrations. 2 ml of hydrogen peroxide solutions in PBS were added to 1 ml of the extracts or standards in methanol. After 10 minutes, the absorbance at 230 nm was measured in comparison to a blank solution made up of extracts in PBS without hydrogen peroxide. The IC₅₀ value is the concentration of the sample required to scavenge 50% free radical (24). The above experiments were carried out (in triplicate), and the percentage inhibition was determined using the formula below:

$$\% \text{Inhibition} = \frac{\text{Abs. of control} - \text{Abs. of sample}}{\text{Abs. of control}} \times 100$$

5. Evaluation Of Anti-inflammatory Activity

5.1 NO (Nitric oxide radical scavenging activity)

Nitric oxide was generated from sodium nitroprusside and measured by the Griess reaction. The reaction mixture (6 mL) contains extract, fractions or standard (1 mL) in DMSO at varied concentrations, phosphate buffer saline (PBS, pH 7.4), and sodium nitroprusside (10 mM, 4 mL). It was incubated at 25° C for 150 min. After incubation, 0.5 mL of the reaction mixture containing the nitrite ion was taken out, 1 mL of sulphanilic acid reagent (0.33 percent w/v), was added, and the mixture was thoroughly mixed before being left to stand for 5 minutes to complete the diazotization process. 1 mL of NEDD was then added, stirred, and let to stand for 30 minutes in diffused light. A chromophore with a pink color was prepared and absorbance was measured at 540nm (25).

5.2 Protein denaturation Assay

A solution of 0.2% w/v of Bovine serum albumin (BSA) was prepared in Tris Buffer (pH 6.8). Both extracts, fractions and standard drugs (diclofenac sodium) were diluted in various concentrations (500, 1000, 1500, 2000 and 2500 µg/ml). 5 ml of 0.2% w/v was transferred to test tube containing 50 µg/ml of extract/fraction/standard. The control tubes contained 5 ml of 0.2% w/v BSA solution with 50 µl methanol (26, 27). The samples were heated at 72°C for 5 min and cooled at room temperature for 15 min. The optical density of the solution was taken at 660nm and percentage inhibition of precipitation (denaturation of proteins) was determined as compared to control using the following formula:

$$\% \text{Inhibition} = \frac{\text{Abs. of control} - \text{Abs. of sample}}{\text{Abs. of control}} \times 100$$

6. Results

6.1 Preliminary (qualitative) phytochemical screening

The n-hexane and hydro-alcoholic extracts of stem bark of *H. pubescens* showed the presence of phytoconstituents like alkaloids, tannins, phenolics, flavonoids, carbohydrate and saponins shown in Table 1.

Table 1
Results of the phytochemical constituents of *H. pubescens* stem bark with n-hexane and hydro-alcoholic extracts.

Phytochemical Constituents	n-Hexane extract	Hydro-alcoholic extract
Alkaloids (Wagner test)	+	+
Flavonoids (H ₂ SO ₄)	+	+
Tannins (Braymer's test)	+	+
Saponins (Foam test)	+	+
Phenols (Ferric chloride test)	+	+
Carbohydrates (Molish test)	+	+
Proteins (Millions reagent test)	+	+
+ Present		

6.2 Quantitative phytochemical screening

The total amount of phytochemicals that were found in both extracts was quantitatively determined by standard procedures. In both the extracts of *Holarrhena* species, the hydro-alcoholic extract showed a higher percentage of phytochemicals than n-hexane extracts. Among the seven phytochemicals, the alkaloid content was highest in the hydro-alcoholic extract which was followed by saponins, flavonoids, phenolics, and tannins. Proteins and carbohydrates content was very low as compared to others (Table 2).

