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Assessment of antimicrobial, free radical scavenging, and anti-inflammatory activities of *Holarrhena pubescens* (Buch.-Ham.) Wall. seeds using *in vitro* methods and evaluation of antiarthritic potential using an *in vivo* method

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ABSTRACT

Introduction: *Holarrhena pubescens* is used traditionally for treating several infectious and inflammatory disorders with no scientific evidence. The present study was designed to evaluate the antimicrobial and free radical scavenging properties of different fractions of ethanolic extract of *H. pubescens* seeds. Furthermore, the potential of the fractions of the plant extract in treating acute and chronic inflammation through *in vitro* and *in vivo* experiments was also evaluated.

Methods: The successive fractions of ethanolic extract of *H. pubescens* seeds, including petroleum ether (HPF), chloroform (HCF), and methanol (HMF) fractions were subjected to *in vitro* study to evaluate the properties to eradicate the microorganisms using disc diffusion method and to scavenge oxidants in the DPPH method. The HCF was further used in an anti-inflammatory activity study by inhibition of bovine serum albumin denaturation assay and an *in vivo* study to evaluate the effect of the drug in a chronic inflammatory condition by adjuvant-induced arthritis in a rats' model.

Results: HCF showed the most potent effect among all the test drugs. The fraction showed a broad-spectrum antimicrobial effect and 80.35 ± 0.46% free radical scavenging effect (IC_{50} value = 28.41 ± 0.33 µg/mL) in the antioxidant study. Further, HCF showed an anti-inflammatory effect with 68.37 ± 1.11% inhibition of denaturation of albumin and 75.16% (0.38 ± 0.16 mL, $P<0.05$) inhibition of rat paw edema in the *in vivo* study.

Conclusion: The study exhibited the antimicrobial, antioxidant and anti-inflammatory potentials of the fractions of ethanolic extract of *H. pubescens* seeds.

Implication for health policy/practice/research/medical education:
The successive solvent fractions of *Holarrhena pubescens* seeds' ethanolic extract showed antimicrobial, antioxidant, and anti-inflammatory potentials in the *in vitro* experiments. The selected bioactive fraction of *in vitro* studies also showed the potential for treating chronic inflammation in an *in vivo* experiment of adjuvant-induced arthritis in a rats' model. Hence, the seeds of this plant might be used as a natural antioxidant, antimicrobial, and anti-inflammatory drug. Nevertheless, further studies are required to understand the mechanism of action of the drug for its anti-inflammatory activity.

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Introduction

Inflammation is a natural physiological response of the body to an injury or any other type of cell damage. The inflammatory process is orchestrated by different immunological cells and the release of chemical mediators (1). The process is evident and unavoidable as it triggers

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the natural repair system of the body to any cellular damage but, in certain conditions, the biological process can lead to the development of various disorders (2).

Inflammation, the biological defense mechanism, allows immunological cells like neutrophils, monocytes, and lymphocytes including natural killer cells to develop an inflammatory matrix at the site of damage by scavenging the cellular debris, releasing cytokines, histamine, prostaglandins, and other chemical mediators to promote the healing process (3,4). Unlike normal circumstances, the process of inflammation is disrupted during infection, chronic diseases, or any immunologically compromised conditions due to an imbalance of cellular or chemical mediators (5). Consequently, chronic inflammatory conditions appear due to this imbalance, which is also the causative factor for the development of associated diseases (6,7). Therefore, there is a need for proper medical interventions to manage the disruption of the normal inflammatory process and to regularize the body's natural defense system.

Free radicals are generated during the inflammatory process and take part in cellular defense by facilitating cytotoxic mechanisms to eliminate unwanted cell fragments. The cytotoxic effect of free radicals also helps in the eradication of microbial growth at the site of injury (8). Microbial accumulation is often observed in the area of tissue damage and the normal inflammatory response is usually able to remove them. Nonetheless, in conditions like chronic inflammation or persistent viral and bacterial infections, the natural defense mechanism fails to provide the proper eradication of microorganisms which leads to the progression of infection. Furthermore, these conditions cause an overproduction of free radicals, resulting in oxidative stress, which also contributes to the development of several diseases. In these circumstances, proper medical intervention is required to reduce oxidative stress by scavenging free radicals and to combat the infection by eliminating pathogenic microorganisms (9,10). Thus, antioxidants and antimicrobial drugs play a significant role in the treatment of inflammatory diseases. Moreover, by controlling excess free radicals, antioxidants help restore cellular homeostasis and revitalize the defense system (11).

