D-5677
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RESEARCH ARTICLE Agricultural Science Digest, Volume Issue : ()

Estimation of Phytochemicals and Antioxidant potency of *Trigonella foenum-graecum* Leaves (Variety HM444)

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10.18805/ag.D-5677

ABSTRACT

Background: Plants contain phytochemicals that are crucial to their growth and development. The potential health benefits of dietary phytochemicals including flavonoids, isoflavones and other polyphenols. Polyphenolic substances, the largest class of phytochemicals, act as strong antioxidants due to abundance of hydroxyl groups. *Trigonella foenum-graceum* also known as Fenugreek is rich source of phytochemicals, therefore possess extraordinary healing and medicinal properties.

Methods: The present study is designed to evaluate chief phytochemical constituents and antioxidant capacity of acetone extract of dried *Trigonella foenum-graecum* leaves (Variety HM 444). The phytochemicals like total phenolic content by Folin-Ciocalteu method, total flavonoids by $AlCl_3$ colorimetric assay, total sugars by method of Dubois and reducing sugars by method of Nelson as modified by Somogyi are determined in the acetone extract of leaves. The antioxidant capacity is determined by using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay and phosphomolybdenum assay.

Result: Various phytochemicals such as total phenolic content (3.17 mg GAE/g), total flavonoids (1.34 mg CE/g), total sugars (32.35 mg/g), Reducing sugars (0.53 mg/g) and Non- reducing sugars (31.82 mg/g) are investigated in acetone extract of leaves. The antioxidant activity of leaves extract is evaluated using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay and reported an IC_{50}value of 737.50 µg/mL. The total antioxidant capacity using phosphomolybdenum assay is 28.21 mg AAE/g.

Key words: Antioxidant capacity, Fenugreek, Phytochemicals.

Abbreviations: AAE: Ascorbic acid equivalent, CE: Catechin equivalent; GAE: Gallic acid equivalent; HM: Hisar mukta; IC_{50}: Half-maximal Inhibitory concentration.

INTRODUCTION

Phytochemical studies are based on finding new ways to use plants to make therapeutic drugs. Phytonutrients are endowed with health advantages. For instance, they may be antibacterial, anti-inflammatory, anti-diabetic, anti-cancer and antihypertensive.Since the dawn of human civilization, people have used plants and plant-based products as medicines.The phytochemical constituents that plants produce, which have specific physiological actions, are what give them their therapeutic worth. Primary constituents, which include amino acids, sugars, protein and chlorophyll, *etc*. and secondary constituents, which include alkaloids, essential oils, flavonoids, tannins, terpenoids, saponins and phenolic compounds, *etc.*, are two main categories of phytochemicals, which are chemical compounds derived from plants and these are non-essential nutrients (Murlidhar and Goswami, 2012). *Trigonella foenum-graceum*, also known as fenugreek, is a spice thathas tremendous medicinal and therapeutic properties. The spice is used as alaxative, to cure indigestion and flatulence and to soothe skin irritation. It also reduces swelling and pain. Carbohydrates, proteins, lipids, alkaloids, flavonoids, fibres, saponins and steroidal saponins, as well as vitamins, amino acids and minerals are the main components of fenugreek.Flavonoids such as apigenin, luteolin, orientin, quercetin, vitexin and isovitexin are abundant in fenugreek. These organic antioxidants support cellular health, the immune system and the reduction of ageing indications.The redox properties of phenol and flavonoids, which are crucial in scavenging and neutralising free radicals, are the fundamental cause of their antioxidant action (Aggarwal *et al*., 2022; Wani and Kumar, 2018; Devi *et al*., 2020). The aim of present study was to estimate phytochemical constituents and evaluate antioxidant capacity of acetone extract of dried *Trigonella foenum-graecum* leaves (Variety HM 444). To the best of our knowledge and literature survey, there was no research work reported on *Trigonella foenum-graceum* L. leaves belonging to variety HM 444. In this study, the leaves were found to be rich source of total phenolics, total flavonoids, total sugars, reducing sugars and non-reducing sugars.The presence of phenolics and flavonoids in leaves

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How to cite this article: Moond, M., Singh, S., Devi, R., Pinki and Rajita (2023). Estimation of Phytochemicals and Antioxidant potency of *Trigonella foenum-graecum* Leaves (Variety HM444). Agricultural Science Digest. doi: 10.18805/ag.D-5677.

