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Research Article

Antidiabetic and antioxidant potential of *Zanthoxylum armatum* DC. leaves (Rutaceae): An endangered medicinal plant

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Abstract

The present study was designed to evaluate the antidiabetic and antioxidant potential of methanolic extract of *Zanthoxylum armatum* leaves using *in vitro* approaches. The concentration of plant extract that inhibited 50% (IC_{50}) of alpha amylase was found to be 89.37±4.68 µg/ml which is higher than standard. Results of this study shows that 2,2-diphenyl-1-picrylhydrazyl scavenging test show high radical scavenging activity as compared to hydrogen peroxide scanvenging method with IC_{50} Value of 57.83 µg/ml and 79.13 µg/ml, respectively. Plant extract found to exhibit enormous amount of phenols and flavonoid content i.e., 140.71 mg GAE/g and 88.53 mg of Quercetin/g of extract respectively. Further phytochemical analysis revealed that plant exhibit glycosides, alkaloids, terpenoids, flavonoids, saponin and tannin that are frequently implicated as having antidiabetic effects. Elemental analysis revealed the presence of essential elements 'Mg', 'Mn', 'Zn', 'Fe', 'K', 'P', 'Ca', 'Cu', 'Mo' and 'Ni' known to play role in regulating blood glucose. It could be speculated that the observed antidiabetic activity of *Z. armatum* might be related to the presence of these phytochemicals, phenolic compounds as well as mineral elements which found to be the important constituent of *Z. armatum.* These results indicate that *Z. armatum* could be an excellent source of natural antioxidants and exhibited antidiabetic activity.

Keywords: *Zanthoxylum armatum*; antidiabetic; DPPH; α-amylase; mineral; antioxidant.

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Introduction

Plants are the richest source of drugs and have been used since ages for their therapeutic value. A large number of new pharmacologically active agents have been explored from plants which are the valuable source of potent and powerful drugs (1). One of the great advantages of medicinal plants is that these are readily available, more effective and have low or no side effects. So, the pharmaceutical industries are directly or indirectly dependent upon the plant material. Among the large diversity of plants, only a small number of plants known for their medicinal purposes and

there is indeed the need for exploration and investigation of plants for pharmacological purposes. Many of them play an important role in the management of diabetes mellitus especially in developing countries where resources are meagre. Diabetes mellitus (DM) is a serious health problem, and very prevalent disease affecting the people of both developed and developing countries. At least 30 million people throughout the world suffer from it and its being the third greatest cause of death (2). DM is associated with oxidative stress, leading to an increased production of reactive oxygen species in the body (3). Plants not only contain the metabolites but show an effective antioxidant property wherein these molecules are capable of preventing the oxidation of free radicals (4). Recently, some medicinal plants have been reported to be useful in diabetes and have been used empirically as antidiabetic and antihyperlipidemic remedies. More than 400 plant species having hypoglycemic activity have been available in literature (5). However, exploration for new antidiabetic drugs from natural origin is still attractive and considered valuable because they contain substances which take alternative and safe effect on DM.

Zanthoxylum armatum DC. of family Rutaceae is an endangered medicinal plant of the Himalayan region. It is a large spiny shrub or small tree found in the temperate Himalayas at an altitude of 1000-2000 meters and commonly known as 'timur' or 'Timru'. It is also known as important magical plant because every part of this plant *viz*. stem, fruits, leaves, bark, and seeds possess medicinal properties and are extensively used in indigenous medicine as a flatulence, stomachic and anthelmintic (6). It is used as antipyretic and anti-diarrheal agent also increases saliva secretion and improves speaking power (7). Seeds and fruits of this plant are used in skin diseases, fever and dyspepsia (8). Due to its tremendous medicinal potential and therefore unsustainable harvest from the wild from several decades has caused severe threat to *Z. armatum* populations, resulting in its listing as 'endangered' in the Indian Himalayan region (9). Therefore characterization of genetic diversity for this plant is needed for mounting the conservation approaches. Hence, there is an imperative necessity to advance reproductive propagation protocol for multiplication and conservation of this plant. Less than 1% out of a total 250000 higher plants has been screened pharmacologically and very few in regard to DM. Hence, an antidiabetic drug with antioxidant potential of herbal origin is therefore needed for treatment of diabetes and its associated diseases. Therefore, it is necessary to look for various other options in herbal medicine for diabetes as well. The present study was undertaken to analyse the active phytochemicals, antidiabetic and antioxidant potential as well as estimation of total phenolic and flavonoid content in the leaves of this plant.

