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ORIGINAL ARTICLE

CRUDE ALKALOIDS FROM FRUIT, BARK, LEAVES AND ROOT OF *ZANTHOXYLUM ARMATUM* SHOWED ANTI-A- GLUCOSIDASE, ANTI-UREASE AND ANTI-LIPOXYGENASE ACTIVITY

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ABSTRACT

Zanthoxylum armatum (ZA) (family: Rutaceae) is one of the commonly used spices, food ingredient and medicinal plant in the traditional medicine in Asia. The plant extracts containing secondary metabolites are good source of enzyme inhibitors and potential source of medicines. The alkaloid fraction isolated from stem bark (ZBA) showed maximum inhibition of 72.61±1.34% with IC_{50} values of 325.62±1.29 µg/mL. This activity was significant when compared to the standard drug Acarbose (IC50 = 375.82±1.76µM). The isolated crude alkaloids were also screened against the urease enzyme. The fruit alkaloids ZFA showed excellent inhibitory activity with 87.28±1.35% with IC_{50} values of 57.95±1.23 µg/mL. Similarly, alkaloids from root bark also showed good activity. Anti-lipoxygenase inhibitory studies of alkaloids fractions were also assessed and the results are tabulated in table I. The alkaloids fractions from root bark and fruit showed maximum inhibition of 5-LOX with percentage inhibition of 91.36±0.87 (IC_{50} ±S.E.M = 42.83±0.58) and 89.42±0.84 (IC_{50} ±S.E.M=32.25±0.67), respectively. The results showed inhibitory activity against the enzymes tested, therefore, motivating the use of natural products as source of enzyme inhibitors due to greater structural diversity. The results showed that alkaloids fractions have the potential to treat diabetes, inflammation, gastritis, ulcers and other related disorders by acting as enzyme inhibitor.

Keywords: Alkaloids, *Zanthoxylum armatum,* glucosidase, urease, lipoxygenase, HPLC

INTRODUCTION

In recent years the compounds with enzyme inhibition activity has attracted the attention of researchers due to the applications of these compounds in treating different diseases. Natural products are always considered the indigenous source of such compounds and a variety of compounds based on enzyme inhibition property has been isolated. The secondary metabolites produced by the plants are the most appropriate pharmacophore for various medicinal agents (Rauf and Jehan, 2017). Since the mid-90s many bioactive molecules have been isolated from plants and are now part of modern medicine. The search of fineness, factual, low priced and simply available natural enzyme inhibitors is the most followed strategy for the search of new drug entities in the world (Rauf *et al*., 2011)

Enzyme inhibitors mainly work by binding to an enzyme and reducing its activity. This can lead to killing of a pathogen or can recover an imbalance in a metabolic process. Others can increase the enzymatic actions by reacting with enzymes and may alter to new products in enzyme catalytic cycle. This reaction can finish a substrate and enzyme acts as catalyst. In irreversible inhibition process the inhibitor

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act on enzyme covalently and adjust the amino acid remnants. The enzyme reversible inhibitors are not bonded covalently and produced enzyme substrate complex (Shapiro and Vallee, 1991). Natural enzyme inhibitors are more specific and have unique effectiveness. The high specificity and potency is related to lower toxicity and less side effects (Shapiro and Vallee, 1991).

Alkaloids are the most important class of secondary metabolites with many therapeutic properties. Many of the alkaloids are enzyme inhibitors and are part of drugs prescribed for various purposes. The alkaloids have been isolated by activity guided isolation process based on their bioactivity against various enzymes like berberine and jatrorrhizine (Rackova *et al*., 2007).

Diabetes is a metabolic disorder and most prevalent in the world today. Diabetes leads to many complications including retinopathy, neuropathy and heart related conditions. To manage the glucose level in the blood is the main focus to control diabetes. Inhibition of enzymes involved in carbohydrate hydrolysis like α-glucosidase and α-amylase results in glucose control. Many research experiments have been conducted to investigate the activity of various secondary metabolites from natural sources as inhibitors of these enzymes (Kumar *et al*., 2012).

