

Anti- α -Glucosidase, Anti- α -Amylase and Anti-Inflammatory Effects of Leaf Extracts of *Ziziphus Spina-christi* (Sedr) Grown in Jordan

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Abstract: *Ziziphus spina-christi* is a famous plant in Jordan available in different regions. It is used traditionally for the treatment of several pathologies. The aim of this study is to investigate and compare the *in vitro* α -glucosidase, α -amylase and inflammation inhibitory activities for different concentration of methanolic and ethanolic leaf extracts of *Z. spina-christi*. Fresh leaves of *Z. spina-christi* were collected and subjected to methanolic and ethanolic extraction and were investigated for their anti- α -glucosidase, anti- α -amylase and anti inflammatory activities. Methanolic extract seems to be a strong potent in both enzymes inhibitory potential compared to ethanolic. The calculated IC_{50} were 8.9 and 305.6 μ g/ml against α -glucosidase activity and 39.12 and 318.4 μ g/mL against α -amylase activity for methanolic and ethanolic leaf extracts, respectively. Anti-inflammatory activity, using protein denaturation method, showed that methanolic extract exhibited a highest anti inflammatory effect compared with ethanolic extract and the standard. At the concentration of 100 μ g/mL, the anti inflammatory effect were 95.3, 25.2 and 20.2% for methanolic extract, ethanolic extract and standard diclofenac sodium, respectively. This study support and agree with the traditional usage of plants of *Z. spina christi* for the control of diabetes and inflammation.

Key words: *Ziziphus spina-christi*, anti- α -glucosidase, anti- α -amylase, anti inflammation, extract, diabetes, inflammation

INTRODUCTION

Since, ancient times, medicinal plants are being used in Jordanian traditional medicinal system for the treatment of numerous human diseases. The medicinal values of plants lie in their phytochemical components which produce definite physiological actions on the human body (Afifi and Kasabri, 2013). The most important of these components are alkaloids, tannins, flavonoid and phenolic compounds (Adzu and Haruna, 2007). *Ziziphus spina-christi* also, known as “Sedr” or “Nabak” is a medicinal plant largely found in the mediterranean region including Jordan (Abu-Hamdah *et al.*, 2005). Different parts of this plant is consumed by local population for the treatment of several pathologies such as cough, malaria, wounds toothache and rheumatic diseases, Alzheimer disease, disease related to Reactive Oxygen Species (ROS) formation, bacterial infection, diabetes and inflammation. The medicinal properties of this plant depend on the part of the plant and the extract type (Asgarpanah and Haghighat, 2012; Khaleel *et al.*, 2016).

Published surveys indicated that more than 1200 plants are used worldwide in traditional medicine for their alleged hypoglycemic activity (Grover *et al.*, 2002). Despite the recommendations by World Health

Organization for further investigation, most of these plants are being used for medical purposes without proper scientific validation (Jung *et al.*, 2006). Most useful drugs derived from plants have been discovered by follow-up of ethno medical uses (Fabricant and Farnsworth, 2001). For instance, many plants are used for diabetes due to the fact that they are natural, effective inexpensive and safe when compared to synthetic hypoglycaemic drugs (Modak *et al.*, 2007).

Diabetes Mellitus (DM) is the most common metabolic disorder affecting millions worldwide. It is recognized as a global major health problem. According to the report of WHO, the number of people with diabetes in 1980 (108 million) has risen to 422 million in 2014 (WHO., 2016). It is reported that, diabetes will be the 7th leading cause of death in 2030 (Mathers and Loncar, 2006). DM is a chronic endocrine disorder that affects the metabolism of carbohydrates, proteins, fat, electrolytes and water. It includes a group of metabolic diseases characterized by hyperglycemia in which blood sugar levels are elevated either because the pancreas do not produce enough insulin or cells do not respond to the produced insulin (Liamis *et al.*, 2014). Primary goal in the management of diabetes is to regulate the blood glucose concentrations to prevent chronic diabetic complications such as retinopathy, nephropathy and cardio vascular

diseases (Sheard *et al.*, 2004). Inhibiting of carbohydrate hydrolyzing enzymes like α -amylase and α -glucosidase is one of the therapeutic approaches to lower blood glucose concentration by decreasing the postprandial rise in the blood glucose (Bojarova and Kren, 2009; Fred-Jaiyesimi *et al.*, 2009).