Table 2
Quantitative analysis of phytochemicals (mg/g) in *H. pubescens*
stem bark extracts (n-hexane and Hydro-alcoholic)

Phytochemicals	n-hexane extract	Hydro-alcoholic extract
Alkaloids	96.01 ± 1.52	451.73 ± 0.76
Saponins	109.9 ± 1.93	407.90 ± 0.96
Flavonoids	102.40 ± 1.09	184.91 ± 0.63
Phenols	84.09 ± 1.22	170.58 ± 0.71
Tannins	33.14 ± 0.84	90.14 ± 1.45
Protein	39.40 ± 0.20	52.13 ± 0.65
Carbohydrates	36.60 ± 0.30	51.73 ± 0.50

n-hexane extract contained 96 mg of alkaloids, 109 mg of saponins, 102 mg of flavonoids, 84 mg of phenols, 33 mg of tannins, 39 mg of protein, and 36 mg of carbohydrates. In the case of hydro-alcoholic extract 451 mg of alkaloids, 407 mg of saponins, 184 mg of flavonoids, 170 mg of phenols, 90 mg of tannins, 52 of proteins, and 51 mg of carbohydrates was present.

6.3 Evaluation of *in-vitro* anti-oxidant activity:

The complexity and diversity of phytochemical substances derived from plant extracts have an impact on their efficacy in various assessments. Therefore, evaluations using a variety of approaches are reliable for estimating the effectiveness of the substances. In the current study, total of three methods from *H. pubescens* stem bark have been used to access anti-oxidant activities i.e., the DPPH radical scavenging assay, reducing power assay and H₂O₂ scavenging assay.

6.3.1. DPPH (1–1 diphenyl-2-picrylhydrazyl radical scavenging) assay

The DPPH assay is a rather quick and effective way to assess free radical scavenging capacity. To create a stable diamagnetic molecule, DPPH can receive an electron or hydrogen radical. A decrease in the absorption of the DPPH radical is indicated by color changes from purple to yellow in terms of IC₅₀ values (Table 3). The results showed the degree of discoloration and indicate significant free radical scavenging activity in terms of IC₅₀ values of ascorbic acid (28.00 ± 0.49), ethyl acetate fraction (34.34 ± 0.86), butanol fraction (57.71 ± 1.06), H₂O fraction (69.19 ± 1.06), chloroform fraction (82.23 ± 1.57) and hydro-alcoholic extract (44.88 ± 1.25). The antioxidant activity of the extract and fractions were compared with ascorbic acid (standard reference) that may inhibit free radicals and was assessed using the percentage of inhibition shown in Fig. 1a.

Table 3
Free radical scavenging activity of extract and fractions

Extract/Fractions	IC50 DPPH (µg/ml)	IC50 H ₂ O ₂ (µg/ml)	IC50 NO (µg/ml)
Ethyl acetate fraction	34.34 ± 0.86	26.05 ± 0.64	48.83 ± 1.13
Butanol fraction	57.71 ± 1.06	99.27 ± 1.38	30.39 ± 1.37
Chloroform fraction	82.23 ± 1.57	109.03 ± 1.08	166.46 ± 0.83
H ₂ O fraction	69.19 ± 1.06	49.06 ± 1.08	80.42 ± 1.19
Hydro-alcoholic extract	44.88 ± 1.25	18.87 ± 0.65	134.61 ± 1.55
Ascorbic acid	28.00 ± 0.49	15.03 ± 1.11	13.16 ± 1.28

6.3.2. Reducing power assay

The electron-donating properties of extract and fractions were found to be comparable to ascorbic acid (standard) in terms of their reducing capacities. The results of the reducing power assay of extract and fractions are summarized in Table 4.

Table 4
Reducing power assay of extract and fractions

Conc. (µg/ml)	Reducing powder assay					Ascorbic acid
	Chl	EA	But	H ₂ O	HA	
20	0.173 ± 0.007	0.305 ± 0.018	0.254 ± 0.010	0.267 ± 0.008	0.286 ± 0.008	0.378 ± 0.005
40	0.169 ± 0.042	0.354 ± 0.009	0.294 ± 0.005	0.387 ± 0.011	0.365 ± 0.009	0.463 ± 0.010
60	0.253 ± 0.006	0.453 ± 0.007	0.363 ± 0.007	0.455 ± 0.009	0.448 ± 0.005	0.615 ± 0.018
80	0.336 ± 0.009	0.565 ± 0.009	0.486 ± 0.008	0.643 ± 0.005	0.536 ± 0.007	0.684 ± 0.008
100	0.481 ± 0.010	0.633 ± 0.009	0.611 ± 0.013	0.692 ± 0.005	0.649 ± 0.0004	0.735 ± 0.010