The crucial function of α-glucosidase is hydrolysis of incurable non-reducing (1-4) attached α-glucose which remains to release a solitary α-glucose molecule. α-glucosidase is primarily a carbohydrate and releases α-glucose through process of hydrolysis. The substrate molecule differentiation of α-glucosidase is exceptional to sub site attractions of the active site of enzymes (Mehrani and Storey, 1993). Ureases are another class of enzymes attracting the attention due to its impact in health and quality of life. It is involved in urea hydrolysis. The persistent of urease activity in living cells results in some diseases and pathogen infections. Secondary metabolites have the ability to inhibit the urease activity to overcome its unwanted effects (Modolo *et al*., 2015). The enzyme urease is a metaloenzyme. It contains nickel. This enzyme caused the hydrolysis of urea into ammonia and CO_2. Due to urease the microbe *Helicobacter pylori* can survive at acidic environment of stomach. Consequently, urease have an important role in development of ulcers in GIT. It may also leads to other complications like hepatic encephalopathy, urolithiasis and urinary catheter encrustation. Therefore, search of new urease inhibitors form natural sources is the need of the day (Arfan *et al*., 2010).

Leukotrienes is an important inflammatory mediator and are released by leukocytes. 5-lipoxygenase (5-LOX) is the important enzyme for the biosynthesis of leukotriene. This enzyme speed up the early steps of the conversion of arachidonic acid to bioactive leukotrienes. The metabolism of arachidonic acid by 5-LOX produced many bioactive lipids that have an important function in inflammation (Steinhilber, 1999). Therefore, LOX are considered crucial for the potential targets for the drug design. The LOX inhibitors based on mechanism can have applications in ailments like asthma, inflammation and cancer related conditions. Hence, search for new LOX inhibitors seems to be a favorable tactic for the development of new drugs (Nie and Honn, 2002). The current study was carried out to screen the indigenous medicinal plant *Zanthoxylum armatum* of Pakistan for the enzyme inhibition activity to provide a base for its potential application in inflammation, ulcer and related conditions.

MATERIALS AND METHODS

Plant collection and extraction

Drugs and Chemicals

All the chemical and drugs were of standard quality and were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA) unless otherwise stated. The test substances were prepared fresh before application in assays.

Table 1: The enzyme inhibitory activity of alkaloid fractions isolated from bark (ZBA), leaves (ZLA), fruit (ZFA) and root bark (ZRA) of *Zanthoxylum armatum.*

S. No.	Sample	α-Glucosidase inhibition		Urease inhibition		Lipoxygenase inhibition	
		Inhibition (%) at 0.5 mg/mL	IC_{50} (µg/mL)	Inhibition (%) at 1 mg/mL	IC_{50} (µg/mL)	Inhibition (%) at 0.5 mg/ml	IC_{50} (µg/ml)
1	ZBA	72.6±1.34	325.6±1.2	57.1±1.8	196.7±1.6	85.2±0.7	97.5±0.5
2	ZLA	37.38±1.5	-	67.54±1.7	191.3±1.5	72.3±0.7	215.4±0.5
3	ZFA	29.5±1.4	-	87.2±1.3	57.9±1.2	89.4±0.8	32.2±0.6
4	ZRA	38.6±1.2	-	69.5±1.67	183.6±1.4	91.3±0.8	42.8±0.5
Standard		Acarbose 65.7±1.9 mM	375.8±1.7 µM	Acetohydroxamic acid 91.2±1.5 µM	44.0±2.8 µM	Quercetin (%Inhibition at 0.25 mM) 89.2±0.6	IC_{50} (µM) 2.3±0.3

Plant collection and preparation of extract

The plant *Zanthoxylum armatum* was collected from Salhad, district Abbottabad, KPK Pakistan in September 2018. The taxonomist Dr. M. Nazir identified the specimen and signed the voucher CUHA-198 at institute herbarium. The collected parts were freed from dirt and other impurities and washed under running water, shade dried, later converted to fine powder and kept in an airtight container. The drug was extracted with 95% methanol at room temperature, filtered and the filtrate was concentrated under vacuum.

Extraction of total alkaloids

100 grams of extract was first treated with pet-ether under reflux to defat and then treated with 25% sodium hydroxide for 4 hrs and again fractionated with chloroform using a soxhlet apparatus for six hrs. The chloroform fraction obtained was shaken well with 2% aqueous sulfuric acid. The acid extracts were then treated thrice with 50 ml of sodium hydroxide (25%) to attain pH 10 to liberate the free alkaloids. It was separated in a separating funnel with dichloromethane and were passed over sodium sulphate. The filtrate was concentrated to dryness under vacuum to obtain crude alkaloids (Mahdeb, Mayouf *et al*., 2013).

