Study of alpha-amylase and urease inhibitory activities of *Melilotus indicus* (Linn.) All.

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Abstract: *Melilotus indicus* (Linn.) All. is a small herb distributed throughout Pakistan and has a number of ethnomedicinal uses. It is also consumed as a vegetable. In the present work, we are reporting the alpha-amylase and urease inhibitory activities of methanolic extract of *M. indicus* and its sub-fractions in different solvents. Both the methanolic extract and its fractions in chloroform, ethyl acetate, n-butanol and water showed remarkable inhibitory activities against alpha-amylase with the IC_{50} values being 1.29, 1.45, 1.07, 1.45 and 2.10 mg/mL respectively. The efficacy of the methanolic extract was comparable with that of acarbose (1.20 mg/mL), while the ethyl acetate fraction was more potent. The urease inhibitory activities of methanolic extract and chloroform, ethyl acetate, n-butanol and water fractions were more prominent with IC_{50} values being 0.95, 0.89, 1.53, 0.98 and 4.90 µg/mL respectively. The activity of methanolic extract was slightly higher than that of thiourea (0.97 µg/mL) which in turn was slightly higher than that of n-butanolic fraction. The chloroform fraction showed the highest anti-urease activity. All the plant samples showed enzyme inhibitory activity in a dose-dependent manner. Moreover, they were manifold more effective against urease than alpha-amylase. The combination of the plant extract with acarbose considerably increased the potency of the latter. The findings suggest that enzyme inhibitory activities of the vegetable *M. indicus* may have pharmacological significance against diabetes mellitus and gastrointestinal ulcers.

Keywords: *Melilotus indicus*, alpha-amylase, urease, inhibitory activities.

INTRODUCTION

The genus Melilotus (family Fabeaceae) comprises about 25 species, and the plant *M. indicus* (also called *M. indica)* is found in many parts of the world including Pakistan, India, Europe and Africa (Ahmed *et al*., 2012; Al Sherif, 2008). The plant, which in Pakistani local languages is called Sinji/Sinjhi (Qureshi *et al*., 2008; Memon *et al*., 2008), is a small herb found in agricultural farms, grassy grounds and lawns, and many people use it as vegetable. The flowering period of the *M. indicus* is March-August. It is known to possess antibacterial, anticoagulant, astringent, emollient, laxative, and narcotic properties. It is also used in infantile diarrhea, and is externally applied as poultice or plaster on swellings (Qureshi *et al*., 2008). A number of secondary metabolites have been isolated from the herb including terpenoids, flavonoids, coumarins, steroids and glycosides (Ahmed *et al*., 2012).

Enzyme inhibition is now an important part of the modern drug discovery research. Alpha-amylase and urease are two important enzymes that are associated with a number of clinical conditions. Inhibition of alpha-amylase has been shown to reduce the bioavailability of glucose (Payan, 2004). It is therefore considered one of the important strategies to control hyperglycemia (Ali *et al*., 2006). Since type II diabetes is anticipated to be one of the major human health concerns in the 21st century (Odhav *et al*., 2010), discovery of safer, cheaper and more effective alpha-amylase inhibitors is crucial. Synthetic alpha-amylase inhibitors are available but they cause harmful side effects (Fred-Jaiyesimi *et al*., 2009). Edible vegetables and fruits that can inhibit alpha-amylase, thus, provide a better strategy to control postprandial hyperglycemia (Sudha *et al*., 2011; Kasote *et al*., 2011).

The microorganism *Helicobacter pylori* that is one of the main causes of gastrointestinal ulcers, produces urease as mechanism to survive in the acidic environment of stomach. It has been estimated that more than 50% of the world population is infected with *H. pylori* worldwide (Logan and Walker, 2001). Since inhibition of urease activity can terminate *H. pylori* infection, it is considered to be a promising therapy for ulcer (Arfan *et al*., 2010; Hassani *et al.*, 2009). Urease inhibitors are also important to suppress activity of microbial urease, which converts fertilizers urea into ammonia and is a cause of great economic and environmental losses (Giocchini *et al.* 2002).

In view of its consumption as a vegetable, we planned to investigate *Melilotus indicus* for its possible alpha-amylase and urease inhibitory activities. As long as we could explore, this study has not yet been conducted on this plant. We evaluated alpha-amylase and urease inhibitory activities of the methanolic extract of the herb and its sub-fractions in hexane, chloroform, ethyl acetate, n-butanol and water.

