

PHYTOCHEMICAL INVESTIGATION AND EVALUATION OF ANTIHYPERGLYCEMIC POTENTIAL OF *PREMNA CORYMBOSA*

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ABSTRACT

Objective: The current study is aimed at evaluating the antidiabetic efficacy of *Premna corymbosa* and to evaluate its phytochemical constituents.

Methods: The leaves of *P. corymbosa* were subjected to extraction with methanol, ethyl acetate and hexane. The extracts were scrutinized for their antidiabetic potential using α amylase inhibitory activity, non-enzymatic glycosylation of hemoglobin, glucose uptake by yeast cells and glucose diffusion assays. Besides the plant extract was also subjected to qualitative and quantitative phytochemical studies followed by thin layer chromatography.

Results: The results of α -amylase inhibition assay revealed that the methanol extract was significant with IC₅₀ of 145.72 μ g/ml, when compared with ethyl acetate and hexane extracts. The inhibition of glycosylation of hemoglobin was in a dose dependent manner, conversely the glucose transport and diffusion differs with the sample and glucose concentration. Qualitative phytochemical analysis of the methanol extract confirms the presence of various phytochemicals like flavonoids, phenol, tannin, alkaloids, carbohydrates, terpenoids, coumarins, quinones and cardiac glycosides. Furthermore, phenols, flavonoids and tannins were quantified to be 734.54 GAE/g, 738.95 QE/g and 224.24 GAE/g, respectively.

Conclusion: The results of the work consequently signify the potential of the extract to manage hyperglycemia. However, these effects need to be confirmed using *in vivo* models and clinical trials for its effective utilization as therapeutic agents.

Keywords: *Premna corymbosa*, Phytochemicals, α -amylase, Glucose uptake, Glucose diffusion, Glycosylation.

INTRODUCTION

Diabetes mellitus (DM) is a faction of metabolic anarchy typified by the presence of an unrelieved hyperglycemia due to defective insulin secretion and/or insulin action. It is allied with dysfunctions in carbohydrate, fat and protein metabolism [1, 2]. Long term obstacle such as renal failure, coronary artery disorder, cerebro-vascular disease, neurological complications, blindness, limb amputation, long term damage, dysfunctions, failure of various organs and ultimately premature death are linked with unremitting hyperglycemia [3]. The numeral of inhabitants enduring from diabetes globally is growing at a disquieting rate. It is envisaged that about 366 million people are liable to be diabetic by the year 2030 [4].

With escalating occurrence of diabetes, exploration for new surrogate therapy for its avoidance and treatment is the need of the hour [5]. Newly, the relevance of natural substances for the prevention, management and treatment of DM has been reported by numerous researchers and there is increasing exploration for herbal hypoglycemic agents [6, 7, 8, 9].

The medicinal plants might provide a useful source of new oral hypoglycemic compounds for development of pharmaceutical entities or as a dietary adjunct to existing therapies. Plant derived drugs came into use in the topical medicine through the uses of plant material as aboriginal cure in myths or traditional systems of medicine. Over 400 traditional plants have been reported for the treatments of diabetes [10].

India has a rich history of using various potent herbs and herbal components for treating diabetes. Many Indian plants have been investigated for their beneficial use in different types of diabetes and reported in numerous scientific journals. *Premna corymbosa* rottl. (Family: Verbenaceae), commonly known as kulamani in Tamil is a small tree or large shrub. Leaves are used to cure weakness of limbs, to alleviate headache and to treat diabetes [11, 12].

MATERIALS AND METHODS

Preparation of the extract

The leaves of *Premna corymbosa* plant were collected from Kelambakkam forest situated at Chennai and identified by Dr. N. Mathivanan, CAS in Botany, University of Madras. The leaves were

shade dried at room temperature and then milled in to coarse powder by a mechanical grinder. 10gm of air dried powder was added to 100ml of methanol, ethyl acetate and hexane, kept on a rotary shaker at 190-220 rpm for 24 hours. After 24 hours the solution was filtered and evaporated to make the final volume one - fourth of the original volume and stored at 4°C in air tight containers.

