

Phenolic contents, elemental analysis, antioxidant and lipoxygenase inhibitory activities of *Zanthoxylum armatum* DC fruit, leaves and bark extracts

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Abstract: *Zanthoxylum armatum* (DC) is a traditional spice and important herb used in Asia as a part of food and household medicine for the treatment of various conditions. The present study was designed to investigate *in vitro* for phenolic/flavonoid contents, antioxidant activities, lipoxygenase inhibitory activity, minerals and heavy-metal contents. The leaves, bark and fruit of *Zanthoxylum armatum* were extracted with organic solvent and evaluated for phenolic contents (Folin-Chiocalteu method), for flavonoids contents (colorimetric method), for *in vitro* antioxidant assays (DPPH and FRAP), lipoxygenase inhibitory activity (5-LOX assay), mineral contents (atomic absorption), and for heavy-metal contaminants (atomic absorption). The fruit contained the highest phenolic (25.6 ± 1.2 mg GAE/g) and flavonoid (26.3 ± 1.4 mg QE/g) contents and therefore, exhibited the most effective antioxidant properties in DPPH ($88.5 \pm 0.9\%$ inhibition) and FRAP ($94.21 \pm 3.2\%$ inhibition) assays. The extracts also inhibited the LOX enzyme, and the fruit showed more inhibition ($63.8 \pm 1.2\%$) as compared with the leaves and bark. *Z. armatum* contain valuable minerals and fortified with magnesium (4.948 ± 0.2 , 3.07 ± 0.03 and 3.53 ± 0.12) and potassium (0.19 ± 0.011 , 1.91 ± 0.003 , and 1.90 ± 0.05) for leaves, fruit, and bark, respectively. The chemical profiling for heavy metals showed that their concentrations were within permissible limits. The data suggest that *Z. armatum* is a safe and valuable natural agent with functional properties for food and pharmaceutical industries.

Keywords: Antioxidant, anti-inflammatory, minerals, phenolics, *Zanthoxylum armatum*.

INTRODUCTION

Ethnomedicine is a branch of medicine which encompass various information, knowledge, sacred therapies, beliefs, approaches, plants and animals sources, or minerals for the treatment and prevention of diseases (Abbasi *et al.*, 2015). The ethnomedicine explored so far for phenolics found to have antioxidants activities and have been exploited to decrease the risk of cancer and heart ailments. The traditional medicinal plants have also been proven to cause inhibition of the biological enzymes for example lipoxygenase, cholinesterase and tyrosinase (Zengin *et al.*, 2015). The plants polyunsaturated fatty acids are converted by enzymes lipoxygenase (LOX) into bioactive metabolites, which are eventually involved in inflammatory and immune responses. This occurs due to the formation of the leukotrienes (LTs). The approaches which cause inhibition of LOX isoforms and/or their bioactive metabolites can be valuable in the dealing with asthma, allergic rhinitis, rheumatoid arthritis, psoriasis, and cancer (Yus Azila *et al.*, 2014).

Due to increasing interest in medicinal plants of traditional origin, it is important from a consumer point of view, to determine that the plant material in use is safe and free of toxic elements. On the other hand, the elements like potassium, sodium and phosphorous are

essential for health and so it is important to quantify these elements for nutritional point of view (Rodushkin I, 1999). *Zanthoxylum armatum* var. *ferrugineum* (Rehder & E.H. Wilson) C.C. Huang is a member of the family Rutaceae consists of about 150 genera and 1,500 species. Its common name in English is 'toothache tree' and in local language it is known as 'timber'. It is a wild plant in different areas of Pakistan like, Dir, Hazara, and Galliyat. *Z. armatum* fruits have stomachic and carminative properties (Alam and QN, 2015). Mostly used in above mentioned areas as spice for the preparation of condiments. This plant has traditional reputation in the folk medicine for the treatment of different ailments. In Ayurveda, *Z. armatum* fruit is mentioned as an appetizer, anthelmintic, analgesic, antitumor and carminative. It is useful in diseases of eye, ear, and in headache, and leukoderma (Kala *et al.*, 2005). The seeds and the bark of plant are considered aromatic tonic and are also employed for treating fevers, cholera and dyspepsia. The different chemical classes reported are terpenes, sterols, flavonoids, alkaloids, saponins, and coumarin (Singh and Singh, 2011).

