Int J Pharm Bio Sci 2013 Apr; 4(2): (P) 494 - 503

Research Article **Pharmacology**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

INVITRO EVALUATION OF α-AMYLASE INHIBITORY ACTIVITY & ANTIOXIDANT POTENTIAL OF *PTERIS VITTATA* L. WITH SPECIAL REFERENCE TO ITS HPTLC PROFILE.

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ABSTRACT

Pteris vittata L., a fern was evaluated for its alpha amylase inhibitory potential owing to its previously reported antihyperglycemic activity. This Pteridophyte was also assayed for its free radical inhibition ability in ABTS decolourisation assay followed by quantification and HPTLC of polyphenols. The Porcine pancreatic alpha amylase inhibition was studied *invitro* and compared with standard drug Acarbose and their IC50 values were determined. For Antioxidant Potential, the ABTS free Radical Scavenging activity was performed and concentration of the test extracts equivalent to ascorbic acid was determined. The phenols and flavonoids being held responsible for antioxidant propertiesof plants lead us to quantify them which were followed by HPTLC fingerprinting of flavonoids to facilitate its identification. Standard Quercetin was used as standard along with the two extracts of *Pteris vittata* L. As compared to aqueous extract, the ethanolic extract showed better results in the parameters undertaken to study.

KEYWORDS:*Pteris vittata* L., Alpha amylase inhibition, ABTS,Phenol, Flavonoid,HPTLC.

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INTRODUCTION

Plants are often used as a drug or supplement to treat cardiovascular diseases, diabetes and other metabolic syndromes that rise due to excessive production of oxidative stress[1]. In diabetes, alpha amylase plays a role in the augmentation of the postprandial blood glucose by directly breaking down the carbohydrate into their respective maltose and maltotriose. The above phenomenon results in a process more commonly known as postprandial hyperglycemia (PPHG) [2]. It has been found that in drug design and development, alpha amylase inhibitors and glucosidase inhibitors are more emphasised now-a-days [3]. Thus, researchers are trying to overcome PPHG by utilising the low cost natural products to inhibit the alpha amylase activity that will reduce the absorption of glucose by the breakdown of starch [4]. Another prospect mostly emphasized is the antioxidant potential of plants which have beneficial effects on prevention of Diabetes and other chronic diseases. According to Sasikumar *et al* [5] antioxidants from natural source play a principal role by stimulating endogenous antioxidants to neutralize oxidative stress. Plants generally contain secondary metabolites like phenolics, flavonoids, glycosides, coumarins, saponins, terpenoids, alkaloids etc. which reveal their specific characteristic properties and attribute to their pharmacological properties [6]. These phytochemicals are qualitatively and quantitatively estimated by different spectrophotometric and chromatographic techniques. HPTLC being one of the powerful analytical tools can be utilized for linking the chemical constituents of the plant with high proficiency which in turn provides unique profiling of that particular plant [7]. *Pteris vittata* L., which is commonly known as the Chinese brake fern is a cosmopolitan pteridophyte found in crevices and in any calcareous substrate [8]. Pteridophytes are the second largest group of vascular plants out of which 61 medicinally important pteridophyte species are found in India [9]. This vascular fern, *Pteris vittata* L. is reported to have antimicrobial activity [10], antitumor activity, platelet aggregation, anti-inflammatory action [11] as well as hypo-tensive, antiviral and anti bacterial activity [12]. Ethnomedicinally, the whole plant is made into a paste and used for healing of wounds by applying externally [13]. Moreover, paste of fresh rhizomes and fronds of this fern is applied for the relief of glandular swelling by the folklores of Orissa [14].Our previous study [15]revealed that *Pteris vittata* L. has hypoglycaemic property. Thus, it was thought necessary to find out the alpha amylase inhibitory activity as well as its antioxidant potential for finding its action in diabetes.

MATERIALS AND METHODS

(i) Collection and Authentication

The fern *Pteris vittata* L. (PV) was collected from Chandraprabha Vanrai, Dapoli in the Ratnagiri district of Maharashtra in the end of monsoon and was authenticated by Dr. P.G Diwakar from Botanical Survey of India, Pune under the number BSI/WC/Tech./2011/306. A specimen of the herbarium was submitted in APT Research Foundation, Pune.

