

Estimation of Phenolic content, Flavonoid content, Antioxidant and Alpha amylase Inhibitory Activity of Marketed Polyherbal Formulation

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ARTICLE INFO

Article history:

Received on: 01/07/2014

Revised on: 24/07/2014

Accepted on: 12/08/2014

Available online: 27/09/2014

Key words:

Gallic acid, Quercetin,

Fenugreek, α -amylase.

ABSTRACT

The objective of this study was to evaluate α -amylase inhibitory activity of a marketed polyherbal formulation. The α -amylase is one of the major secretory products helps in digestion of starch and glycogen. The polyherbal extract were prepared with aqueous, hydroalcohol and ethanol. In addition, total phenolic content, total flavonoid content and in vitro antioxidant activity was evaluated. Total phenolic content was found to be 3.5725 ± 0.2336 mg of GAE/ 100 g (aqueous extract), 2.9616 ± 0.2563 mg of GAE/ 100 g (hydroalcohol extract), 4.6683 ± 0.4199 mg of GAE/ 100 g (ethanol extract). Total flavonoid content was found to be 96.1556 ± 4.2664 mg of quercetin equivalent/ 100 g (aqueous extract), 85.1881 ± 4.2135 mg of quercetin equivalent/ 100 g (hydroalcohol extract), 96.0122 ± 2.9972 mg of quercetin equivalent/ 100 g (ethanol extract). In vitro antioxidant activity was found to be 6.436 ± 0.3638 mg of ascorbic acid equivalent / 100 g (aqueous extract), 6.7242 ± 0.2461 mg of ascorbic acid equivalent / 100 g (hydroalcohol extract), 5.4616 ± 0.6696 mg of ascorbic acid equivalent / 100 g (ethanol extract). α -amylase inhibitory activity was found to maximum in water extract followed by ethanol extract and hydroalcohol extract.

INTRODUCTION

Diabetes mellitus is a metabolic disorder of multiple etiology characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both. According to World Health Organization (W.H.O) report, number of diabetic patients is expected to increase from 171 million in year 2000 to 366 million or more by the year 2030. There are two main types of diabetes, namely type I diabetes, type II diabetes and gestational diabetes. In type I diabetes (juvenile), β -cell of pancreas does not produce insulin. These patients have absolute insulin deficiency and are dependent on insulin replacement for life. Type II diabetes is accounting for about 90-95%. The key components of type II diabetes are β -cell dysfunction causing impaired insulin secretion and increased need for insulin due to insulin resistance (American Diabetes Association, 2008; Arif *et al.*, 2014). The cause of diabetes remains mystery although both genetics and

environmental factors such as obesity, changing life style, eating habits and lack of exercise appear to play a role. The treatment with insulin or oral hypoglycemic agent on long term usage leads to increase blood sugar, drug resistance, adverse effects and complications which will further affect the immune system of the body. To avoid such problems, it seems beneficial to use ayurvedic formulations for better management of diabetes mellitus (Mishra, 2003). The phytoconstituents have reported to exert biological effects, including carbohydrate hydrolyzing enzyme inhibition and antioxidant activity. The α -amylase (α -1, 4-glucan-4-glucanohydrolases) is one of the major secretory products of the pancreas and salivary glands, playing a role in digestion of starch and glycogen and can be found in microorganisms, plants and higher organisms. The α -amylase enzyme catalyzes the initial step in hydrolysis of starch to a mixture of smaller oligosaccharides consisting of maltose, maltotriose and branched oligosaccharides of α -(1-6) and α -(1-4) oligoglucans. These are the acted on by α -glucosidases and further degraded to glucose which on absorption enters the blood stream. Rapid degradation of dietary starch by α -amylase leads to elevated postprandial hyperglycemia (PPHG).

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Inhibition of α -amylase limits postprandial glucose levels by delaying the process of carbohydrate hydrolysis and absorption. Another prospect mostly emphasized is the antioxidant potential of plants which have beneficial effects on prevention of diabetes and other chronic diseases. The antioxidants from natural source play a principal role by stimulating endogenous antioxidants to neutralize oxidative stress. Plants generally contain secondary metabolites like phenolics, flavonoids, glycosides, coumarins, saponins, terpenoids, alkaloids etc. which reveal their specific characteristic properties and attribute to their pharmacological properties (Sudha *et al.*, 2011; Paul and Banerjee, 2013).