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MATERIALS AND METHODS

Plant collection and extract preparation

The areal parts of the herb *Melilotus indicus* (Linn.) All. were collected from Hazara region of Pakistan, in February, 2010. The plant material was dried under shade for 15 days, and the dried material was ground to obtain a powder. The dried powder (200 g) was soaked in 100% methanol (1500 mL) and kept at room temperature for two weeks to ensure maximum extraction. The extract was filtered, and the solvent was evaporated on a rotary evaporator under reduced pressure at 30°C to get the crude methanolic (MeOH) extract (9.01 g). The MeOH extract was then suspended in water (20 mL) and sequentially fractionated with solvents of increasing polarity, i.e., hexane, ethyl acetate (EtOAc), chloroform ($CHCl_3$) and n-butanol (n-BuOH) respectively. Each fraction was then dried under reduced pressure and weighed.

Chemicals and apparatus

Urease (Jack Bean) was purchased from Avonchem, alpha-amylase (*Aspergillus oryzae*) from Unichem and acarbose from Baeyer, Pakistan. All other chemicals used in the study were of analytical grade. UV-Visible Spectrophotometer UVD-3200 Labomed, Inc. was used to determine absorbance. Rotavapor R-210/A (Buchi) was used for evaporation of solvents at low pressure.

Alpha-amylase inhibitory assay

Alpha-Amylase inhibitory activities in our study were determined using the procedure reported by Nickavar and Yousefian (2009), which was originally proposed by Bernfeld (1955). Briefly, 1 mg of alpha-amylase was dissolved in 100 mL of 20 mM phosphate buffer (pH 6.9) to prepare the enzyme solution. The solutions of the plant extract/ fractions were prepared in DMSO to give concentrations from 0.1-3.6 mg/mL. The starch solution (0.5% w/v) was used as substrate and was prepared by boiling potato starch in distilled water for 15 min. The DNS solution (20 mL 96 mM 3,5-dinitrosalicylic acid, 12 g sodium potassium tartrate in 8 mL of 2 M sodium hydroxide and 12 mL deionized water) was used as the coloring reagent. In a test tube, 0.5 mL of a plant sample was mixed with 0.5 mL of enzyme solution and the mixture was incubated at 25°C for 30 min. After adding 1 mL of the starch solution, the mixture was again incubated at 25°C for 3 min. Then, 1 mL of the DNS solution was added and the mixture was heated in a water bath at 85°C for 15 min. Then, after cooling the tube, the mixture was diluted with 9 mL of distilled water. It was thoroughly mixed and the absorbance was recorded at 540 nm. For the blank, the method was same except that the DNS solution was added before the addition of starch solution. For the control, the plant extract was replaced with the same volume of DMSO. Acarbose was used as positive control / standard. The percentage enzymatic inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = [(A_c - A_s) / A_c] \times 100$$

In the formula, A_c and A_s are the absorbances of control and sample respectively. To calculate the IC_{50} (the concentration of a sample required to inhibit the activity of a given enzyme by 50%) value for each sample, the %inhibition was plotted against the sample concentration and a logarithmic regression curve was established.

Urease inhibitory assay

Urease inhibitory activities of the methanolic extract of *Melilotus indicus* and its sub-fractions in different solvents were determined according to a reported method (Berthelot, 1859; Ghous *et al.*, 2010). The given plant extract or fraction was dissolved in 25 mL of phosphate buffer (pH 7.0) and stored at 4°C. Different concentrations (0.1-20 µg/mL) of plant samples were prepared. One mL of a sample or standard was taken in a test tube and 15 µL of urea (0.08 g/mL), 485 µL of phosphate buffer and 2.5 mL of Reagent 1(consisting of phosphate buffer 120 mmol/L, sodium salicylate 60 mmol/L, sodium nitroprusside 5 mmol/L, EDTA 1 mmol/L, and urease 5 mg/L) was mixed. The mixture was incubated at 37°C for 5 min, and 2.5 mL of Reagent 2 (consisting of phosphate buffer 120 mmol/L, sodium hydroxide 400 mmol/L, and sodium hypochlorite 10 mmol/L) was mixed. Then, after incubating the reaction mixture at 25°C for 10 min, absorbance was measured at 625 nm against a blank. The blank contained 500 µL of buffer and 2.5 mL of Reagent 1 and without prior incubation of the mixture, 2.5 mL of Reagent 2 was added. The control contained all the reagents except the sample. The percent inhibition was determined using the following formula:

$$\% \text{ Urease Inhibition} = [(A_c - A_s)/A_c] \times 100$$

Here A_c and A_s are the absorbances of the control and the sample respectively. Thiourea was used as positive control. The IC_{50} values for urease inhibition were calculated by the same method used for the calculation of alpha-amylase.

STATISTICAL ANALYSIS

Each experiment was performed in triplicate and the results were expressed as mean of three readings. One way ANOVA was applied and the results were correlated.

