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PHYTOCHEMICAL INVESTIGATION AND IN VITRO EVALUATION OF HYPOGLYCEMIC POTENTIAL OF GREWIA HIRSUTA

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ABSTRACT

Diabetes is a metabolic disorder which results due to deficiency in insulin and its metabolism. A search for effective medication with lesser or no side effects is the need of the hour. Hence the current study is focused at evaluating the anti-diabetic potential of *Grewia hirsuta.* The leaves of *G. hirsuta* were subjected to solvent extraction with methanol, ethyl acetate and hexane and analyzed by various *in vitro* assays such as inhibition of carbohydrate digesting enzymes, non-enzymatic glycosylation of hemoglobin, glucose diffusion and uptake of glucose by yeast cells. The best screened extract was further evaluated for it phytochemical profile by qualitative and quantitative phytochemical analysis. The extract was also subjected to TLC. The results of the study suggest that *Grewia hirsuta* possesses significant hypoglycemic potential. With further mechanistic studies it can be proved as a better source of natural anti-diabetic agents.

KEYWORDS: ***Grewia hirsuta*, anti-diabetic activity, phytochemical analysis, thin layer chromatography.**

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INTRODUCTION

Diabetes Mellitus (DM) is a systemic metabolic disease characterized by hyperglycemia, abnormal elevated levels of lipid and fat in blood and hypoinsulinaemia[1]. According to WHO, the global prevalence of diabetes is estimated to increase from 4% in 1995 to 5.4% by the year 2025 majorly in the developing countries[2]. India presently has the largest number of diabetic patients in the world and has been infamously known as the 'diabetic capital of the world'[3]. The classical symptoms of type 1 diabetes are polyuria (frequent urination), polydipsia (increased thirst), polyphagia (increased hunger) and weight loss. In recent years, drug therapies have been in use for the treatment of diabetes. Some of the standard synthetic drugs used for the treatment of diabetes are sulfonylureas, biguanides, α-glucosidase inhibitors and glinides etc. These drugs lend to cause side effects like nausea, vomiting, abdominal pain, diarrhoea, head ache, abnormal weight gain, allergic reaction, low blood glucose, dark urine, fluid retention or swelling. Moreover, they are not safe for use during pregnancy[4]. Active research has been performed on traditional available medicinal plants for discovery of new antidiabetic drug as an alternative for synthetic drugs. Hence the current study is focused to evaluate the antidiabetic potential of selected medicinal plants.

MATERIALS AND METHODS

(i) Extraction of Plant material

Fresh leaves of *Grewia hirsuta* were collected from the fields located in Malachery forest, Gingee, Thiruvannamalai District, TamilNadu. Coarsely powdered leaves were subjected to direct extraction using solvents of varying polarity such as hexane, ethyl acetate and methanol by following the method[5]. 10g of the leaf powder were immersed in 100ml of respective solvents (1:10 w/v) and kept under shaking condition for 24hrs with intermittent filtration. The filtrates were collected and condensed to obtain the crude extract.

(ii) Evaluation of Anti-diabetic Potential

a. ***α- Amylase inhibition method***

In α -amylase inhibition method, the enzyme solution was prepared by dissolving α -amylase in 20mM phosphate buffer (6.9) at the concentration of 0.5mg/ml. 1ml of the extract of various concentrations (250, 500, 750, 1000 µg/ml) and 1ml of enzyme solution were mixed together and incubated at 25°C for 10min. After incubation, 1ml of starch (0.5%) solution was added to the mixture and further incubated at 25°C for 10min. The reaction was then stopped by adding 2ml of dinitro salicylic acid (DNS, color reagent) heating the reaction mixture in a boiling water bath (5min). After cooling, the absorbance was measured colorimetrically at 565 nm[6]. The inhibition percentage was calculated using the given formula,

$$\%\text{inhibition} = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Sample}} \times 100}{\text{Abs}_{\text{Control}}}$$

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.