Evaluation of antidiabetic potential of *P. corymbosa*

α -amylase inhibition assay

The assay mixture containing 200 μ l of 0.02M sodium phosphate buffer, 20 μ l of α -amylase (1%) and the plant extracts in concentration range 20-100 μ g/ml were incubated for 10 min at room temperature followed by addition of 200 μ l of starch (1%) in all test tubes. The reaction was terminated by adding 400 μ l DNS reagent and placed in boiling water bath for 5 minutes, cooled and diluted with 15 ml of distilled water and absorbance was measured at 540 nm. The control samples were prepared without any plant extracts [13]. The % inhibition was calculated according to the formula.

$$\text{Inhibition (\%)} = \left[\frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \right] \times 100$$

Acarbose was used as the standard alpha amylase inhibitor. All tests were performed in triplicate.

Inhibition of Glucose Diffusion Assay

This model was adapted from a method described by Edwards *et al.*, [14] which involved the use of a sealed dialysis tube into which 15 ml of a solution of glucose and NaCl (0.15 M) was introduced and the appearance of glucose in the external solution was measured. The model used in the present experiments [15] consisted of a dialysis tube (6 cm \pm 15 mm) into which 2 ml of 0.15 M NaCl containing 0.22 mM D-glucose was added. The dialysis tube was sealed at each end and placed in a 50 ml centrifuge tube containing 45 ml of 0.15 M NaCl. The tubes were placed on an orbital shaker and kept at room temperature (20 \pm 2°C). The movement of glucose into the external solution was monitored at set time intervals. The effects of 50 g/l

plant extracts on glucose diffusion were compared to control tests conducted in the absence of plant extract. At the end of the experimental period, the concentrations of glucose within the dialysis tubing were measured. All tests were carried out in triplicate.

Non-enzymatic glycosylation of hemoglobin

Antidiabetic activity of leaf extract of *P. corymbosa* was investigated by estimating degree of non-enzymatic hemoglobin glycosylation, measured colorimetrically at 520nm [16]. Glucose (2%), hemoglobin (0.06%) and Gentamycin (0.02%) solutions were prepared in phosphate buffer 0.01 M, pH 7.4. 1 ml each of above solution was mixed. Methanol leaf extract of *P. corymbosa* was weighed and dissolved in DMSO to obtain stock solution and then 1-5 µg/ml solutions were prepared. 1 ml of each concentration was added to above mixture. Mixture was incubated in dark at room temperature for 72hrs. The degree of glycosylation of hemoglobin was measured colorimetrically at 520nm. Trolax was used as a standard drug for assay. % inhibition was calculated as-

$$\text{Inhibition (\%)} = \left[\frac{\text{Abs sample} - \text{Abs control}}{\text{Abs sample}} \right] \times 100$$

Glucose uptake by Yeast cells

Yeast cells were prepared according to the method of Cirillo, 1962 [17]. Briefly, commercial baker's yeast was washed by repeated centrifugation (3,000×g; 5 min) in distilled water until the supernatant fluids were clear and a 10% (v/v) suspension was prepared in distilled water. Various concentrations of extracts (1-5 mg) were added to 1 mL of glucose solution (5, 10 and 25 mM) and incubated together for 10 min at 37 °C. Reaction was started by adding 100 µl of yeast suspension, vortex and further incubated at 37 °C for 60 min. After 60 min, the tubes were centrifuged (2,500 × g, 5 min) and glucose was estimated in the supernatant [18]. Metronidazole was taken as standard drug. The percentage increase in glucose uptake by yeast cells was calculated using the following formula-

$$\text{Inhibition (\%)} = \left[\frac{\text{Abs sample} - \text{Abs control}}{\text{Abs sample}} \right] \times 100$$

Where, Abs control is the absorbance of the control reaction (containing all reagents except the test sample), and Abs sample is the absorbance of the test sample. All the experiments were carried out in triplicates.

Phytochemical screening

Qualitative phytochemical analysis

Chemical tests were carried out on the methanol leaf extract using standard measures to recognize the constituents [19, 20, 21, 22].

