**SUPPLEMENTARY MATERIAL**

***In vitro* evaluation reveals the nutraceutical properties of edible lichen**

***Parmotrema tinctorum* beneficial to diabetic complications**

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**Abstract**

The present study evaluated the inhibitory potential of ethyl acetate extract of edible lichen, *Parmotrema tinctorum* (PTEE) against aldose reductase and carbohydrate digestive enzymes such as α-glucosidase and α-amylase. It was also screened for antioxidant activities employing DPPH, ABTS, superoxide and hydroxyl radical scavenging assays.PTEE exhibited α-glucosidase, α-amylase and aldose reductase inhibition along with significant antiglycation potential with an estimated IC50 value of 58.45 ± 1.24, 587.74 ± 3.27, 139.28 ± 2.6 and 285.78 ± 1.287 µg/ml, respectively. Antioxidant activity of PTEE against DPPH (IC50 396.83 ± 2.98 μg/ml), ABTS (151.34 ± 1.79 μg/ml), superoxide (30.29 ± 1.17 μg/ml) and hydroxyl (35.42 ± 1.22 µg/ml) radicals suggests the antioxidant potential of *P. tinctorum.* Significant antioxidant activity and inhibitory potential against carbohydrate digestive enzymes and aldose reductase suggests that *P. tinctorum* can be developed as functional food/nutraceuticals for diabetes after detailed study.

**Keywords**: Lichen, nutraceuticals, antioxidants, α-amylase, α-glucosidase, hyperglycemia

**1. Experimental**

**1.1. *Chemicals and reagents***

1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,20-azinobis-3- ethylbenzothiazoline-6-sulfonic acid (ABTS), deoxyribose, nitrotetrazolium blue (NBT) and phenazine methosulphate (PMS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trolox (6-hydroxy- 2,5,7,8- tetramethyl chroman-2- carboxylic acid), gallic acid (3,4,5- trihydroxy benzoic acid), quercetin, α-glucosidase, α-amylase, acarbose, ascorbic acid and p-nitrophenol-a-d-glucopyranoside (PNPG) were obtained from Sigma Aldrich Chemical Co. (Milwaukee, WI, USA). Nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide- phosphate (NADPH), dl-glyceraldehyde, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4’,4”- disulfonicacid (ferrozine), Folin-Ciocalteau reagent, thiobarbituric acid (TBA), dinitro salicylic acid, potassium sodium tartarate and starch from Sisco Research Lab (India). All the other chemicals used, were of standard analytical grade and solvents were of HPLC grade.

**1.2. *Preparation of lichen extract***

The lichen sample (*P. tinctorum)* was collected during the month of January 2013 from Wayanad district (Kerala, India) and was identified and authenticated by Dr. H. Biju, taxonomist, Jawaharlal Nehru Tropical Botanical Garden and Research Institute (JNTBGRI), Palode, Kerala, India. Proper sanction has been obtained from forest department, Govt. of Kerala for collecting lichen samples (No.WL10-35151/12). A voucher specimen (No.P1213AGP/CSIR-NIIST) was kept in our herbarium for future reference.

The lichen was dried, ground to powder and extracted (250 g) with hexane (1 L) to remove low polarity contaminants. Then the powder was extracted with ethyl acetate (2.5 L) at ambient temperature (27 ± 1 °C) under stirring for 6 h, and the extraction process was repeated until the solvent became colourless. The supernatant was ﬁltered through Whatman No.1 ﬁlter paper and concentrated *in vacuo* under reduced pressure in a rotavapor (Heidolph) followed by lyophilisation. The lyophilised *P. tinctorum* extract (PTEE) was stored at 4 °C until use.

**1.3. *Analysis of proximate composition***

Proximate composition, total fatty matter, carbohydrate, protein and ash content of the *P.* *tinctorum* were analyzed according to the AOAC (2000) procedures.

  Dry weight of *P. tinctorum* was determined by oven dry method. About 10 g of the material was accurately weighed in a pre weighed petri dish and placed in hot air oven and dry for 2 hours at 100ºC. The dish with sample was cooled in a desicator and weight was noted. Heated again at 100ºC in air oven for 30 minutes cooled and weighed. This process of heating for 30 minutes was repeated till the difference in weight between two successive weighing was less than 1 mg. From the loss weight during the drying amount of moisture was calculated.