The present study was grounded upon the fact that *Zanthoxylum armatum* have been traditionally as a spice and anti-inflammatory agent, therefore, this plant was assessed *in vitro* for antioxidant and anti-lipoxygenase activities and to evaluate its safety as a traditional and functional nutriment and as medicine.

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Table 1: Comparison of phenolic, flavonoids contents, DPPH, FRAP assays and Lipoxygenase inhibition of *Zanthoxylum armatum* extracts.

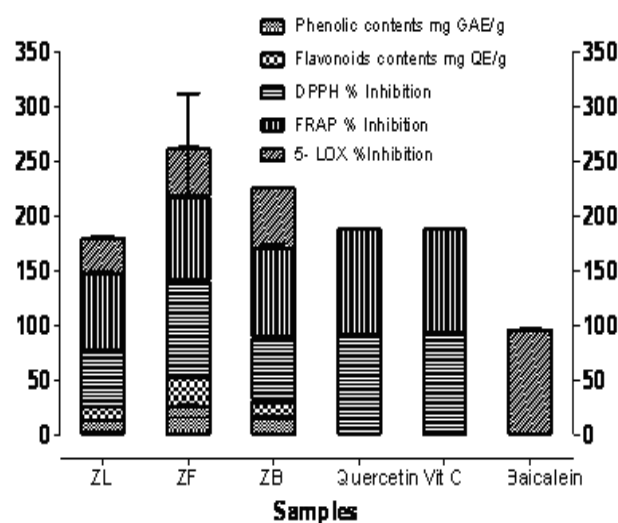
Samples	Phenolic contents mg GAE/g of extract	Flavonoids contents mg QE/g of extract	DPPH % inhibition 0.5 mg/ml	IC ₅₀ µg/ml	FRAP % inhibition 0.5mg/ml	IC ₅₀ µg/ml	5- LOX Inhibition	
							% Inhibition at 0.5mg/ml	IC ₅₀ µg/ml
ZL	13.1±0.8	12.7±0.8	51.2±0.3	150±1.6	69.6±2.81	44.2±0.3	35.3±1.5	-
ZF	25.6±1.2	26.3±1.4	88.5±0.9	70.4±0.5	94.21±3.2	14.4±0.6	63.8±1.2	70.3±2.1
ZB	15.5±1.5	16.3±0.9	57.4±0.7	130±1.3	81.11±3.4	32.7±0.6	54.9±1.5	90.5±3.6
Quercetin	-	-	92.1±0.0	16.9±0.1	95.21±0.1	6.04±0.00	-	-
Vitamin C	-	-	93.1±0.0	13.5±0.1	94.39±0.0	5.82±0.0	-	-
Baicalein	-	-	-	-	-	-	93.7±1.2	22.4±1.3

Values are given as standard error mean (SEM±) of three determinations.

Table 2: The concentration of minerals and heavy metals in samples of *Zanthoxylum armatum*