The leaf extracts were analyzed for alkaloids, flavonoids, phlobatannins, glycosides, phenols, saponins, fat, tannins, anthraquinones, quinones, cardiac glycosides, coumarins acids, steroids, phytosterols, proteins, carbohydrates.

Quantitative phytochemical analysis

The phytochemicals which are present in the methanol extracts of *Premna corymbosa* were determined and quantified by standard procedures.

Determination of total phenols

100 mg of the extract of the sample was weighed accurately and dissolved in 100 ml of distilled water. 1 ml of this solution was transferred to a test tube, then 0.5 ml 2N of the Folin-Ciocalteu reagent and 1.5 ml 20% of Na₂CO₃ solution was added and ultimately the volume was made up to 8 ml with distilled water followed by vigorous shaking and finally allowed to stand for 2 hours after which the absorbance was taken at 765 nm. These data were used to estimate the total phenolic content using a standard calibration curve obtained from various diluted concentrations of gallic acid [23].

Determination of total flavonoids

Total flavonoids were determined by Aluminium Chloride Colorimetric method [24]. 0.5ml of plant extract was mixed with 1.5ml of ethanol, 0.1ml of 10% aluminium chloride, 0.1ml of 1M potassium acetate and 2.8ml of distilled water. It was kept at room temperature for 30mins. The absorbance of the reaction mixture was measured at 415nm. The results were expressed as milligrams quercetin equivalents (QE) per gram of extract (mg QE/g extract). The standard curve was prepared by quercetin in different concentrations (12.5, 25, 50, 80 and 100 mg/L).

Determination of tannins

The tannins were determined by Folin and Ciocalteu method [25]. 0.1 ml of the sample extract was added with 7.5 ml of distilled water and adds 0.5 ml of Folin Phenol reagent, 1 ml of 35% sodium carbonate solution and dilute to 10 ml with distilled water. The mixture was shaken well, kept at room temperature for 30 min and absorbance was measured at 725 nm. Blank was prepared with water instead of the sample. A set of standard solutions of gallic acid is treated in the same manner as described earlier and read against a blank. The results of tannins are expressed in terms of gallic acid mg/g of extract

Thin Layer Chromatography

The TLC was performed on precoated 20×20 cm and 0.25 mm thick plates. The plates were dried and developed in suitable solvents for rapid screening chloroform/ methanol in the ratio 1:9, 0.5:9.5 and 0.25:9.75. The plates were run in the above solvent systems and dried at room temperature. Derivatisation of TLC plates was done by UV light at 254nm and also under iodine the plates are visualized. Different bands were observed and corresponding R_f values are determined. R_f value of each spot was calculated as:-

$$R_f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

RESULTS AND DISCUSSION

Inhibitory of alpha amylase

The plant based α amylase inhibitors offers a prospective therapeutic approach for the management of diabetes [26]. The inhibitory activity of methanol leaf extracts of *Premna corymbosa* on alpha amylase was investigated in this study and the results are shown in fig 1. In the alpha amylase inhibition assay, methanol extract (145.72µg/ml), ethyl acetate extract (176.60 µg/ml), and hexane extract (183.95µg/ml) showed 50% alpha amylase inhibition activity at the mentioned concentrations.

Inhibition of Glucose Diffusion

Antihyperglycemic behaviors of most efficient plants were in part explicated by the ability of the phytoconstituents to increase glucose transport and metabolism in muscle and to stimulate insulin secretion [27]. Glucose concentrations inside the dialysis tubing in the absence or presence of plants were inversely related to the glucose concentrations in the external solution. Without the plant extract (Control), glucose movement out of dialysis had reached a plateau with a mean glucose concentration in the external solution. The Methanol leaf extract of *P. corymbosa* signifies inhibitory potential of glucose diffusion (Fig 2).

Non-enzymatic glycosylation of hemoglobin

The methanolic leaf extract of *P. corymbosa* shows an excellent antidiabetic activity. The percentage inhibition of glycosylation is dose dependent, as dose increases; inhibition increases (Fig 3). Because as the concentration of drug increases formation of glucose-hemoglobin complex decreases and free hemoglobin increases and shows the inhibition of glycosylated hemoglobin. The hemoglobin present in the red blood corpuscles has a tendency to get bound to glucose and form an abduct Alc. The greater the blood-glucose concentration, the greater is the amount of glucose-bound (glycosylated) hemoglobin. Such glucose

hemoglobin linkage is rather constant and lasts for 60 to 120 days (the life-span of red blood corpuscles). Thus the amount of glycosylated hemoglobin is a convinced steer to the concentration

of glucose in the blood. Amount of glycated hemoglobin should not be more than 12% [28].

