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Phytochemical profiling and in vitro α-amylase inhibitory activity of *Glycosmis pentaphylla* (Retz.) DC.

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Abstract

The present work has been done with an objective to analyze the phytochemical composition responsible for plausible antidiabetic effect of leaf, stem and root of *Glycosmis pentaphylla* (Retz.) DC. Fresh leaf, stem and root of *Glycosmis pentaphylla* were extracted with ethanol (EEGPle, EEGPst, EEGPro) and evaluated by phytochemical analysis for their infinite primary and secondary metabolites *viz.* carbohydrates, proteins, alkaloids, flavonoids, terpenoids, glycosides, saponin and phenols. The antiabetic effect was done with the help of alpha amylase inhibitory assay. Estimation of total flavonoid content (TFC) and total phenolic content (TPC) was also done to confirm the presence of these phytochemicals. The phytochemical analysis of EEGPle revealed that carbohydrates, proteins, alkaloids, flavonoids, glycosides, tannins, phenols and saponins were present, whereas, EEGPst showed the presence of proteins, alkaloids, flavonoids, phenols and terpenoids. Presence of carbohydrates, alkaloids, phenol and terpenoids were found in EEGPro. A significant hypoglycemic activity was revealed by the EEGPle [EC_{50}: (23.14±0.006) µg/mL], compared to EEGPst and EEGPro. Quantity of TFC and TPC was highest in EEGPle (112.96±3.89 mg QRE/g and 96.6±1.08 mg GAE/g extract) rather than EEGPst and EEGPro extracts. The present work suggests that EEGPle has a significantly higher anti-diabetic property than EEGPst and EEGPro. These extracts can help in preventing or slowing down the occurance of diabetes, but a detailed analysis of these extracts is required to determine the presence of promising compound(s) responsible for their anti-diabetic potential.

Keywords: *Glycosmis pentaphylla*, Phytochemicals, Anti-diabetic activity, Hyperglycemic

1. Introduction

The demand and acceptance for medicinal plants is progressively increasing. In fact, the existence of human race depends more or else on herbal drugs. Today, most of the under-developed nations are still known to practice traditional systems of herbal medicine (Singh, 2002). This is so, as overconsumption of allopathic drugs can cause severe side effects like cardiovascular disorders, memory loss, stress, anxiety and so on (Firenzuoli and Gori, 2007). Even though the role of medicinal plants is highly appreciated, thorough knowledge has to be made before being accepted for medication. Hence, there is a need to determine the actual content and composition of crude drug extracts. Also, standardization of the active components present in plants can help in the emergence of a new millennia of preventive medicine to treat human diseases in future.

Diabetes is a serious health issue that affects millions of people and is known as the fifth leading to death (Mukesh and Namita, 2013). This disease is caused due to the metabolic disorders of proteins, fats and carbohydrates (Osadebi *et al.* 2014). It results from either insulin deficiency or malfunction as it causes an increase in blood glucose after any type of meal (Modak *et al.* 2007). Treatments like the use of insulin, pharmaceutical drugs and controlled diet have enabled specialists to control diabetes to some extent. Modern systems of medicine have also identified several types of glucose-lowering drugs that can decelerate diabetes. These drugs have some disadvantages, including drug resistance (reduction of efficiency), side effects, and even toxicity (Hui *et al.* 2005). Medicinal plants are being used in the treatment of diabetics as they have the ability to improve the performance of pancreatic tissue by the production of insulin or reducing the glucose absorption in intestine.

The plant *Glycosmis pentaphylla* (Retz.) DC. (*G. pentaphylla*) of the citrus family, Rutaceae, is an evergreen shrub or small tree that grows up to 5 metres tall. The plant is native to China, India, Sri Lanka, Thailand, Cambodia, Vietnam, Malaysia, Indonesia and Philippines. The detailed description of plant is available elsewhere (Yoganarasimhan and Jadhav, 1996). The fruit of the plant is edible and the juice of leaves is used to treat diarrhoea, coughs, rheumatism, anaemia and jaundice (Chopra, 1969). Several authors investigated the plant for anti-inflammatory efficacy (Ahamad and Aqil, 2007), hepatoprotective activity (Nayak *et al.* 2013) antimicrobial effect (Amran *et al.* 2011) and antipyretic potential (Sarkar and Mandal, 2011).