No	Metal	Leaves (ppm)	Fruit (ppm)	Bark (ppm)
1	Ni	0.237±0.02	0.216±0.01	0.193±0.01
2	Pb	0.380±0.01	0.386±0.02	0.429±0.02
3	Hg	0.50±0.03	0.53±0.03	0.51±0.002
4	Cu	0.0108±0.001	0.014±0.002	0.02±0.001
5	Co	0.0034±0.0001	0.035±0.002	0.035±0.001
6	Cr	Not detected	Not detected	Not detected
7	As	Not detected	Not detected	Not detected
8	Cd	0.0171±0.001	0.0171±0.001	0.0180±0.0012
	Minerals	Leaves Contents (mg/100 g)	Fruit Contents (mg/100 g)	Bark Contents (mg/100 g)
9	Na	0.518±0.02	0.29±0.01	0.396±0.02
10	Ca	0.226±0.03	0.48±0.031	0.529±0.003
11	Mg	4.948±0.21	3.07±0.03	3.53±0.12
12	Fe	0.0795±0.02	0.068±0.001	0.129±0.003
13	K	0.19±0.011	1.91±0.003	1.90±0.05
14	Zn	0.026±0.001	0.072±0.002	0.050±0.001
15	Mn	0.015±0.002	0.012±0.001	0.009±0.001
16	Si	0.2419±0.02	0.646±0.01	0.531±0.01

Values are given as standard error mean (SEM±) of three determinations.

**Fig. 1:** Comparison of phenolic, flavonoids contents, DPPH, FRAP assays, and Lipoxygenase inhibition of *Zanthoxylum armatum* extracts (ZL= leaves, ZF = fruit, ZB = bark).

MATERIALS AND METHODS

Plant material and extraction

Zanthoxylum armatum leaves, fruits and bark were collected in August 2014 from Tanawal area (coordinates are 34°21'30" N and 73°40' E in DMS of District Haripur, KPK Pakistan. It was validated by Taxonomist Prof. Dr. Manzoor Ahmed, Department of Botany, postgraduate college, Abbottabad and a voucher specimen (PG/B/ZA, 2014) was deposited at the college herbarium. The plant materials were shade dried and ground to coarse powder and were kept in an air closed and light resistant bottle.

100g of powder material was extracted with methanol using Soxhlet extraction apparatus for 20hrs. Whatman Grade-I filter paper was used for filtration of extract. The evaporation of filtrate was carried out under vacuum at 40°C. The percent extractive yield measured for the methanol extracts were; bark (ZB): 21±1% leaves (ZL):19±1%, and fruit (ZF): 14.34±1%.

Chemicals

DMSO dimethyl sulphoxide (PubChem CID: 679), and Methanol (CID: 887) (analytical) were purchased from Merck, Germany. 1, 1-diphenyl, 2-picryl hydrazyl (CID: 2735032) (DPPH), Ascorbic acid (CID: 54670067) (Vitamin C), Gallic acid (CID: 370), Quercetin (CID: 5280343), tetrazolium chloride (CID: 9853362), Folin Chiocalteu's reagent, 5-LOX enzyme from soybean, KH_2PO_4 (CID: 516951) buffer and Baicalein (CID: 5281605) were all procured from Sigma-Aldrich, St. Louis, MO, USA. HClO_4 , H_2SO_4 and Standards for Fe, Pb, Na, K, Ca, Cr, Zn, Mn, Co, Hg, Cd and Ni acquired from Merck, Germany

Determination of total phenolic contents (TPC)

TPC of methanol extract of *Zanthoxylum armatum* (leaves, fruit and bark extracts) was measured by Folin-Chiocalteu method (Abbasi *et al.*, 2015) with some amendment. The extracts were diluted with distilled water to obtain the readings inside the standard curve range of 0.0 to 600 μg of gallic acid/ml. A mixture was obtained by adding 250 μl of gallic acid, 1ml of distilled water, 250 μl of Folin-Chiocalteu reagent in a test tube. After mixing, it was kept standing for 5min at normal temperature. Then, 7% sodium carbonate aqueous solution (2.5ml) and distilled water were added to make up the volume to 6ml. The mixture was kept in an incubator for 90 minutes. The absorption of the subsequent blue color solution produced, was measured at 760nm by spectrophotometer. The results were calculated as mg of gallic acid (Standard) equivalents (GAE)/g of extract by means of an equation from the standard gallic acid graph. Three readings were noted for each extract.

