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An investigation of enzyme inhibitory activity of different leaf extracts of *Taxus baccata* Linn.

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Abstract
Around 80% of the world's population utilises traditional herbal medicines as stated by World Health Organization (WHO). Use of herbal remedies is on the rise in developed as well as developing countries. The current study was undertaken to investigate the leaf extracts of *Taxus baccata* Linn. For their enzyme inhibitory activity (α-amylase and urease) in three different solvents i.e. methanol, acetone and aqueous. The plant showed good α- amylase enzyme inhibition (57.40±2.12) at a concentration of 1 mg/mL in methanol extract whereas acetone and aqueous extracts showed moderate inhibition against α- amylase (46.30±0.66 and 37.90±0.80 respectively). In case of urease inhibition, methanol extract exhibited best inhibitory activity (52.40±2.10) followed by acetone and aqueous extracts at a concentration of 1 mg/mL. The plant extracts showed concentration dependent inhibition of α-amylase and urease enzymes. The inhibitory activity increased with increasing the concentration of each plant extract in the range of 0.2-1.0 mg/mL. The results further indicated that methanol leaf extracts exhibited maximum inhibitory effects than other solvent extracts. This tends to show that the active metabolites of the different plant parts are better extracted with methanol than other solvents. Therefore, the leaf extracts of *T. baccata* can be selected for further investigation to determine their ultimate therapeutic potential.

Keywords: *Taxus baccata*, leaf extract, α-amylase, urease

1. Introduction
The biological activity of plant-derived substances may be considered as a source of new anti-enzyme drugs. Therefore, traditional Indian plants which are commonly used as remedies to control different diseases were screened to discover possible plant-derived α- amylase and urease inhibitors.

α- Amylase and its inhibitors are drug design targets for the treatment of certain disorders like diabetes, obesity and hyperlipaemia (Eichler *et al*. 1984) [2]. Majority of anti-diabetic drugs presently available in the market exert their action mainly by stimulation of insulin absorption and its release from pancreas or by the inhibition of carbohydrate degrading enzymes such as α- amylase and α- glucosidase (Rang *et al*. 2003) [10]. But the usage of these commercially available drugs leads to drug resistance and many side effects after prolonged treatment (Fujisawa *et al*. 2005) [3]. To overcome these detrimental effects and identify natural inhibitors of α- amylases from plant based sources is now the primary concern of scientific research.

Ureases are important enzymes in some human and animal pathogenic states. They are responsible for kidney stones entailed in urolithiasis that contributes toward the acute pyelonephritis with other urinary tract infection through urea splitting bacteria namely *Helicobacter pylori*, *Proteus mirabilis*, *Klebsiella pnuemoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Rahman *et al*. 2003*;* Juszkiewicz *et al.* 2004) [9, 7]. Furthermore, urease contributes to arthritis and gastric intestinal infections and ultimately the urease imbalance leads to peptic ulcers (Macegoniuk, 2013) [8]. Additionally, urease inhibitors may be effective therapies for the treatment of various diseases caused by urease-dependent pathogenic microbes. However, the commercially available urease inhibitors are of low stability and toxic which prevents their clinical use (Azizian *et al*. 2012) [1]. Therefore, the search for novel urease inhibitors with improved stability and low toxicity is necessary to improve life quality of human beings and other animals.

Taxus baccata Linn. (Family: Taxaceae) is an evergreen conifer native to western, central and southern Europe, northwest Africa, northern Iran and southwest Asia. In India, the plant is distributed in the states of Jammu and Kashmir, Himachal Pradesh, Uttar Pradesh, Sikkim, West Bengal, Arunachal Pradesh, Meghalaya, Nagaland and Manipur (Shukla *et al*. 1994) [11].

All the parts of *T. baccata* except the fleshy fruits possess certain properties i.e. antispasmodic, cardiotonic, diaphoretic, emmenagogue, expectorant, narcotic and purgative. Plant contains valuable substance "Taxol" with anticancer properties. The leaves are also used internally in the treatment of various illnesses like asthma, bronchitis, hiccup, indigestion, rheumatism and epilepsy. Externally, the leaves of this plant are used in a steam bath as a treatment for rheumatism (Guenard *et al*. 1993; Gurbuz *et al*. 2004) [5, 6]. In view of its above mentioned useful properties, we planned to analyse *T. baccata* for its enzyme inhibitory activity.

2. Materials and methods
Collection of plant material
Leaves of *Taxus baccata* Linn. Were plucked and collected from Churdhar area of District Sirmaur, Himachal Pradesh, India. The collected plant material was brought to the

laboratory for further analysis.