Previous research has revealed that *G. pentaphylla* possesses anti-diabetic activity in its leaves (Gupta *et al.* 2011). But there are no reports showing comparison of the

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hypoglycemic activity of the plant in its leaf, stem and root. Therefore, an attempt to explore the phytochemical composition and in vitro α-amylase inhibitory activity of ethanolic extract of *G. pentaphylla* leaf (EEGPle), stem (EEGPst) and root (EEGPro) is made.

2. Materials and Methods

2.1. Chemicals

Acarbose, Folin-Ciocalteu's reagent, quercetin (QT) gallic acid (GA), 3,5 dinitro salycylic acid (DNSA), Dimethylsulfoxide (DMSO) and α-amylase from *Aspergillus oryzae* were purchased from Sigma-Aldrich and other remaining chemicals were used of analytical grade unless otherwise specified.

2.2. Collection and authentication of plant materials

Fresh leaf, stem and root (about 5 kg) of *G. pentaphylla* were collected from Thodupuzha (Latitude: 9.8959°N; Longitude: 76.7184°E) of Kerala, India during April, 2018 to October, 2018. The leaves, stem and roots were thoroughly washed to remove foreign matters, and then shade dried for 2 weeks. Later, the identification was done as *G. pentaphylla* (Accession number KUBH 6073) by an expert taxonomist from Department of Botany, University of Kerala, India.The leaf, stem and root of *G. pentaphylla* (about 2 kg each) were seperated manually, followed by shade dried. Dried leaf, stem and root were grounded with the help of a mechanical grinder into coarse powder. The generated powders (about 300 g each) were preserved in airtight containers and placed in a cool, dry and dark place until extraction.

2.3. Preparation of plant extracts

Based on literature review, it was found that ethanol possesses higher extraction efficiency due to its polarity (Snyder and Kirkland, 1979). About200 g individual powdered sample was taken in clean, flat-bottomed amber colored glass container and soaked in 700 mL of 95% ethanol. The container was sealed and kept for several days with occasional shaking. The whole mixtures then underwent coarse filtration by pieces of cotton. Thereafter, the mixture was filtered through filter (Whatman No.1) paper and the solvent was made to evaporate under reduced pressure with the help of a rotary evaporator at 50° C to yield crude extracts. (*i.e.* 10.75 g for EEGPle, 6.18 g for EEGPst and 8.25 g for EEGPro). The crude ethanolic extracts thus obtained were kept at 4° C for further studies.

2.4. Qualitative phytochemical screening

Crude extracts were screened to identify the presence of primary and secondary metabolites, *viz.* carbohydrates, proteins, alkaloids, flavonoids, terpenoids, glycosides, tannins and saponins, using standard screening tests and phytochemical procedures (Sofowora, 1982; Harborne, 1973; Suriyamoorthy *et al.* 2014; Al-Daihan *et al.* 2013; Kapoor *et al.* 1969; Smolenski *et al.* 1974; Krishnamoorthi, 2015; Blois, 1958; Tiwari *et al.* 2011).