Determination of total flavonoid contents

The total flavonoid content of *Z. armatum* (leaves, fruit and bark) was measured by using a colorimetric method as mentioned by (Zhishen *et al.*, 1999) with little modification. The dry crude extract (25mg) was ground with 10ml methanol (80%) in a mortar. The homogenized mixture was kept standing for 20 minutes at normal temperature. The mixture was filtered through G4 filter. A part (0.4ml) of filtrate was added with 0.6ml distilled water, 0.06ml NaNO_2 solution (5%) and mixed. This mixture was kept undisturbed for 5 minutes. Then 0.06ml AlCl_3 solution (10 %) was added to the mixture. Instantly added 0.4ml NaOH (1 N) and 0.45ml distilled water to the mixture and kept standing for 30 minutes. Absorbance of the mixture was measured at 510nm. The values were taken as milligram of Quercetin (Standard) equivalents per 1-gram dry weight. Data was noted as mean $\pm$ SD for triplicates.

DPPH radical scavenging assay

Test solution in triplicates (10 μl) and DPPH (100 μM) solution in methanol (90 μl) added to the 96-wells plate and incubated for 30 minutes at 37°C. Then using Synergy

HT BioTek® USA micro plate reader the absorbance was measured at 517nm. The test solutions were analyzed at various dilutions, i.e. 0.5, 0.25, 0.125, 0.0625, 0.0313, 0.015mM to determine the IC_{50} values. The data obtained was computed on Statview version 5.0. Ez-fit computer software. Quercetin and Vitamin C were used as positive controls. The inhibition was denoted in percent and were measured by comparing with a control group (DMSO). The data was employed to calculate the concentration of the sample needed to scavenge 50% of the DPPH-free radicals (Shen *et al.*, 2010). The radical scavenging activity was calculated by the following equation:
Inhibition (%) = (Absorbance of control - Absorbance of test solution/ Absorbance of control) $\times$ 100

Ferrous reducing antioxidant power (FRAP)

In a 96 well microplate 25 μl of each of test sample and of phosphate buffer (pH 7.2) were mixed and then added 50 μl of 1% potassium ferricyanide solution into it followed by incubation for 10 minutes at 50°C. Then trichloroacetic acid (10 % w/v) solution (25 μl) and distilled water (100 μl) were added into it and measured the absorbance as a pre read value at 540nm in the micro plate reader. Finally, freshly prepared ferric chloride (0.2% FeCl_3) solution (25 μl) was added to the mixture and absorbance was noted at 700 nm. Quercetin was used as a standard (Saini *et al.*, 2011). The ferrous reducing power was measured by the following equation:

% inhibition = (absorbance of sample/absorbance of control) $\times$ 100

5-LOX assay

5-LOX activity was analyzed by the method of (Ben-Nasr *et al.*, 2015) with slight modifications. A total volume of 200 μl containing 140 μl of KH_2PO_4 buffer (100mM, pH 8.0), 20 μl test compound, and 15 μl purified LOX enzyme (127 units per well) were mixed and pre-read at 234nm. The mixture was pre-incubated at 25°C for 10 minutes. The reaction was originated by the addition of 25 μl substrate solution. The absorbance was noted every 10min at 234nm for any change in absorbance. Baicalein was used as a positive control. The IC_{50} values were determined at 0.5, 0.25, 0.125, 0.0625, 0.0313, 0.015mg dilutions. The resulted data was computed on Ez-fit software. All extracts assays carried out in triplicate. The radical scavenging activity was calculated by the equation:

Inhibition (%) = (Absorbance of control - Absorbance of test solution/Absorbance of control) $\times$ 100

Determination of minerals and heavy metals

The minerals (Na, K, Fe, Ca, Mg, Mn, Zn, and Si) were analyzed by using an atomic absorption spectrophotometer (Perkin Elmer AAnalyst700, USA). The samples were digested in mixture of H_2SO_4 , HNO_3 and HClO_4 before analysis. All the experiments were carried out in triplicate. The mineral contents were expressed as mg/100g of sample (Oumarou *et al.*, 2013).