α -Amylase is secreted by the pancreas and the salivary glands. It is a key enzyme in the carbohydrate digestion which catalyses the initial hydrolysis of starch by acting on the interior α -D-1,4 glucosidic linkages. α -Amylase converts starch in to dextrins, maltose and maltotriose (Sales *et al.*, 2012). α -Glucosidase is an intestinal brush border enzyme. It catalyzes the liberation of absorbable monosaccharides such as glucose from the substrate which eventually facilitates the absorption by the small intestine (Kumar *et al.*, 2011). Inhibition of these enzymes delays carbohydrate digestion, decreasing the rate of glucose absorption and therefore blunting the post-prandial plasma glucose rise. α -Amylase and α -glucosidase inhibitors are the potential targets in the development of lead compounds for the treatment of diabetes (Bojarova and Kren, 2009). However, the current used synthetic enzyme inhibitors cause gastrointestinal side effects such as bloating, abdominal discomfort, diarrhea and flatulence, making them less attractive as therapeutic agents (Kimmel and Inzucchi, 2005). On the other hand, natural α -amylase and α -glucosidase inhibitors from the dietary plants have minimal side effects, so, they can be used as an effective therapy for treating post prandial hyperglycemia (Nair *et al.*, 2013; Hamza *et al.*, 2015; Zaklos-Szyda *et al.*, 2015).

Inflammation is a normal protective response to tissue injury and it involves a complex array of enzyme activation, mediator release, fluid extravasations, cell migration, tissue breakdown and repair. It is a complex process which is frequently associated with pain and involves occurrences such as increase in vascular permeability increase of protein denaturation and membrane alterations (Vane and Botting, 1995). Many non-steroidal anti-inflammatory drugs are routinely prescribed for the management of inflammatory conditions but their use is associated with many undesirable side effects such as gastric irritation, ulcer, etc. (Umapathy *et al.*, 2010; De *et al.*, 2017). Different studies reported associations between elevated (but 'normal range') levels of circulating acute phase inflammatory markers and indices of insulin resistance and the development of type 2 diabetes mellitus (Greenfield and Campbell, 2006; Luft *et al.*, 2013; Jung *et al.*, 2014). On the other hand, different studies reported that medical plants can be used to treat anti-inflammatory conditions and they are safe and effective (De *et al.*, 2017).

Z. spina-christi is a plant, widely available in Jordan, used traditionally to treat diabetes. The aim of this study is to investigate and compare the *in vitro* α -glucosidase, α -amylase and inflammation inhibitory activities for different concentration of methanolic and ethanolic leaf extracts of *Z. spina-christi*. *Z. spina-christi* is widely available, so, it is a reliable source to manage and control diabetes and other diseases.

MATERIALS AND METHODS

Sample collection: Fresh leaves of *Ziziphus spina-christi* were collected from different locations of Jordan valley. The collected leaves were rinsed with distilled water to remove any particulate matter and dried at room temperature for a few days.

Preparation of *Ziziphus spina-christi* leaf extracts: The dried powdered leaves (500 g) were extracted by maceration with absolute ethanol or methanol at 20% (w/v) concentration for 2 days at the room temperature with occasional shaking. The mixtures were filtered through whatman no: 4 and then membrane filter (0.45 μ m). The excess solvents from the filtrate were evaporated under vacuum using a rotary evaporator (Buchi, Switzerland) at 40°C. The crude concentrated extract was transferred to brown colored sample vial and stored in a refrigerator until used (Khaleel *et al.*, 2016).