Aim of the present was to evaluate α -amylase inhibitory potential of marketed polyherbal formulation which consists of nineteen herbs namely *Acacia arabica* Wild. (Leguminosae), *Asphaltum*, *Bombax ceiba* Linn. (Bombacaceae), *Butea monosperma* Wild. (Fabaceae), *Emblica officinalis* Gaertn. (Euphorbiaceae), *Eugenia jambolana* Lam. (Myrtaceae), *Ficus bengalensis* Linn. (Moraceae), *Gymnema sylvestre* Retz. (Ascepidaceae), *Holarrhena antidysentrica* Wall. (Apocynaceae), *Momordica charantia* Linn. (Cucurbitaceae), *Pistacia integerrima* Stew. ex Brand (Anacardiaceae), *Plumbago zeylanica* Linn. (Plumbaginaceae), *Pongamia glabra* Linn. (Fabaceae), *Pterocarpus marsupium* Roxb. (Leguminosae), *Santalum album* Linn. (Santalaceae), *Swertia chirata* Buch Ham. (Gentianaceae), *Terminalia chebula* Retz. (Combretaceae), *Tribulus terrestris* Linn. (Zygophyllaceae) and *Woodfordia fruticosa* Kurz. (Lythraceae).

MATERIALS AND METHODS

The marketed D-Diabetes S.M.A.R.T powder was obtained from Shree Maruti Herbals, Mumbai.

Folin Ciocalteu's reagent was procured from Qualigens Fine Chemicals, Bombay. Sodium carbonate, sodium nitrite, aluminium chloride, sodium hydroxide, potassium dihydrogen orthophosphate, sodium hydroxide, potassium ferricyanide, trichloroacetic acid, ferric chloride, Potato starch, diastase (α -amylase), 3, 5-dinitro salicylic acid, sodium potassium tartrate (Rochelle's salt), disodium hydrogen orthophosphate, sodium dihydrogen orthophosphate, sodium chloride were procured from S.D. Fines Chemicals Pvt. Ltd., Mumbai. Gallic acid and Quercetin standards were procured from Dr. K. S. Laddha, ICT, Matunga as a gift samples. Fenugreek seed powder was purchased from Sheetal Ayurved Kendra, Thane.

Preparation of standard solutions, buffer, reagent and extract

Gallic acid solution (100 $\mu\text{g/ml}$): 10 mg of gallic acid was dissolved in 100 ml of distilled water in volumetric flask.

Quercetin solution (1000 $\mu\text{g/ml}$): 1000 $\mu\text{g/ml}$ stock solution was prepared by dissolving 100 mg of quercetin in 100 ml of absolute alcohol.

Ascorbic acid solution: Ascorbic acid was prepared in distilled water of different concentration such as 60, 120, 180, 240, 300, 360, 420, 480 $\mu\text{g/ml}$.

Fenugreek seed powder extract: 20 g of *Trigonella foenum-graecum* Linn. (Leguminosae) seed powder was weighed and kept for maceration in 100 ml of hydroalcohol (1.0: 1.0) [98 % ethanol] solvent overnight and was filtered and filtrate was used for assay.

Preparation of 0.02 M sodium phosphate buffer pH 6.9 with 6.7 mM sodium chloride: 0.02 M disodium hydrogen phosphate and 0.02 M sodium dihydrogen phosphate was prepared separately and check individual pH. The one that has lower pH was added to the one that has higher pH until pH reaches 6.9, if necessary adjust pH with 1 M sodium hydroxide. 6.7 mM sodium chloride was added (Rushabhy).

DNSA reagent preparation: 1 g of 3, 5-dinitro salicylic acid (DNSA) was dissolved in 50 ml of distilled water and then add 30 g of sodium potassium tartrate in small lots; the solution turns milky yellow in color. To the above, add 20 ml of 2 N sodium hydroxide; it turns to transparent orange yellow color. Volume was made up with 100 ml of distilled water. The reagent bottle was wrapped in brown paper and stored in dark and cool place (Amrita, 1956).