Submitted: 17-09-2022 **Accepted:** 17-01-2023 **Online:** 06-02-2023

were responsible for their good antioxidant potential which was evaluated using DPPH free radical scavenging assay and phosphomolybdneum assay.

MATERIALS AND METHODS

Collection of plant material

Leaves of *Trigonella foenum-graecum* L. (Fenugreek) of variety HM 444 were collected from Research field of Vegetable science, Chaudhary Charan Singh Haryana Agricultural University, Hisar shown in Fig 1. Before processing, the leaves were dried under the shade at room temperature.

Preparation of acetone extract of fenugreek leaves for phytochemical analysis and antioxidant activity

10 grams powdered samples of Fenugreek leaves were taken in thimble made up of Whatman No.1 filter paper. This thimble was placed in a conventional Borosil soxhlet apparatus accompanied with 500 mL round bottom flask. Approximately 250 mL acetone was added up to one and a half siphons. Thus, the powdered sample of leaves was percolated by using soxhlet apparatus using acetone as solvent. This acetone extract was used for determination of total phenolic content, total flavonoids, total sugars, reducing sugars, non-reducing sugars, DPPH free radical scavenging activity and total antioxidant capacity.

Quantitative analysis of phytochemicals

Determination of total phenolic content

Folin-Ciocalteu method was used for the determination of total phenolic content by using Gallic acid as standard (Singleton and Rossi, 1965). 1.0 mL of acetone extract was taken in a test tube to which, 1.0 mL of 1mol/L Folin-Ciocalteu reagent and 2.0 mL of Na_2CO_3 (20%, w/v) was added,mixed and final volume was made up to 10.0 mL with water. The reaction mixture was centrifuged at 6000 rpm for 10 minutes and then incubated for 8 minutes. Using UV-Vis spectrophotometer, the absorbance of supernatant solution was taken at 730 nm against a blank prepared in the same way having respective solvent instead of extract. The concentration of total phenolic content in the acetone extract was calculated from regression equation obtained from the standard curve of gallic acid and the result was expressed as: mg GAE/g.

Determination of total flavonoids

Catechin was used as a standard for determination of total flavonoids by using $AlCl_3$ colorimetric assay (Marinova *et al*., 2005). In a test tube having 4.0 mL of distilled water, added 1.0 mL of acetone extract and then 0.3 mL of 5% $NaNO_2$ was added. After 5 min, 0.3 mL of 10% $AlCl_3$ was added followed by 2.0 mL of 1M NaOH and then dilutions with distilled water was done upto 10.0 mL volume. The reaction mixture was thoroughly mixed and a UV-Vis double beam spectrophotometer was used to measure the absorbance at 510 nm against a blank prepared in a similar manner but having acetone instead of extract. The total flavonoids concentration in extract was calculated from the regression equation obtained from the standard curve of catechin and result obtained was expressed as: mg CE/g.

Determination of total sugars

Modified method of Dubois was used for total sugars determination (Dubois *et al*., 1956). 1 mL acetone extract of Fenugreek leaves was taken in test tube and added 2.0 mL of phenol solution to it. Then 5.0 mL of conc. H_2SO_4 was poured directly in the reaction mixture followed by cooling of solution for 30 minutes and then UV-Vis double beam Spectrophotometer was used to measure absorbance of the reaction mixture at 490 nm against a blank prepared in a similar way but having acetone in place of extract. The total sugars concentration in extract was calculated from the regression equation obtained from the standard curve of D-glucose and results obtained was expressed as: mg/g.