α -glucosidase inhibition assay: The enzymatic activity of α -glucosidase (EC 3.2.1.20) was determined colorimetrically by monitoring the release of p-Nitrophenol from appropriate P-Nitro Phenol Glycoside (PNPG) substrate. The assay was performed using a modified protocol by Ghareeb *et al.* (2014). A sample of 100 μ L from plant extract (25, 50, 100, 250 and 500 μ g/mL) (sample) organic solvents (control) or dH₂O (blank) was diluted with 2.5 mL and 0.1 M phosphate buffer with pH value of 7.4. An equal amount (100 μ L) of purified yeast α -glucosidase (Cat. No. G 5003, Sigma Aldrich Chemical Co, USA) was added, mixed well and incubated in a water bath with the reaction mixture at 30°C for 5 min. Next, 500 μ L PNPG (5 mM) was added and the reaction was allowed to proceed for 15 min. The reaction was then stopped by the addition of 2 mL of 1 M Na₂CO₃. The produced color was spectrophotometrically detected at 400 nm. A unit of enzyme activity was defined as the released n moles of p-nitrophenol/min. α -Glucosidase inhibition activity was calculated using the following equation:

$$\alpha\text{-glucosidase inhibition activity (\%)} = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}} \times 100}$$

Where:

A control = The absorbance of control reaction (without plant extract)

A sample = The absorbance in the presence of a plant extract

The inhibitory concentration of the extract required to inhibit the activity of the enzyme by 50% (IC_{50}) was calculated by regression analysis. Experiments were performed in triplicate for statistical analysis.

α -amylase inhibition assay: α -amylase (EC 3.2.1.1) inhibition assay was carried out according by Xiao *et al.* (2006) with a slight modification based on the starch-iodine test. Ethanolic or methanolic extracts from the *Ziziphus* at varied concentrations (25, 50, 100, 250 and 500 μ g/mL) in 500 μ L were added to 500 μ L of 0.02 M sodium phosphate buffer (pH 6.9 containing 6 mM sodium chloride) containing 0.04 units of porcine α -amylase (Cat. No. 10080, Sigma Aldrich Chemical Co, Steinheim, Germany) solution. The mixed solution was incubated at 37°C for 10 min, then 500 μ L of the soluble starch (1%, w/v) was added to each reaction and incubated at 37°C for 15 min. One M HCl (20 μ L) was added to stop the enzymatic reaction, followed by the addition of 100 μ L of iodine reagent (5 mM I_2 and 5 mM KI). The color change was noted and the absorbance was read at 620 nm. The control reaction representing 100% enzyme activity did not contain any plant extract. To eliminate the absorbance produced by plant extract, an appropriate extract controls without the enzyme were also included. Percentage of inhibition was calculated using the equation:

$$\alpha\text{-amylase inhibition activity (\%)} = \frac{1 - [\text{Abs2} - \text{Abs1} / \text{Abs4} - \text{Abs3}]}{1} \times 100$$

Where:

Abs1 = The absorbance of the incubated mixture containing plant sample, starch and amylase

Abs2 = The absorbance of incubated mixture of sample and starch

Abs3 = The absorbance of the incubated mixture of starch and amylase

Abs4 = The absorbance of incubated solution containing starch

IC_{50} was calculated by regression analysis. Experiments were performed in triplicate.

Evaluation of *in-vitro* anti-inflammatory activity: Anti-inflammatory activity of the *Z. spina-christi* extracts

were evaluated by protein denaturation method (Alhakmani *et al.*, 2014). The reaction mixture consisting of 2 mL of plant extract at different concentrations (50, 75 and 100 μ g/mL) or standard diclofenac sodium and 2.8 mL of phosphate buffered saline (pH 7) was mixed with 2 mL of egg albumin (from fresh hen's egg) and incubated at 27°C for 15 min. Denaturation was induced by keeping the reaction mixture at 7°C in a water bath for 10 min. After cooling, the absorbance was measured at 660 nm by using double distilled water as blank. Each experiment was done in triplicate and the average was taken. The percentage inhibition of protein denaturation was calculated by using the following equation:

$$\text{Protein denaturation inhibition activity (\%)} = \frac{(\text{A sample} - \text{A control}) / \text{A control}}{1} \times 100$$

