Research Article

Screening of Free Radical Scavenging, Anticancer Potential and GC-MS Analysis of *Trigonella foenum graecum* Leaves.

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ABSTRACT

The main objective of this study was to evaluate antioxidant and anticancer potential of the extract and fractions from *Trigonella Foenum graecum* (fenugreek) leaves. The methanolic extract of the fenugreek leaves was sequentially fractioned in petroleum ether, chloroform, ethyl acetate, butanol and aqueous fractions. Qualitative screening of these fractions suggested presence of alkaloids, tannins, flavonoides, terpenoides, saponins, steroids and glycosides. The total phenolic content (38.48 mg gallic acid equivalent/g dry weight) and antioxidant activity (log IC_{50} 1.57±14.3 µg/ml) was found to be highest in butanol fraction of fenugreek leaves. Anti-proliferative assay revealed potent inhibitory effect of chloroform fraction (log IC_{50} 2.93±5.5 µg/ml) on breast carcinoma cells (MCF-7). The gas chromatography mass spectrometry (GC-MS) analysis of the chloroform fraction showed 20 peaks representing 81.72% of the total fraction. Di-(2-ethylhexyl) phthalate was identified as a major compound in chloroform fraction, suggesting its role in killing cancer cells. It is evident from these results that *T. Foenum graecum* contains bioactive compounds having anticancer and antioxidant potential and is recommended as a plant of phytopharmaceutical importance.

Keywords: *Trigonella foenum graecum*, Leaf extract, anticancer, antioxidant, gas chromatography mass spectrometry.

INTRODUCTION

Cancer is one of the major causes of deaths worldwide, together with cardiac and cerebrovascular diseases.[1] The 2016 reports of the National Cancer Institute revealed breast cancer as the most common cancer in females.[2] The cancer treatment deals with chemotherapy, radiotherapy and surgery.[3]Chemotherapy is considered as the most effective methods for cancer treatment, but these chemicals also affect healthy cells. Therefore, the plant derived natural products have received considerable attention in recent years due to their diverse pharmacological properties.[4]

Protein and DNA damage are caused by oxidative stress initiated by free radicals like hydroxyl, peroxyl and superoxide radicals. This damage contributed to the pathogenesis of cancer, diabetes, atherosclerosis, cardiovascular diseases, ageing and inflammatory diseases.[5] Fruits and vegetables contain polyphenolic phytochemicals that are known to lower the risk of several diseases, such as cancer, cardiovascular diseases and strokes.[6] Phenolic compounds that are widely distributed in nature are well known for their antioxidant activity.[7] Antioxidants are the substances that prevent cell damage caused by free radicals by supplying electrons to these free radicals.[8]

The *Trigonella foenum graecum* (fenugreek) is an annual plant that belongs to *Leguminosae* family.[9] Traditionally, fenugreek has been used to treat diabetes, cancer, wounds, arthritis, bronchitis, allergies, high cholesterol, anemia, to ease labor pains, menstruation pain, and as an appetite stimulant.[10] The biological and pharmacological properties of fenugreek are accredited to the diversity of its constituents like polyphenolic substances, volatile constituents, amino acids, etc.[11] Till date, there is no report available on the fenugreek leaves fractionation and analysis of antioxidant and anticancer activities of these fractions. Therefore, the current work was designed to study the antioxidant and anticancer effects of fenugreek leaves extract and fractions. Furthermore, the bioactive fraction of fenugreek leaves was subjected to GC-MS analysis to identify phytocompounds that were responsible for the anticancer activity.

MATERIALS AND METHODS

Collection of plant material

Trigonella foenum graecum (Fenugreek) leaves were collected in the month of February from the Solan region of Himachal Pradesh and then the leaves were carefully separated from whole plant. The botanical identity was confirmed by Department of Forest Products, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.). Voucher specimens were deposited with the Herbarium and are entered in UHF- Herbarium Field book no. 13548, Receipt No. 083.