Materials and Methods

Plant collection and identification

Plant was collected in the month of August from Solan which is located at 30.92º North and 77.12º East with an average elevation of 1502 metre. Himachal Pradesh and identified by comparing with authenticated herbarium specimens deposited in Panjab University Chandigarh (PAN).

Extract preparation

Leaves of *Zanthoxylum armatum* was washed thoroughly with running tap water then shade dried for 2-3 days and grounded into course powder. 15 g of powder then subjected to extraction with 30 ml of methanol solvent for 24 hrs in the orbital shaker. Later extract was filtered through Whatman paper and the filtrate was allowed to evaporate at room temperature until a very concentrated extract was obtained. The residue left was used as plant extract.

Qualitative analysis

Qualitative phytochemical analysis was carried out to identify phytochemical constituents of the plant in various extracts *viz*., aqueous, methanol, ethanol and chloroform depending on their solubility. Following phytochemicals were tested Alkaloids (10), Flavonoids (11), Glycosides (12), Terpenoids (12), Steroids (12), Tannins (11) and Saponins (11).

In vitro antidiabetic activity

Study of *in vitro* antidiabetic activity was checked by Alpha-amylase inhibition assay by the method modified from Sigma-Aldrich (13). Extract of various concentration ranging from 50-250 µg were prepared in DMSO. 25 ml of 1% (w/v) starch solution was prepared with pH 6.9 in phosphate buffer and solubilisation of starch solution was done by heating for 15 min. with constant stirring, solution was allowed to cool down at room temperature and then it was brought to original volume 25 ml by addition of water. 0.001 g of alpha-amylase was prepared in 100 ml of sodium phosphate buffer (20 mM) with pH 6.9 containing 6.7 mM sodium chloride. The color reagent was prepared by mixing 20 ml 3,5- dinitrosalicylic acid (96 mM), 8 ml (5.31 M) sodium potassium tartrate in 2 M sodium hydroxide and then color reagent was diluted to 40 ml by adding 12 ml deionized water. Absorbance was observed at 540 nm using acarbose solution as positive control. Milligram of maltose liberated was calculated by using the standard curve of maltose and alpha-amylase inhibition % was calculated according to formula.

$$\%\, inhibition = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} x\ 100$$

In vitro antioxidant activity

DPPH radical scavenging assay

The free radical scavenging activity was determined by DPPH (Diphenyl- 2-picrylhydrazyl) assay (14). The ability to scavenge DPPH radical was calculated as percentage inhibition. IC_{50} represents the level where 50% of the radicals were scavenged by test samples.

The % inhibition was calculated by the formula:

$$\%\ inhibition = \frac{Control - test}{Control} \times 100$$

Hydrogen peroxide radical activity

The ability of the extract to scavenge the hydrogen peroxide was determined by the standard method (15). Methanol was taken as blank and ascorbic acid served as standard. IC_{50} represents the level where 50% of the radicals were scavenged by test samples. The % of hydrogen peroxide scavenged by the plant extract and the standard was calculated by the formula:

$$\%\ \text{Scavenged } (H_2O_2) = [(AO - A1)/AO] \times 100$$

Where AO was the absorbance of the control and A1 was the absorbance of the test.

Determination of total phenolic content (TPC)

Total phenolic content (TPC) of the plant extract was determined by using the Folin-Ciocalteu reagent (16).

Determination of total flavonoids content (TFC)

TFC of the plant extract was determined by using $AlCl_3$ colorimetric assay as proposed by (17).

Elemental analysis

Analysis of various micro and macro elements such as Magnesium (Mg), Iron (Fe), Chromium (Cr), Zinc (Zn), Copper (Cu), Molybdenum (Mo), Manganese (Mn), Potassium (K), Calcium (Ca), Strontium (Sr), Phosphorus (P), Selenium (Se), Nickel (Ni), Bromine (Br) and Sulphur (S) of *Z. armatum* were done by using WDXRF (Wavelength Dispersive X-Ray Fluorescence) Spectrometer (Burker, S8 TIGER). This test was performed at SAIF, CIL, Panjab University, Chandigarh.