Where:

A sample = The absorbance in the presence of a plant extract

A control = The absorbance of control reaction (without plant extract). Experiments were performed in triplicate

Statistical analysis: All experiments were performed in three different sets each in triplicates ($n = 3$). Results were represented using the mean and Standard Deviation (SD). Statistical difference using Student's t-test and linear regression analysis were performed using SPSS Statistical Software (Version 10). Results were statistically significant if $p < 0.05$. The half-maximal Inhibitory Concentration (IC_{50}) values were determined from linear regression plots of percent inhibition versus inhibitor concentration of the mean inhibitory values.

RESULTS AND DISCUSSION

***In vitro* α -glucosidase inhibitory effect:** The percentage α -glucosidase inhibition (%) of *Z. spina christi* ethanolic and methanolic leaf extracts at varying concentrations are shown in Table 1. The results revealed that both extracts were able to inhibit α -glucosidase and their inhibitory pattern was dose-dependent. The methanolic extract showed a strong inhibitory potential with percentage inhibitions ranging from 98.2-51.9% for concentrations ranging from 500-25 μ g/mL ($p < 0.05$). Accordingly, the half-maximal Inhibitory Concentration (IC_{50}) of methanolic and ehanolic extracts were found to be 8.9 and 305.6 μ g/mL, respectively.

***In vitro* α -amylase inhibitory effect:** Table 2 illustrates the inhibitory activity of *Z. spina christi* ethanolic and

Table 1: *In vitro* α -glucosidase inhibitory effect of ethanolic and methanolic leaf extracts of *Z. spina christi*

Inhibition of α -glucosidase (%)			
Concentration ($\mu\text{g/mL}$)	Ethanolic extract (mean $\pm$ SD)	Methanolic extract (mean $\pm$ SD)	*p-values
25	5.1 $\pm$ 2.0	51.9 $\pm$ 5.7	0.0000
50	9.3 $\pm$ 1.8	55.5 $\pm$ 5.4	0.0000
100	17.4 $\pm$ 3.9	62.5 $\pm$ 7.9	0.0003
250	41.7 $\pm$ 3.7	82.3 $\pm$ 7.7	0.0006
500	81.1 $\pm$ 8.3	98.2 $\pm$ 1.9	0.0320

*p<0.05 is significant

Table 2: *In vitro* α -amylase inhibitory effect of ethanolic and methanolic leaf extracts of *Z. spina christi*

Inhibition of α -amylase (%)			
Concentration ($\mu\text{g/mL}$)	Ethanolic extract (mean $\pm$ SD)	Methanolic extract (mean $\pm$ SD)	*p-values
25	7.1 $\pm$ 3.3	10.5 $\pm$ 2.6	0.1240
50	12.2 $\pm$ 2.8	32.9 $\pm$ 6.9	0.0042
100	13.9 $\pm$ 2.8	72.8 $\pm$ 6.1	0.0000
250	44.6 $\pm$ 5.7	83.9 $\pm$ 6.4	0.0000
500	75.3 $\pm$ 13.0	91.4 $\pm$ 3.2	0.0551

*p<0.05 is significant

methanolic leaf extracts at varying concentrations against α -amylase activity. In both extracts studied, ethanolic extract is less potent in α -amylase inhibitory potential compared to methanolic. The percentage inhibition of α -amylase activity at 25, 50, 100, 250 and 500 $\mu\text{g/mL}$ concentrations of the plant extracts ranged from 7.1-75.3 and 10.5-91.4% for ethanolic and methanolic extracts, respectively. The calculated IC_{50} values differ significantly ($p<0.05$) and were found to be 39.12 and 318.4 $\mu\text{g/mL}$ for methanolic and ethanolic extracts, respectively.