b. Non-enzymatic glycosylation of haemoglobin method

Glucose (2%), haemoglobin (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 and 1ml of the methanol extract of varying concentrations were added to it. The reaction mixture was incubated in dark at room temperature for 72 hrs and then the degree of glycosylation of haemoglobin was measured colorimetrically at 520 nm[7]. Metformin was used as a standard drug for assay and percentage inhibition was calculated using the formula,

$$\%\text{inhibition} = \frac{\text{Absorbance}_{\text{Sample}} - \text{Absorbance}_{\text{Control}}}{\text{Absorbance}_{\text{Sample}}} \times 100$$

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.

c. Glucose uptake by Yeast cells method

Yeast suspension was prepared by repeated washing (by centrifugation 3,000×g; 5 min) in distilled water until the supernatant fluids were clear[8]. A 10% (v/v) suspension was prepared with the supernatant fluid. 1mL of glucose solution (5, 10 and 25 mM) was added to various concentrations of methanol extract (250, 500, 750 and 1000 µg) and incubated for 10 min at 37 °C. Reaction was started by adding 100 µl of yeast suspension, vortexed and further incubated at 37 °C for 60 min. After 60 min, the reaction mixture was centrifuged (2,500×g, 5 min) and glucose was estimated in the supernatant[7]. Metformin was taken as standard drug. The percentage increase in glucose uptake by yeast cells was calculated using the following formula-

$$\%\text{inhibition} = \frac{\text{Absorbance}_{\text{Sample}} - \text{Absorbance}_{\text{Control}}}{\text{Absorbance}_{\text{Sample}}} \times 100$$

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.

d. Glucose diffusion assay method

This assay was performed as described by[9] with minor modifications. 2 ml of 0.15 M NaCl containing 0.22mM D-glucose was loaded into a dialysis tube containing plant extract (50g/L) and the dialysis tube was sealed. The sealed tube was then placed in a centrifuge tube containing 45 ml of 0.15 M NaCl and kept in an orbital shaker at room temperature. The diffusion of glucose into the external solution was monitored by measuring the glucose in the external solution every 60minutes.

(iii) Qualitative phytochemical analysis

The methanol extract of *G. hirsuta* was subjected to various biochemical tests to screen for the presence of phytochemicals such as alkaloids, flavonoids, phenols, saponins, tannins, carbohydrates and glycosides following the methods[10].

(iv) Quantitative photochemical analysis

a. Determination of total phenols

The total phenolics content in different solvent extracts was determined with the Folin-Ciocalteu's reagent (FCR) following the assay provided[11]**.** 0.5 ml of the extract was mixed with 0.1ml FCR (diluted 1:10 v/v), incubated for 15min, followed by the addition of 2.5 ml of saturated sodium carbonate solution. The final volume of the tubes were made up to 10 ml with distilled water, allowed to stand for 30 min at room temperature and the absorbance was measured against reagent blank at 750nm. A calibration curve was constructed using gallic acid solutions as standard and total phenolic content of the extract was expressed as Gallic Acid Equivalents/g sample.

b. Estimation of total flavonoids

Total flavonoid content was determined by Aluminium chloride method using Quercetin as a standard. 1ml of methanol extract of *G. hirsuta* was added to 4 ml of distilled water and incubated for 5min. After incubation, 0.3 ml of $NaNo_2$ (5%) and 0.5 ml of $AlCl_3$ were added and the mixture was re-incubated at room temperature for 6min followed by the addition of 0.5ml of 1M NaOH. The final volume was made up to 10ml with distilled water and the absorbance of the reaction mixture was measured[12] at 510 nm.

c. Estimation of total alkaloids

The total alkaloid content of *G. hirsuta* was estimated using the method specified by Harborne. 5 g of the sample (leaf powder) was weighed and added to 200 ml of acetic acid (10% in ethanol), covered and allowed to stand for 4h. The solution was filtered and the

filtrate was concentrated on a water bath to one-quarter of the original volume. To the concentrate NH_4OH was added drop wise until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected, washed with dilute ammonium hydroxide and filtered. The residue is the alkaloid, which was dried and weighed[13].

(v) Thin layer chromatography

The methanol extract of *G. hirsuta* was further subjected to TLC to study its compound profile. The extract was spotted on pre-coated silica plates and developed with methanol: chloroform mixture in varying ratio. The run TLC plates were visualized under UV illumination and Iodine vapors. The ratio in which distinct bands appeared was optimized and Rf values of the bands was calculated[14].

