



In vitro antidiabetic and anti-inflammatory activities of the bark of *Ehretia acuminata* R.Br.

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Ehretia acuminata is native to Southeast Asia, America, and Africa. It is commonly used as fodder and in quite a variety of medicinal applications. *E. acuminata* is widely used in India, but there are few reports in the literature of studies on its biological properties. In this study, the antidiabetic and anti-inflammatory activities of plant *E. acuminata* was evaluated. The antidiabetic activity of the different extracts was measured by α -amylase and α -glycosidase inhibition method. The anti-inflammatory activity was evaluated by the egg albumin of hen. Petroleum ether, chloroform, ethyl acetate, ethanol and water extracts of bark of *E. acuminata* was evaluated for *in-vitro* anti-inflammatory and antidiabetic activity. Ethyl acetate and ethanol extract showed significant anti-inflammatory effects (IC_{50} 170 and 172 μ g/mL respectively). Chloroform is the only extract that showed significant inhibition against the antidiabetic effect (IC_{50} 42-45 μ g/mL). The findings suggest that the bark of *E. acuminata* contains potential antidiabetic and anti-inflammatory compounds, which could be tested as drug candidates against diabetic and inflammation-related pathological processes in medicinal chemistry studies.

Keywords: Antidiabetic, Anti-inflammatory, *Ehretia acuminata* R.Br., α -Amylase, α -Glucosidase.

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Introduction

The use of secondary metabolites present in plants represents a large source of traditional medicine that could be serving as leads for the development of novel drugs. Several anti-inflammatory, digestive, antinecrotic, neuroprotective, and hepatoprotective drugs have recently been shown to have radical scavenging mechanism as part of their activity¹. Diabetes mellitus is a metabolic disease, characterized by hyperglycemia together with impaired metabolism of glucose and other energy-yielding fuels, such as lipids and proteins². This metabolic disorder is the result of a deficiency in insulin secretion or a resistance to insulin action or both³. More than 220 million people worldwide have diabetes and by the year 2030, this number might be more than double⁴. Diabetic patients also exhibit oxidative stress, which leads to lipid peroxidation and tissue damage including retinopathy, nephropathy, and coronary heart disease^{5,6}. Dyslipidemia or hyperlipidemia is also involved in the development of cardiovascular complications, which are a major cause of morbidity and mortality⁷.

Inflammation and oxidative stress have an important role in various diseases. Inflammation is an immunological defence mechanism elicited in response to mechanical injuries, burns, microbial infections, allergens and other noxious stimulus⁸. The use of anti-inflammatory agents may therefore be helpful in the treatment of inflammatory disorders⁹. Steroidal or non-steroidal anti-inflammatory drugs are usually used to treat different inflammatory diseases. The contrary effects of the presently usable anti-inflammatory drugs however pose a major problem in their clinical use, therefore naturally originated agents with very minor side effects are desirable to replace chemical therapeutics¹⁰. There has been a rising interest in phenolic components of natural resources like vegetables and fruits, which may promote human health or decrease the risk of disease. Recent studies have focused on the health functions of phenolics including flavonoids from fruit and vegetables¹¹. In search of sources of natural antioxidants, some medicinal plants have been extensively studied for their antioxidant activity and radical scavenging activity^{12,13}. Regenerate attention to alternate medicines and natural treatment has raised researchers' interest in traditional herbal medicine.

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Because of their perceived effectiveness, with minimal side effects in clinical experience and relatively low costs, herbal drugs are prescribed widely, even when the contents of the biologically active constituents are unknown.

Ehretia acuminata is a wild medicinal plant found in Asia, America, Australia, and Africa. Many traditional claims are reported as medical treatments for various diseases like some kind of fever, dysentery and other health ailments¹⁴. The bark of *E. acuminata* is useful in sores on the tongue, ripe fruit is edible and unripe fruit is used as a pickle by tribal communities.

Ethno-botanical surveys on genus *Ehretia* also suggest snake antivenom activity and anti-tubercular activity of different parts of different species^{15,16}, antioxidant and antiarthritic activity¹⁷. An antitrypanosomal¹⁸, antiprotozoal activity¹⁹, and anti-inflammatory of leaves and root extract of *Ehretia*^{20,21} were also reported. Such activities can be due to the presence of phytochemicals alkaloids^{22,23}, flavonoids²⁴⁻²⁶, and fatty acids²⁷.