Processing of plant material

Leaves of *T. baccata* were washed thoroughly under tap water and then with 2% Mercuric chloride. After that the leaves were cut into smaller pieces for quick drying. Cleaned leaves were shade dried for 15-20 days. The dried plant material was crushed into fine powder with the help of pestle mortar. Finally the fine powder was stored in an air tight container at room temperature.

Enzyme inhibitory activity assays

α- Amylase inhibition assay

α- amylase inhibition activity of different plant extracts was determined by some modifications in the method proposed by Giancarlo *et al*. (2006) [4]. The starch solution (1% w/v) was obtained by boiling and stirring 1 g of potato starch in 100 mL of sodium phosphate buffer for about 30 minutes. The porcine pancreatic α-amylase enzyme (EC 3.2.1.1; purchased from Sigma Aldrich-3176) was prepared by mixing 0.01 g of α-amylase in 10 mL of sodium phosphate buffer (pH 6.9) containing 0.0006 mM sodium chloride. The extracts were dissolved in DMSO to give concentrations from 0.2 to 1.0 mg/mL (0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL). The colour reagent was a solution containing 0.1 g of 3, 5-dinitrosalicylic acid plus 2.99 g sodium potassium tartrate in 0.16 g sodium hydroxide and phosphate buffer (10 mL). Fifty microliter of each plant extract and 150 µL of starch solution as well as 10 µL of enzyme were mixed in a 96 well plate and incubated at 37 ºC for 30 min. Then 20 µL of sodium hydroxide and 20 µL of colour reagent were added and the closed plate placed into a 100°C water bath. After 20 min, the reaction mixture was removed from the water bath and allowed for cooling thereafter α- amylase activity was determined by measuring the absorbance of the mixture at 540 nm using a UV-VIS spectrophotometer. Blank samples were used to correct the absorption of the mixture, in which the enzyme was replaced with the buffer solution. Also, a control reaction was used, in which the plant extract was replaced with 50 µL of DMSO and the maximum enzyme activity was determined. Acarbose solution was used as a positive control in the concentration range of 0.2-1.0 mg/mL. The assay was performed in triplicate and the mean absorbance was used to calculate percentage of α- amylase inhibition. The inhibition percentage of α- amylase was assessed by the following formula:

$$\% \text{ α- Amylase Inhibition} = \left(\frac{\Delta A control - \Delta A sample}{\Delta A control}\right) \times 100$$

Where,

$\Delta A_{control} = A_{test} - A_{Blank}$

$\Delta A_{sample} = A_{test} - A_{Blank}$

The concentration of the extract (inhibitor) required for 50% of enzyme inhibition (IC_{50}) for all the crude extracts was determined from corresponding dose-response curves of percentage inhibition versus inhibitor concentration and compared to acarbose, a known inhibitor of α-amylase. The % Inhibition was plotted against the concentration of a sample and a logarithmic regression curve was established to calculate the IC_{50} value for each sample which is the concentration of the given sample required to inhibit the activity of urease enzyme by 50%. Data were expressed as mean ± standard deviation (SD).

Urease inhibition assay

The enzyme inhibition was determined through catalytic effects of urease on urea by measuring change in absorbance in the absence and in the presence of inhibitor at 640 nm, using UV-VIS spectrophotometer. All the plant extracts were tested for their urease inhibitory activity at a concentration of 1.0 mg/mL. Herbal extracts that exerted significant inhibition, were tested in a concentration range of 0.2 to 1.0 mg/mL (0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL). For urease inhibition assays after addition of 10 mL of phosphate buffer to accurate weight of enzyme, sonication was performed for about 60 seconds, followed by centrifugation and absorbance of upper solution was measured at 280 nm. By using equation A= εbc, where c is concentration of solution (mol/L), b is length of the UV cell and ε represents molar absorptivity, one can calculate the concentration of initial urease solution. After proper dilution, the concentration of enzyme solution was adjusted to 2 mg/mL. Reaction mixture containing 1.2 mL of phosphate buffer solution (10mM potassium phosphate, 10 mM lithium chloride and 1 mM EDTA, pH 8.2 at 37ºC), 0.2 mL of urease enzyme solution, and 0.1 mL of test compound was subjected to incubation for 5 minutes. After pre-incubation 0.5 mL (66 mM) of urea was added to the reaction mixture and incubated for about 20 minutes. Urease activity was determined by measuring the ammonia released during the reaction by modified spectrophotometric method as described by Weatherburn (1967) [12]. Briefly, 1 mL each of phenol reagent (1% w/v sodium nitroprusside) and an alkaline reagent (1% w/v NaOH and 0.075% active chloride NaOCl) were added to each test tube. The control contained all the reagents except the sample. The increase in absorbance at 640 nm was measured after 30 minutes. The percent inhibition was determined using the formula:

$$\% \text{ Urease Inhibition} = \left(\frac{Ac - As}{Ac}\right) \times 100$$

Here A_s is the absorbance of the sample under study while A_c is the absorbance of the control. Each experiment was repeated thrice and average was calculated. Thiourea was used as a positive control. Data are expressed as mean ± standard deviation (SD). IC_{50} values were determined from the dose response curves.

3. Results and Discussions

In the present investigation, the leaf extracts (methanol, acetone and aqueous) of *T. baccata* were tested for their enzyme inhibitory activity against α-amylase and urease and it was observed that the plant extracts exhibited significant inhibition at concentration of 1 mg/mL as shown in Table 1. At a concentration of 1 mg/mL, the activity of α-amylase was 57.40±2.12, 46.30±0.66 and 37.90±0.80% for methanol, acetone and aqueous extract respectively. The inhibitory activity increased with increasing the concentration of each plant extract in the range of 0.2-1.0 mg/mL. The results further indicated that methanol extracts exhibited maximum

inhibitory effects than other solvent extracts. This tends to show that the active metabolites of the different plant parts are better extracted with methanol than other solvents. As per literature survey, there is no previous report found on α-amylase inhibitory activity of this plant, Furthermore, the urease inhibitory activities of leaf extracts were studied against jack bean urease by using phenol hypochlorite method as compiled in Table 2. All the extracts showed inhibition ≥35% at concentration of 1 mg/mL. All the three extracts of *T. baccata* were reported to exert inhibitory effects on jack bean urease enzyme. Among these, methanol extract showed maximum inhibition of 52.40±2.10% followed by acetone (46.30±0.65) and aqueous extracts (39.90±0.80). This is the first report on enzyme inhibitory activity of *T. baccata* as there is no literature found regarding α- amylase and urease inhibitory activity.

Table 1: α-Amylase inhibitory activity of *T. baccata* leaf extracts at different concentrations

A-Amylase inhibitory activity (%)				
Concentration (mg/mL)	**Methanol extract**	**Acetone extract**	**Aqueous extract**	**Acarbose**
0.2	19.60±0.45	11.90±0.35	10.00±0.20	29.50±0.70
0.4	27.18±1.20	20.10±0.28	15.20±0.35	40.85±2.15
0.6	38.90±1.35	32.45±0.80	21.00±0.55	56.45±1.25
0.8	49.82±0.45	40.10±0.10	30.50±0.20	66.22±0.52
1.0	57.40±2.12	46.30±0.66	37.90±0.80	78.56±0.45
IC_{50} (mg/mL)	0.83	0.91	1.36	0.53

Values are given as mean ± S.E.

Table 2: Urease inhibitory activity of *T. baccata* leaf extracts at different concentrations

Urease inhibitory activity (%)				
Concentration (mg/mL)	**Methanol extract**	**Acetone extract**	**Aqueous extract**	**Acarbose**
0.2	15.90±0.46	12.20±2.05	10.60±0.50	28.38±0.78
0.4	21.18±1.20	20.10±1.20	18.20±0.35	41.58±0.55
0.6	28.90±1.35	31.45±0.80	25.00±0.70	56.30±1.20
0.8	39.87±0.66	40.00±0.10	34.50±1.20	69.20±0.50
1.0	52.40±2.10	46.30±0.65	39.90±0.80	81.26±1.25
IC_{50} (mg/mL)	1.00	1.05	1.25	0.51

Values are given as mean ± S.E.

4. Conclusion

The results of α- amylase inhibitory studies are encouraging as all the tested leaf extracts of *Taxus baccata* showed inhibition in the range of 37.90±0.80 to 57.40±2.12% at concentration of 1 mg/mL. Similarly, urease inhibitory activity ranged from 39.90±0.80 to 52.40±2.10% suggesting a strong α- amylase and urease inhibitory effects of this plant. Hence it is clear from the results that leaf extracts under study displayed variable enzyme (α- amylase and urease) inhibitory activities thereby confirming their roles in the treatment of various diseases caused by the malfunctioning of these enzymes.

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