RESULTS

1. Extraction of Plant material

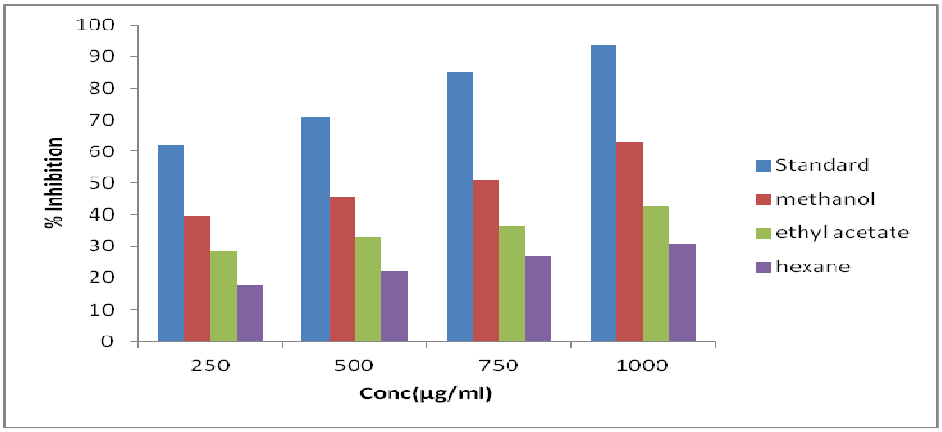
Extracts of the *Grewia hirsuta* was obtained using coarse powdered leaves extracted with different solvents of varying polarity such as chloroform, ethyl acetate and methanol. These extracts were filtered, re extracted with same solvents respectively, condensed to dryness to obtain crude extracts.

2. α- Amylase inhibition method

Alpha amylase is an enzyme that hydrolyses alpha-bonds of alpha linked polysaccharide such as starch to yield high levels of glucose and maltose. Alpha amylase inhibitors bind to alpha- bond of polysaccharide and prevent break down of polysaccharide into mono and disaccharide. *In vitro* inhibitory assay of α-amylase was performed using the methanol extracts of *Grewia hirsuta*. From the following data obtained, results suggest that methanol extract showed significant inhibitory activity and compared with the standard drug metformin. The percentage inhibition varied from 39 to 63 in the concentration range of 250µg/ml to 1000µg/ml which was higher when compared to that of the ethyl acetate and hexane extracts (Fig. 1). Hence the methanol extract was selected for further investigation.

Figure 1

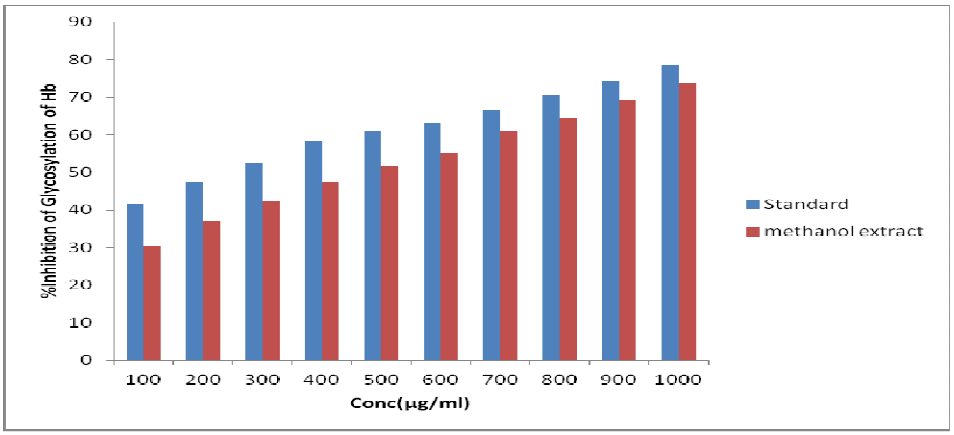
Inhibitory effect of G. hirsuta extracts on alpha amylase activity



3. Non-enzymatic glycosylation of haemoglobin method

The inhibitory activity of methanol extract of *Grewia hirsuta* was found to be up to 73%. It was significant when compared to the standard used which possessed 78% inhibition on glycosylation of hemoglobin (Fig. 2). The hemoglobin has a tendency to get bound to glucose which is present in the red blood cell. The greater the blood-glucose concentration, the greater is the amount of glucose-bound haemoglobin. As the concentration of drug increases formation of glucose-haemoglobin complex decreases and free haemoglobin increases, which show the inhibition of glycosylated haemoglobin.