Heavy metals (Hg, Ni, Co, Pb, Cr, Cu, As and Cd) contents were determined using an atomic absorption spectrophotometer (Meena *et al.*, 2010). From the standard stock solutions (1000ppm) different dilutions were made ranging from 1ppm to 10ppm and stored at 4°C. The acidity of solutions was maintained with 0.1% nitric acid. The test solutions were digested in 20ml mixture of concentrated nitric and perchloric acids (9:1 ratio) for 3hrs in a water bath maintained at 70°C until a clear brownish solution was obtained. When the solutions cool down, these re-formed with deionized autoclaved water to 20ml. Whatman filter paper (0.45µ, Axiva) was used for filtration and stored in closed acid-washed glass vials. All the samples were analyzed in triplicate.

RESULTS

Determination of phenolic and flavonoid contents

The fruit, bark and leaves extracts of *Zanthoxylum armatum* were assessed for phenolic and flavonoids contents and it was observed that the fruit extract (ZF) showed the highest contents followed by the bark (ZB) extract. The leaves (ZL) extract showed lowest contents among the extracts. All the values are tabulated in table 1. In our earlier HPLC investigation we have reported the presence of phenolic compounds in extracts of *Z. armatum* (Alam and QN, 2015)

Determination of antioxidant activities

The fruit, bark and leaves of *Z. armatum* were assessed by DPPH method and as expected ZF extract showed the maximum inhibition followed by the ZB extract. Similar pattern of observations was noted when antioxidant activity of *Z. armatum* fruit, bark and leaves were evaluated by FRAP assay table 1.

Anti-lipoxygenase activity

The LOX enzymes are associated with inflammation and allergic reactions due to the creation of the leukotrienes (LTs) (Pontiki and Hadjipavlou-Litina, 2007). It has been observed that in the inflammatory conditions like asthma, psoriasis, rheumatoid arthritis, colitis and allergic rhinitis the levels of leukotrienes are increased. LTs production can be inhibited by inhibiting the LOX pathway (Martel-Pelletier *et al.*, 2003). The extracts of *Z. armatum* exhibited activity against the 5-LOX enzyme. The fruit extract showed maximum inhibitory activity followed by the bark extract. All the results are presented in fig. 1 and table 1.

Fig. 1 revealed a clear correlation of total phenolic and flavonoids contents to the antioxidant and anti-inflammatory (LOX inhibition) activities in a concentration dependence manner.

Minerals and heavy metal contents

Table 2 present the mineral contents of *Z. armatum* fruit, bark and leaves extracts.

The major minerals content in ZL, ZF and ZB found were magnesium and potassium. In structure and function of the human body magnesium and potassium have a very vital role (Sodamode, 2013).

DISCUSSIONS

Reducing power is the key index of antioxidant effects and proved as an important indicator of antioxidant measurement (Wang *et al.*, 2009). Among the components of plant, the phenolic and flavonoids are mostly proved responsible for antioxidant activity and consequently analyzed for this purpose. Research on phenolics so far indicated that their antioxidant properties are attributed to the abilities to donate hydrogen, to quench the singlet oxygen and to scavenge free radical (Rice-Evans *et al.*, 1997). Among the antioxidant screening methods the DPPH assay has been proven to be most sensitive method and it is fast and easy (Koleva *et al.*, 2002). In our experiment, the antioxidant activity shown by samples may be attributed to their ability of hydrogen transfer or may be due to electron transfer to neutralize the DPPH free radical. The maximum antioxidant power exhibited by extract of *Z. armatum* fruit extracts which was almost comparable with the positive control.