***In-vitro* anti-inflammatory activity:** The inhibitory effect of different concentrations of ethanolic and methanolic leaf extracts of *Z. spina christi* on protein denaturation are shown in Fig. 1. The *in vitro* anti-inflammatory activity of the extracts was comparable to the reference drug (diclofenac sodium). A significant difference in the inhibition of thermally induced protein denaturation was observed in case of methanolic extract when compared to the ethanolic extract and the standard at the same concentrations of 50, 75 and 100 $\mu\text{g/mL}$. At the concentration of 100 $\mu\text{g/mL}$, methanolic extract showed the highest anti inflammatory effect (95.3%), followed by ethanolic extract (25.2%) and the standard diclofenac sodium (20.2%). No significant differences were noticed between ethanolic extract and diclofenac sodium at any concentration.

The present study is aimed to investigate and compare the α -glucosidase, α -amylase and inflammation inhibitory activities for different concentrations of the methanolic and ethanolic leaf extracts of *Z. spina-christi* grown in Jordan.

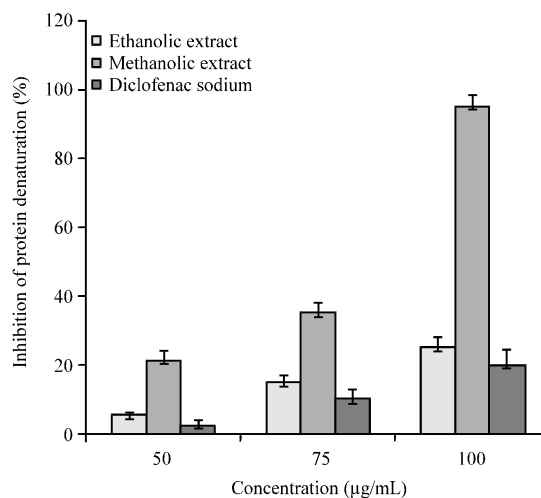


Fig. 1: *In vitro* anti inflammatory effect of ethanolic and methanolic leaf extracts of *Z. spina christi* by protein denaturation method. Values are mean $\pm$ SD, n = 3

The results of this *in vitro* study revealed that both methanolic and ethanolic leaf extracts of *Z. spina-christi* inhibited α -glucosidase (Table 1) and α -amylase (Table 2) enzymes activities in a dose-dependent manner. However as revealed by the IC_{50} values, methanolic extract seems to be a strong potent in both enzymes inhibitory potential compared to ethanolic. The calculated IC_{50} values differ significantly ($p<0.05$) and were found to be 8.9 and 305.6 $\mu\text{g/mL}$ against α -glucosidase activity and 39.12 and 318.4 $\mu\text{g/mL}$ against α -amylase activity for methanolic and ethanolic leaf extract, respectively. The differences between two types of extracts may be attributed to the nature of the extracting solvent as well as the chemical nature and availability of the compounds extracted (Felhi *et al.*, 2017). As it was reported previously, the presence of various bioactive compounds with different chemical characteristics and polarities may or may not be soluble in a particular solvent (Turkmen *et al.*, 2006). For example, ethanol has been known as a good solvent for polyphenol extraction and is safe for human consumption whereas methanol has been generally found to be more efficient in extraction of lower molecular weight polyphenols (Dai and Mumper, 2010).

Our findings with *Z. spina christi* leaf extracts on α -amylase and α -glucosidase inhibition agreed with those of a previous studies. Gholamhoseinian *et al.* (2008) demonstrated that methanolic extract from fruits hull of *Pistacia vera* has strong inhibitory effect (97%) on the α -glucosidase whereas other extracts had moderate potency. Another study for Jung *et al.* (2014) reported that methanolic extracts of green and red *Kohlrabi*

cultivars and their different fractions showed potent anti-diabetic, anti-inflammatory and inhibitory activity and is therefore, a promising candidate for DM treatment. While, ethanolic extract of *P. marsupium* showed a very high inhibitory activity against porcine pancreatic α -amylase (IC_{50} 5.16 μ g/ mL) and yeast α -glucosidase (IC_{50} =1.06 μ g/ mL) (Gulati *et al.*, 2012). The results of the lower inhibitory effects observed with *T. cordifolia* leaves from previous study did not match the findings in this study. Only 16% inhibition was obtained even with 1 mg/mL methanol extract of *T. cordifolia* (Poongunran *et al.*, 2015).