6.3.3. H₂O₂ (Hydrogen peroxide scavenging) assay

Plants extract or fractions can scavenge hydrogen peroxide due to the presence of phenols and tannins, which might donate electrons and neutralize hydrogen peroxide into water. The results showed that varying concentrations of *H. pubescens* stem bark extract and fractions cause a scaled rise in the scavenging of H₂O₂. Calculated IC50 values are presented in Table 3 along with a percentage inhibition graph (Fig. 1b). The IC 50 values are ascorbic acid (15.03 ± 1.11), ethyl acetate fraction (26.05 ± 0.64),

butanol fraction (99.27 ± 1.38), H₂O fraction (49.06 ± 1.08), chloroform fraction (109.03 ± 1.08) and hydro-alcoholic extract (18.87 ± 0.65). Ethyl acetate fraction showed promising H₂O₂ scavenging activity.

6.4 Evaluation of *in-vitro* anti-inflammatory activity:

6.4.1. NO (Nitric oxide radical scavenging activity)

The Griess-Ilosvoy reaction can be used to measure the amount of nitric oxide scavenging activity (Garrat, 1964). It is known that the chemical sodium nitroprusside breaks down in an aqueous solution at physiological pH (7.2), producing NO. The formation and scavenging of NO by plant extract and fractions were shown to be equivalent to standard drugs and presented as IC₅₀ values: ascorbic acid (13.16 ± 1.28), ethyl acetate fraction (48.83 ± 1.13), butanol fraction (30.39 ± 1.37), H₂O fraction (80.42 ± 1.19), chloroform fraction (166.46 ± 0.83) and hydro-alcoholic extract (134.61 ± 1.55) in Table 3 and percentage inhibition is shown in Fig. 1c.

6.4.2. Albumin denaturation assay

The present results showed that there is a scaly increase in albumin denaturation assay in extract and fractions of *H. pubescens* stem bark. The results of the albumin denaturation assay are summarized in Table 5.

Table 5
Albumin denaturation assay of extract and fractions

Conc. (µg/ml)	% Inhibition					Diclofenac sodium
	Chl	EA	But	H ₂ O	HA	
500	69.48 ± 1.91	74.56 ± 1.91	55.81 ± 2.05	64.71 ± 2.24	40.50 ± 2.04	79.76 ± 0.79
1000	76.29 ± 1.87	79.74 ± 1.84	61.69 ± 1.90	71.63 ± 1.67	46.73 ± 2.16	84.96 ± 1.41
1500	81.26 ± 1.97	85.11 ± 1.44	65.80 ± 2.15	78.07 ± 2.56	53.55 ± 2.18	90.96 ± 1.53
2000	84.29 ± 2.63	87.81 ± 1.88	73.78 ± 2.05	83.48 ± 1.98	63.59 ± 1.16	95.62 ± 0.83
2500	89.69 ± 1.73	92.64 ± 1.89	82.14 ± 2.14	85.73 ± 2.21	76.54 ± 1.94	98.55 ± 0.88

8. Discussion

Plants have medical value because they contain certain chemicals that have distinct physiological effects on people. Numerous phytochemicals have been discovered to have a variety of functions that may aid in the prevention of chronic diseases. Alkaloids protect against chronic illnesses. Saponins have