Preparation of polyherbal extract: The sample was prepared by macerating 5 g of polyherbal powder in three different solvent such as aqueous, hydroalcohol (1.0: 1.0) [98 % ethanol] and ethanol (absolute) for 4 hours. The extract was filtered and filtrate was used for assay.

Determination of total phenol content

Total phenolic content was estimated by Folin Ciocalteu's method. 1 ml of aliquots and standard gallic acid (10, 20, 40, 60, 80, 100 $\mu\text{g/ml}$) was positioned into the test tubes and 5 ml of distilled water and 0.5 ml of Folin Ciocalteu's reagent was mixed and shaken. After 5 minutes, 1.5 ml of 20 % sodium carbonate was added and volume made up to 10 ml with distilled water.

It was allowed to incubate for 2 hours at room temperature. Intense blue color was developed. After incubation, absorbance was measured at 750 nm spectrophotometer using UV-visible Jasco V- 630 instrument. The extracts were performed in triplicates. The blank was performed using reagent blank with solvent. Gallic acid was used as standard. The calibration curve was plotted using standard gallic acid. The data for total phenolic contents of polyherbal formulation were expressed as mg of gallic acid equivalent weight (GAE)/ 100 g of dry mass (Bhalodia *et al.*, 2011; Patel *et al.*, 2010)

Determination of total flavonoid content

Total flavonoid content was measured with the aluminium chloride colorimetric assay. 1ml of aliquots and 1ml standard quercetin solution (100, 200, 400, 600, 800, 1000 $\mu\text{g/ml}$) was positioned into test tubes and 4ml of distilled water and 0.3 ml of 5 % sodium nitrite solution was added into each. After 5 minutes, 0.3 ml of 10 % aluminium chloride was added. At 6th minute, 2 ml of 1 M sodium hydroxide was added. Finally, volume was making up to 10 ml with distilled water and mix well. Orange

yellowish color was developed. The absorbance was measured at 510 nm spectrophotometer using UV-visible Jasco V-630 instrument. The blank was performed using distilled water. Quercetin was used as standard. The samples were performed in triplicates. The calibration curve was plotted using standard quercetin. The data of total flavonoids of polyherbal formulation were expressed as mg of quercetin equivalents/ 100 g of dry mass (Patel *et al.*, 2010; Pallab *et al.*, 2013; Satish Kumar *et al.*, 2008; Patel *et al.*, 2012).

In vitro antioxidant assay

In vitro antioxidant assay was estimated by ferric reducing antioxidant power method. To the 2.5 ml of extract, 1ml of 0.2 M phosphate buffer pH 6.6 and 1 ml of 1 % potassium ferricyanide was added. The reaction mixture was incubated in water bath at 50°C for 20 minutes. Afterward, reaction mixture was rapidly cooled and 2.5 ml of 10 % trichloroacetic acid was added to stop the reaction and was centrifuged for 10 minutes. 2.5 ml of aliquots was pipetted out and 2.5 ml of distilled water and 0.5 ml of 0.1 % ferric chloride solution was added. The color changes to green.

The mixture was allowed to stand for 10 minutes and absorbance was measured at 593 nm spectrophotometrically using UV- visible Jasco V-630 instrument. The blank was performed using reagent blank and solvent. Ascorbic acid was used as standard. The extracts were performed in triplicate (Patel *et al.*, 2010; Patel *et al.*, 2012).

In vitro inhibitory alpha amylase assay

The 500 µL of plant extract was incubated with 500 µL of α-amylase solution (enzyme solution (2 units/ml) was obtained by dissolving 0.001 g of α-amylase in 100 ml of 0.02 M sodium phosphate buffer pH 6.9 with 6.7 mM sodium chloride) at room temperature (32°C) for about 10 minutes. After incubation, 500 µL of 1 % starch solution (dissolving 1 g of potato starch in 100 ml of distilled water with boiling and stirring for 15 minutes) was added and was incubated at room temperature (32°C) for about 10 minutes. To the above, 1 ml of DNSA reagent was added to stop the reaction and was incubated in hot water bath (85°C) for 5 minutes. After 5 minutes, reaction mixture color changed to orange-red and was removed from water bath and cooled to room temperature.