Statistical analysis

All determination were replicated three times and the results expressed as mean ± SD.

Results

Qualitative analysis

Phytochemical investigations are the tools which evaluate the active components in the plants that can be further explored in the production of useful plant based medicines. Results revealed that all the phytochemicals tested were found in aqueous, methanolic and ethanolic extract except for chloroform where only alkaloids, glycosides and terpenoids were detected which may be due to their non-polar nature. Because of their prominence in pharmaceuticals industry these phytochemical were selected for study (Table 1).

Table 1. Preliminary qualitative analysis of phytochemical in various leaves extracts of *Zanthoxylum armatum*

Sr. No.	Phytochemical tests	Aqueous extract	Methanol extract	Ethanol extract	Chloroform extract
1.	Alkaloids	+	+	+	+
2.	Flavonoids	+	+	+	-
3.	Glycosides	+	+	+	+
4.	Terpenoids	+	+	+	+
5.	Steroids	+	+	+	-
6.	Tannins	+	+	+	-
7.	Saponin	+	+	+	-

*(+) indicate presence, (-) indicate absent

In vitro antidiabetic activity

Alpha-amylase inhibition activity

Methanolic extract of leaves of *Zanthoxylum armatum* was evaluated for their possible alpha-amylase inhibitory activity with acarbose as a positive control. IC_{50} value for acarbose was found to be 171.15±3.49 µg/ml and 89.37±4.68 µg/ml for methanolic extract of *Z. armatum*. The ability of methanolic plant extract of *Z. armatum* to inhibit the alpha amylase was calculated as percentage inhibition which was found to be 88.08% at 250 µg/

Table 2. Percentage inhibition of alpha amylase and IC_{50} value by methanolic leaves extract of *Zanthoxylum armatum* at various concentrations

Concentration (µg/ml)	% Inhibition of *Z. armatum*	IC_{50} (µg/ml) of *Z. armatum*	% Inhibition by Acarbose	IC_{50} (µg/ml) of Acarbose
50	39.89±0.1008	89.37±4.68	19.52±0.022	171.15±3.49
100	55.75±0.076		29.75±0.032	
150	61.78±0.027		44.43±0.064	
200	74.04±0.027		59.12±0.032	
250	88.08±0.055		69.74±0.063	

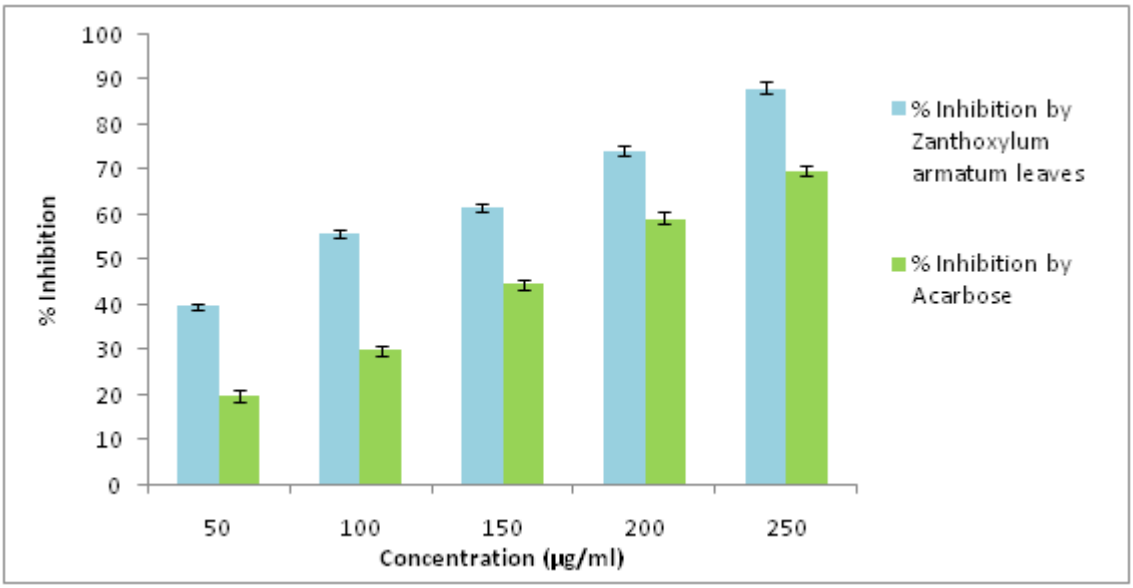

Fig. 1. Differences in the % inhibition of alpha amylase by methanolic leaves extract of *Zanthoxylum armatum* and Acarbose