α- glucosidase inhibitory assay

The method adopted for this assay was carried out earlier by (Matsui, Yoshimoto *et al*., 1996) with some modification. The samples were dissolved in DMSO. An enzyme solution containing α -glucosidase (0.8 units/ml) in 50mM phosphate buffer with pH 7, containing 100 mM NaCl was prepared instantly before use. The solution was kept cold during the procedure. The substrate, pNP-G (0.7mM) in phosphate buffer, was made fresh earlier to use. The test solution (20µL) and enzyme solution (80µL) was pre-incubated for 5 minutes at 37°C. The reaction was instigated with 1.9mL of substrate solution and incubated for 15 minutes at 37°C. The reaction was stopped by adding 2.0mL (0.5M) aqueous Tris solution and the absorbance of PNP released from PNP-G was noted at 400 nm. 20µL DMSO was kept as blank. Acarbose was used as a positive control. Analysis was carried out in triplicates and the results were calculated as ±SEM. Percent α-glucosidase inhibition was calculated as follows: (1-B/ A) × 100, where A is the absorbance of control and B is the absorbance of samples containing extracts.

Urease inhibitory assay

The reaction mixture consisting of 55-µL phosphate buffer solution (3mM, 4.5 pH), 25µL of urease enzyme solution and sample (5µL) were incubated for 15 min (30°C) in 96-well plates.

During the reaction the amount of ammonia released corresponds to urease activity. In brief, 40µL of each phenol reagent, comprising a mixture of 1% phenol, 0.005% of sodium nitroprussside and appropriate amount of sodium hydroxide reagents were put in 96 well plates. After fifty minutes the absorbance (at

630 nm) of the reaction mixture was noted in a micro-plate reader equipped with built-in software. Percent inhibition was measured by determining the optical density (OD) using the formula: 100-(OD) test well/(OD) control. Thiourea was used as a control. The IC50 values were determined using EZ-Fit kinetic database (Perrella Scientific Inc., Amherst, NH, USA) (Amin *et al*., 2013).

Lipoxygenase inhibitory activity

Lipoxygenase activity was determined by the method previous employed (Ben-Nasr *et al*., 2015) with few modifications made. A total volume of 200µl containing 140µl of KH_2PO_4 buffer (100mM, pH 8.0), 20µl crude alkaloids and 15µl purified LOX enzyme (127 units per well) were mixed and pre-read at 234 nm. This mixture was incubated for ten minutes at 25°C. 25µl of substrate solution was added to initiate the reaction. The absorbance was noted at 234 nm every ten minutes and change was noted. 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 results were computed on Ez-fit software. The tests were carried out in triplicate. The radical scavenging activity was calculated by the equation;

Inhibition (%) = (Absorbance of control - Absorbance of test solution/Absorbance of control) × 100

STATISTICAL ANALYSIS

Experimental data were analyzed with three replicates for analysis of variance. Statistical analysis was done by One Way ANOVA. The p values less than 0.05 were considered to be statistically significant.

HPLC chromatogram

Crude alkaloidal contents were dissolved in methanol and then filter by using syringe filters. HPLC (Analytical, SHMADZU, Japan) coupled with UV/Vis detector are used. The analytical column used was Shim pack GIST C18 (150mm×4.6mm, 5µm). The temperature of was kept at 25°C. Mobile phase consist of two solvent A (water) and solvent B (Acetonitrile). The solvent is then filtered by using 0.45µm filter and then degassed. The sample injection volume was 10µl. The samples were then injected into HPLC and with mention specification. The elution was; at 5 min (A 100% vs B 0%), at 15 min (A 85% vs B 15%), at 25 min (A 65% vs B 35%), at 30 min (A 0% vs B 100%).

RESULTS AND DISCUSSION

The substances with enzyme inhibition properties are considered attractive targets for management of different diseases. A plethora of compounds have been isolated which showed a variety of enzyme inhibition activities. Alkaloids and other metabolites are exceptional source of pharmacophore. Finding such metabolites for the development of drug are dynamics in discipline of pharmacology and biochemistry. Such inhibitors from natural sources are expected to have very few adverse effects and lower toxicities (Shapiro and Vallee, 1991).

One of such enzyme inhibitors is α-glucosidase inhibitors which are involved in diabetes management. The alkaloids have been reported to have such activities and in this case alkaloids fractions from fruit, bark, leaves and roots were tested for yeast α-glucosidase inhibition. The alkaloid fraction isolated from stem bark (ZBA) showed maximum inhibition of 72.61±1.34% with IC_{50} values of 325.62±1.29µg/mL. This activity was significant when compared to the standard drug Acarbose (IC50 = 375.82±1.76µM). The results obtained are shown in table 1.

Evidences are available from the previous studies which strengthen the fact that alkaloids have the α-glucosidase inhibitory activity. Two new pyrrolidine alkaloids, radicamines A and B were isolated as inhibitors of a-glucosidase from *Lobelia chinensis* LOUR. (Campanulaceae). Radicamines A and B. (Shibano *et al*., 2001).