Determination of reducing Sugars

Method of Nelson as modified by Somogyi (1952) was used for the determination of reducing sugars (Nelson, 1944). 1 mL acetone extract of Fenugreek leaves was taken in test tube and 1.0 mL of distilled water was added to it. Thereafter, 1.0 mL of alkaline copper reagent was added, properly mixed and covered with aluminium foil and heated in hot water bath for 20-25 minutes. After the boiling, tubes had cooled to room temperature, 1.0 mL of the arsenomolybdate reagent was added and then properly mixed and the reaction mixture was diluted with distilled water upto 10.0 mL volume. As a result, the reaction mixture's absorbance was measured at 520 nm using a UV-Vis double beam spectrophotometer against a blank prepared in a similar manner but having 1.0 mL distilled water in place of extract. The reducing sugars concentration present in the acetone extract was calculated from the standard curve of D-glucose and the result was expressed as: mg/g.

Determination of non-reducing sugars

The non-reducing sugars were determined as the difference between the concentration of total sugars and that of reducing sugars.

Non-reducing sugars = Total sugars-Reducing sugars

Evaluation of antioxidant capacity of acetone extract of fenugreek leaves (HM 444)

DPPH free radical scavenging activity

Method given by Hatanowas used to evaluate the DPPH free radical scavenging activity of the Fenugreek leaves extract (Hatano *et al*., 1988). 0.2 mL of acetone extract of particular concentration was taken in test tube followed by adding of 3.0 mL of DPPH (0.1 mM in methanol) and properly mixed for five minutes. After incubation of the reaction mixture in dark for 30 minutes at room temperature, absorbance of the extract and control was noted at 517 nm by using the UV-Vis double beam Spectrophotometeragainst a blank containing respective solvent.A graph was drawn

by plotting DPPH free radical scavenging activity (%) against extract concentration (ìg/mL).Using the formula from the equation $ax^2+bx+c = 0$, IC_{50} was calculated by

$$x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$$

Where,
$x = IC_{50}$ (µg/mL).

Calculation

The percentage of DPPH scavenged (% $DPPH^*_{sc}$) was calculated using:

$$\% \text{ DPPH } *_{sc} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Where,
$A_{control}$ = Absorbance of control.
A_{sample} = Absorbance of sample.

Phosphomolybdneum assay

Modified phosphomolybdenum method by Prieto was used to evaluate the antioxidant capacity of the Fenugreek leaves extracts (Prieto *et al*., 1999). The three mL of phosphomoly bdenum reagent was added to 1 mL of extract in glass vial and the solution was mixed thoroughly, covered with lid. It was incubated for 90 minutes at 95°C. Following this, the vial's contents were allowed to cool before the UV-Vis Double Beam Spectrophotometer was used to detect absorbance at 695 nm. In a similar manner, a blank was made and in place of the extract, the appropriate solvent was utilised. A standard curve was plotted between absorbance against concentration of ascorbic acid (µg/mL).

RESULTS AND DISCUSSION

Quantitative analysis of phytochemicals

Quantitative analysis of various phytochemicals such as total phenolic content, total flavonoids, total sugars, reducing sugars and non-reducing sugars were carried out in acetone extract of Fenugreek leaves and results were reported in Table 1.

Total phenolic content

The total phenolic content in acetone extract of the Fenugreek leaves was estimated with the help of a standard curve using gallic acid as a standard (Fig 2). The Folin-Ciocalteu reagent is used to oxidise phenolic compounds. This reagent is obtained from a mixture of phosphotungstic acid and phosphomolybdic acid which after oxidising 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 730 nm and intensity of colour produced is directly related to the amount of phenolic compounds present in the extract.

The regression equation showed that the absorbance and amount of gallic acid were linearly related. Using the regression equation, ($y = 0.011x+0.0184$, $R^2 = 0.9941$), the total phenolic content in acetone extract of leaves was found to be 3.17 mg GAE/g.

Table 1: Phytochemicals in acetone extract of the Fenugreek leaves.

Phytochemicals	Concentration
Total phenolic content	3.17 mg GAE/g
Total flavonoids	1.34 mg CE/g
Total sugars	32.35 mg/g
Reducing sugars	0.53 mg/g
Non-reducing sugars	31.82 mg/g

Fig 1: Fenugreek leaves of variety HM 444.