(ii) Preparation of Plant Extracts

The fronds were cleaned and shade dried in a dryer and the dried fronds obtained were crushed and kept in the Soxhlet apparatus for obtaining ethanolic and aqueous extract by the respective solvents. The aqueous as well as ethanolic extracts obtained were concentrated in rotary evaporator under vacuum and the percent yields were determined.

(iii) Chemicals and reagents

Porcine Pancreatic Amylase, ABTS (2, 2'-azino-bis- 3-ethyl benzthiazoline-6-sulphonic acid), 3, 5, Dinitrosalicylic acid, Gallic acid, Quercetin were procured from Sigma Aldrich (St. Louis USA). All the chemicals and reagents used were of analytical grade.

(iv) Alpha amylase inhibition assay

The alpha amylase inhibitory activity was determined according to the method of Jyothi *et al* [16]. Briefly, the total assay mixture containing, 200 μl of 0.02M sodium phosphate buffer, 20 μl of enzyme (Porcine Pancreatic Amylase), and the plant extracts in the concentration range 10-

100 μg/ml were incubated for 10 minutes at room temperature followed by addition of 200μl of 1 % starch in all the test tubes. The reaction was terminated by the addition of 400μl of 3,5,-dinitrosalicylic acid (DNSA) , which was made by dissolving 1 gm of DNSA in 50 ml of distilled water and adding 30g of Sodium potassium tartarate tetrahydrate in small lots; the solution turns milky yellow in colour when the reagent is added. Then 20 ml of 2N Sodium hydroxide was added, which turned the solution to transparent orange yellow colour. The final volume was made with 100 ml with distilled water and stored in amber coloured bottle away from sunlight.After addition of DNSA the test tubes were placed in a boiling water bath for 5 minutes, cooled at room temperature and diluted with 15 ml of distilled water. The absorbance was measured at 540 nm. The control tubes were prepared without any plant extracts and the results were expressed as % inhibition which was calculated using the formula:

$$\text{Inhibition activity (\%)} = \frac{\text{Abs (control)-Abs (test)}}{\text{Abs of control}} \times 100$$

The IC50 values of the plant extracts were determined by performing the assay as above with varying concentrations of the plant extracts ranging from 10-100 μg/ml by using the software Graphpad Prism version 5.

(v) Determination of antioxidant potential by invitro ABTS+ decolourisation assay

The antioxidant effect of the extracts was studied using ABTS (2, 2'-azino-bis- 3-ethyl benzthiazoline-6-sulphonic acid) radical cation decolourisation assay according to the method of Shirwaikar *et al*. (2006) [17]. ABTS radical cations (ABTS+) were produced by reacting ABTS solution (7mM) with 2.45mM ammonium persulphate. The mixture was allowed to stand in dark at room temperature for 12-16 hours before use. The absorbance was read at 735nm in a spectrophotometer (Chemito) and the concentration equivalent to ascorbic acid was determined from the standard curve of Ascorbic acid (y = mx+c). The percent inhibition was calculated using the formula:

$$\text{Inhibition (\%)} = \frac{\text{(Abs of Control – Abs of test)}}{\text{Abs of Control}} \times 100$$

(vi) Quantification of Total Phenolic content

The total phenolic content in the two extracts viz. aqueous and ethanolic were determined with Folin-Ciocalteu (FC reagent) using Gallic acid (50-250 μg/ml) as a standard. A 1mg/ml of the extracts were prepared and diluted to 45 ml with distilled water in a volumetric flask. A 1 ml of FC reagent was then added and the content of the flask mixed properly. After three minutes, 3 ml of 20 % sodium carbonate was added to the mixture and it was allowed to stand for 2 hour with occasional shaking. The absorbance of the blue colour that developed was read at 760nm. The concentration of total phenols was expressed as Gallic acid equivalents in mg/g of dry extract [18].