E. acuminata is a tree that belongs to the family Boraginaceae. Previous literature provides meagre information about therapeutic uses of this plant²⁸. Branchlets of *E. acuminata* are used traditionally in Chinese herbal medicines. Antioxidant activities are attributed to the fact that the bark of *E. acuminata* contain a variety of secondary metabolites, such as flavones, sterols, several hydrocarbons, anthraquinones, glycosides, etc. Among these flavones, glycosides, and sterols are considered to be useful as anti-inflammatory and antidiabetic agents.

Materials and Methods

Plant materials

Bark material of plant *E. acuminata* was collected from Pantnagar (in the daytime, at temperature 26–27.9 °C), Udham Singh Nagar district, Uttarakhand in Kumaun region of India in the month of April 2017 and authenticated from Botanical Survey of India (BSI) Dehradun (Voucher specimen number 117138 05/2017). Bark was dried for 10–20 days under shade until the bark material was ready for grinding and stored at room temperature. It was ground in a laboratory grinder and stored at 5 °C.

Chemicals

Nitrophenyl- α -D-glucopyranoside (SRL Pvt., Ltd), tris buffer (Merck), α -amylase ex porcine pancreas (SRL Pvt., Ltd), dimethyl superoxide (DMSO) (Merck), 3,5-dinitrosalicylic acid (DNSA) (SRL Pvt.,

Ltd), α -glucosidase for biochemistry ex microorganism (SRL Pvt Ltd); and acarbose (Bayer India Limited), diclofenac sodium, sodium carbonate (CDH) were purchased. All other chemicals, solvents and reagents used were of analytical or HPLC grade.

Preparation of crude plant extract

Collection and extraction of plant material were done by the method used by the authors earlier²⁹. Bark material of *E. acuminata* was collected from the Kumaun region Uttarakhand. Bark was dried under shade and powdered using a grinder. The extraction was done by soxhlet method at room temperature by taking 100 g of dried material. It was soaked in different solvents (600 mL) according to increasing order of polarity {petroleum ether (60-80 °C), chloroform, ethyl acetate, ethanol, water} for 6-8 days with stirring every 20 h by using a sterilized glass rod. The final extracts were filtered through Whatman filter paper No.1. The filtrates obtained were concentrated under vacuum on a rotary evaporator at 42 °C and stored at low temperature for further experiment.

Phytochemical screening

Phytochemical screening of solvent extracts of *E. acuminata* bark was done to test for the presence of alkaloids, glycosides, flavonoids, carbohydrate, tannins, terpenoids, anthraquinones, fat and oil³⁰.

Evaluation of *in vitro* antidiabetic activity

Inhibition assay of α -amylase enzyme

The enzymes inhibition procedure of α -amylase was according to the method of Kazeem *et al.* with some modification³¹. The α -amylase activity can be measured by the determination of the reducing group arising from hydrolysis of soluble starch by isolated pancreatic α -amylase. Reduction of 3,5- dinitrosalicylic acid to nitroaminosalicylic acid produces a colour shift which is followed photometrically by a change in the absorbance at 540 nm. Inhibition of starch hydrolysis by the α -amylase inhibitor results in a diminished absorbance at 540 nm in the comparison with control. Acarbose (AC) was used as a standard drug in α -amylase antidiabetic assay. The percentage (%) of inhibition for α -amylase was also expressed as the half-maximal inhibitory concentration (IC₅₀). The formula for % of inhibition is as given below:

$$\% \text{ of inhibition} = \frac{\text{Absorbance of control} - (\text{Absorbance of extract})}{\text{Absorbance of control}} \times 100$$

Inhibition assay of α -glucosidase enzyme

The enzymes inhibition procedure of α -glucosidase was according to the method of Kim *et al.* with slight modification³². The activity is based on the fact that when the enzyme α -glucosidase is incubated with the substrate *p*- nitrophenyl- α -D-glucopyranoside (p-NPG), it hydrolyses the substrate to *p*- nitrophenol and D- glucose respectively. Acarbose (AC) was used as a standard drug for α -glucosidase inhibition assay. The absorbance of the released *p*-nitrophenol was measured at 410 nm. Each experiment was performed in triplicates. The percentage (%) of inhibition for all the enzymes except for α -glucosidase was also expressed as the half-maximal inhibitory concentration (IC₅₀). The formula for % of inhibition is as given above.