There are several existing anti-inflammatory medicines which are mostly containing synthetic chemicals. Despite the effectiveness of these medications, their usage in the long-term treatment process is limited due to their associated side effects (12). Moreover, anti-inflammatory drugs are found to be insufficient to produce their due effect in chronic inflammatory disease conditions, such as infections, cancer, and arthritis, which compels the use of multiple medicines for the treatment (13). Hence, there is a need to search for safe and effective anti-inflammatory medicines that can showcase their efficacy even in chronic disease conditions. In this regard, plant-based medicines have consistently shown their potential to treat various ailments, including chronic diseases. The effectiveness of plants in treating various diseases is attributed to the diverse range of phytochemicals present in them (14). With this perspective, the present study was also planned with the objectives of evaluating the antimicrobial, antioxidant, and anti-inflammatory potential of the seed of the Indian medicinal plant *Holarrhena pubescens* (Buch.-Ham.) Wall.

H. pubescens is an Indian deciduous tree of the Apocynaceae family. The plant is known as kurchi, kuataja, and indrajava in India. The plant is observed to be used traditionally for various infection-related diseases, including leprosy and dysentery, as well as inflammation-related disorders like diabetes, eczema, colic, dyspepsia, and asthma (15,16). Previous experiments on this plant reported antiplasmodial, anthelmintic, antidiarrheal, antimalarial, febrifuge, anti-inflammatory, antihyperlipidemic, and antidiabetic effects (17-19). The phytochemical studies reported the presence of different classes of phytoconstituents, like steroidal alkaloids, glycosides, triterpenoids, steroids, and others (20). Nevertheless, no previous studies have reported the antimicrobial, antioxidant, and anti-inflammatory potential of *H. pubescens* seeds in chronic inflammation. Thus, the present study was designed to assess the pharmacological effect of the plant material against microorganisms, the scavenging potential of free radicals, and the anti-inflammatory effects in acute and chronic inflammation.

Materials and Methods

Reagents and chemicals

Analytical reagent (AR) grade chemicals were used in this research work. The procurement of all chemicals, Tween 80 and dimethyl sulfoxide (DMSO), were from SD Fine-Chem Limited, Mumbai, India. Bovine Serum Albumin, Ascorbic acid, and Agar media were procured from Himedia Laboratories Pvt. Ltd., Mumbai. 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Loba Chemie Pvt. Ltd., Mumbai), and Freund's Complete Adjuvant (FCA) (Sigma Aldrich, St. Louis, USA) were also procured. Diclofenac sodium was obtained as a gift sample from Cipla Pharmaceuticals, Ahmedabad, Gujrat, India. *Staphylococcus aureus* (MTCC 96), *Bacillus subtilis* (MTCC 441). *Escherichia coli* (MTCC 739), and *Salmonella typhi* (MTCC 733) were procured from CSIR-Institute of Microbial Type Culture Collection and Gene Bank, Chandigarh, India. Instruments like, the UV-VIS spectrophotometer (Shimadzu UV-1800) and Rotary vacuum evaporator (Remi, India) were used for the study.

Collection and authentication of seed sample

The collection of *H. pubescens* seeds was carried out at Mangaluru of Dakshin Kannada district of Karnataka state, India. Prof. Dr. Krishna Kumar from the Department of Applied Botany of Mangalore University, Mangaluru, identified and authenticated the plant material. A voucher

specimen (D-82) was submitted to the institution, the working place of this research work.

Preparation of extraction and fractionation of seeds

The maceration method was used for the extraction of the collected, shade-dried, and coarsely powdered seeds of *H. pubescens* using ethanol (95%). The rotary flash evaporator was used to concentrate the extract, and it was further dried in a water bath to remove all solvents. Then, successive fractionation of the seed extract was carried out using petroleum ether, chloroform, and methanol, according to their increasing polarity. These fractions, petroleum ether fraction (HPF), chloroform fraction (HCF), and the fraction of methanol (HMF) of the seed extract, were dried and used further in the pharmacological studies to assess their potential.