(vii) Quantification of Total Flavonoid content

The total flavonoid content was determined by following the Aluminium chloride colorimetric method described by Lobo *et al* [19]. In brief, 1ml of plant extract (1mg/ml) was added to 2 ml of water and after 5 minutes 3 ml of 5% sodium nitrite and 0.3ml of 10% aluminium chloride were added. After 6 minutes, 2ml of 1M sodium hydroxide was added to the solution and the volume was made upto 10 ml with distilled water. The red coloured complex formed was measured at 510nm. The percentages of total flavonoids were calculated from the standard calibration curve of Quercetin (10-250μg/ml) and total flavonoids were expressed as Quercetin equivalents in mili grams per gram sample.

(viii) HPTLC profiling of flavonoids

HPTLC studies were carried out following Harborne [20] and Wagner *et al* [21]. The extracts were dissolved with HPLC grade methanol 100mg/0.5ml.The extracts were dissolved using a waterbath sonicator for 15 minutes and used for HPTLC analysis. Later, 10, 20 and 30 μl of the samples were loaded as 8mm band length in the 20X10 Silica Gel 60 F_{254} TLC Plate using

Hamilton Syringe and CAMAG Linomat 5 instrument. The sample loaded plate was kept in a TLC saturation chamber for saturation with the mobile phase. The mobile phase used for separation of flavonoids was Ethyl Acetate: Formic acid: Glacial Acetic Acid: Water in the ratio of 10:0.5:0.5:1.3. The developed plate was dried using hot air to evaporate solvents from the plate and sprayed with Anisaldehyde Sulphuric Acid reagent (ASA). The plate was kept in photo documentation chamber CAMAG Visualizer: 150503 and images were captured at 254nm, 366nm, visible light and after spraying with ASA using a Digital camera DXA252: 306921208, 16 mm scanner & Lens f4.0

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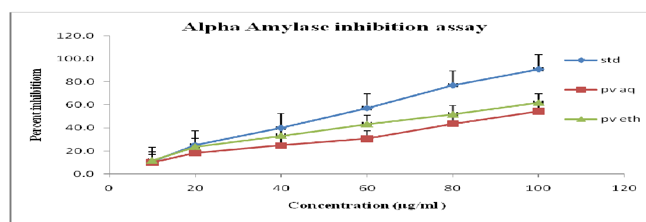
RESULTS

(i) *Alpha amylase inhibition assay*

In the porcine pancreatic alpha amylase enzyme inhibition assay, the aqueous extract of *Pteris vittata* L. showed a 10-52% inhibition at concentrations of 10, 20, 40, 60, 80 and 100µg/ml respectively. At similar concentrations the ethanolic extract of *Pteris vittata* L. showed 11-70% inhibition. The aqueous extract of *Pteris vittata* L. at a concentration of 100µg/ml showed an inhibition of 54.54±0.46% while the ethanolic extract of *Pteris vittata* L. at a concentration of 100µg/ml showed 70.18±0.74% inhibition.

Graph 1
Alpha Amylase inhibition assay



Graph 1 Percent inhibition of α- amylase enzyme by aqueous and ethanolic extracts of Pteris vittata L.
Std = Acarbose standard drug, PV aq = Aqueous extracts of Pteris vittata L., PV eth= Ethanolic extracts of Pteris vittata L.

(ii) *Determination of antioxidant potential by invitro ABTS+ decolourisation assay*

The ABTS radical scavenging ability was studied of the two extracts of *Pteris vittata*.L where the aqueous extract at 500µg/ml showed 82.29 % inhibition while the ethanolic extract showed 84.33 % inhibition at the same concentration. The 500µg/ml of the ethanolic extract was equivalent to 44.71 µg/ml of ascorbic acid while the concentration of 500µg/ml of the aqueous extract was equivalent to 43.64 µg/ml of ascorbic acid. (Table 1)

Table 1
ABTS radical scavenging activity of Aqueous and Ethanolic extracts of Pteris vittata L.