It was diluted up to 5 ml of distilled water. Extracts at different concentrations (2, 5, 10, 15, 20 mg/ml) were performed in triplicates. Individual blank was performed by replacing enzyme with buffer. Control was performed by replacing extract with solvent. Fenugreek seed powder (4, 10, 20, 30, 40 mg/ml) was used as positive control. Absorbance was measured at 540 nm in JascoV-630 spectrophotometer. Enzyme unit is defined as one unit of enzyme will liberate 1mg of maltose from 1 % starch in 5 minutes under defined condition i.e. room temperature. Logarithmic regression curve was established by plotting percentage of alpha amylase inhibition against sample concentration in order to calculate IC₅₀ (inhibitory concentration)

value. This represents sample concentration (mg/ml) required to decrease the absorbance by 50 % of alpha amylase (Paul and Banerjee, 2013; Parimelazhagan *et al.*, 2011).

The inhibition percentage of α-amylase was assessed by the following formula:

$$\text{The } \alpha\text{-amylase inhibitory activity} = \frac{(Ac^+) - (Ac^-) - (As - Ab)}{(Ac^+) - (Ac^-)} \times 100$$

Where,

Ac⁺ that absorbance of 100% enzyme activity (only solvent with enzyme)

Ac⁻ that absorbance of 0% enzyme activity (only solvent without enzyme)

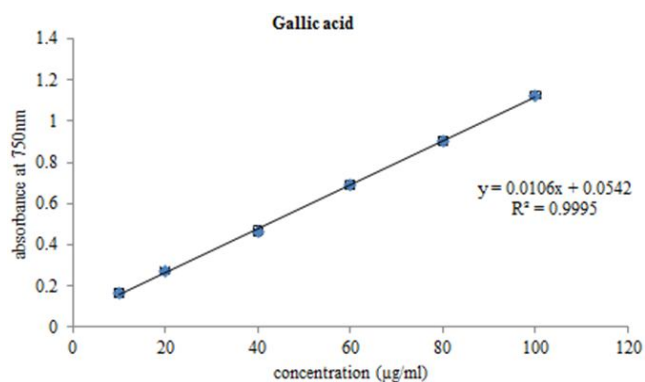
As that absorbance of test sample with enzyme

Ab that absorbance of test sample without enzyme

RESULTS AND DISCUSSION

Determination of total phenolic content

The total phenolic content for aqueous, hydroalcohol and ethanol extracts were estimated by Folin Ciocalteu's method using gallic acid as standard. The reagent is formed from a mixture of phosphotungstic acid and phosphomolybdic acid which after oxidation of the phenols, is reduced to a mixture of blue oxides of tungsten and molybdenum. The blue coloration produced has a maximum absorption in the region of 750 nm and proportional to the total quantity of phenolic compounds originally present. The gallic acid solution of concentration (10-100 ppm) conformed to Beer's Law at 750 nm with a regression co-efficient (R²) = 0.9995. The plot has a slope (m) = 0.0106 and intercept = 0.0542. The equation of standard curve is y = 0.0106x + 0.0542 (Fig. 1).



R² values represented mean data set of n=3

Fig. 1: Total phenolic content for standard gallic acid.

Table 1: Results of total phenolic content for polyherbal formulation.

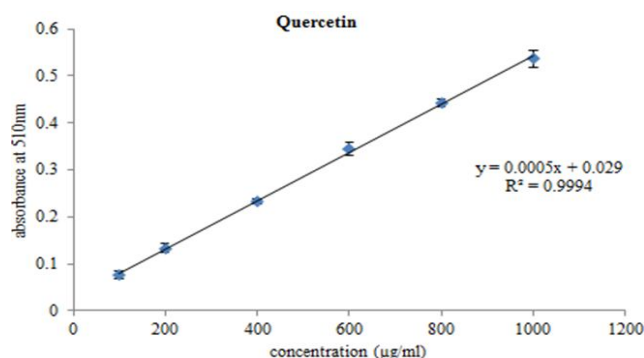
Concentration of extracts	Phenolic content (mg of gallic acid equivalent/ g dry material)
Aqueous, 4 mg/ml	3.5725 ± 0.2336
Hydroalcohol, 1 mg/ml	2.9616 ± 0.2563
Ethanol, 20 mg/ml	4.6683 ± 0.4199