In vitro studies

Antimicrobial activity study

The antimicrobial study for the fractions (HPF, HCF, and HMF) of seed extract was evaluated by the Disc diffusion method using two gram-positive bacteria (*Staphylococcus aureus* MTCC 96 and *Bacillus subtilis* MTCC 441) and two gram-negative bacteria (*Escherichia coli* MTCC 739 and *Salmonella typhi* MTCC 733). The agar medium was prepared, and then all bacteria strains were inoculated in that. This medium along with bacterial strains was then transferred to the petri plates aseptically and left at room temperature for solidification. After the solidification, 6 mm diameter cups were made on each plate. A 100 µg/mL concentration of all test drugs (HPF, HCF, and HMF) was prepared by dissolving them in DMSO. This freshly prepared solution of each test drug (0.1 mL), DMSO as a control group, and the standard drug (amoxycillin) were transferred to each designated cup in all Petri plates. All these plates were then incubated at the temperature of 37 °C. After 48 hours of incubation, the diameters of the zone of inhibition for test and standard drugs, and control group were measured. The averages of diameters of the zone of inhibition (n=3) were calculated (21).

Antioxidant activity study

DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging potential

A stock solution (0.2 mM) of DPPH was prepared in methanol. A series of concentrations of 10-50 µg/mL of Ascorbic acid as a standard drug, and test drugs (HPF, HCF, and HMF) were prepared by dissolving them in methanol. 100 µL of the stock solution of DPPH was then added in each concentration (10-50 µg/mL) of drug samples. These solutions were placed in a dark area and allowed to stand for 30 min. Then, the absorbance of all solutions was taken at 517 nm, and the percentage of activity of test drugs of scavenging free radicals was calculated using the following formula 1 (22).

$$Percentage\ of\ activity = \frac{A_{control} - A_{Sample}}{A_{control}} \times 100$$

Where, A= Absorbance

Anti-inflammatory activity study

Bovine serum albumin denaturation inhibition potential

The anti-inflammatory effect of the test drug (HCF) was evaluated by this method of screening the potential of HCF to inhibit the denaturation of bovine serum albumin (BSA). An aqueous solution of BSA (1%), and a series of concentrations of 10-50 µg/mL of diclofenac sodium as a standard drug, and test drug (HCF) were prepared during the study. BSA solution (1%) and hydrochloric acid (1N, for maintaining pH 6.3) were then mixed with each concentration (10-50 µg/mL) of standard and test drugs. Further, all solutions were incubated (at 37 °C for 20 miutesn), followed by heating (at 57 °C for 3 min.), and cooling. Then, 2.5 mL of phosphate buffer was added to all mixtures. The absorbance of all samples (Standard and test drugs) and a blank sample (phosphate buffer) was taken at 416 nm. The percentage of activity of inhibition of denaturation of bovine serum albumin of the test drug was calculated by using the above-mentioned formula 1 (23).

In vivo studies

Experimental animals

Wistar albino rats of the male variant of 180-200 g weight were procured to carry out the *in vivo* experiment. All animals were divided into groups, housed in cages, and maintained with a 12-hour light-dark cycle at the temperature of 24 ± 2 °C. All animals were fed with pellets (Hindustan Lever Co., Bombay, India) and water. The food and water were available throughout the time in the cage and experimental animals were taken as per their need. The experiment with animals was carried out by following the guidelines of the institutional animal ethics committee.