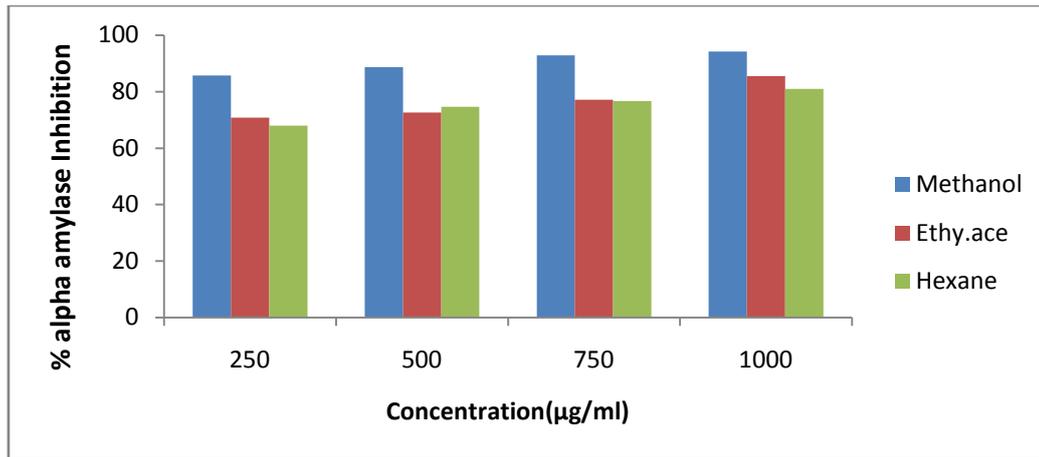


Fig. 1: Inhibition of α amylase activity by extracts of *Premna corymbosa* leaves

