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Metal ion chelation and anti-glycation properties of polysaccharides of *Phyllanthus amarus* plant

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Abstract

The study was conducted to investigate the in vitro anti-glycation activity of *Phyllanthus amarus* plant extract. The above plant has been used for centuries for its useful health benefits in variety of inflammatory diseases. In current study, antioxidant, metal chelating and ferric ion reducing and antiglycation property was studied for the polysaccharides isolated from *Phyllanthus amarus* plant extract. At 25 µg/mL sugar concentration and Vitamin C, gave 57 % and 65 % inhibition in hydroxyl radical scavenging assay and in Antiglycation assay the extract and vitamin C showed 65% and 87% respectively. It also had metal ion chelating (PAPE 74%, Ascorbic acid 88%) and ferric ion reducing activity showed by PAPE IS about 68% when compared to standard metal ion chelator EDTA 90%. It inhibited fructosamine formation by 52% after 3 days of incubation. The above studies gave an opinion that the inhibition of glycation exhibited by extract was due to its free radical scavenging property but also due to the modification in the amino or carbonyl groups resulted in the inhibition of fructosamine formation. The polysaccharides of the extract may be donating the hydrogen atom to the free radical, and exhibiting the antioxidant activity. So polysaccharide isolated from *Phyllanthus amarus* plant may use in preventing many free radicals induced diseases and also in delaying or preventing complications of diabetes.

Keywords: *Phyllanthus amarus*; Antioxidant; Antiglycation; Advanced glycation end products; Diabetes; Free radicals

1. Introduction

Reactive Oxygen species (ROS) plays a major role in many chronic diseases like cancer, alzheimer disease, parkinson disease, diabetes, cardio vascular diseases, inflammation, viral infections, autoimmune pathologies, etc [1-3]. The exogenous and endogenous sources induces free radicals generation. Due to the metabolic process and mitochondrial respiratory chain and other reactions of the body, free radicals are generated in the body [4-5]. Added to the above, a wide variety of environmental pollutants, radiations, ROS may initiate the peroxidation of lipids and hence, the glycation of proteins which leads to long term complication of immune based diseases [6-7]. Hence, a search for nontoxic, inexpensive, dietary source of anti-glycative and antioxidant source attaining more importance. Studies on extracts of guava leaves, Turmeric, Piper longum, Ginger, *Muntingia calabura*, Star Anise, *Coleus aromaticus* providing a great value in the prevention or reduction of glycation-associated complications in diabetes [8-12]. *Phyllanthus amarus* herb is in

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traditional medicine belongs to a family of Euphorbiaceae and commonly known by the name of carry me seed, stone breaker, gala of wind and locally Nela nalli [13-14]. It is a branching annual herb of 10 - 20 cm height widely spread throughout tropics and sub-tropics as a weed. As per the literature, *Phyllanthus amarus* is gaining attention for its hepatoprotective, anti-carcinogenic, anti-bacterial, anti-viral, anti-inflammatory, and anti-diabetic and more activities due to the presence of different combinations of secondary metabolites. Herein, we made an attempt to extract the crude sugars of the whole plant and studied its anti-glycation, metal ion chelating activity including its antioxidant activity in appropriate methods [15-16].

2. Material and methods

All required plant materials, chemicals and reagents used were of analytical grade were collected / procured from authentic source, Merck Co, and S.d. fine chem., Mumbai, India.

2.1. Preparation of extract

The *Phyllanthus amarus* plant procured from Herbal Park maintained by SAC College of Pharmacy. The extract was prepared using double distilled water. The extract was kept at 4°C overnight to precipitate sugars in the extract. Extract was centrifuged at 10,000 rpm at 4°C for 20 min, and then the pellet was re-dissolved in minimum amount of distilled water, filtered through 0.22 µm filter and stored at -20°C for further use. The extract obtained was called as *Phyllanthus amarus* plant extract (PAPE). Protein [17] and Sugar estimation [18] was done for the extract.

2.2. Hydroxyl radical scavenging activity by *2*-Deoxy D-ribose assay

The 2-Deoxy D-ribose assay is done to determine the hydroxyl radical scavenging activity of PAPE and polysaccharide fraction in an aqueous medium [19-20]. The reaction mixture containing $FeCl_3$ (100µM), ascorbic acid (100µM), EDTA (104µM), H_2O_2 (1mM), 2-deoxy-D-ribose (2.8mM) were mixed with 20µg of extract in 20mM potassium phosphate buffer, pH 7.4 and incubated for 1hr at 37°C. A similar assay was done with other known antioxidants such as ascorbic acid (Vit-C) at 400µM concentration serving as positive controls. The reaction mixture was heated at 95°C in boiling water bath for 15min following the addition of 1mL of TBA (0.5%). At the end, the reaction mixture was cooled in ice and optical density was measured at 535nm using UV Visible spectrophotometer. The assay was carried out with appropriate blanks and controls. Antioxidant activity was expressed as percent inhibition of hydroxyl radical formation.