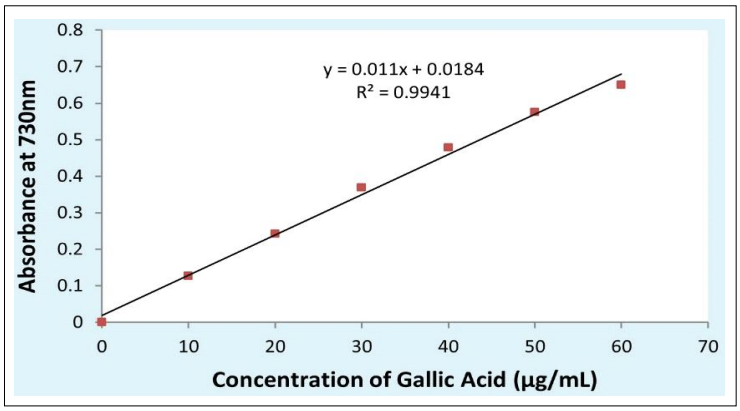

Fig 2: Standard curve of total phenolic content using standard as gallic acid.

Total flavonoids

Using catechin as a standard, a standard curve was used to determine the total flavonoids in the acetone extract of the Fenugreek leaves (Fig 3). Determination of total flavonoids is based on the principle that C-3, C-5 hydroxyl group and C-4 keto group of flavones and flavonols form acid stable complex with $AlCl_3$. In addition to this, orthodihydroxyl groups of the A or B ring in flavonoids also form acid labile complexes by reacting with $AlCl_3$.

The regression equation showed that the absorbance and concentration of catechin were linearly related. Using the regression equation, ($y = 0.0019x+0.0055$, $R^2 = 0.9898$), the total flavonoids in acetone extract of leaves was found to be 1.34 mg CE/g.

Total sugars

Using D-glucose as a standard, a standard curve was used to determine the total sugars in the acetone extract of the Fenugreek leaves (Fig 4). D-glucose got dehydrated in acidic medium to form Hydroxymethyl furfural which converts into yellow-brown coloured solution by reacting with phenol and had maximum absorbance (λ_{max}) at 490 nm.

The regression equation showed that the absorbance and concentration of D-glucose were linearly related. Using the regression equation, ($y = 0.0051x - 0.0215$, $R^2 = 0.9927$), the total sugars in acetone extract of leaves was found to be 32.35 mg/g.

Reducing sugars

Reducing sugars in acetone extract of the Fenugreek leaves was estimated with the help of a standard curve using D-glucose as a standard (Fig 5). When reducing sugars are heated in the presence of alkaline copper tartrate, cupric ions are reduced to cuprous state and subsequently cuprous oxide is produced. Reduction of molybdic acid to molybdenum blue occur when cuprous oxide is mixed with arsenomolybdic acid, which is analysed at 520 nm by using UV-Vis spectrophotometer.

The regression equation showed that the absorbance and amount of D-glucose were linearly related. Using the regression equation, ($y = 0.0049x - 0.0068$, $R^2 = 0.9929$), the total sugars in acetone extract of leaves was found to be 0.53 mg/g.

Non-reducing sugars

The difference between the concentration of total sugars and that of reducing sugars was used to evaluate the non-reducing sugars in the acetone extract of the Fenugreek leaves. It was found to be 31.82 mg/g.

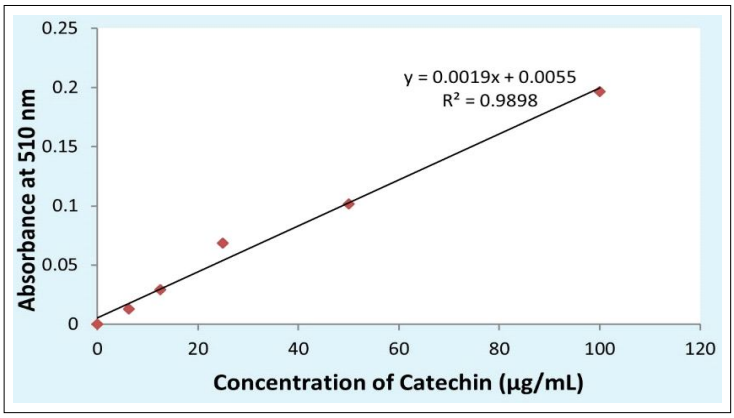

Fig 3: Standard curve of total flavonoids using catechin as standard.