Evaluation of *in vitro* anti-inflammatory activity

Inhibition by albumin method

The ability of plant extract against protein denaturation was determined according to the method described by Chandra *et al.* with some modification³³. The reaction mixture contained 2 mL of varying concentrations of different extract, 0.2 mL of egg albumin (fresh hen's egg) and 2.8 mL of phosphate-buffered saline (pH 6.4) and the final concentrations are 25, 50, 100, 250, and 500 μ g/mL. Then the reaction mixtures were incubated at room temperature in the BOD incubator for 15 min and then, heated at 72 °C for 5 min. Many kinds of biological substances are observed in visible light (400-700 nm) in spectrophotometry. Due to the appearance of viscosity in the tested samples, absorbance was taken at the high visible range 660 nm (Systronic 118, UV-VIS) by using a blank and their viscosity was determined by using Ostwald viscometer. Sodium Diclofenac was used as the reference drug and treated similarly as plant extracts for determination of absorbance. The following

formula was used to calculate the percentage inhibition of protein denaturation:

$$\% \text{ inhibition} = 100 \times \frac{V_t (\text{absorbance of test sample})}{V_c (\text{absorbance of control})} - 1$$

Statistical analyses

All the experiments were done in triplicates and the results were expressed as Mean \pm SD. The data were statistically analyzed using one way ANOVA followed by Duncan's test. Mean values were considered statistically significant when $P > 0.05$.

Results and Discussion

Extracts of *E. acuminata* bark in different solvents gave different colours and different yields. The lowest yield was found in chloroform (0.467%) and the highest yield was found in water (2.96%). Table 1 gives the total yield and colour of all extracts.

The qualitative phytochemical screening of all the extracts of *E. acuminata* revealed the presence of alkaloids, terpenoids, and glycosides in chloroform extract and tannins, saponins, flavanoids in polar solvents ethyl acetate and ethanol extract.

The *in vitro* α -amylase inhibitory activity of *E. acuminata* bark extracts compared with acarbose (Table 1 & Fig. 1) represents the alpha-amylase

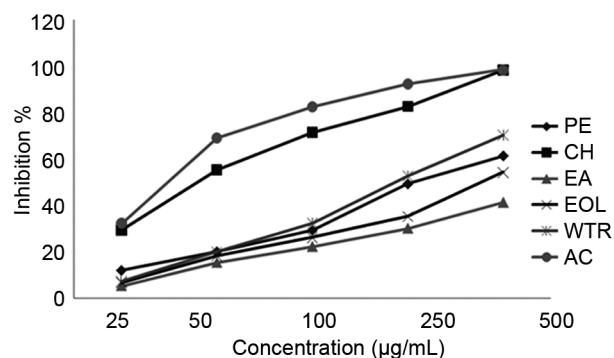


Fig. 1 — Correlation between extract concentration and percentage of α -amylase enzyme inhibition.

Table 1 — Yield, colour of extracts and IC₅₀ of antidiabetic & anti-inflammatory activity of *Ehretia acuminata* bark

Plant extract	Bark extraction		Results of α -amylase antidiabetic activity	Results of α -glucosidase antidiabetic activity	Results of anti-inflammatory activity by egg albumin	
	yield %	Colour	IC ₅₀ (μ g/mL)	IC ₅₀ (μ g/mL)	IC ₅₀ (μ g/mL)	Viscosity (cp)
Petroleum ether	0.508	Light yellow	270	270	680	0.75
Chloroform	0.467	Pale yellow	45.35	42.90	1150	0.71
Ethyl acetate	1.785	Yellow	580	550	170	0.88
Ethanol	2.735	Brown	475	480	180	0.80
Water	2.96	Dark brown	242.90	250.80	185	0.79
Acarbose			40.25	38.45		
Sodium diclofenac					120	0.84