    Total fatty matter in the dried samples was extracted by soxhlet apparatus using hexane as solvent. 5g of dried sample was taken in a thimble and placed in the soxhlet fitted with pre-weighed round bottom flask and filled over 1/4th part with hexane. Extraction with solvent is carried out for 4-6 hours. The solvent containing the fat fraction obtained in the round bottom flask was evaporated. From the weight of the residue total fat soluble matter can be determined.

To determine the crude fiber content, residue from crude fat determination was used. 2g of sample was taken and refluxed consecutively with 200ml 1.25% H2SO4 and 200ml 1.25% NaOH solution. The digested material was filtered, washed with hot distilled water several times until the washings were free of alkali. Washed with 25ml 95% alcohol, dried and transferred to sintered crucible then dried it in a hot air oven at 110ᵒC until it attain a constant weight. It was allowed to cool in desiccators and weighed. The gained weight represents the crude fiber.

To determine the ash content, 5g sample was accurately weighed into a pre-weighed, clean crucible. The crucible heated to the point of charring of the sample on a hot plate and the crucible with the carbon residue was placed in muffle furnace at temperature of 650º C until the carbon residue disappears. Allowed to cool and then weighed.

10 g of defatted *P. tinctorum* was dried and dissolved in 80% ethanol and refluxed for 6 hours. Then the mixture was made up to known volume. Pipetted out different volumes of sample solution in the test tubes and made up the solution to 1ml with distilled water. 4ml of anthrone reagent was added to test tubes and heated in a boiling water bath for 15 minutes. Cooled under tap water and the absorbance were measured at 620nm in UV–vis spectrophotometer (UV-2450PC, Shimadzu, Japan). The amount of carbohydrates in the sample was determined by plotting the calibration curve using authentic standard of glucose.

In order to determine the total protein content in *P. tinctorum*, about 1 gm of defatted sample was dissolved in 3.5 ml of ice cold extraction buffer (175 mM Tris HCl, 5% SDS, glycerol 15%, 0.07% 2-mercaptoethanol at pH 8.8), centrifuged ans the supernatant was collected and protein was precipitated using ice cold acetone containing tricholoro acetic acid (TCA) and 2-mercaptoethanol. Stored at -20ᵒC and centrifuged and pellets were dried at room temperature and used as protein source. The amount of protein was estimated using Lowry’s method.

**1.4 *Antioxidant assays***

1.4.1 *Total phenolic content*

Total phenolic content (TPC) of PTEE was quantified using Folins-Ciocalteau phenol reagent method of Singleton and Rossi (1965) with slight modifications. Briefly, 100 µl of PTEE was added to 500µl Folin-ciocalteu reagent and kept at room temperature for 10min, then added 1ml Na2CO3(20%) and incubated at ambient temperature for 90 min. The color developed was measured at 765 nm. Results are expressed as mg Gallic acid equivalents per g of extract (mg GAE/g).

 1.4.2 *Total flavonoid content*

Total flavonoid content (TFC) was quantified according to the method of Jia et al. (1999). 0.5 mL PTEE, 100 μL aluminum chloride (10%), 100 μL potassium acetate (1M) and 2.3 mL distilled water were incubated at room temperature for 30 min. The absorbance was measured at 415 nm. Results are expressed as mg quercetin equivalents per g of extract (mg GAE/g).

1.4.3 *DPPH radical scavenging assay*

The antioxidant activity of PTEE was determined according to the method of Shimada et al. (1992). Briefly, a 3mL reaction mixture contained 2.9mL of methanolic DPPH˙ and 0.1mL of extract at various concentrations. In the control, methanol was used in the place of the sample. When DPPH˙ reacts with an antioxidant compound that can donate hydrogen, it gets reduced, and the resulting decrease in absorbance at 517nm after 30 min was recorded using a UV–Vis spectrophotometer (UV-2450PC, Shimadzu, Japan).

1.4.4 *ABTS radical scavenging assay*

ABTS decolorisation assay (Re et al. 1999) involves the generation of ABTS+• chromophore by the oxidation of ABTS with potassium persulfate. It is applicable for both hydrophilic and lipophilic compounds. The ABTS radical cation (ABTS+•) was produced by

reacting 7 mM stock solution of ABTS with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark for at least 16 h at room temperature before use. The ABTS+• solution was diluted to an absorbance of 0.7 ± 0.05 at 734 nm (UV-2450PC; Shimadzu, Kyoto, Japan). Absorbance was measured 7 min after the initial mixing of different concentrations of the PTEE with 1 ml of ABTS+• solution at 734 nm.