The LOX enzymes are associated with inflammation and allergic reactions due to the creation of the leukotrienes (LTs) (Pontiki and Hadjipavlou-Litina, 2007). The extracts of *Z. armatum* exhibited activity against the 5-LOX enzyme. The fruit extract showed maximum inhibitory activity followed by the bark extract.

The usage of medicinal plant without proper monitoring and quality control of heavy metal contents can have serious consequences when present beyond permissible limits. Hence, it is necessary to analyze the heavy metals contents and other pollutants and this practice will consequently helpful in selecting the appropriate site of collection of medicinal plants. There are some proposed limits for different heavy metals content in raw materials in some countries. In Canada, for example, the minimum permissible limits for arsenic, lead, cadmium, mercury and chromium are 5.0, 10.0, 0.3, 0.2 and 2.0ppm respectively (WHO, 2007).

Similarly, sodium has imperative role to maintain the electrolyte balance in the human body. The other most vital mineral is calcium, which is essential to develop bones and teeth. The content of calcium was more in bark as compared with fruit and leaves. It is well known that iron is essential for the formation hemoglobin and deficiency may lead to conditions like Anemia. Manganese (Mn) and Zinc (Zn) are recognized as essential trace element, which acts as cofactor for various enzymes. The content of Mn was more in leaves as compared with fruit and bark (Rosario Goyeneche *et al.*,

2015). Likewise, other minerals also have their role in working of human body like Silicon is known as a beautifying mineral and there are also many health benefits associated with it. This study clearly justifies the traditional nutritional use of *Z. armatum* beside its medicinal values because of high nutrient content.

The results revealed that the concentrations of heavy metals in *Z. armatum* were either low or were within the limits as defined by (WHO, 2007). Chromium (Cr), is known as one of the most toxic metal. The permissible limit for Cr in raw herbal materials is 2.0 ppm and that for finished products is 0.02 mg/day (WHO, 2007). The samples ZL, ZF and ZB were free of Cr, and were not detected. Lead (Pb) is very toxic to plants, animals and microorganisms which intensify in pollution due to enlarged fertilizer utilization, fuel combustion and sewage sludge. The samples of *Z. armatum* disclosed to contain very low concentrations of Pb compare to the permissible limit of 10 ppm defined by (WHO, 2007). Cadmium (Cd) is which is found commonly in medicinal plants is a dangerous heavy metal. The main sources which lead to the accretion of cadmium in soil and plants are phosphate fertilizers, non-ferrous smelters, lead and zinc mines, sewage sludge application and combustion of fossil fuels.

In our study the samples of *Z. armatum* analyzed showed that the Cd concentration was within the acceptable range of 0.3 ppm as endorsed by (WHO, 2007) for the herbal raw material. Ni is considered as allergenic and interact with proteins directly and Co is typically consumed as a component of cyanocobalamin vitamin (Zhang Y, 2009). Though no limit has been established for Nickel in food stuffs (Bhat R, 2010). The presence of cobalt, nickel and chromium are among the main causes of contact dermatitis (Basketter DA, 2003). The concentration of Ni and Co in *Z. armatum* samples were much low. The content of mercury in ZF, ZB and ZL were approximately 0.5ppm, which is equivalent to the maximum limit approved in china (0.5ppm). The concentration of as was negligible and not detected in the samples, its limits as given by WHO is 10ppm table 2.

CONCLUSION

This study is the first to report on the elemental analysis, lipoxigenase inhibition, and antioxidant activities of different parts of *Z. armatum*. The results showed that there is a positive association between phenolic contents and antioxidant activity. Based on the experimental findings, it can be concluded that methanol extract of *Z. armatum* fruit is more concentrated in the phenolic and flavonoids, therefore, have more DPPH and FRAP reducing activities. The results also revealed that *Z. armatum* has strong antioxidant and anti-inflammatory potential and can be a source of significant therapeutic agent. Moreover, it is also investigated that the plant

contains important minerals and is safe as a drug and food.

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