Values are mean ± S.E.M, n=3

Determination of total flavonoid content

The total flavonoid content for aqueous, hydroalcohol and ethanol extracts were measured with the aluminium chloride

colorimetric assay using quercetin as standard. Aluminium chloride forms acid stable complexes with the C-4 keto groups and either the C-3 or C-5 hydroxide group of flavones and flavonols. In addition it also forms liable complexes with ortho dihydroxide groups in A/B rings of flavonoids. The quercetin solution of concentration (100-1000 ppm) conformed to Beer's Law at 510 nm with a regression co-efficient (R^2) = 0.9994. The plot has a slope (m) = 0.0005 and intercept = 0.029. The equation of standard curve is $y = 0.0005x + 0.029$ (Fig. 2).



R^2 values represented mean data set of $n=3$

Fig. 2: Total flavonoid content for standard quercetin.

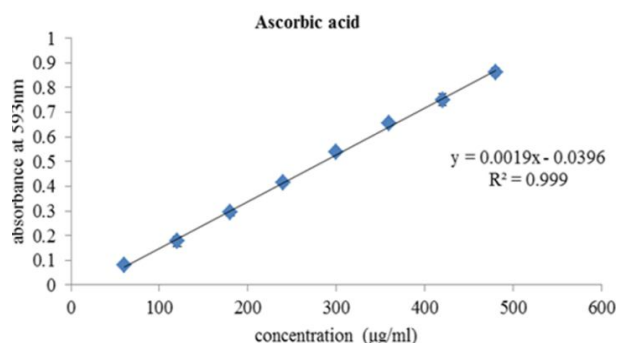
Table 2: Results of total flavonoid content for polyherbal formulation.

Concentration of extracts	Flavonoid content (mg of quercetin equivalent/ g dry material)
Aqueous, 40 mg/ml	96.1556 ± 4.2664
Hydroalcohol, 15 mg/ml	85.1881 ± 4.2135
Ethanol, 20 mg/ml	96.0122 ± 2.9972

Values are mean ± S.E.M, $n=3$

In vitro antioxidant assay

In vitro antioxidant assay for aqueous, hydroalcohol and ethanol extracts were estimated by ferric reducing antioxidant power method using ascorbic acid as standard. Reducing power is a measure of ability of the extract to reduce Fe^{+3} to Fe^{+2} . Substance which have reduction potential react with potassium ferricyanide (Fe^{+3}) to form potassium ferrocyanide (Fe^{+2}) and then react with ferric chloride to form ferric-ferrous complex. The ascorbic acid solution of concentration (60-480 µg/ml) conformed to Beer's Law at 593 nm with a regression co-efficient (R^2) = 0.999. The plot has a slope (m) = 0.0019 and intercept = 0.0396. The equation of standard curve is $y = 0.0019x - 0.0396$ (Fig. 3).



R^2 values represented mean data set of $n=3$

Fig. 3: *In vitro* antioxidant assay for standard ascorbic acid.

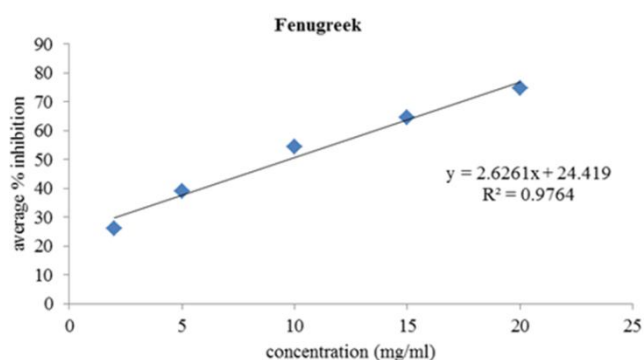
Table 3: Results of *in vitro* antioxidant assay for polyherbal formulation.

Concentration of extracts	Flavonoid content (mg of quercetin equivalent/ g dry material)
Aqueous, 5 mg/ml	6.4360 ± 0.3638
Hydroalcohol, 0.8 mg/ml	6.7242 ± 0.2461
Ethanol, 2 mg/ml	5.4616 ± 0.6696

Values are mean ± S.E.M, $n=3$

In vitro inhibitory alpha amylase assay

The IC_{50} value of α -amylase inhibition for water extract, hydroalcohol extract and ethanol extract was found to be 6.476, 12.395 and 8.690 mg/ml respectively. The IC_{50} value of α -amylase inhibition for hydroalcohol extract of fenugreek seed powder was found to be 9.741 mg/ml (Fig. 4). It was found that water extract shows maximum α -amylase inhibitory activity followed by ethanol extract and hydroalcohol extract (Fig. 5).