Preparation of *Trigonella foenum graecum* leaves extracts

Leaves of *T. foenum graecum* were shade dried for weeks to remove moisture content. The dried material was ground into fine powder and packed in air tight bottle. Powder of fenugreek leaves was dissolved in methanol and kept at 37° C for 3 days with continuous stirring. After 3 days the supernatant was filtered through Whatman filter paper. Filtrates were concentrated using a rotary

evaporator at 40° C and then freeze-dried in a vacuum. The methanolic extract was dissolved in water, followed by sequential fractionation using petroleum ether, chloroform, ethyl acetate and butanol.[12]

Phytochemical screening of methanolic plant extract and fractions

Preliminary phytochemical tests were carried out to identify phytocompounds by adopting standard procedures.[13]

Analysis of total phenolic content

Total phenolic content was estimated by Folin Ciocalteu's method. Gallic acid (100 µg/ml) was used as standard at different concentrations (5-30 µg/ml). The plant extract and fractions were dissolved in 1 ml of Follin Ciocalteu's reagent, after 5 min, 2 ml of 7.5 % sodium carbonate was added. After 30 min incubation in the dark, absorbance of the mixture was measured at 765 nm and compared to a Gallic acid calibration curve. The results were expressed as mg of Gallic acid equivalent weight (GAE) / g of dry weight of plant material.[14]

Free radical scavenging assay

2, 2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) free radical scavenging assay was used to estimate antioxidant potential of plant extract and fractions. The relative capacity of antioxidants to scavenge the $ABTS^{\bullet+}$was compared to the antioxidant potential of ascorbic acid. The $ABTS^{\bullet+}$ radicals were induced by mixing ABTS (7.4 mM) with 2.46 mM $K_2S_2O_8$ solution in 1:1ratio and then the solution was kept in dark for 24 hours. The $ABTS^{\bullet+}$working solution was prepared by diluting in methanol in 1:25 ratio. To 3ml of $ABTS^{\bullet+}$ solution, sample extracts containing antioxidant were added at the various concentrations and absorbance was measured at 734 nm.[15] Percentage inhibition of $ABTS^{\bullet+}$ was calculated using the formula:

% scavenging activity = A_O-A_S/A_O×100

Where, A_O is the absorbance of control and A_S is the absorbance of sample. The inhibitory radical scavenging concentration (IC_{50}) of samples were calculated using regression analysis by Graph pad prism 5.0.2.

Maintenance of cell culture

MCF-7 (breast carcinoma) cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India. Cells were cultured in DMEM supplemented with 10% heat inactivated FBS, 1% penicillin-streptomycin and amphotericin B (5µg/ml) in a humidified atmosphere of 5% CO_2 at 37°C.

***In vitro* Cytotoxicity Assay**

Cell viability was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay.[16] $1X10^4$ cells were seeded per well in a 96 well plate followed by overnight incubation. These cells were treated with various concentrations (25, 50, 100, 200 µg/ml) of extract/fractions for 48 hrs. Vincristine sulfate (10 µg/ml) was taken as positive control and DMSO was taken as a negative control. MTT assay was conducted by adding 10µl MTT (5 mg/ml) for formazan generation followed by 4-hour incubation. The media was discarded and 100µl of DMSO was added into each well for dissolving the purple colored complex. The optical density of each well was noted at 595 nm using a microplate reader.[17] Percentage cytotoxicity was calculated by the following formula:

Percentage of cytotoxicity = (OD of Control- OD of Sample/ OD of Control) X 100.

Statistical analysis

Results were expressed as mean ± standard error. The one-way ANOVA and multiple comparisons of 2-tails of Student's t test (2 groups) were used to evaluate the difference between the control and test samples by the Graphpad prism software for windows.

Gas chromatography-Mass spectroscopy of bioactive compounds

The GC-MS analysis was done with ionization energy of 70 eV. The injector temperature was kept at 250° C, with a helium carrier flow of 1 ml/min in a split ratio (10:1). Total GC-MS running time was at 33.10 min and relative percentage amount of each component was calculated by comparing its average peak area to the total areas.[18]

RESULTS AND DISCUSSION

Phytochemical analysis

The qualitative analysis of *T. Foenum graecum* leaves extract and fractions showed a wide range of phytochemicals which were both polar and non-polar in nature (Table 1). Butanol fraction was found to contain most of the phytochemicals followed by aqueous fraction. Steroids were found in only chloroform or ethyl acetate fractions. However, flavonoids were depicted in all fractions except petroleum ether or chloroform. Earlier also, it was reported that methanolic leaves extracts of *T. Foenum graecum* contain tannins, saponins, flavonoids, alkaloids, steroids, phlobatannins.[19]Rajan et al., 2014, also reported that methanolic leaves extract of *T. foenum graecum* contain alkaloids, flavonoids, tannin, saponins, glycosides, resin, thiols and steroids.[20]

Determination of total phenolic content

The total phenolic content of methanolic extract and other fractions was estimated by Folin-Ciocalteu's method using Gallic acid as a standard. The equation of standard curve is y = 0.108x + 0.16 with regression co-efficient (R^2) =0.896. The total phenolic content of methanolic extract and fractions of the fenugreek leaves isgiven in Table 2. As compared to other fractions, highest phenolic content was obtained in a butanol fraction (38.48 mg GAE/g dry weight). Previously, Premanath et al., 2011, has also reported the presence of polyphenols in ethanolic, hexane, chloroform and aqueous extracts of

fenugreek leaves.[21]

Table1: Phytochemical analysis of different extracts/fraction of Fenugreekleaves.