Concentration (µg/ml)	PV aq		PV eth	
	% inhibition	Conc. Equivalent to ascorbic acid	% inhibition	Conc. Equivalent to ascorbic acid
10	09.67	05.57	10.90	06.21
50	14.03	07.86	15.53	08.64
100	21.93	12.00	26.57	14.43
150	38.83	20.86	45.10	24.14
200	46.05	24.64	52.59	28.07
250	60.90	32.43	68.12	36.21
500	82.29	43.64	84.33	44.71

(iii) Quantification of Total Phenolic content

In the present study, the total phenolic content was estimated and it was found that the aqueous extract of *Pteris vittata* L. contained 202±2.82 mg/gm and ethanolic extract of *Pteris vittata* L. contained 284±1.41mg/gm of dried extract equivalent to Standard Gallic acid [R^2 value 0.996]

(iv) Quantification of Total Flavonoid content

Preliminary phytochemical screening suggested the presence of Flavonoids in both aqueous and ethanolic extracts of *Pteris vittata* L. which led us to quantify the flavonoids content of this fern. The flavonoid content was found to be 66.07±1.10 mg/gm in aqueous extract and 70±1.0 mg/gm in the ethanolic extract respectively when compared with standard Quercetin. [R^2 value 0.994] (Table 2)

Table 2
Total phenol and flavonoid content of Pteris vittata L. (Mean ± SD)

Plant Extract Concentrations	Phenol content (mg/gm)	Flavonoid content (mg/gm)
PV aq 1 mg/ml	202 ± 2.82	66.07 ± 1.10
PV eth 1 mg/ml	284 ± 1.41	70.00 ± 1.00

Data : (n=3)

(v) HPTLC profiling of flavonoids

The HPTLC profile of flavonoids of aqueous and ethanolic extracts of *Pteris vittata* L. was undertaken which has been depicted in (Table3).

Table 3
HPTLC details of Aqueous and Ethanolic extracts of Pteris Vittata L.

PV aqueous			PV ethanolic			Quercetin(Standard)		
Peak	**Max Rf**	**Area %**	**Peak**	**Max Rf**	**Area %**	**Peak**	**Max Rf**	**Area %**
1	0.02	6.23	1	0.03	4.48	1	0.81	100
2	0.07	29.13	2	0.06	2.15			
3	0.17	27.81	3	0.09	2.44			
4	0.25	32.37	4	0.15	4.35			
5	0.78	3.82	5	0.24	20.84			
6	0.85	0.65	6	0.35	6.46			
			7	0.44	8.67			
			8	0.73	8.00			
			9	0.78	1.90			
			10	0.82	13.60			
			11	0.88	3.86			
			12	0.91	23.27			

The 2D spectrum of standard Quercetin showed a single peak with an area of 100% and maximum Rf of 0.81.(Fig.2) The aqueous extract showed six peaks with maximum Rf values starting from 0.02 to 0.85.(Fig.3) The ethanolic extract showed twelve peaks with maximum Rf values starting from 0.03 to 0.91 (Fig.4).

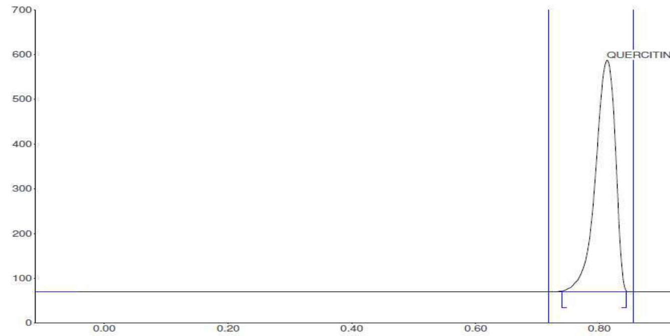


Figure 2
2D display of chromatogram of Standard Quercetin.

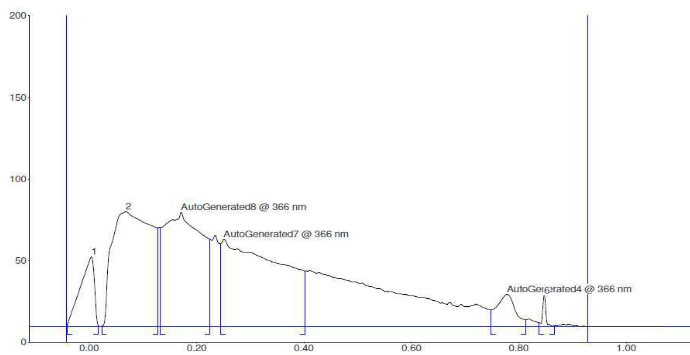


Figure 3
2D display of chromatogram of aqueous extract of Pteris vittata L. at 366nm.