1.4.5 *Hydroxyl radical scavenging activity*

The hydroxyl radical scavenging activity of PTEE was measured by the deoxyribose method (Halliwell et al. 1987) and compared with that of catechin. The reaction mixture containing PTEE at various concentrations, deoxyribose (3.75mM), H2O2 (1mM), potassium phosphate buffer (20mM, pH 7.4), FeCl3 (0.1mM), EDTA (0.1mM) and ascorbic acid (0.1mM) was incubated in a water bath at 37ᵒC for 1h. The extent of deoxyribose degradation was measured by adding TBA (1%) and TCA (2%) and heated in a water bath at 100ᵒC for 20 min. The absorbance of the resulting solution was measured spectrophotometrically at 532 nm (UV-2450PC; Shimadzu, Kyoto, Japan).

1.4.6 *Superoxide radical scavenging activity*

Superoxide anions were generated in a non-enzymatic phenazine methosulphate (PMS)–NADH system through the reaction of PMS, NADH and oxygen (Liu e al. 1997) and it was assayed by the reduction of NBT. In this assay, the superoxide anions were generated in Tris-HCl buffer (100 mM, pH 7.4) containing 0.75mL of NBT (300 mM) solution, 0.75mL of NADH (936 mM) solution and 0.3mL of different concentrations of PTEE. The reaction was initiated by adding 0.75mL of PMS (120 mM) solution to the mixture. After 5 minutes of incubation at room temperature, the absorbance at 560nm was measured in a spectrophotometer (UV-2450PC; Shimadzu, Kyoto, Japan).

1.4.7 *Determination of total reducing power*

Total reducing power (TRP) of PTEE was determined according to the method of Oyaizu (1986). Different amounts of PTEE were mixed with phosphate buffer (0.2M, pH 6.6) and 1% potassium ferricyanide [K3Fe(CN)6]. The mixture was incubated at 500C for 20 min. TCA (10%) was added to the mixture, which was then centrifuged for 10 min. The upper layer of solution was mixed with distilled water and FeCl3 (0.1%), and the absorbance was measured at 700 nm (UV-2450PC; Shimadzu, Kyoto, Japan).

**1.5. *α-Glucosidase and α-amylase inhibitory assay***

 α-glucosidase was assayed (Riya et al. 2013) by using 50 μl of extract (PTEE) and 100 μl of 0.1 M phosphate buffer (pH 6.9) containing α- glucosidase solution (1.0 U/ml) and incubated in 96 well plates at 25ᵒC for 10 min. After incubation, 50 μl of 5 mM p-nitrophenyl-α -D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added. The reaction mixtures were then incubated at 25ᵒC for 5 min. Before and after incubation, absorbance was recorded at 405 nm by multimode reader (Synergy 4, Biotek Instruments, Vermont, USA) and compared to a control which had 50 μl of buffer solution in place of the extract. The α-glucosidase inhibitory activity was expressed as % inhibition and was calculated as follows:

% Inhibition = [ΔA Control-ΔA Extract] x 100

 ΔA control

 For α-amylase inhibition assay, 500 μl of PTEE at different concentrations and 500 μl of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) containing α-amylase solution (0.5 mg/ml) were incubated at 25ᵒC for 10 min (Riya et al. 2013). After incubation, 500 μl of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) was added to each tube. The reaction mixtures were then incubated at 25ᵒC for 10 min. The reaction was stopped with 1.0 ml of dinitrosalicylic acid reagent. The test tubes were then incubated in a boiling water bath for 5 min and cooled to room temperature. The reaction mixture was then diluted after adding 10 ml distilled water and absorbance was measured at 540 nm using multimode reader (Synergy 4, Biotek Instruments, Vermont, USA). The results were expressed as % inhibition of enzyme activity and calculated according to the following equation.