Antiarthritic effect in a chronic inflammation study of FCA-induced arthritis in rats

The study was carried out to evaluate the potential of the test drug (HCF) to reduce chronic inflammation, in the form of arthritis (24). In the study, the test drug (HCF) and standard drug (diclofenac sodium) were used as a suspension using a 1% solution of tween 80. Twenty four rats were divided into four groups (n = 6) and named Control, which received vehicle (1% solution of tween 80), Diclo (Diclofenac sodium of 10 mg/kg oral dose as per body weight), HCF 200 (HCF of 200 mg/kg oral dose as per body weight), and HCF 400 (HCF of 400 mg/kg oral dose as per body weight). All animals received respective drugs and vehicles on day 0 of the experiment. After 2 hours of administration of oral doses, on the same day (Day 0), 0.1 mL of FCA was injected to induce arthritis in animals of all the groups. All animals were observed

and received respective treatment for 14 days. The paw volume of animals was measured for all 14 days and the percentage inhibition was calculated using the following formula 2 (25).

$$Percentage\,inhibitaion = 1 - \frac{PV_t}{PV_c} \times 100$$

Where, PV_t and PV_c were considered as relative changes in paw edema of the test and control groups, respectively.

Statistical analysis

Values of all findings of the present research work were presented as mean ± standard error means (SEM). The linear regression was used to analyze the IC_{50} values of *in vitro* studies for antioxidant and anti-inflammatory activity studies. One-way ANOVA, followed by Dunnett's *t*-test was used to determine the statistical significance of the *in vivo* study for evaluating the antiarthritic effect of the fraction of the seed extract in the FCA-induced arthritis in rats; the $P<0.05$ value was considered to be significant.

Results

Antimicrobial activity study

The antimicrobial study of different fractions (HPF, HCF, and HMF) of ethanolic extract of the seed of *H. pubescens* was carried out by the disc diffusion method against gram-positive and gram-negative bacteria. The result, as shown in Table 1, suggested the broad-spectrum antimicrobial potential of test drugs against all test microorganisms at the concentration of 100 µg/mL. The chloroform fraction (HCF) was found to be the most active among all test drugs at the same concentration. Hence, the study proved the broad-spectrum antimicrobial effect of seed of *H. pubescens,* and chloroform fraction was found to be the most effective.

Antioxidant activity study

The antioxidant activity of different fractions (HPF, HCF, and HMF) of the seed extract of *H. pubescens* was carried out by DPPH free radical scavenging activity study. The result of the study as described in Table 2, indicates the antioxidant potential of plant materials, and HCF showed the potent effect (80.35 ± 0.46% and IC_{50} value 28.41 ± 0.33 µg/mL) among the test drugs at 50 µg/mL concentration. HPF and HMF with the same concentration also showed the potential of free radical scavenging with 42.21 ± 0.36% and 61.09 ± 0.3%, respectively. The study also showed that the potential of test drugs to scavenge free radicals was directly proportionate to the concentration of those samples. The IC_{50} value of HCF was also indicative of its potential compared to other test drugs i.e., HPF (42.53 ± 0.24 µg/mL) and HMF (36.18 ± 0.17 µg/mL), as shown in Figure 1.

Anti-inflammatory activity study

The results of antimicrobial and antioxidant studies showed that the effect of HCF was the most potent in both studies in comparison to other test drugs (HPF and HMF). Hence, the HCF, the chloroform fraction of *H. pubescens* seed extract, was selected to study its anti-inflammatory potential by *in vitro* experiment, the assay of inhibition of

Table 1. Antimicrobial effect of different fractions of *Holarrhena pubescens* seed extract against gram-positive and gram-negative bacteria in a Disc diffusion assay

Groups	Zone of inhibition of microorganisms (mm)			
	Staphylococcus aureus **(MTCC 96)**	***Bacillus subtilis*** **(MTCC 441)**	***Escherichia coli*** **(MTCC 739)**	***Salmonella typhi*** **(MTCC 733)**
Amoxycillin	29 ± 1.18	33 ± 1.03	28 ± 1.21	27 ± 1.09
HPF	14 ± 1.23	19 ± 1.17	13 ± 1.14	11 ± 1.13
HCF	36 ± 1.03	40 ± 1.15	32 ± 1.12	31 ± 1.27
HMF	17 ± 1.16	21 ± 1.19	16 ± 1.00	15 ± 1.18

Data are presented as mean ± SEM by carrying out experiments in triplicate. HPF, HCF, and HMF: Petroleum ether, chloroform, and methanol fractions of *H. pubescens* seed extract, respectively, used at the concentration of 100 µg/mL.