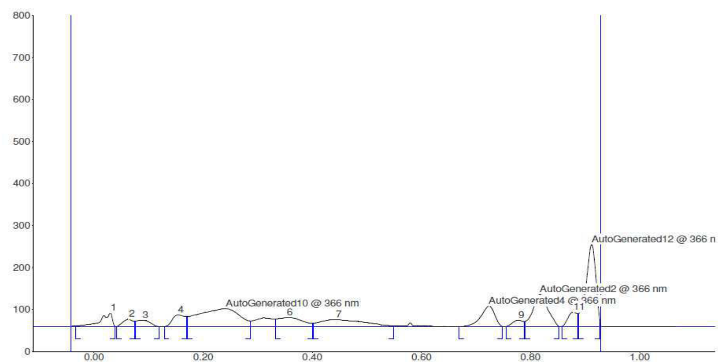


Figure 4
2D display of chromatogram of ethanolic extract of Pteris vittata L. at 366nm.

HPTLC CHROMATOGRAM

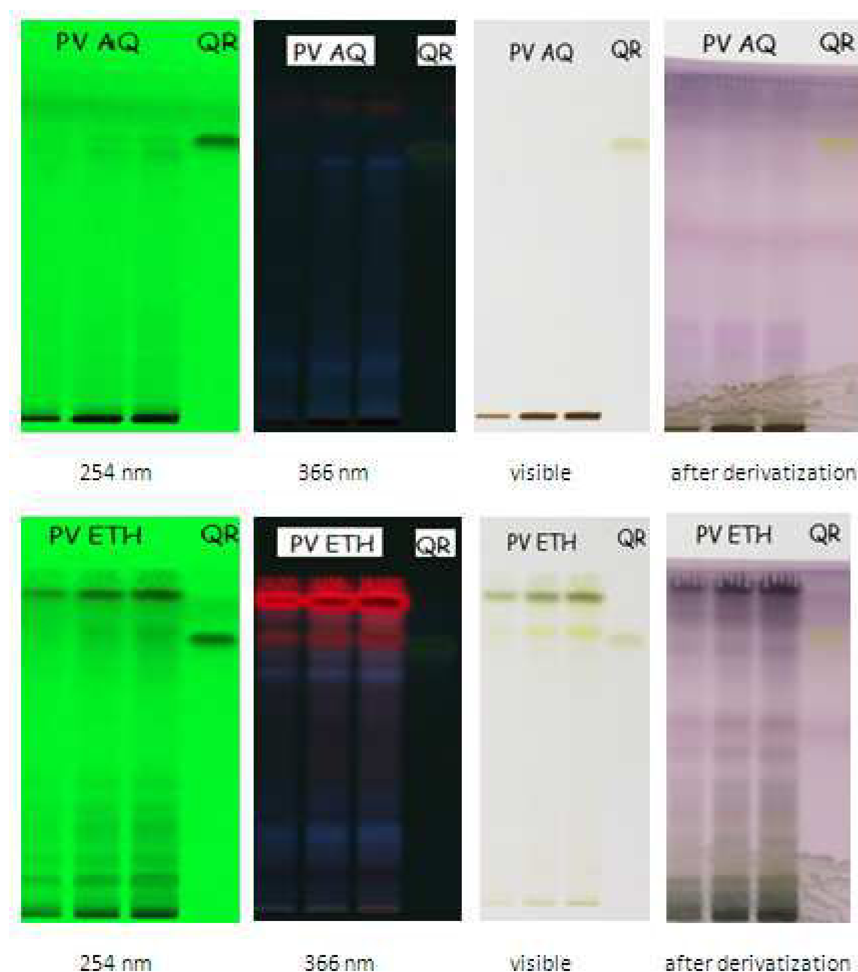


Figure 5

Chromatograms of Pteris vittata L. extracts and standard Quercetin on Silica Gel 60 F_{254} TLC plates. PV aq = Aqueous extracts of Pteris vittata L., PV eth = Ethanolic extracts of Pteris vittata L, QR= Standard Quercetin.