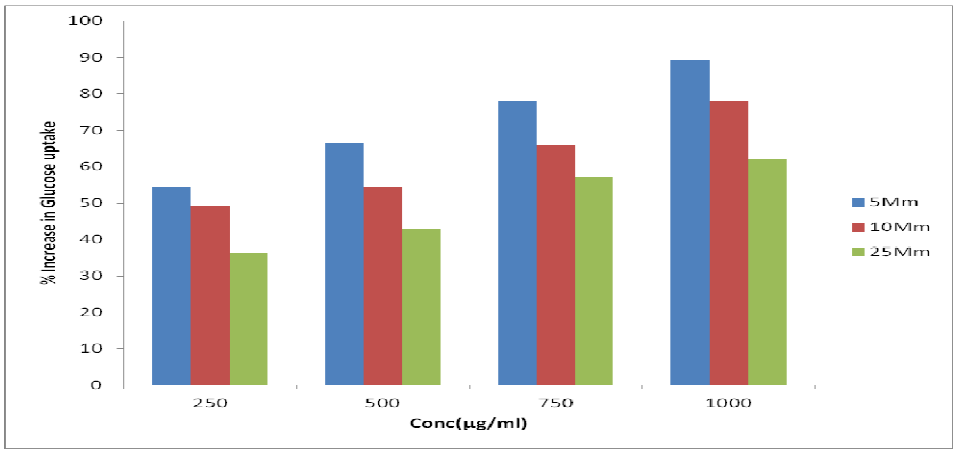
Figure 2
Inhibitory effect of methanol extract of G. hirsuta on glycosylation of haemoglobin



4. Glucose uptake by Yeast cells method

It was studied that the glucose uptake rate increased with increasing concentration of the plant extract and decreased with increasing extracellular glucose. The methanol extract of *G. hirsuta* showed up to 89% increase in glucose uptake by yeast cells (Fig. 3). It is stated that transport of glucose across yeast cell membrane occurs by facilitated diffusion down the concentration gradient. Hence glucose transport occurs only if the intracellular glucose is effectively reduced (utilized)[15]. The data obtained clearly suggests that the plant extract is capable of effectively enhancing glucose uptake which in turn suggests that it is capable of enhancing effective glucose utilization thereby controlling blood glucose level.

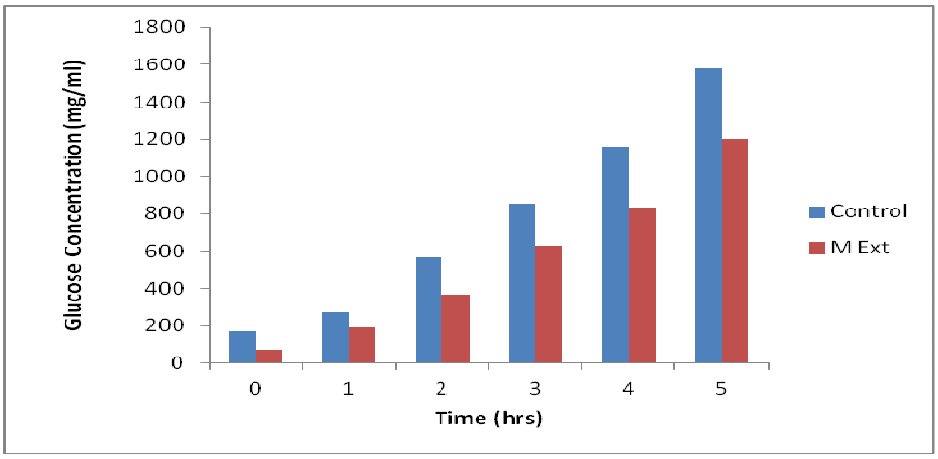
Figure 3
Effect of methanol extract of G. hirsuta on glucose uptake by yeast cells



5. Glucose diffusion assay method

Methanol extract significantly decreased the glucose movement across the membrane when compared to control. The results of the glucose diffusion assay are depicted in Fig. 4. The data clearly suggests that *G. hirsuta* is significant in inhibiting glucose diffusion which in turn states that the plant is capable of regulating glucose movement out of the cells into the blood stream thereby controlling post prandial glucose levels.