2.5. In vitro α-amylase inhibitory studies

The α-amylase inhibition assay was performed using 3,5 dinitro salycylic acid (DNSA) method (Miller, 1959) with slight modifications. The crude extract EEGPle, EEGPst and EEGPro was dissolved in a minimum amount of 10% DMSO and was further dissolved in buffer ($(Na_2HPO_4/NaH_2PO_4$ (0.02 M), NaCl (0.006 M) at pH 6.9) to give concentrations ranging from 25 to 200 µg/mL. A volume of 200 µL of α-amylase solution (2 units/mL) was mixed with 200 µl of each of the extracts and was incubated for 10 min at 30 °C. Thereafter, 200 µl of the starch solution (1% in water (w/v)) was added to each tube and incubated for 3 min. The reaction was terminated by the addition of 200 µL DNSA reagent (12 g of sodium potassium tartrate tetrahydrate in 8.0 mL of 2 M NaOH and 20 mL of 96 mM of 3,5-dinitrosalicylic acid solution) and was boiled for 10 min in a water bath at 85–90 °C. The mixture was cooled to ambient temperature and was diluted with 5 mL of distilled water, and the absorbance was measured at 540 nm using a UV-Visible spectrophotometer. The blank with 100% enzyme activity was prepared by replacing the plant extract with 200 µL of buffer. A blank reaction was similarly prepared using plant extracts at each concentration in the absence of the enzyme solution. A positive control sample was prepared using acarbose (100 µg/mL µg/ml), and the reaction was performed similarly to the reaction with plant extract as mentioned above. The % α-amylase inhibition was plotted against the extract concentration and the IC_{50} values were obtained from the graph. The α-amylase inhibitory activity was calculated using the equation given below:

$$\%\ alpha\ amylase\ inhibition = 100 \times \frac{Abs\ 100\%\ control - Abs\ sample}{Abs\ 100\%\ control}$$

2.6. Total Phenolic Content (TPC)

The TPC was estimated according to Cheung *et al.* 2003. The crude extracts of *G. pentaphylla* were mixed with methanol (95%) for preparation of the stock solution (1 mg/mL). A standard, GA was also mixed with 95% methanol to prepare the 1 mg/mL concentration standard solution. For this test, 1 mL of crude extract with 1000 µg/mL concentration was mixed along with 1 mL Folin-Ciocalteu's reagent, 5 min later 10 mL volume of sodium carbonate (7%) solution was added to the mixture, and then deionized distilled water (13 mL) was added and thoroughly mixed. This mixture was kept for 90 min in the dark at 23^0 C and then the absorbance was recorded at 750 nm by UV spectrophotometer (Thermo Fisher Scientific G10S). Standard curve for estimation of TPC was prepared using GA standard solution (i.e. 6.25 µg/mL to 200 µg/mL) using the similar procedure as described earlier. The TPCs were expressed as mg of gallic acid equivalents (GAE) per g of the dried sample.

2.7. Total Flavonoid Content (TFC)

The TFC was estimated by method described by Park *et al.* 2008. The stock solution was prepared as mentioned in TPC. Similarly, the standard solution of QT was prepared through mixing it with 95% methanol (i.e.1 mg/L). To estimate the TFC, 0.3 mL of the crude extract (1000 µg/mL), 3.4 mL of methanol (30%), 0.15 mL of 0.5 moL/L sodium nitrate and 0.15 mL of 0.3 moL/L aluminum chloride were mixed. Then after 5 min, 1 mL of 1 moL/L sodium hydroxide was supplemented. The obtained solution was thoroughly mixed, and absorbance was recorded at 506 nm against the reagent blank. TFCs were expressed as mg of quercetin equivalents (QRE) per g of the dried sample.

2.8. Statistical Analysis

All experimental results are expressed as mean ± standard error (SE), and data were analysed by one-way analysis of variance (P< 0.001) using SPSS software (ver. 22.0; SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Estimation of phytoconstituents

The phytochemical analysis in leaf, stem and root of *G. pentaphylla* revealed the existence of many important bioactive molecules in different extracts, such as carbohydrates, proteins, alkaloids, flavonoids, terpenoids, glycosides, tannins and saponins that were confirmed by colour reaction tests as shown in Table 1. Based on the intensity of the colour reaction, the EEGPle contained the highest amount of alkaloids, phenols, flavonoids and saponin, compared to EEGPst and EEGPro.