Table 2. Antioxidant effect of the fractions (HPF, HCF, and HMF) of *Holarrhena pubescens* seed extract in DPPH free radical scavenging assay

Concentration (µg/mL)	Percentage of scavenging of DPPH free radical (%)			
	Standard drug	HPF	HCF	HMF
10	25.36 ± 0.13	4.10 ± 0.25	9.63 ± 0.32	7.88 ± 0.16
20	40.68 ± 0.42	6.42 ± 0.31	20.5 ± 0.11	12.00 ± 0.07
30	91.29 ± 0.09	12.04 ± 0.46	39.17 ± 0.14	28.68 ± 0.41
40	94.41 ± 0.26	23.73 ± 0.11	63.44 ± 0.28	39.16 ± 0.12
50	98.22 ± 038	42.21 ± 0.36	80.35 ± 0.46	61.09 ± 0.30

Data are presented as mean ± SEM by carrying out experiments in triplicate. HPF, HCF, and HMF: Petroleum ether, chloroform, and methanol fractions of *H. pubescens* seed extract, respectively. Standard drug: Ascorbic acid.

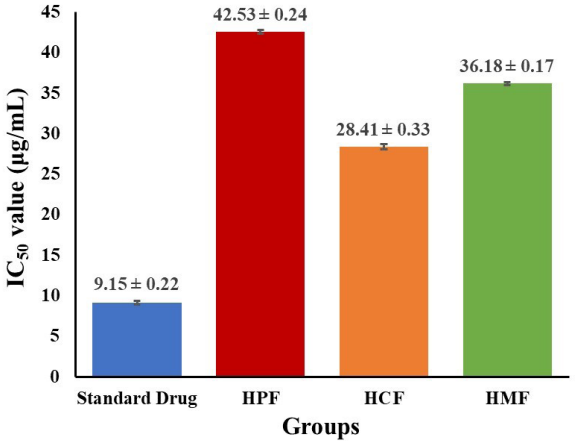

Figure 1. The IC_{50} value (µg/mL) of antioxidant effect of the fractions of *Holarrhena pubescens* seed extract in DPPH free radical scavenging assay. HPF, HCF, and HMF: Petroleum ether, chloroform, and methanol fractions of *H. pubescens* seed extract, respectively. Standard drug: Ascorbic acid. The linear regression was used to analyze the IC_{50} values and presented here as mean ± SEM by carrying out experiments in triplicate.

bovine serum albumin denaturation. As shown in Table 3, the result of the study showed the protein denaturation inhibition potential of HCF. The highest potential of the test drug was recorded at the concentration of 50 µg/mL (68.37 ± 1.11%). The inhibitory potential of drugs was seen to be directly proportionate to the concentration of samples used during the study. The standard drug (diclofenac sodium) showed a potent anti-inflammatory effect (76.22 ± 0.48%) at the same 50 µg/mL concentration. The IC_{50} values of both drugs were calculated, which indicated their anti-inflammatory potential.

Antiarthritic effect in a chronic inflammation study of FCA-induced arthritis in rats

The potential of chloroform fraction (HCF) of the plant material in chronic inflammation was evaluated through an *in vivo* arthritis experiment, which was induced in rats by injecting FCA. During the study, HCF was administered in two oral doses of 200 mg/kg and 400 mg/kg based on the body weight of the animals. The results of the present study (Table 4) showed the dose-dependent anti-inflammatory response of the test drug. The oral dose of 400 mg/kg of HCF showed a statistically significant ($P < 0.05$) response in rat paw edema inhibition, with a value of 0.38 ± 0.16 mL on the 14th day of the study. Whereas, 200 mg/kg oral dose of HCF showed a statistically non-significant effect in inhibiting rat paw edema, with a value of 0.81 ± 0.22 mL. The statically significant result of inhibitory effect on paw edema was shown by diclofenac sodium (standard drug) with an oral dose of 10 mg/kg on the 7th day (0.49 ± 0.24 mL, $P < 0.05$) and on the 14th day (0.19 ± 0.03 mL, $P < 0.01$) of the study. The inhibitory effect of the oral dose of 400 mg/kg of HCF was statistically non-significant on the 7th day of the study.