Table 3. % Inhibition of DPPH by methanolic leaves extract of *Zanthoxylum armatum* and standard (ascorbic acid)

Concentration (µg/ml)	DPPH scavenging activity	IC_{50} Value of plant extract (µg/ml)	% Inhibition by Ascorbic acid	IC_{50} value (µg/ml) of Ascorbic acid
50	48.87±0.100	57.83±3.97	28.47±0.043	159.42±3.12
100	57.47±0.050		36.09±0.032	
150	73.76±0.026		42.51±0.040	
200	86.54±0.003		60.18±0.056	
250	94.67±0.014		72.26±0.026	

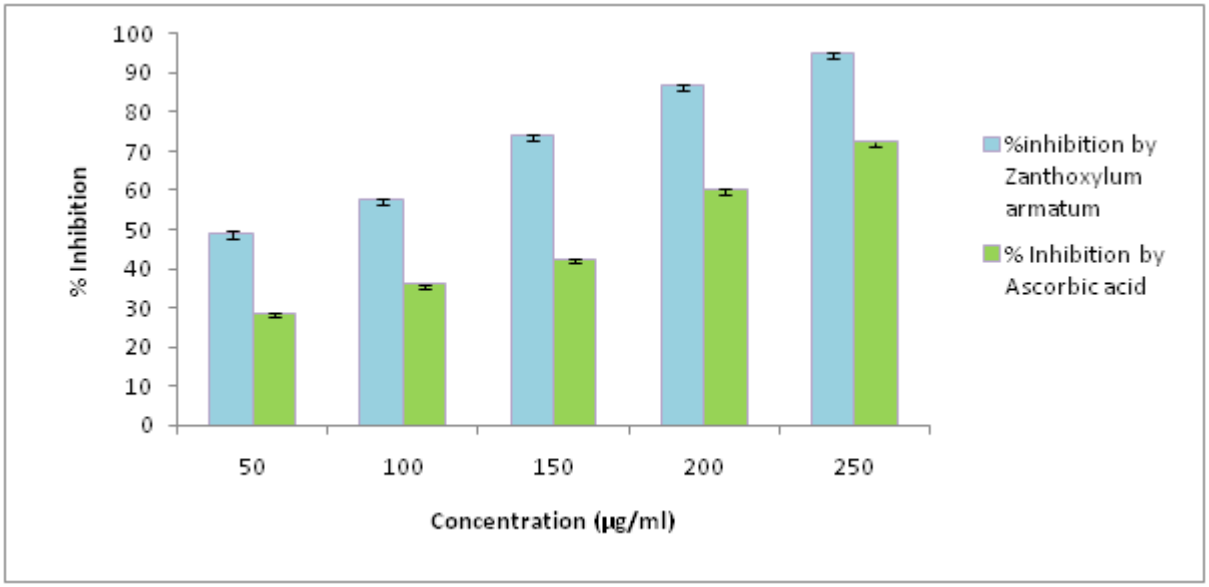

Fig. 2. Difference in % inhibition of DPPH by methanolic leaves extract by *Zanthoxylum armatum* and ascorbic acid at various concentrations

ml, whereas the % inhibition for acarbose at the same concentration was 69.74% (Table 2 and Fig. 1). The percentage inhibition of alpha amylase by plant extract was found to be higher than standard.

In vitro antioxidant activity

DPPH free radical scavenging activity

DPPH, a highly stable free radical has been widely used to assess the antioxidant potential of many natural products. The methanolic extract of *Zanthoxylum armatum* leaves showed better antioxidant potential when compare to standard ascorbic acid by DPPH scavenging assay method. Percentage inhibition was found to be 94.67% and 72.26% for plant extract and standard (ascorbic acid) respectively. IC_{50} value obtained was 57.83 µg/ml for plant extract and for ascorbic acid it was 159.42 µg/ml. It means methanolic extract of plant at higher concentration captured more free radicals formed by DPPH resulting into fadedness

Table 4. % Inhibition of H_2O_2 by methanolic extract of *Zanthoxylum armatum* leaves and standard ascorbic acid