Figure 4
Inhibition of glucose diffusion by methanol extract of G. hirsuta



6. Qualitative phytochemical analysis

The phytochemical analysis of methanol extract of *G. hirsuta* showed the presence of alkaloids, flavonoids, phenols and terpenoids in major amounts (Table 1) and was quantified.

Table 1
Qualitative phytochemical analysis

PHYTO COMPOUNDS	RESULTS
Alkaloids	+++
Phenols	+++
Flavonoids	+++
Tannins	++
Saponins	-
Glycosides	+
Reducing sugars	-
Proteins	+

+ Present in minor amounts ++ present in moderate amounts +++ present in major amount

7. Quantitative photochemical analysis

Quantitative tests proved that total alkaloids, total flavonoids and total phenols were estimated to be 0.319g/ gram of sample; 2mg QE/g of the extract and 0.95mg GAE /g of extract, respectively (Table 2).

Table 2
Quantitative phytochemical estimation

PHYTO COMPOUND	COMPOSITION
Total Alkaloids	0.319 mg/g sample
Total Flavonoids	2 mg QE/g sample
Total Phenolics	0.95 mg GAE/g sample

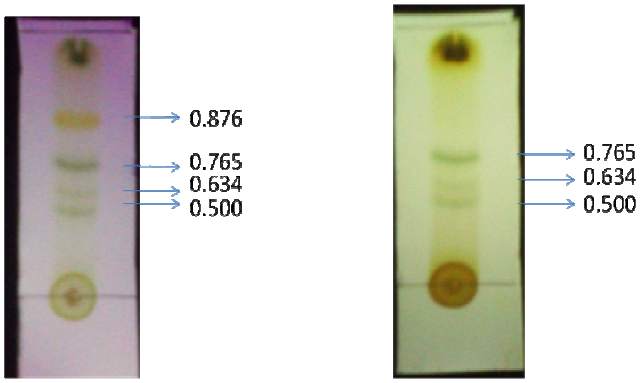
8. Thin layer chromatography

In the ratio 1:9 (methanol:chloroform v/v), the separation of compounds was most distinct and clear. Under the influence of UV four distinct compounds (1, 2, 3 and 4) with R_f values 0.876, 0.765, 0.634 and 0.500 were recognized. When treated with iodine compounds with R_f values 0.765, 0.634 and 0.500 were seen (Fig. 5). The compound 1 with R_f value 0.876 shows higher α-Amylase inhibitory activity. The α-Amylase inhibitory activities of the four compounds with different concentrations are tabulated in table 1.

Table 3
Comparison of the α-Amylase inhibitory activities of the compounds 1, 2, 3 and 4 with extract.

S.No	Concentration (µg/ml)	Inhibition (%)				
		Extract	1	2	3	4
1	250	39.85	33.46	5.40	11	8.83
2	500	45.67	40.63	6.18	17.15	17.80
3	750	50.71	48.79	9.24	28.44	25.07
4	1000	63.14	52.73	14.78	34.18	29.79

Figure 5
Compound separation by thin layer chromatography



CONCLUSION

The anti-diabetic potentials of Grewia hirsuta are evaluated in vitro by several methods such as alpha amylase inhibition, glycosylation of the haemoglobin, glucose uptake and glucose diffusion methods. It was observed that the plant extracts inhibited glycosylation of haemoglobin and since helps in the inhibition of the formation of glycated end products. The studies show that the presence of the phytochemicals in these plants might be the reason for these inhibitions and that the plants may essentially contain herbal bioactive compounds which require further structural elucidation and characterisation methodologies to identify the bioactive compounds.

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