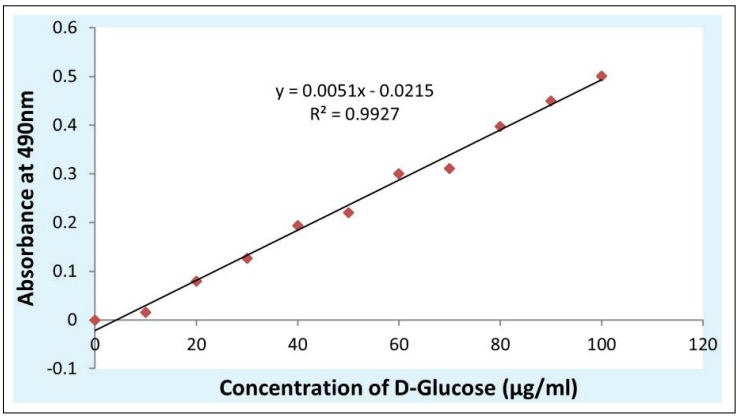

Fig 4: Standard curve of total sugars using D-glucose as standard.

Antioxidant capacity of acetone extract of fenugreek leaves (HM 444)

DPPH free radical scavenging activity

By preventing the propagation of the oxidative chain reaction, antioxidants stop the oxidation of vital biological macromolecules. Researchers had focused their attention on extracting natural antioxidants due to the negative consequences of synthetic antioxidants. A purple stable free radical called DPPH (2, 2-diphenyl-1-picrylhydrazyl) reacts with a hydrogen donor. The entire molecule has delocalized spare electrons, which prohibit dimerization and also give the molecule of DPPH its colour, with an absorption maxima at about 517 nm in UV/Vis spectra. After reacting, the DPPH radical produces the reduced form of DPPH (hydrazine form), which changes the colour from purple to pale yellow. The amount of purple colour disappearance is influenced by the antioxidant concentration. As a result, the degree of solution discoloration revealed scavenging. Antioxidant activity was expressed as IC_{50} value. DPPH free radical scavenging activity (%) was plotted against concentration of extract as shown in Fig 6.

The amount of antioxidants required to decrease the initial DPPH radical by 50% is known as the IC_{50} and was evaluated for acetone extract based on the % of DPPH free radical scavenged (Table 2). The IC_{50} value of acetone extract of Fenugreek leaveswas found to be 737.50 µg/mL.

Table 2: DPPH free radical scavenging activity of acetone extract of Fenugreek leaves.

Concentration of ethyl acetate extract (µg/mL)	% DPPH free radical scavenging activity
100	2.09
300	18.01
500	38.26
700	49.69
900	71.18
1100	78.43
IC_{50} value (µg/mL)	737.50

Phosphomolybdneum assay

The total antioxidant capacityof acetone extract of the Fenugreek leaves was estimated with the help of a standard curve using ascorbic acid as a standard (Fig 7). The antioxidants present in extract reduce the Mo (VI) to Mo (V). Mo (V) react with the phosphate group of sodium phosphate to form a green coloured complex *i.e.* Mo (V)-phosphate complex (phospho molybdenum complex) in acidic medium.