inhibition on changing the concentration of each extract and helps in the estimation of IC_{50} value of each extract as well as standard acarbose. The IC_{50} value is the concentration of each extract or standard drug which is required to inhibit 50 per cent of the enzyme in reaction. Acarbose showed percentage α -amylase inhibition of 32.24-98.45% on varying concentration from 25-500 $\mu\text{g/mL}$ with an IC_{50} value 40.25 $\mu\text{g/mL}$. A lower IC_{50} value corresponds to greater potency and better therapeutic efficacy. Chloroform extract reflects the highest α -amylase inhibitory activity ($IC_{50} = 45.35 \mu\text{g/mL}$) followed by water extract ($IC_{50} = 242.90 \mu\text{g/mL}$). The IC_{50} value of chloroform extract is nearly comparable with acarbose and thus can be regarded as an excellent α -amylase inhibitor. The α -amylase inhibitory activity shown by all extracts might be due to various phytoconstituents present in each extract. Furthermore, the potent activity of chloroform extract must be attributed to its high potential and the majority of phytoconstituents present which are responsible for antidiabetic effect. The available literature depicts that alkaloids, terpenoids, and glycosides are known to show α -amylase inhibitory activity³⁴.

The *in vitro* α -glucosidase inhibitory activity of *E. acuminata* bark extracts compared with acarbose (Table 1 & Fig. 2) represents the α -glucosidase inhibition on changing the concentration of each extract and helps in the estimation of IC_{50} value of each extract as well as standard acarbose. Again the highest α -glucosidase inhibitory activity was demonstrated by chloroform extract (IC_{50} 42.90 $\mu\text{g/mL}$) followed by aqueous extract (IC_{50} 250.80 $\mu\text{g/mL}$). The IC_{50} value of chloroform extract is even close to acarbose (IC_{50} 38.45 $\mu\text{g/mL}$) indicating its

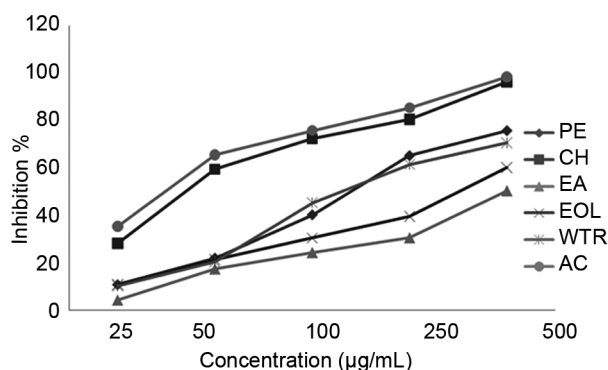


Fig. 2 — Correlation between extract concentration and percentage of α -glucosidase enzyme inhibition

extremely potent nature. Previous literature evidenced alkaloids and terpenoids as potent α -glucosidase inhibitors and these phytoconstituents could be well cited in chloroform extract. These active extracts could be used as antidiabetic agents in comparison to synthetic drugs with numerous side effects like abdominal discomfort, bloating, flatulence, and diarrhoea³⁵.

Polar solvents have been proved as effective solvents to extract phenolic compounds. Considering the ethyl acetate and ethanol extracts, ethanol extract is more polar than ethyl acetate. Both extracts are also a good source for obtaining polyphenolic compounds and are safe for human consumption. Anti-inflammatory potential of *E. acuminata* may be due to the extractability of some bioactive compounds in ethyl acetate and ethanol extracts. Previous work of the authors on total phenolic content revealed that *E. acuminata* bark can scavenge free radicals and that the ethyl acetate and ethanol extracts showed good antioxidant activity²⁸. Therefore, the different extracts were selected for further *in vitro* anti-inflammatory studies. In the present investigation, the *in vitro* anti-inflammatory effect of *E. acuminata* was evaluated against the denaturation of egg albumin. Results are summarized in Table 1. This study shows a concentration-dependent inhibition of protein (albumin) denaturation by *E. acuminata* throughout the concentration range of 25 to 500 $\mu\text{g/mL}$. Diclofenac sodium (25 to 500 $\mu\text{g/mL}$) which is used as the standard drug, also exhibited concentration-dependent inhibition of protein denaturation. However, diclofenac sodium was highly effective when compared with *E. acuminata* and more polar solvent extracts ethyl acetate and ethanol show high inhibition as comparison to other solvents. This is confirmed by comparing their IC_{50} values in Table 1 and inhibition in Fig. 3. From antioxidant and anti-inflammatory activities of *E. acuminata* bark, it is proved that extracts have a high content of phenolic and flavonoid compounds.