DISCUSSIONS

The commercially available drug like Acarbose and Voglibose are exponentially used to lower the post prandial hyperglycemia (PPHG) levels since it delays digestion of carbohydrates. Along with their ability to lower PPHG they also have some side effect like abdominal discomfort [22]. In the present study, owing to the potential of *Pteris vittata* L to lower blood glucose, we have assayed the alpha amylase inhibitory activity to monitor its involvement to inhibit the pancreatic alpha amylase enzyme *invitro*. Our findings showed that ethanolic extract of *Pteris vittata* L. at a concentration of 100µg/ml produced 70.87% inhibition of Porcine pancreatic amylase with an IC50 value of 70µg/ml whereas the Aqueous Extract at a concentration of 100µg/ml showed 54.54% inhibition with an IC50 value of 91µg/ml. The standard drug, Acarbose however, showed 90.91% inhibition at 100µg/ml concentration with an IC50 value of 51µg/ml. The traditional herbal remedies mostly employed for treatment of diabetes have been found to exhibit potential antioxidant activity [23]. In the present study ABTS radical scavenging activity of both aqueous and

ethanolic extracts of *Pteris vittata* L. was performed. The percent inhibition displayed by aqueous extract was almost similar to that of ethanolic extract. However, the percent inhibition values shown by both the extracts at various concentrations were less than that shown by standard ascorbic acid.Phenolics and polyphenols are important secondary metabolites present in plants which are responsible for treating various metabolic diseases by their antioxidant potential [24, 25]. They are basically aromatic ring structured with one or more hydroxyl substitutes [26]. Phenolic compounds when react with phosphotungstic acid and phosphomolybdic acids present in Folin-Ciocalteu reagent produce a blue coloured complex by a redox reaction which can be measured spectrophotometrically at 760 nm [27]. Both the extracts of the fronds of fern, *Pteris vittata* L. have shown presence of phenolic content in the form of mg/gm of Gallic acid equivalent. Previously it was reported that Flavonoids are effective scavengers for most types of oxidizing molecules, which is possible due to their hydrogen-donating ability [28]. Thus, in the present study the flavonoids present, were quantified and it was found that the amount of flavonoids present were 5.61% higher in the ethanolic extract as compared to aqueous extract.HPTLC being an important tool for screening the plants and plant products was conducted to further validate the presence of flavonoids. HPTLC is a fast and accurate method to identify different secondary metabolites present in plant species with better resolution and in a cost effective manner [29]. The chromatogram obtained was analysed under 254nm UV, 366nm UV and in the visible light with and without spraying the derivatization reagents. In the visible light, the plate spotted with extracts showed yellowish–green bands. In the 366nm UV it showed Blue and red bands while at 254nm UV it showed brownish-black bands (Fig 5). The standard Quercetin showed yellow bands at the visible light as well as after derivatization with Anisaldehyde Sulphuric acid (ASA). In the 366nm Quercetin appeared as greyish green band and in 254 nm brownish-black band was observed, In the chromatogram Quercetin showed a single peak at Rf value 0.81 after every three tracks of *Pteris vittata* L. of both aqueous and ethanolic extracts. Similar bands positioned near the Quercetin with Rf value 0.81 were seen in the 10th track of ethanolic extract and 5th track of aqueous extract of *Pteris vittata* L. Besides those Rf values 0.25 in PV aqueous extract and 0.24 in PV ethanolic extract have shown notable % area of 32.37 & 20.84 respectively.

CONCLUSION

Previous studies conducted [30, 31] have reported that phenolic compounds inhibit the amylase enzymes by modulating the enzymatic breakdown of carbohydrate. Thus, it can be concluded from the above mentioned results that the alpha amylase inhibitory potential of both the aqueous and ethanolic extracts of *Pteris vittata* L. might be due to its phenolic content. Further analysis of the active compounds will be carried out which might provide more information about the role of polyphenols and flavonoids present in *Pteris vittata* L. towards management of diseased conditions where there is an increase in the oxidative stress.

ACKNOWLEDGEMENTS

The authors are thankful to Dr. Ramesh C Saxena of SSL Jain P.G. College, Vidisha for providing valuable information. We are also thankful to Anchrom Laboratory, Mulund (E), Mumbai, for their help extended to perform and analyse the HPTLC profiling of the fern species.

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