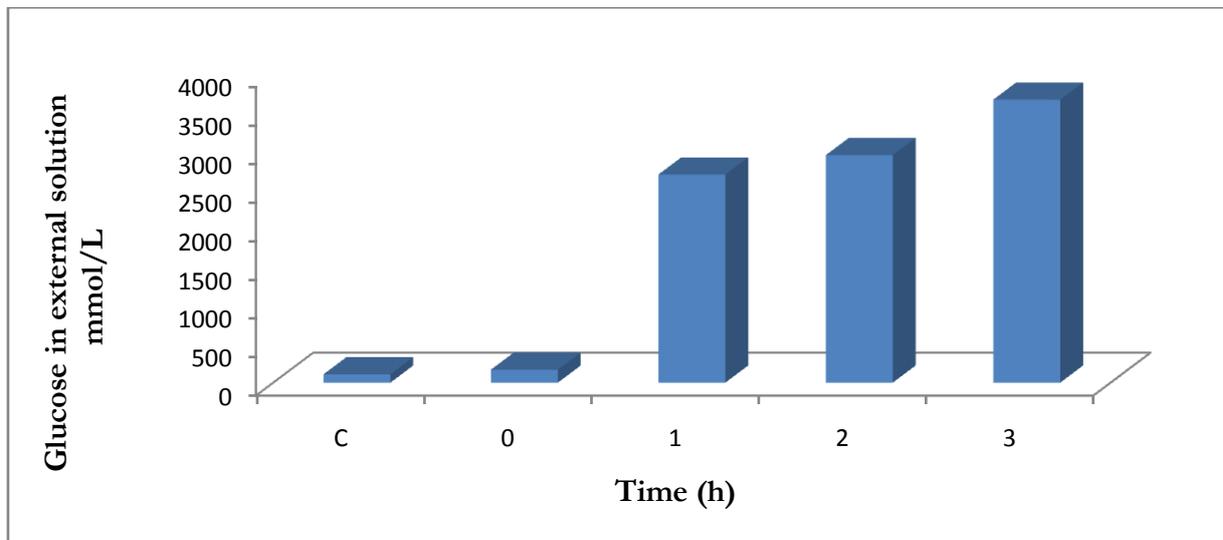


Fig. 2: Effect of methanol leaf extract of *P. corymbosa* on glucose diffusion

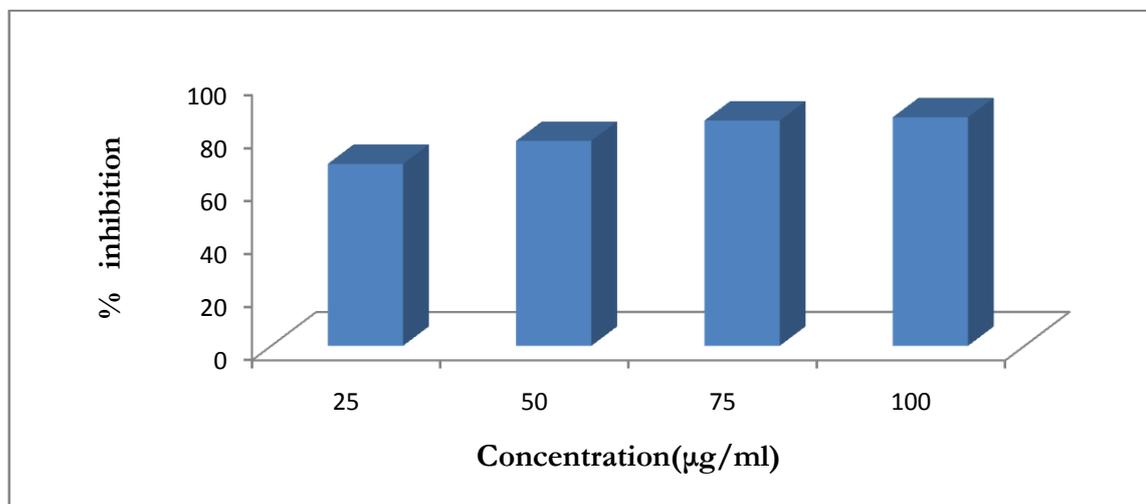


Fig. 3: Inhibition of non-enzymatic glycosylation of hemoglobin by methanol extract of *P. corymbosa*