antibacterial and hypercholesterolemic protection. Triterpenoids and steroids exhibit analgesic properties. The saponins and steroids protect from central nervous system diseases (28–30). *H. pubescens* stem bark extract and fractions were tested for their potential as an anti-inflammatory, and analgesic activity to demonstrate scientifically how it is used in folklore to treat inflammatory disorders. According to (31, 32), the pharmacological activities of *H. pubescens* are due to the presence of anti-oxidant components such as phenols, phenolic diterpenes, and flavonoids. According to our study, the preliminary analysis of *H. pubescens* showed the presence of phenols, flavonoids, alkaloids, carbohydrates, saponins, proteins, and tannins (33–35). *H. pubescens* stem bark extract and fractions were subjected to phytochemical screening to determine the presence of alkaloids, carbohydrates, proteins, flavonoids, saponins, tannins, and phenols. These compounds also have a variety of medicinal benefits, including anti-inflammatory, antioxidant, and analgesic properties. Free radicals are continuously produced by biological systems, and when they are in excess, they can severely harm tissues and biomolecules, which can result in pathological conditions like inflammation, cell death, and organ failure. Antioxidants have ability to scavenge free radicals which may protect against oxidative stress and cell damage (36–38). DPPH radical scavenging activity of ethyl acetate fraction showed positive control as compared with ascorbic acid (standard). The highly reactive species known as the hydroxyl radical, which forms in biological systems, has been linked to numerous free radical pathologies and is capable of causing significant damage to nearly every molecule present in living cells. Additionally, by removing hydrogen atoms from unsaturated fatty acids, this species is one of the rapid initiators of the lipid peroxidation process. It will further damage the bio-membrane that damages cells. So, scavenging of hydroxyl radical is crucial (39). Ethyl-acetate fraction of stem bark of *H. pubescens* resists hydroxyl radical generation. The NO assay is premised on the theory that, in an aqueous solution of sodium nitroprusside at physiological pH, nitric oxide spontaneously produces and combines with oxygen to produce nitrite ions that may be evaluated using the Griess reagent. Nitric oxide scavengers compete with oxygen, which lowers the formation of nitrite ions (40). The effectiveness of plant extract and fractions in preventing the production of nitric oxide was compared to that of the standard drug in which butanol fraction showed decreased production of nitric oxide (41). According to the reducing power assay, the yellow test solution was converted into green and blue colors. Due to the presence of reducers, the ferricyanide complex is reduced to a ferrous form. We can therefore track the Fe²⁺ content by observing the development of Perl's Prussian blue at 700 nm. Reductones, which have been demonstrated to exert an antioxidant effect by breaking the free radical chain by donating a hydrogen atom, are typically linked to the existence of reducing characteristics. The reducing power of extract and fractions were increased and compared well with increasing concentrations shown in the following order: Ethyl acetate > Hydro-alcoholic > H₂O > Butanol > Chloroform. Protein denaturation methods are used to study the *in-vitro* anti-inflammatory activity of plant extract and fractions (42). *Holarrhena* plant extract and fractions prevented the lysis of the erythrocyte membrane caused by hypotonicity and stabilised the lysosomal membrane, which significantly reduced inflammation.

Hence, the present study suggests that the extract and fractions of *H. pubescens* can neutralize free radicals and reduce inflammatory mediators. These biological characteristics might be attributed to the

presence of several substances, such as phenolics, terpenes, flavonoids, etc. Ethyl acetate and butanol fractions showed good anti-oxidant and anti-inflammatory results as compared to other fractions. These preliminary results may serve as a basis for further research being done in our laboratory to assess the *in-vivo* pharmacological potential of extracts and fractions from the *Holarrhena* species for conditions like hemorrhoids, oxidative stress, and inflammation.

9. Conclusion

There are few scientific studies on the biological potential of stem bark of *H. pubescens*. The present investigation on different extracts and fractions of this plant has been tested for various methods of anti-oxidant and anti-inflammatory activities which may help in preventing the various inflammatory and oxidative-stress-related diseases. In conclusion, the study of the active phytoconstituents from *Holarrhena* plant species is very important to create novel treatments for the benefit of humanity.

Abbreviations

ROS- reactive oxygen species, HCl- hydrochloric acid, NaOH- sodium hydroxide, DMSO- Dimethyl sulfoxide, AlCl₃- aluminum chloride, GAE- gallic acid, BSA- bovine serum albumin, DIO- diosgenin, Na₂CO₃- sodium carbonate, TA- tannic acid, DPPH- 1-1 diphenyl-2-picrylhydrazyl radical scavenging, NO- nitric oxide, H₂O₂- Hydrogen peroxide scavenging, IC₅₀- inhibitory concentration, µg- micro gram, ml- milli litre, mg- milli gram, FeCl₃-ferric chloride

Declarations

Ethics and consent to participate: We confirm that all methods were carried out in accordance with relevant guidelines and regulations.

Conest for publication: Not Applicable.

Availability of data and materials: The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no known competing interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author's contributions: SD, PB and RG designed the experimental protocol. SD carried out the experimentation and drafted the manuscript. RN and RT performed the statistical analysis and interpreted the results. All authors read and approved the final manuscript.