RESULTS

Alpha-amylase inhibitory assay

Alpha-amylase inhibitory activity of the plant samples of *Melilotus indicus* was analyzed according to a reported method (Bernfeld, 1955; Nickavar and Yousefian, 2009) with slight modification and the results are shown in the table 1. In this method, starch was used as a substrate, which was converted by alpha-amylase into maltose which in turn reacted with 3,5-dinitrosalicylate to form colored product (λ_{max} 540 nm). The methanolic extract of

M. indicus and its fractions in different solvents exhibited remarkable alpha-amylase inhibitory activity, which was dose dependent. The alpha-amylase inhibitory activity of methanolic extract of *M. indicus* was comparable to that of acarbose, having IC_{50} values 1.29 and 1.20 mg/mL respectively.

Urease inhibitory assay

Urease, an enzyme that contains nickel, is found in plants, fungi and bacteria. In this work, the urease inhibitory activity of the plant samples was evaluated using a reported method (Berthelot, 1859; Ghous *et al*., 2010) and the results are exhibited in table 2. The reaction that occurs as per the following equations is monitored spectrophotometrically (λmax, 625 nm).

$$Urea + H_2O \xrightarrow{Urease} 2NH_3 + CO_2$$

$$NH_3 + Salicylate \xrightarrow[NaOCl\,/\,OH^-]{Nitroprusside} Dicarboxy\ indophenol$$

The methanolic extract displayed slightly higher potential to inhibit urease than thiourea with IC_{50} values being 0.95 and 0.97 µg/mL respectively. Among the sub-fractions, the chloroform fraction was most powerful with IC_{50} value being 0.89 µg/mL. It was more powerful than thiourea. The urease inhibitory activity of n-butanolic fraction was also good and almost equal to that of the standard. The aqueous fraction showed the lowest ability to inhibit urease activity.

Table 1: Percent alpha-amylase inhibitory activities of methanolic extract of *Melilotus indicus* and its fraction in different solvents as a function of concentration using the drug acarbose as positive control

Conc. mg/mL*	Plant extract / fractions					Standard
	Methanol	Chloroform	Ethyl acetate	n-Butanol	Aqueous	Acarbose
0.10	01.02± 0.31	---------	02.65±0.15	01.83±0.72	---------	03.53±0.40
0.20	09.06±0.29	05.88±0.11	10.23±0.39	08.13±0.19	06.14±0.28	14.11±0.17
0.40	18.76±0.09	13.2±0.21	22.47±0.38	14.43±022	10.45±0.34	20.63±0.34
0.60	26.31±0.24	24.15±0.11	34.56±0.28	21.78±0.37	20.32±0.19	29.72±0.48
0.80	38.98±0.46	32.46±0.37	43.71±0.31	31.64±0.45	27.79±0.09	37.44±0.15
1.18	43.51±0.52	39.65±0.46	56.89±0.11	42.21±0.44	31.05±0.23	45.51±0.34
1.47	55.51±0.35	51.07±0.41	68.31±0.26	50.27±0.24	38.66±0.12	54.89±0.37
1.84	63.85±0.39	61.55±0.22	75.17±0.32	66.12±0.21	46.07±0.36	66.77±0.46
2.30	71.87±0.38	66.24±0.31	82.21±0.12	71.95±0.38	59.98±0.24	78.00±0.21
2.80	80.33±0.49	69.47±0.09	89.59±0.42	78.11±0.33	70.24±0.27	86.90±0.25
3.60	89.79±0.31	73.61±0.32	90.12±0.51	84.59±0.46	82.37±0.21	93.88±0.39
IC_{50} (mg/mL)	1.29	1.45	1.07	1.45	2.10	1.20

*Concentrations were selected based on method reported by Nickavar and Yousefian (2009).

Table 2: Percent urease inhibitory activities of methanolic extract of *Melilotus indicus* and its fractions in different solvents and standard thiourea as a function of concentration