The isolated crude alkaloids were also screened against the urease enzyme. The fruit alkaloids ZFA showed excellent inhibitory activity with 87.28±1.35% with IC_{50} values of 57.95±1.23µg/mL. Similarly, alkaloids from root bark also showed good activity. The results are tabulated in table I.

A lot of experiment have been conducted in the past utilizing the ethnomedicinal plants to search the treatment *H. pylori* caused gastritis, ulcers and other GIT disorders. It resulted in the identification and isolated of molecules responsible for urease inhibitory activity and were considered useful to control *H. pylori* strains growth (Modolo *et al*., 2015). Urease enzyme in the background of *H. pylori*, which increase the medium pH by the deposit of NH3, is a urease trait of great medical value (Kerr, Blevins *et al*., 1983). Hence, the main public health issue is related to *H. pylori* which likes to live in acidic environment of stomach (pH 2-4). H. pylori can produce risk of various GIT ulcers and lymphomas by causing gastric irritation initially. There are studies available which revealed that alkaloids have the potential to overcome such problems. These enzyme showed good urease inhibitory activity. In one study four alkaloids isolated from *Corydalis govaniana* Wall namely, govaniadine, caseadine, caseamine and protopine exhibited a good urease enzyme inhibition with IC_{50} ±S.E.M values of 20.2±3.6, 38.9±2.8, 66.7±1.2 and 54.1±1.2mM, respectively, which were comparable to the standard inhibitor, acetohydroxamic acid (IC_{50} = 42.0 mM) (Shrestha *et al*., 2013). In our experiments crude alkaloids from fruit extract showed promising anti-urease activity and showed the potential to treat the complications caused by urease.

Anti-lipoxygenase inhibitory studies of alkaloids fractions were also assessed and the results are tabulated in table 1. The alkaloids fractions from root bark and fruit showed maximum inhibition of 5-LOX with percentage inhibition of 91.36±0.87 (IC_{50} ±S.E.M = 42.83±0.58) and 89.42±0.84 (IC_{50} ±S.E.M = 32.25±0.67), respectively. This showed that alkaloidal contents of this plant has the ability to potentiate the effect of lipoxygenase enzyme. Previous studies showed that alkaloids isolated from plant extracts showed lipoxygenase inhibitory activity. One of example is that of sanguinarine and chelerythrine from *Chelidonium majus*, L. (Papaveraceae), that showed very potent inhibitory activity against 5-lipoxygenase (Vavreckova *et al*., 1996). Similarly, *Mahonia aquifolium* alkaloid fractions containing protoberberine and bisbenzylisoquinoline alkaloids indicated the lipoxygenase inhibition (Rackova *et al*., 2007).

The LOX enzymes are associated with inflammatory and allergic reactions due to the formation of the leukotrienes (LTs) (Pontiki and Hadjipavlou-Litina, 2007). The inflammatory conditions like asthma, psoriasis, rheumatoid arthritis, colitis and allergic rhinitis it is observed that the levels of leukotrienes are increased. LTs production can be inhibited by inhibiting the LOX pathway (Martel-Pelletier *et al*., 2003). The alkaloids fractions from *Z. armatum* showed activity against the 5-LOX and are therefore can be the potential candidate as anti-inflammatory agent.

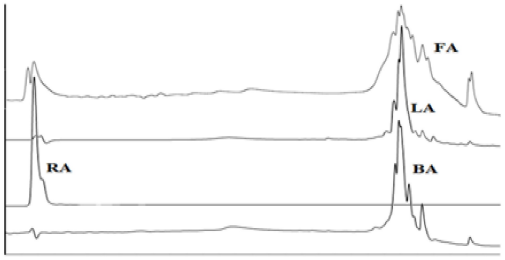

Fig. 1: The overlay shows a comparison of alkaloids components of four samples. The chromatogram clearly shows that fruit (FA), bark (BA) and (LA) contain more alkaloids as compared roots (RA).

HPLC chromatogram was developed to compare the alkaloids contents of fruit (FA), bark (BA), root bark (RA) and leaves (LA) of *Z. armatum*. The fig. 1 shows the matching peaks which stands for similar alkaloidal

contents as well. The leaves and bark showed more number of peaks as compared to fruit and roots. The plant is known to have different alkaloidal contents.

CONCLUSION

The results showed inhibitory activity against the enzymes tested, therefore, motivating the use of natural products as source of enzyme inhibitors due to greater structural diversity. The results showed that alkaloids fractions have the potential to treat diabetes, inflammation, gastritis, ulcers and other related disorders by acting as enzyme inhibitor.

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