Inhibition of α -glucosidase and α -amylase, enzymes involved in the digestion of carbohydrates, can significantly decrease the postprandial increase of blood glucose after a mixed carbohydrate diet, so it can be an important strategy in the management of postprandial blood glucose level in type 2 diabetic patients and border line patients (Ali *et al.*, 2006; Bojarova and Kren, 2009). However, the drugs available currently as inhibitors of α -amylase and α -glucosidase show gastrointestinal side effects such as bloating, abdominal discomfort, diarrhoea and flatulence making them less attractive as therapeutic agents (Kimmel and Inzucchi, 2005; Olaokun *et al.*, 2013). Recently, plant secondary metabolites have been investigated as a natural source of alternative drugs for the treatment of various ailments such as DM because of their reduced toxicity (Nair *et al.*, 2013; Hamza *et al.*, 2015; Zaklos-Szyda *et al.*, 2015).

The results of this study are consistent with previous studies which have been performed yielding a potential α -glucosidase inhibitors from various food components and plants, like *C. nutans*, *C. formosana* and *H. diffusa* (Wong *et al.*, 2014), red and white ginger (Obboh *et al.*, 2010) and α -amylase inhibitors from cranberry extract (Apostolidis *et al.*, 2006), *F. racemosa* stem bark, *P. emblica* fruit, *P. debilis* whole plant and *P. marsupium* latex showed higher inhibitory effects on both α -amylase and α -glucosidase activities (Wong *et al.*, 2014), etc. Therefore, natural α -glucosidase and α -amylase inhibitors from plant sources offer an attractive strategy for the control of postprandial hyperglycemia.

Furthermore, the ability of the two extracts to inhibit inflammation *in vitro* was evaluated against heat induced protein denaturation (Fig. 1). The present findings exhibit concentration dependant anti protein denaturation by the methanolic leaf extracts of *Z. spina christi* (95.3%). And though the ethanolic extract showed a moderate inhibitory effect (25.2 %) its activity was found to be better than the standard non-steroidal anti-inflammatory drug diclofenac sodium (20.2%). Denaturation of proteins is well documented and is caused by inflammation process,

mostly in conditions like arthritis (Umapathy *et al.*, 2010; Alhakmani *et al.*, 2014). Thus, protection against protein denaturation, may play an important role in the antirheumatic activity of *Z. spina christi*.

The result of this study supported by similar previous studies on *Aloe vera*, *Bacopa monnieri*, *Moringa oleifera* and rhizome of *Zingiber officinale* (Padmanabhan and Jangle, 2012) and *Phyllanthus amarus* (Chopade *et al.*, 2012). Gambhire *et al.* (2009) reported that methanol extract of *Murraya koenigi* leaves produces significant anti-inflammatory activities in dose dependent manner in membrane stabilization and inhibition of protein denaturation. Umapathy *et al.* (2010) confirmed that aqueous extract of *Albuca setosa* possess membrane stabilization properties, limiting protein denaturation process and white blood cell anti-migration properties.

Qualitative analysis revealed presence of several phytochemicals like alkaloids, flavonoids, polyphenols, sterols, carbohydrate and tannin in various amount in most *Ziziphus* species (Elaloui *et al.*, 2016). Among these bioactive compounds several have well known potential biological properties. The anti α -glucosidase, anti α -amylase and anti inflammatory properties of *Z. spina christi* may be due to the presence of these bioactive compounds.

CONCLUSION

The search for α -glucosidase, α -amylase and inflammation inhibitors in plants give rise to reliable, cheap and safe medicine in the management and control of diabetes and other diseases. *Z. spina-christi* is a famous plant in Jordan is used traditionally for the treatment of several pathologies such as cough, malaria, wounds toothache and rheumatic diseases, Alzheimer disease, disease related to Reactive Oxygen Species (ROS) formation, bacterial infection. This study investigated and reported scientific evidence for the usage of the *Z. spina-christi* to control diabetes and inflammation.

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