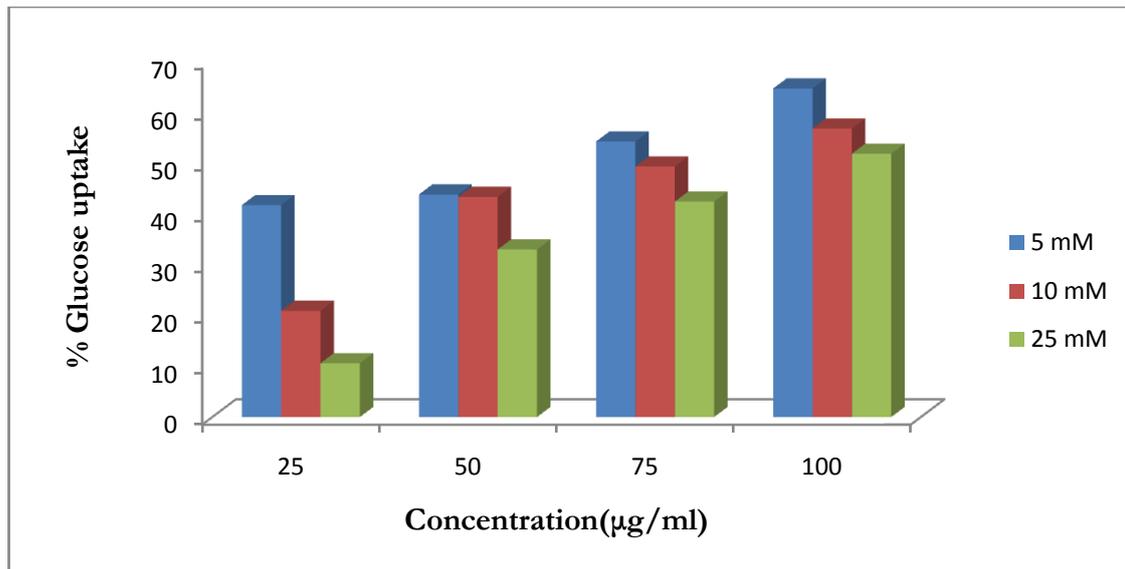


Fig. 4: Inhibition of Glucose uptake by yeast cells

Glucose uptake by Yeast cells

The rate of glucose transport across cell membrane in yeast cells system was explored and the results are given in fig 4. The amount of glucose lingering in the medium after a specific time serves as a marker of the glucose uptake by the yeast cells. The rate of uptake of glucose into yeast cells was linear in all the 3 glucose concentrations. The distinctiveness of the sugar-transport system in yeast has been getting rehabilitated concentration in several laboratories [29]. Recent studies on the transport of non metabolizable sugars and certain metabolizable glycosides suggest that sugar transport across the yeast cell membrane is mediated by stereo specific membrane carriers [30, 31]. It is reported that in yeast cells (*P. corymbosa*) glucose transport is extremely complex and it is generally agreed that glucose is transported in yeast is by a facilitated diffusion process. Facilitated carriers are specific carriers that transport solutes down the concentration gradient. This means that effective transport is only attained if there is removal of intracellular glucose [32].

Table 1: Qualitative phytochemical screening

Phytochemicals	Result
Alkaloids	+
Carbohydrates	+++
Glycosides	---
Saponins	---
Proteins and Amino acids	---
Phenols	+++
Tannin	+++
Coumarin	+++
Phytosterols	---
Flavonoids	+++
Quinone	+++
Cardiac glycosides	+++
Terpenoids	+++
Acids	---
Phlobatannins	+++
Antraquinone	---
Steroids and Phytosteroids	---
Fixed oils	---
Fat	---
Gum & Mucilages	---

+++ : Presence of Constituents,

--- : Absence of Constituents.

Qualitative and quantitative phytochemical analysis

The outcome of qualitative phytochemical screening of methanol extract of *P. corymbosa* showed the presence of flavonoids, phenol, tannin, alkaloids, carbohydrates, terpenoids, coumarins, quinones and cardiac glycosides; whereas the test for protein, saponins and amino acids showed negative results. Among these the phytoconstituents viz. phenols, flavonoids and tannins which are acknowledged to be present in the leaf extract are quantitatively determined.

Thin Layer Chromatography

Methanolic extract was subjected to TLC in order to identify the bioactive compounds. In the present study, the most appropriate TLC system for analysis was shown to be methanol: chloroform (1:9) with the largest discriminating power. Seven bands (Figure 5) were found with Rf values of 0.22, 0.35, 0.43, 0.59, 0.88, 0.94 and 0.98 under Ultra Violet light and eight bands (Figure 5) were found with Rf values of 0.22, 0.35, 0.43, 0.59, 0.88, 0.84, 0.94 and 0.98 under iodine visualization.

Table 2: Quantitative phytochemical estimation

Phytochemical Constituent	Amount
Total Phenols	734.54 GAE/g
Total Flavonoids	738.95 QE/g
Tannins	224.24 GAE/g
GAE - Gallic Acid Equivalents QE - Quercetin Equivalents	

Under UV Under iodine

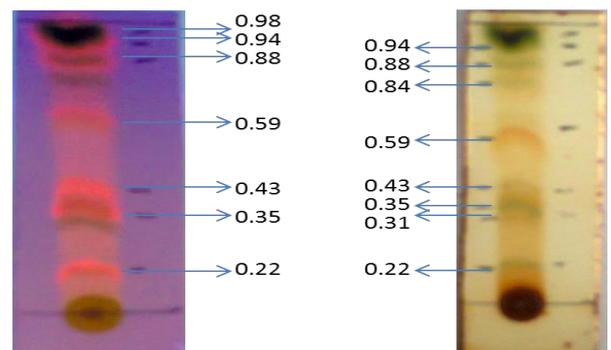


Fig. 5: Thin layer chromatography of methanol extract of *P. corymbosa*

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