Antidiabetic activity of *E. acuminata* was based on two biochemical assays conducted *in vitro*. There could be some problems in using animals in experimental *in vivo* research, such as ethical issues and the lack of rationale for their use when other suitable methods are available. The maltose standard curve for α -amylase inhibitory assay was plotted using various concentrations of maltose. The extracts were tested at different concentrations for inhibition of α -amylase. In

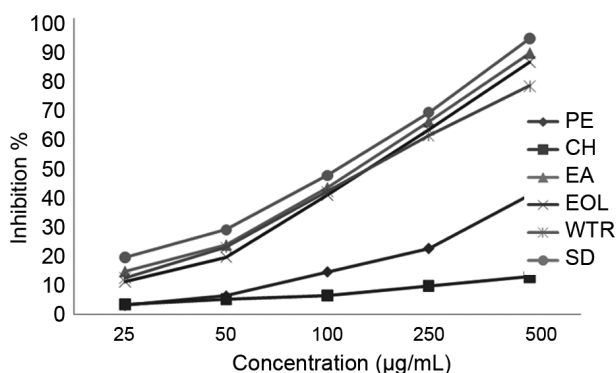


Fig. 3 — Correlation between extract concentration and percentage of protein inhibition.

this assay, the positive control, acarbose gave the IC_{50} value as 40.25 µg/mL. In the α -amylase method, the inhibition effect by phytoconstituents responsible for antidiabetic potential of each extract based on the change in colour intensity was monitored at 540 nm. IC_{50} values obtained for chloroform extract was lower than the other extracts. Antidiabetic potential by α -glucosidase was measured with the help of *P*-nitrophenyl- α -D-glucopyranoside (NPG), tris buffer and 50% inhibition of chloroform extract was reached at a concentration much less than ethyl acetate extract.

E. acuminata was also evaluated for anti-inflammatory activity using the egg albumin method and the protein denaturation bioassay was selected for the *in vitro* assessment. Reason for protein denaturation are diseases like inflammatory and arthritic. The formation of antibodies in some arthritic diseases may be due to the denaturation of proteins³⁶. Factors that can forbid protein denaturation therefore would be favourable for anti-inflammatory drug development.

The increments in absorbances of test samples with respect to control, indicated stabilization of protein i.e., inhibition of heat-induced protein (albumin) denaturation by different extracts and reference drug sodium diclofenac. From the IC_{50} values, it becomes evident that ethyl acetate extract was most active after sodium diclofenac, being effective in lower concentrations. Changes in viscosities also indicate denaturation. It is documented that the viscosities of protein increase on denaturation³⁷. The presence of different extracts of the plant depicts the inhibition of protein denaturation. Here, the viscosities decreased when compared with blank where no test extract/drug was added. However, the viscosities were found to decrease with a co-occurrence decrease in the concentration of plant extract and standard drug as well. Viscosities will decrease with the decrease in the

concentration of plant extracts and standard drug in reaction. It is reported that at pH 6.2-6.5, many non-steroidal anti-inflammatory drugs can stabilize (forbid denaturation) heat-treated egg albumin³⁸. Hence, from this experiment, it can be concluded that *E. acuminata* bark showed a significant *in vitro* anti-inflammatory effect against the denaturation of protein.

Conclusion

The present study revealed that different types of extracts had a big influence on the antidiabetic and anti-inflammatory properties. The results showed that chloroform extract of *E. acuminata* could be a natural source of antidiabetic and ethyl acetate and ethanol extracts could have greater importance as therapeutic agents in preventing or slowing oxidative stress and inflammation-related disorders. Further studies are currently underway to assess the *in vivo* biological activities and to identify the active component responsible for their antidiabetic and anti-inflammatory properties.

Conflict of interest

The authors declare no conflict of interest.

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