Table 1. Phytochemical composition of *G. pentaphylla* leaf, stem and root extracts

Phytochemical constituents	EEGPle	EEGPst	EEGPro
Alkaloids	-	-	
Wagner's Test	++	+	+
Mayer's Test	++	+	-
Test for carbohydrates			
Benedict's Test	++	-	++
Fehling's Test	+++	-	+
Test for glycosides	++	-	-
Phenols	-	-	-
Ferric Chloride Test	+++	++	++
Flavonoids	-	-	-
Lead Acetate Test	+++	++	-
Alkaline reagent Test	+++	+	-
Saponins	-	-	-
Froth Test	+++	-	-
Foam Test	++	-	-
Terpenoids	-	-	-
Libermann Test	+++	++	++
Horizon Test	+++	++	++
Proteins	-	-	-
Xanthoproteic Test	+	-	-
Ninhydrin Test	+	-	+

Where, +: present (in mild amount), ++: present (in moderate amount), +++: present (in large amount), - absent, based on the power of colour generated in the reaction.

3.2. In vitro α-amylase inhibitory studies

Evaluating the plot of % α-amylase inhibition as a function of extract concentrations (Fig. 1), the EC_{50} values were calculated (Table 2). The crude EEGPleexhibited the lowest EC_{50} of 23.14±0.006µg/mL and that of EEGPst and EEGPro were 47.15±0.003 µg/mL and 96.15±0.006 µg/mL, respectively. The EC_{50} value of α-amylase inhibitory assay was acarbose< EEGPle, EEGPst and EEGPro. In comparison with the EC_{50} value acarbose, EEGPle was significantly higher (P<0.001). Thus, the present result reveals that, among the three extracts, EEGPle exerted a 72.2 % α-amylase inhibition at 200 µg/mL concentration.

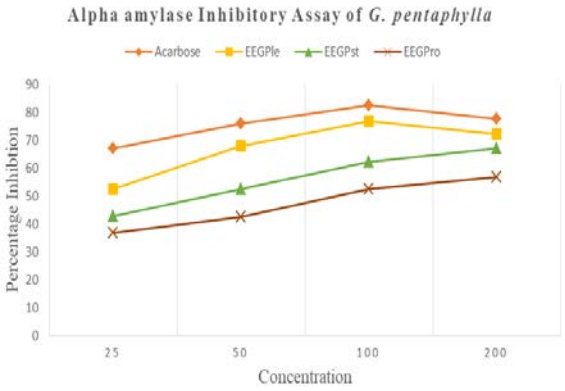

Figure 1. Percentage of α-amylase inhibition as a function of extract concentrations

Table 2. The α-amylase inhibition assay of *Glycosmis pentaphylla*

Standard/ Extract	α-amylase inhibition assay				EC $_{50}$ value for α-amylase inhibition assay
	25 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL	
Acarbose	67.0±0.70	76.0±0.70	82.4±0.50	77.6±0.50	18.12±0.005
EEGPle	52.4±0.50	67.8±0.58	76.8±0.86	72.2±0.58	23.14±0.006
EEGPst	42.8±0.58	52.4±0.50	62.2±0.58	67.0±0.70	47.15±0.003
EEGPro	36.8±0.58	42.4±0.74	52.4±0.50	56.6±0.74	96.15±0.006
Treatment df (n-1)	482.028***	549.293***	467.492***	192.988***	3854.772***

The representative experiment is a mean standard error followed by different superscript lowercase letters indicate significant difference between each parameter as evaluated by Duncan's Multiple Range Test. f value significant at *P≤0.001 level, NS-non-significant.

3.3. Estimation of TPC and TFC

The TPC of *G. pentaphylla* extracts was estimated from GAstandard curve (y = 0.0106x + 0.0542), and the results were represented in milligrams of GAE. Table 5 shows that the TPC in the EEGPle, EEGPst and EEGPro varied largely and EEGPle exhibited the highest TPC. The content of flavonoid was estimated from the QCT standard curve (y = 0.0005x + 0.029), and the results were expressed as mg of QE (Table 3). The EEGPle showed the maximum amount of flavonoid contents followed by EEGPst and EEGPro.

Table 3. Total phenolic and flavonoid contents of *G. pentaphylla* extracts

Extracts	Total phenolic (mg GAE/g)	Total flavonoid (mg QE/g)
EEGPle	112.96±3.89	96.6±1.08
EEGPst	56.16±0.54	32.1±0.8
EEGPro	23.5±0.55	11.16±0.54

Values were expressed as mean±SD (n=3).