2.3. Ferric ion reducing power of PAPE

100µl of 4mM potassium ferricyanide solution was mixed with 200µL of 20mM phosphate buffer pH 6.5 in the presence or absence of PAPE and polysaccharide fraction [21]. A similar assay was done with Ascorbic acid at 40µM concentration. The contents were incubated at 50^0C for 20min. 200µL of 10% TCA was added to the reaction mixture and centrifuged at 5000rpm for 10min at room temperature. The resulting supernatant was taken and mixed with 100µL of 2mM ferric chloride solution and final volume was made up to 1mL with distilled water and then incubated at 37^0C for 10min. The absorbance was recorded at 700nm. Absorbance increases with increase in reducing power.

2.4. Ferrous ion chelating ability

Ferrous ion chelating activity was measured for the PAPE. The reaction solution contained ferrous chloride (200µM) and potassium ferricyanide (400µM) with or without out PAPE and polysaccharide fraction [21]. A similar assay was done with Ethylene Diamine Tetra Acetic acid (EDTA) at 40µM concentration. The components in the reaction mixture were added in final volume of 1 mL distilled water and mixed. The reaction mixture was incubated at 20°C for 10min. Formation of the potassium hexacyano ferrate complex was measured at 700nm. The assay was carried out at 20°C to prevent Fe^{2+} oxidation. Lower absorbance indicated higher iron chelating capacity.

2.5. *In vitro* non enzymatic glycation of bovine serum albumin

Bovine serum albumin (BSA, 20 mg/mL) was incubated in glucose (500 mM) and sodium azide (0.02%) with or without $CuSO_4$ (100µM) in 0.2 M phosphate buffer (pH 7.4). The test compound was added to the reaction mixture, and the reaction mixture was incubated for 3 days at 37°C. After incubating, the fluorescent reaction products were assayed in a fluorescence spectrophotometer with an excitation wavelength of 350 nm and an emission wavelength of 450nm [22]. Results were expressed as percentage inhibition of formation of the glycated protein.

2.6. Spectrophotometric analysis of fructosamine

The procedure of fructosamine assay followed the method of Baker, et al 1994 with minor modifications. The reaction mixture which contained 0.2 mL glycated material and 0.8 mL nitro blue tetrozolium (NBT) reagent (300μM) in sodium carbonate buffer (100 mM, pH 10.35) was incubated at ambient temperature for 15 min, and the absorbance was read at 530 nm against a blank [22].

2.7. Statistical analysis

Statistical analysis was done using students *t*-test. All the values represent mean of triplicates and are expressed as Mean ± SD. $p<0.05$ was considered as significant.

3. Results and discussion

Table 1: Antioxidant and antiglcation studies:

	% Inhibition of Hydroxyl radicals	% Inhibition of glycation activity
Phyllanthus amarus plant extract (25μg/ml)	57	65
Ascorbic acid (10μg/ml)	65	87

3.1. Isolation *Phyllanthus amarus* plant extract (PAPE)

The proximate analysis showed that, PAPE was rich in of carbohydrates with negligible amount of proteins.

3.2. Evaluation of hydroxyl radical scavenging potential of PAPE

PAPE showed an inhibition of formation of hydroxyl radicals by 57% at 25 μg/Ml concentration. Ascorbic acid (Vit. C) at 400μM was able to inhibit hydroxyl radical by 65%. Test for antioxidant ability, PAPE is a potential free radical scavenger than vitamin C (Table 1). The antioxidant property of PAPE could be due to the supply of hydrogen which combined with radicals and thus forming a stable radical to terminate the radical chain reaction by acting as a chain break antioxidant. To know the exact mechanism of action, further studies required.

3.3. Measurement of reducing power and chelation property

Because of the effectiveness of PAPE on hydroxyl radical scavenging, it was further tested to find out its efficacy for reducing activity and chelation properties. PAPE (25 μg/mL) showed 74% reducing power in comparison to ascorbic acid (88%) at 40μM concentration. The results obtained in the present investigation showed that the reducing power of PAPE was due to the antioxidant effect. The ferrous ion–chelating effect was studied showed PAPE (25 μg/mL) showed 68% reducing power in comparison to 0% by EDTA (40μM) concentration.

3.4. Evaluation of anti-glycation activity

The inhibition study for the production of AGEs was carried out for PAPE. The extract was able to inhibit the production AGEs by 65% in comparison to Vit C (87%) at much lower dose (Table 1). PAPE acted as a glycation inhibitor because of its free radical scavenging property. The effectiveness of PAPE in inhibiting hydroxyl radical formation and AGEs formation and hence showed its potential uses for diabetic patients.

4. Conclusion

The results of the above work showed that polysaccharides isolated from PAPE possessed a considerable antioxidant, reducing power, metal ion chelation and anti-glycation properties. Since, it has antiglycating activity; it can be used in delaying or preventing complications of diabetes and aging. However, the *in vivo* activity and the mechanism of action need to be further studied.

Compliance with ethical standards

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Disclosure of conflict of interest

All the authors declared no conflict of interest

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