Concentration (µg/ml)	% Inhibition of *Z. armatum*	IC_{50} (µg/ml) of *Z. armatum*	%Inhibition by Ascorbic acid	IC_{50} (µg/ml) of Ascorbic acid
50	42.42±0.030	79.13±2.65	30.76±0.082	104.24±2.87
100	51.21±0.037		58.02±0.019	
150	73.27±0.073		61.33±0.031	
200	86.3±0.048		70.85±0.051	
250	92.17±0.032		79.41±0.066	

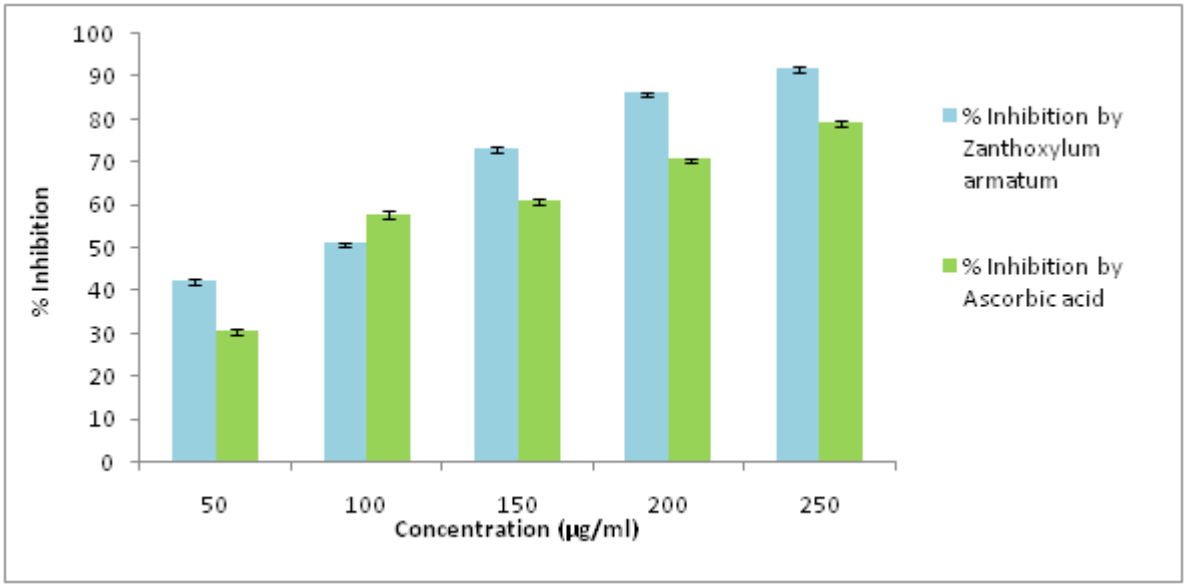

Fig. 3. Difference in % inhibition of H_2O_2 by methanolic leaves extract of *Zanthoxylum armatum* and ascorbic acid at various concentrations

of purple color to yellow results in decrease in absorbance and also IC_{50} value (Table 3 and Fig. 2).

H_2O_2 scavenging activity

Hydrogen peroxide scavenging assay is another useful method for determination of antioxidant activity. Hydrogen peroxide scavenging activity of plant was evaluated and it was observed that significantly higher (P<0.05) antioxidant activity exists in the leaves extract of *Zanthoxylum armatum* at different concentrations (50 to 250 µg/ml) as comparable to ascorbic acid (Table 4 and Fig. 3). Scavenging activity by plant extract was observed to be 92% whereas for standard it was 79.41% at 250 µg/ml concentration. Percentage inhibition was 15.57% higher in plant extract as compare to ascorbic acid. The IC_{50} value was found to be 79.13±2.65 for *Z. armatum* and 104.24 ±2.87 for ascorbic acid.

Total phenolic content

The total phenolic content (TPC) of the plant extract measured according the Folin–Ciocalteau method. The total phenolic content of this plant was varied from 36.79±0.21 to 140.71±0.10 mg gallic acid equivalent per gram of extract from 50 µg/ml to 250 µg/ml concentarion. It is observed to be statistically significant and comparatively higher than the standard (Gallic acid) (Table 5 and Fig. 4).