Conc. (µg/mL)	Plant extract / fractions					Standard
	Methanol	Chloroform	Ethyl acetate	n-Butanol	Aqueous	Thiourea
0.1	02.61± 0.78	09.24±0.09	----------	03.60±0.31	-------	03.87±0.23
0.2	12.93±0.32	17.65±0.13	09.21±0.34	14.34±0.12	05.28±0.41	16.45±0.40
0.4	23.56±0.29	24.06±0.48	21.56±0.28	25.12±0.46	13.34±0.20	28.68±0.36
0.6	32.11±0.40	40.65±0.26	30.91±0.37	30.34±0.21	22.61±0.37	39.83±0.17
0.8	40.76±0.34	47.07±0.33	40.74±0.19	44.42±0.53	28.45±0.15	46.09±0.33
1.0	51.23±0.27	54.19±0.38	41.04±0.33	53.98±0.39	36.09±0.19	51.04±0.24
2.0	61.46±0.46	62.37±0.30	51.85±0.09	59.41±0.25	43.73±0.09	55.21±0.16
4.0	65.26±0.34	69.78±0.14	58.32±0.27	63.03±0.40	49.41±0.12	59.45±0.26
6.0	69.56±0.19	71.78±0.08	62.92±0.31	69.27±0.48	56.66±0.43	64.23±0.22
8.0	74.32±0.36	76.73±0.36	68.05±0.26	74.09±0.36	59.62±0.32	70.23±0.37
10	80.89±0.37	81.93±0.33	71.86±0.13	78.13±0.47	63.38±0.14	74.98±0.18
12	84.26±0.40	83.58±0.21	74.39±0.35	81.81±0.40	67.51±0.35	79.67±0.41
14	88.08±0.16	87.72±0.30	76.72±0.40	85.48±0.31	70.58±0.10	83.61±0.33
16	91.26±0.26	92.88±0.43	79.71±0.21	88.29±0.08	73.61±0.32	89.50±0.45
18	93.61±0.11	94.11±0.12	82.39±0.31	90.77±0.11	77.83±0.34	92.13±0.41
20	95.35±0.15	96.36±0.32	86.74±0.22	92.39±0.29	80.29±0.33	95.03±0.17
IC_{50} µg/mL	0.95	0.89	1.53	0.98	4.90	0.97

DISCUSSION

Alpha-amylase is an important enzyme responsible for hydrolysis of dietary starch in our body. Inhibition of this enzyme can help in keeping the blood glucose level low. As a result, alpha-amylase inhibition can contribute in controlling type II diabetes and obesity. *Melilotus indicus* showed excellent anti-amylase activity. Ethyl acetate fraction was most potent among all the fractions with IC_{50} value being 1.07 mg/mL. It exhibited higher inhibitory power than acarbose, a common alpha-amylase inhibitory drug. The high anti-amylase activity of ethyl acetate fraction may in part be due to the presence of phenolics (Ahmed *et al*., 2012), which have been shown to inhibit this enzyme (Pulok *et al*., 2006; Funke and Melzig, 2005). Consumption of *M. indicus*, therefore, may play a role in managing serum glucose level and related ailments.

The enzyme urease produced by *Helicobacter pylori* in gastrointestinal track has serious clinical significance. The discovery of its inhibitors has, therefore, gained growing interest with time. The methanolic extract of *M. indicus* and its fractions in solvents of varying polarity showed considerable anti-urease activity. The highest activity of the chloroform fraction indicated the involvement of slightly polar compounds in the inhibition of urease.

The plant was more effective against urease than alpha-amylase as, for example, IC_{50} against urease was 0.95 µg/mL as compared to 1.29 mg/mL against alpha-amylase. Against both the enzymes, aqueous fraction proved to be least potent which indicated that the water soluble constituents of *M. indicus* have little ability to inhibit these enzymes (fig. 1). The combined effect of the methanolic extract and acarbose was also tested which showed pronounced synergistic effect. Other researchers have found the same trend (Randhir and Shetty, 2007).

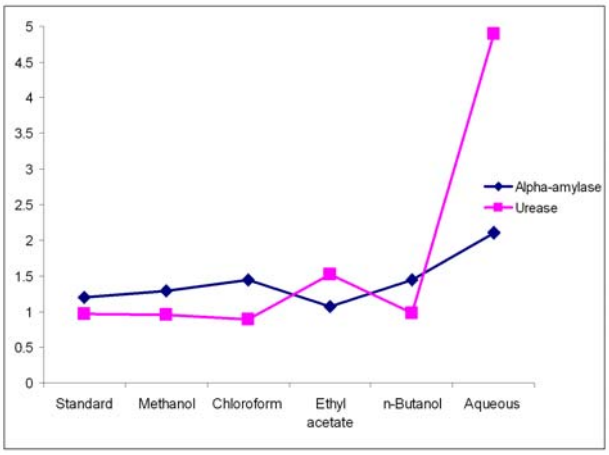

Fig. 1: Comparison of IC_{50} values of melilotus indicus methanolic extract and its fraction in different solvents against urease and alpha-amylase in units of g/ml and mg/ml respectively. IC_{50} values of the standards used are also shown.

CONCLUSION

The methanolic extract of *Melilotus indicus* and its sub-fractions in chloroform, ethyl acetate, n-butanol and water showed remarkable enzyme inhibitory activities against alpha-amylase and urease. Consumption of this edible vegetable may possibly show therapeutic efficacy against postprandial hyperglycemia and gastrointestinal ulcers. In pursuance to our *in vitro* analysis, detailed *in vivo* study and clinical trials are strongly proposed. The assay-guided phytochemical analysis might result in the isolation of effective anti-amylase and anti-urease natural products. The plant, therefore, can be a good source of new, affordable, and safer remedies for diseases related to these enzymes.

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