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Figures

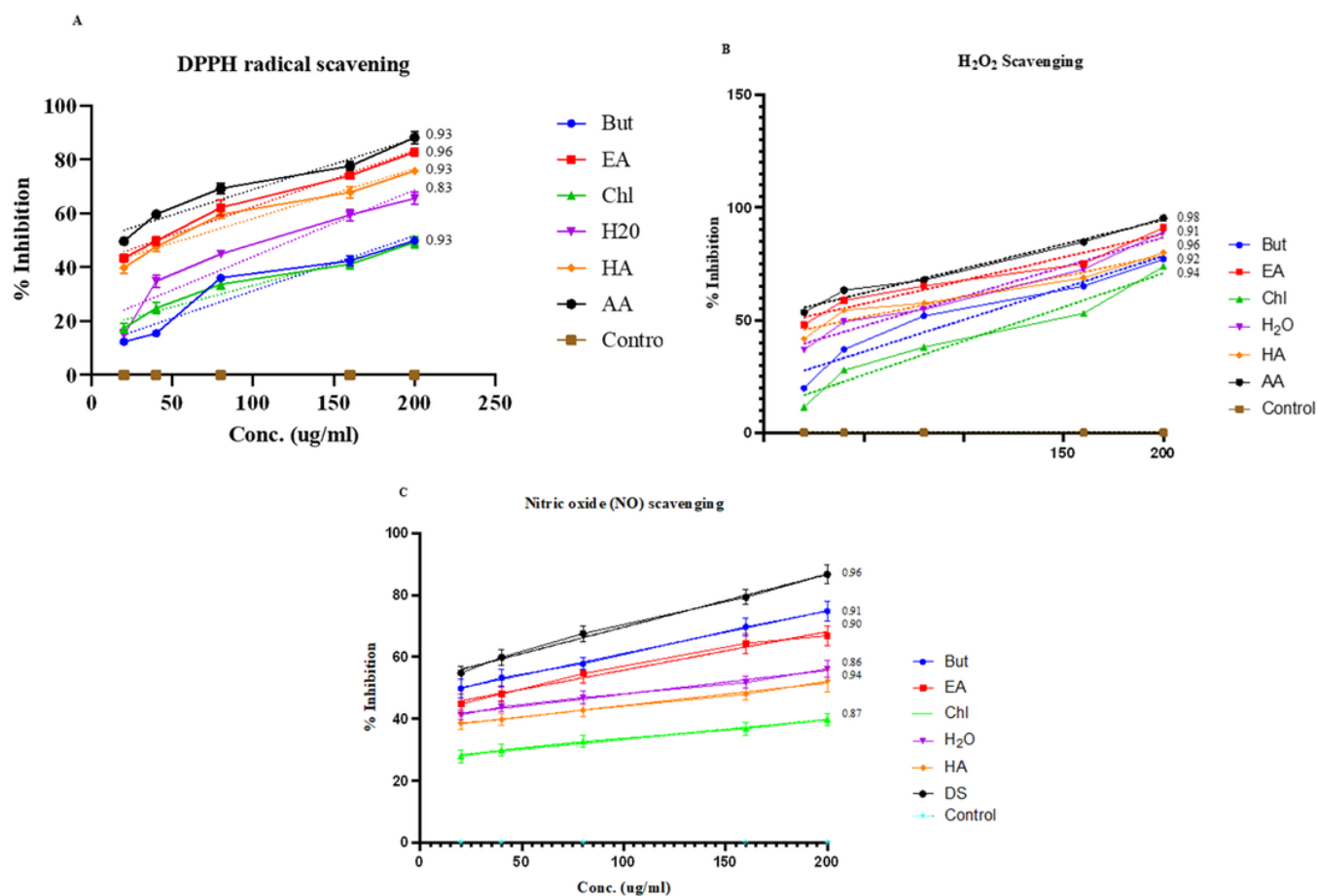


Fig. 1 Antioxidant activities of the plant extract and fractions estimated using: **(a)** DPPH radical scavenging activity, **(b)** Hydrogen peroxide radical scavenging activity, and **(c)** Nitric oxide Radical scavenging activity

Figure 1

See image above for figure legend.