% Inhibition = [Absorbance of control-Absorbance of extract] x 100

 Absorbance of control

**1.6. *Aldose reductase assay***

Lenses from streptozotocin induced diabetic rats (male Sprague- Dawley strain, weighing between 150-200 g) obtained from Cochin University of Science and Technology after proper ethical clearance was used as aldose reductase enzyme source for the study. The lens homogenate (10%) was prepared in 0.1 M phosphate buffered saline, centrifuged in a refrigerated centrifuge and the supernatant was used for experiments.

Aldose reductase inhibitory potential of PTEE was determined by a method described by Hayman and Konoshita (1965) with some modifications using zopolrestat as standard. Briefly 290 µl of reaction mixture containing 0.046 M sodium phosphate buffer (pH 6.2), 50 mM DL-Glyceraldehyade, 27.5x10-5 M NADPH and lens homogenate was taken in a 96 well plate. To this, 10 µl of samples at various concentrations dissolved in 0.1M PBS was added. 10 µl each of PBS and zopolrestat was used as negative and positive control respectively. It was incubated at 37 °C for 10 min and the reaction was initiated by the addition of DL-glyceraldehyde. The decrease in the absorbance was read at 340 nm in a microplate reader (Biotek Synergy 4, USA) for 10 min at 1 min interval.

**1.7. *Antiglycation assay***

The antiglycation property of PTEE was examined according to the method of Brownlee et al. (1986) with slight modifications, BSA (5 mg/mL) was incubated with glucose (500 mM) in phosphate buffered-saline (PBS) (1mL total volume, pH 7.4) and extract (0.05 to 0.5mg/mL) containing 0.02% sodium azide. Aminoguanidine was used as positive control. Reaction mixtures were kept in the dark at 37 °C for 7 days. After 7 days of incubation, fluorescence intensity (excitation and emission wavelength of 370 nm and 440 nm) was measured.

**1.8. *Statistical Analysis***

The experimental results were expressed as mean ± SD (standard deviation) of 3 independent experiments with duplicates (n=6). The data were subjected to one-way analysis of variance (ANOVA) and the significance of differences between means was calculated by Duncan’s multiple range tests using SPSS for Windows, standard version 7.5.1, with the significance accepted at P≤ 0.05.

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**Table.S1.** Proximate composition of *P. tinctorum*

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| **Parameters** | **Yield (%)** |
| Crude Fiber | 19.0 ± 0.5 |
| Dry weight | 0.87 ± 0.2 |
| Ash content | 11.65 ± 1.2 |
| Total Carbohydrate | 32.35 ± 3.4 |
| Total Protein | 31.33 ± 2.3 |
| Total Fat | 1.8 ± 0.8 |

**Table.S2**. Estimated IC50 values of DPPH, ABTS, Superoxide and Hydroxyl radical scavenging activities exhibited by standard compounds and PTEE.

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| --- | --- |
| Sample |  IC50 values of radical scavenging assays (µg/ml)  |
| DPPH | ABTS | Superoxide | Hydroxyl |
| PTEE | 396.83±2.98 | 151.34±1.79 | 30.29±1.17 | 35.42±1.22 |
| GA | 2.43±0.36 | - | - | - |
| Trolox | - | 3.14 ±0.58 | 5.54±0.93 | - |
| Catechin | - | - | - | 9.86±0.79 |

PTEE – *Parmotrema tinctorum* ethyl acetate extract, GA- Gallic acid. Results expressed as mean ± SD of 3 independent experiments (n=6).

**Fig. S1** Total reducing power of PTEE, gallic acid and ascorbic acid

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Results expressed as mean±SD of three independent experiments with duplicates (n=6) and the significance accepted at P≤0.05. ‘\*’ indicates values are significantly different from each other for PTEE, GA and AA.

PTEE- *Parmotrema tinctorum* ethyl acetate extract; GA-gallic acid; AA-ascorbic acid

**Fig. S2** Aldose reductase inhibitory potential of PTEE

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Results expressed as mean±SD of three independent experiments with duplicates (n=6) and the significance (\*) accepted at P≤0.05 when compared to the control.

PTEE- *Parmotrema tinctorum* ethyl acetate extract

**Fig. S3** Antiglycation potential of PTEE

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Results expressed as mean±SD of three independent experiments with duplicates (n=6) and the significance (\*) accepted at P≤0.05 when compared to the control.

PTEE- *Parmotrema tinctorum* ethyl acetate extract