Discussion

In the present study, the seed of the plant material was used to evaluate antimicrobial, antioxidant, and anti-inflammatory activity study by *in vitro* method and chronic inflammation study by inducing arthritis in rats' model.

Microbial contamination is always a reason for the

Table 3. Anti-inflammatory effect of chloroform fraction (HCF) of *Holarrhena pubescens* seed extract in the BSA denaturation inhibition assay

Concentration (µg/mL)	Percentage inhibition of BSA denaturation (%)	
	Standard drug	HCF
10	23.12 ± 0.82	21.57 ± 0.15
20	33.31 ± 1.06	32.59 ± 0.43
30	46.24 ± 0.63	44.79 ± 0.70
40	62.14 ± 0.52	57.26 ± 0.93
50	76.22 ± 0.48	68.37 ± 1.11

Data are presented as mean ± SEM by carrying out experiments in triplicate. BSA: Bovine serum albumin. Standard drug: Diclofenac sodium.

Table 4. Antiarthritic effect of chloroform fraction (HCF) of *Holarrhena pubescens* seed extract in a study of Freund's complete adjuvant-induced arthritis in rats

Groups	Oral dose (mg/kg)	Increase in paw volume (mL)		
		Day 0	Day 7	Day 14
Control	-	0.47 ± 0.13	0.97 ± 0.5	1.53 ± 0.28
Diclofenac	10	0.43 ± 0.17	0.49 ± 0.24*	0.19 ± 0.03**
HCF 200	200	0.53 ± 0.15	0.76 ± 0.10	0.81 ± 0.22
HCF 400	400	0.44 ± 0.31	0.63 ± 0.19	0.38 ± 0.16*

The changes in rat paw thickness were calculated by plethysmometer and expressed as mean ± SEM (n = 6). The statical significance of the values was calculated by One-way ANOVA followed by Dunnett's t-test. The values in the table marked with * ($P < 0.05$), and ** ($P < 0.01$) were considered as statistically significant when values were compared with the control group.

development of various infections or other diseases. The involvement of microorganisms during any inflammation usually causes a chronic condition. There are several microorganisms present in nature, like, viruses, fungi, bacteria, and protozoa (26). Antimicrobial agents are generally found to be effective to any of these species of microorganisms, where plants have shown broad-spectrum effects on various species of microbes (27). Based on this concept, in the present study, different fractions (HPF, HCF, and HMF) of ethanolic extract of seed of *H. pubescens* were used to evaluate their antimicrobial properties against gram-positive and gram-negative bacteria. The results of the study proved the broad-spectrum antibacterial effect of the test drugs at 100 µg/mL. The study further proved that the chloroform fraction (HCF) of the plant material was the most potent among the test drugs. Hence, the study suggested the antimicrobial properties of *H. pubescens*.

Free radicals, like reactive oxygen species are produced during biological processes and they help in the different defense mechanisms of the body. Nevertheless, these natural conditions can be altered due to the excess production of free radicals during any microbial infection or chronic disease state (28). The oxidative stress condition can affect the body reversely and may lead to the development of various diseases (29). An antioxidant can help to control such conditions by removing excess free radicals. In the present study, the antioxidant potential of test drugs (HPF, HCF, and HMF) was evaluated using an *in vitro* study of the DPPH free radical scavenging method. The DPPH reagent produces free radicals in prepared test samples, and the antioxidant effect of test drugs can be measured by evaluating their potential to scavenge those free radicals. The results of the study suggested the potent antioxidant potential of HCF with a 50 µg/mL concentration (80.35 ± 0.46% and IC_{50} value 28.41 ± 0.33 µg/mL). Protein denaturation is a common phenomenon observed during inflammation. The anti-inflammatory agents show their potential to inhibit that denaturation process (30). This concept was used in the present study to evaluate the anti-inflammatory activity of a test drug (HCF). The chloroform fraction (HCF) of the plant material showed the most potent effect in antimicrobial and antioxidant studies. Therefore, only the chloroform fraction (HCF) among all test drugs was further subjected to anti-inflammatory activity studies. In the study, the anti-inflammatory effect of HCF was evaluated based on its potential to inhibit the denaturation of bovine serum protein (albumin). The result showed the percentage inhibition with 68.37 ± 1.11% for HCF at 50 µg/mL concentration during the study. The IC_{50} value of the test drug (27.41 ± 1.16 µg/mL) and standard drug (18.14 ± 1.74 µg/mL) further suggested their potentials.