R^2 values represented mean data set of $n=3$

Fig. 4: *In vitro* alpha amylase inhibitory assay for standard fenugreek.

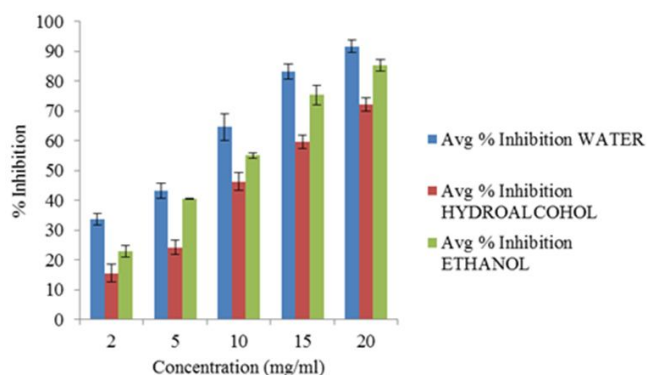


Fig. 5: *In vitro* alpha amylase inhibitory assay for aqueous, hydroalcohol and ethanol polyherbal extracts.

The α -amylase enzyme, catalyses the hydrolysis of α -1-4 glycosidic linkages from the non-reducing ends of polysaccharides (starch-amylase, amylopectine and glycogen) to yield maltose units. This enzyme does not hydrolyze α -1-6-glycosidic linkages present in branch chain polysaccharide (amylopectin, glycogen). Alpha amylase inhibitor is a proteinaceous substance, which binds with alpha amylase enzyme and forms the alpha amylase enzyme-inhibitor complex and thereby inhibit the alpha amylase activity, which resulted in slow digestibility of starch and reduce rate of glucose absorption. Thus, postprandial rise in glucose is decreased. Preliminary screening of D- Diabetes polyherbal extracts such as aqueous, hydroalcohol and ethanol revealed the presence of

carbohydrates, alkaloids, glycosides, phytosterols, phenols, tannins and flavonoids. Quantitative estimation of polyherbal aqueous, hydroalcohol and ethanol extract also showed the presence of phenol, flavonoid and antioxidant. Diabetes mellitus is a metabolic disorder may be due to enhanced cellular oxidative stress and reduced antioxidant activity. Polyphenols and flavonoids are natural antidiabetic agents, which interferes the production of free radicals, reduce oxidative stress and inhibit digestive enzyme, thus lowering postprandial glucose. The aqueous polyherbal extract shows high α -amylase inhibitory activity followed by ethanol and hydroalcohol extract. This suggests that these extracts, rich in flavonoids and phenolics have potential to contribute to the management of diabetes.

CONCLUSION

D-Diabetes polyherbal formulation shows the presence of polyphenols, flavonoids and antioxidant compounds. The polyherbal extracts shows good α -amylase inhibitory activity. The highest α -amylase inhibitory activity shown by aqueous polyherbal extract followed by ethanol and hydroalcohol extracts. This suggests that these extracts, contains flavonoids and phenolics which have potential to contribute in the management of diabetes and its complications. Hence, the parameters such as chemical and *in vitro* pharmacological methods may be considered as a tool for assistance to the scientific organization and manufacturers in developing standards.

ACKNOWLEDGEMENTS

The authors are thankful to Shree Maruti Herbals, Mumbai for gift sample of D-Diabetes S.M.A.R.T powder and Dr. K. S. Laddha, I.C.T, Matunga, Mumbai for gift samples of Gallic acid and Quercetin.

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How to cite this article:

Samidha Kamtekar, Vrushali Keer, Vijaya Patil. Estimation of Phenolic content, Flavonoid content, Antioxidant and Alpha amylase Inhibitory Activity of Marketed Polyherbal Formulation. *J App Pharm Sci*, 2014; 4 (09): 061-065.