4. Discussion

In the recent past, there has been an exponential growth in the field of herbal medicine, and these drugs are gaining popularity both in developing and developed countries. This is so, as being natural entails less side effects. The traditional medicines in use are derived from medicinal plants, minerals and organic matter (Grover *et al*. 2002). Inclusion of fruits and vegetables in diet has decelerated the occurrence of chronic diseases associated with aging such as cancer, cardiovascular diseases, brain dysfunction and cataract. Also, plants serve as sources for the development of drugs in contemporary medicine. Still, there is a need to determine the safety efficacy and stability of plant derived products before being marketed. Hence, researchers are focusing on the potential of medicinal plants to be used as crude drugs. As part of this, in the present study, the phytoconstituents, and α-amylaseinhibitory activities of the ethanolic extracts of leaf, stem and root of *G. pentaphylla* were evaluated.

Secondary metabolites like flavonoids, alkaloids, tannins, terpenoids, saponins and coumarin metabolites were introduced with major impact on diabetes (Bahmani *et al.* 2014). The present study on *Glycosmis pentaphylla* has shown that several polyphenolic compounds like flavonoids, phenolic acids, and tannins are deemed as the chief constituents of plants. These metabolites have profound activity to enhance glucose utilization by regulating glucagon (Valette*et al.* 1984) and insulin secretion, thus implicating its role in hypoglycemic action in medicinal plants. Alkaloids have been shown to exhibit cytotoxic effect on tumour cell lines emphasizing its role in prevention of cancer, neurodegenerative diseases and chronic inflammation (Lamkadem *et al.* 2001). Bio-functionalities of these secondary metabolites present in the extracts influence the biological activities of the plants. In addition, protective ability of plant extracts against the pathological diseases is related to total phenolics and flavonoids in the plant samples as they have been recognized to exhibit various biological activities (Oyedemi*et al*. 2011). This study suggested that among the ethanolic crude extracts of leaf, stem and root of *G. pentaphylla*, EEGPle have superior anti-diabetic potential owing to the presence of higher amount of phenols, flavonoids, and saponins. These are significantly associated to the anti-diabetic potential of these extracts.

Hyperglycemia has been a classical risk in the development of diabetes and the complications associated with diabetes. Therefore, control of blood glucose levels is critical in the early treatment of diabetes and reduction of macro- and microvascular complications. One therapeutic approach is the prevention of carbohydrate absorption after food intake, which is facilitated by inhibition of enteric enzymes including *α*-glucosidase and *α*-amylase present in the brush borders of intestine. The α-amylase inhibitory studies performed demonstrated that the extracts of *G. pentaphylla* had significant inhibitory potentials. The EC_{50} value (23.14 ± 0.006 µg/mL) of EEGPle is almost similar to that of acarbose (18.12 ± 0.005 µg/mL) a widely used and marketed anti-diabetic drug. EEGPst and EEGPro also exhibited α- amylase inhibition in a dose dependent fashion. These α- amylase inhibitors terminate or decelerate the absorption of starch into the body by blocking the hydrolysis of 1,4-glycosidic linkages of starch and other oligosaccharides into maltose, maltriose and other simple sugars (Kumar *et al.* 2010). The highest α amylase inhibitory activity in EEGPle is mostly due to polar compounds and is worth further investigating and isolating pure active compounds. Hence the above results suggest that EEGPle could be greatly beneficial in reducing the absorption of starch into the body; also, it can be effectively used in ayurvedic treatments.

5. Conclusion

Plant parts have been used individually or in formulations for treatment of diabetes and its complications. One of the major problems with this herbal formulation is that the active ingredients are not well-defined. Efforts are now being made to investigate mechanism of action of some of these plants using model systems. The present study suggests that ethanolic leaf extract of *G. pentaphylla* have anti-diabetic property. As a result, the leaf extracts of *G. pentaphylla* leaf may serve as a possible source of natural anti-diabetic drug. It is important to know the active components and their molecular interaction, which will help to analyse therapeutic efficacy of the product and to standardize the product.

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