The *in vitro* studies can provide a preliminary understanding of the potential of a drug. These studies demonstrate the effect of drugs on damaged cells or physiological molecules (31). However, the physiological response of the human body during disease involves various cellular and biochemical mechanisms. The physiological environment cannot be fully replicated in the *in vitro* experiments (32). Moreover, the potential of a drug in treating any disease depends on the systemic factors of the human body, such as absorption, metabolism, and distribution. The influence of the intrinsic physiological factors also cannot be observed in *in vitro* studies. Unlike *in vitro* methods, *in vivo* studies can mimic the human physiological characteristics in experimental animals, which also play an important role in understanding the mechanism of action of a drug (33). Hence, the activity of a drug observed in an *in vitro* study needs to be confirmed by an *in vivo* study to evaluate its effect under the complex physiological conditions of an animal body (34).

In the present research, the findings from the *in vitro* study on the anti-inflammatory activity of the test drug (HCF) were further corroborated by an *in vivo* study using FCA-induced arthritis model. The adjuvant induces polyarthritis as a chronic inflammation in animals within 14 days (35). Hence, the study assessed the anti-inflammatory potential of HCF in chronic inflammatory conditions. Even though inflammation is a fundamental protective response of the body, it is often observed to be a causative factor for several associated chronic diseases. Arthritis is one of the chronic inflammatory diseases, and it is observed that many people throughout the world are suffering from this disease which makes it a significant concern for the healthcare system (36). In this context, the effect of the test drug on chronic inflammatory conditions was evaluated in this research work. HCF (test drug) with the oral dose of 400 mg/kg inhibited the chronic inflammation (75.16%) in rats on day 14^{th}, while on the same day, diclofenac sodium (standard drug) showed a significant ($P < 0.01$) 87.58% of inhibition of inflammation. Hence, the findings of the experiments support and provide insight into the previously reported effects of *H. pubescens* seeds, such as its anthelmintic, antidiarrheal, antidiabetic, and antinociceptive properties (17,19,37-39). Additionally, the significant potential of chloroform fraction of the plant material to counter oxidative stress indicates its therapeutic potential against microorganisms and treating inflammation. Nevertheless, more studies need to be conducted to comprehend the mechanisms behind the pharmacological activities of the test drug.

Conclusion

The present research work proved the effects of *H. pubescens* seed on microorganisms, free radicals scavenging, and inflammation treatment using experimental models. Also, the chloroform fraction of this plant material (HCF) exhibited the most significant effect among all fractions in the pharmacological activities mentioned. The effect of the test drug (HCF) to inhibit chronic inflammation was also observed in an

in vivo study. Overall, the results of this research may support the rationale for ethnopharmacological uses of this plant drug. In addition, this work suggests that the antimicrobial and antioxidant properties of the plant drug might enhance its anti-inflammatory effect, which could make it useful in treating various chronic diseases. Hence, it is concluded that the study validated the hypothesis and objectives of the present work. However, further research is recommended to identify the therapeutically potent phytocompounds in this chloroform fraction to better understand its bioactivity.

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Authors' contribution

Conceptualization: Santanu Saha, EVS Subrahmanyam.
Data curation: Santanu Saha.
Formal analysis: Santanu Saha.
Investigation: Santanu Saha.
Methodology: Santanu Saha.
Software: Santanu Saha.
Writing–original draft: Santanu Saha, EVS Subrahmanyam.
Writing–review & editing: Santanu Saha, EVS Subrahmanyam.

Conflict of interests

The authors declare that there is no conflict of interest.

Ethical considerations

The animal experiment in the present study was conducted following the guidelines of the institutional animal ethics committee (KSHEMA /AEC/077/2008).

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