Total flavonoid content

The total flavonoid content of *Zanthoxylum armatum* extract was measured spectrophotometrically by aluminium chloride colorimetric assay. The flavonoid content of the extract was expressed as mg quercetin equivalent per gram of the extract. Flavonoid content varied from 16.26±0.88 to 88.53±0.10 mg/g when concentration of plant extract ranges from 50 µg/ml to 250 µg/ml and it seems to be increased with increasing plant concentration (Table 5 and Fig. 4).

Elemental analysis

Mineral element plays an important role in controlling diabetes and their related diseases. Some of them are ‘Ca’, ‘Cl’, ‘Mn’, ‘K’, ‘Si’, ‘Mg’, ‘S’, ‘Fe’, ‘Ni’, ‘Na’, and ‘Zn’ and all of these elements were observed in *Zanthoxylum armatum* in the present study and shown in Table 6.

Discussion

Most of the plants have been found to contain substances like glycosides, alkaloids, terpenoids, and flavonoids etc. that are frequently implicated as having antidiabetic effects (18). Present study confirmed the presence of these entire phytochemicals in studied plant (Table 1). Alpha-amylase is one of the main enzymes in human that

Table 5. Total amount of phenolic and flavonoid content of methanolic leaves extract of *Zanthoxylum armatum*

Concentration of leaves Extract of *Z. armatum* (µg/ml)	Total Phenolic Content (mg GAE/g)	Total Flavonoid Content (mg of Quercetin/g of extract)
50	36.79±0.21	16.26±0.88
100	58.83±0.26	27.01±0.61
150	83.71±0.34	49.78±0.43
200	115.94±0.74	66.97±0.14
250	140.71±0.10	88.53±0.10

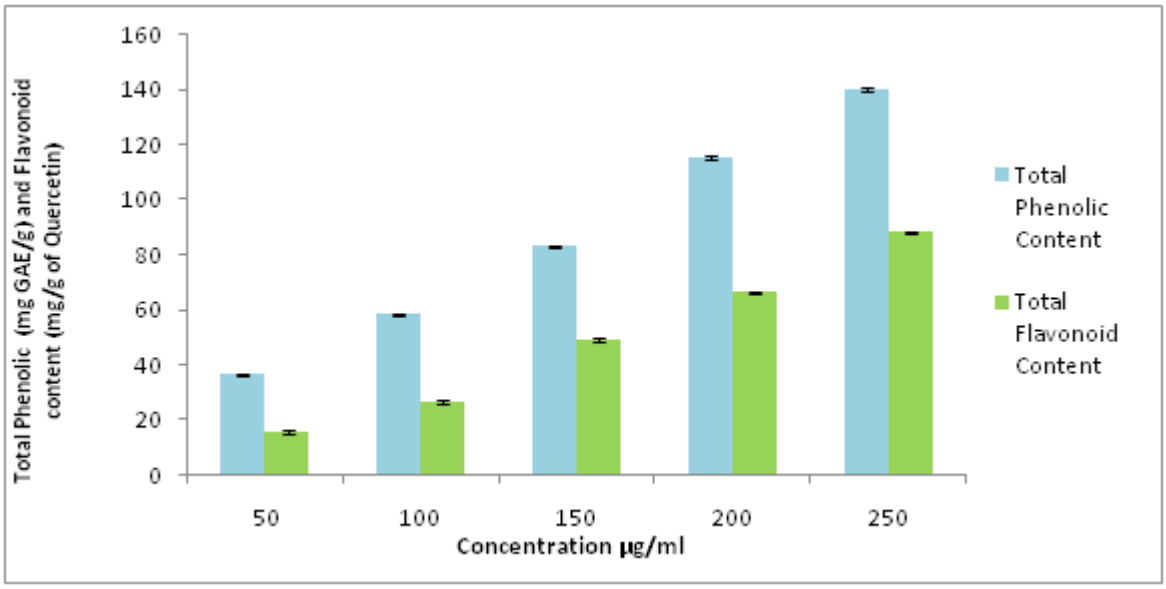

Fig. 4. Total phenolic content represented as mg equivalent of Gallic acid and total flavonoid content represented as mg equivalent of Quercetin of methanolic leaves extract of *Zanthoxylum armatum*