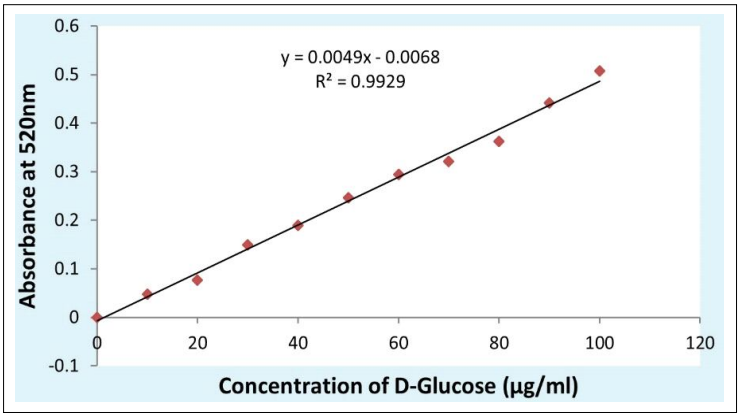

Fig 5: Standard curve of reducing sugars using standard as D-glucose.

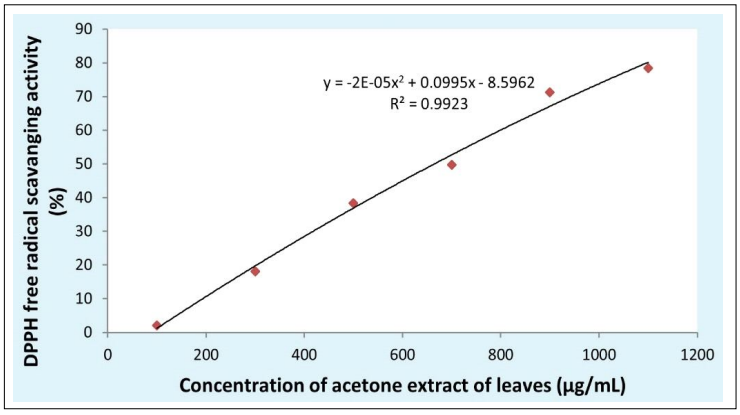

Fig 6: Quadratic regression equation for IC_{50} value of acetone extract of fenugreek leaves.

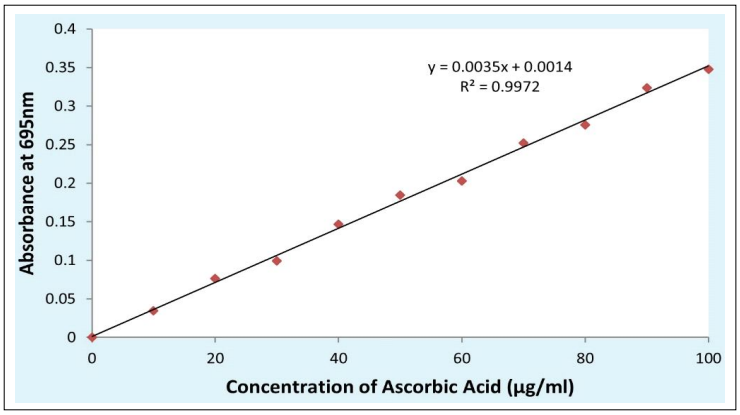

Fig 7: Standard curve for total antioxidant capacity using ascorbic acid as standard.

This complex is measured using spectrophotometer at λ_{max} and the reaction is highly time dependent.

The regression equation showed that the absorbance and amount of ascorbic acid were linearly related. Using the regression equation, ($y = 0.0035x + 0.0014$, $R^2 = 0.9972$), the total antioxidant capacity in acetone extract of leaves was found to be 28.21 mg AAE/g.

The leaves extracts' antioxidant properties are often explained by their overall phenolic and flavonoid content. The phenolics in leaves are one of the principal classes of phytochemicals serving as primary antioxidants include their primary redox characteristics, which include free radical scavenging, hydrogen donation and singlet oxygen quenching.

CONCLUSION

The present study indicated the presence of phytochemicals in acetone extract of Fenugreek leaves of variety HM 444 which might be the key players in scavenging of oxidative stress inducing species. The quantitative analysis of phytochemicals and antioxidant capacity would be helpful in understanding the pharmacological actions of fenugreek leaves. As Fenugreek leaves are rich source of phytochemicals so its applications in the medicinal, pharmaceutical and nutraceutical fields need to be explored in the future. However, more investigation is required to identify the specific compounds that constitute the antioxidant system and develop applications for the pharmaceutical and food industries.

Conflict of interest: None.

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