Table 6. Elemental analysis of *Zanthoxylum armatum* leaves using WDXRF

Elements	Na	Mg	P	K	Ca	Cl	Si	Fe	S	Mn	Cu	Zn	Ni	Mo	Sr
Values (mg/kg)	400	7100	2000	12300	30100	13300	2200	600	2200	95	14	42	8	12	58

is responsible for the breakdown of the starch to more simple sugar. Inhibition of α-amylase activities by *Zanthoxylum armatum* was found to be 88.08% which is 18.34% higher than standard i.e. acarbose and IC_{50} value calculated for *Z. armatum* was found to be more effective than acarbose i.e. 89.37 for *Z. armatum* and 149.96 for acarbose.. Consumption of phenols prevents many diseases especially related to oxidative stress. Diabetes is also related to oxidative stress therefore the phenolic compounds become a strategy for the treatment of diabetes. *Z. armatum* was found to contain enormous amount of phenolic compounds i.e. 140.71±0.10 mg/g. Flavonoids play an active role in quenching of free radicals due to their redox potential, amount of flavonoid content in *Z. armatum* was observed to be 88.53±0.01 mg/g. The ability of methanolic leaves extract of *Z. armatum* to scavenge DPPH free radical and H_2O_2 scavenging ability was found to be statistically significant and it was 22.41% and 12.76% higher than their standard respectively (Table 3 and 4). DPPH radical scavenging and H_2O_2 radical scavenging activity is due to the presence of phenols which readily donate hydrogen atom to the free radical (19). Hydrogen peroxide itself is not very reactive, but it can be toxic due to the increased hydroxyl radicals in the cells thus, removing H_2O_2 is very important (20). IC_{50} value of 50% and above corresponds to a larger scavenging activity and is considered significant. IC_{50} value was observed to be 57.83±3.97 for DPPH and 79.13±2.65 for H_2O_2. Hence, based on the IC_{50} values, *Z. armatum* extract exhibited a stronger scavenging activity than that of standard at all concentrations. A similar study revealed that the methanol extract of *Pittosporum viridiflorum* Sims (Pittosporaceae) bark in Cameroon showed an excellent inhibitory activity of 68.82% against DPPH radical at a concentration of 250 µg/ml (21). Antoxidants were found to be most active to reduce serum glucose level in *Emblica officinalis* Gaertn. (Phyllanthaceae) and *Terminalia chebula* Retz. (Combretaceae) (22). The elements which are reported from this plant include K, Ca, Mg, Na, P, Fe, Zn, Mn, Cu, Mo and Ni. These elements are very helpful in reducing glucose intolerance and insulin resistance as well as proven insulin secretagogue

in the isolated pancreas and intact organism (23-31). Many of these elements are known to have insulin-like property. The concentration of these elements in *Z. armatum* was found to be 7100 mg/kg for 'Mg', 2000 mg/kg for 'P', 12300 mg/kg for 'K', 30100 mg/kg for 'Ca', 600 mg/kg for 'Fe', 95 mg/kg for 'Mn', 14 mg/kg 'Cu', 42 mg/kg 'Zn' and 8 mg/kg 'Ni' (Table 6). These elements can be given as supplements for the diabetic patients. Therefore plants with these mineral elements are more useful for the diabetics and also some of these elements such as 'Cu', 'Mn' and 'Zn' are commonly referred as antioxidant minerals that are required for the activity of some antioxidant enzymes. Therefore, this plant can be used for the treatment of diabetes and its related diseases.

Conclusion

The leaves extract shows the good inhibitory effect on alpha-amylase and has effective antioxidant activity. Oxidative stress in diabetes may partially be reduced by antioxidants and as seen antioxidants have been prescribed to reduce the long term complications seen in diabetes. Results suggested that the content of phenolic compounds and flavonoids are directly related with the antioxidant activity. The studied plant contain considerable amount of all the essential elements which play an important role in controlling diabetes. Conforming to the above findings, *Zanthoxylum armatum* can be serves as a better antioxidant and useful for treating diabetes mellitus and its related metabolic damages. Therefore, the search of antioxidant and antidiabetic agent of plant origin with no side effects provide an alternative drug. Present study provides scientific evidence of the *in vitro* antioxidant and antidiabetic potential of *Z. armatum*. It adds to the overall value to its medicinal potential can further be used for the analysis of *in vivo* activity.

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Authors' contributions

SK conducted the experimental work and compiled the results. RJ and HK helped in plant collection and manuscript writing. RP helped in design of research analysis and approval of final manuscript.

Conflicts of interest

There are no conflicts of interest.

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