Test	Methanolic Extract	Petroleum Ether	Chloroform	Ethyl acetate	Butanol	Aqueous
Carbohydrate	--	--	--	--	+	+
Alkaloids	+	+	+	--	+	--
Saponins	--	+	+	+	+	+
Steroids	--	--	+	+	--	--
Terpenoids	--	+	+	+	+	+
Tannins	+	+	--	+	+	+
Flavonoids	+	--	--	+	+	+
Protein	+	+	--	--	--	+
Glycosides	--	--	--	--	+	+

Table 2: Total phenolic content of methanolic fraction and extracts of *T. foenum graecum.*

S.No.	Extract/Fractions	Phenolic content
1.	Methanolic extract	10.52±0.02
2.	Petroleum Ether	4.27±0.08
3.	Chloroform	4.60±0.09
4.	Ethyl acetate	12.82±0.01
5.	Butanol	38.48±0.05
6.	Aqueous	14.14±0.21

***In vitro* anti-oxidant assay**

The free radical scavenging activity was analyzed by ABTS assay.

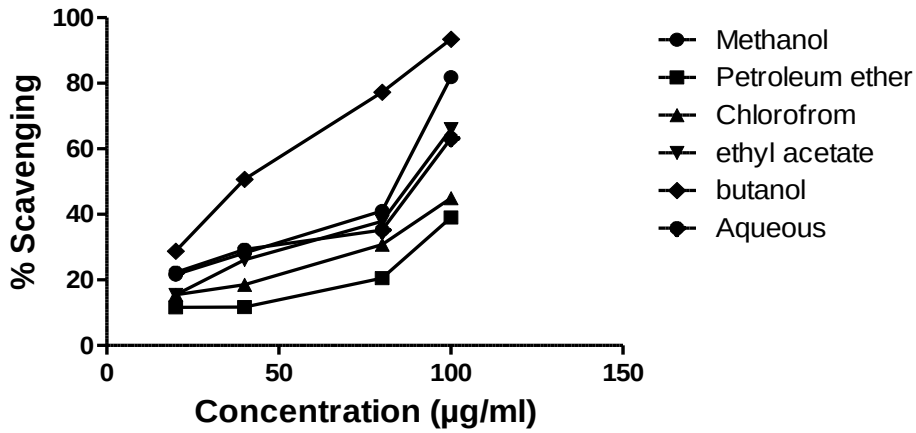

Figure 1: *In vitro* antioxidant activity of extract and fractions of *T. foenum graecum* was done in triplicate fractions. Data is significant as p value< 0.05.

All fenugreek leaves fractions displayed antioxidant potential which increased with the increase in concentration (Fig. 1). The butanol fraction of fenugreek leaves showed the highest antioxidant activity as compared to the other fractions and result was comparable to that of ascorbic acid (Table 3). The antioxidant potential of these fractions can be attributed to their phytochemical constituents, especially phenols, flavonoids and alkaloids. Polyphenolic compounds are known to have antioxidant activities and this activity is believed to be mainly due to their redox properties, which help in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides.[22,23]

Table 3: Free radical scavenging activity of extract/fractions and ascorbic acid (standard) expressed as IC_{50} value.

S.No.	Extract/Fractions	Log IC_{50}±SE
1.	Methanolic extract	1.82±13.5
2.	Petroleum ether	2.43±6.4
3.	Chloroform	2.33±6.7
4.	Ethyl acetate	2.01±10.8
5.	Butanol	1.57±14.3
6.	Aqueous	2.04±8.9
7.	Ascorbic acid	1.43±11.0

Our data also confirmed the correlation between the phenolic content and the antioxidant activity, as both phenolic content and the free radical scavenging activity were found to be highest in the butanol fraction of fenugreek leaves. It has been reported earlier that the aqueous fraction of germinated fenugreek seeds exhibited anti-oxidant activity and authors also suggested that phenolics and flavonoids present in the aqueous fraction could be responsible for the anti-oxidant activity.[24]

Anticancer activity

The anticancer potential of *T. foenum graecum* leaves extract/fractions was analyzed on MCF-7 cancer cell line via MTT cytotoxicity assay at a concentration range of 10-800 µg/ml. The MTT assay revealed that *T.foenum graecum* leaves extract/fractions exhibited cytotoxicity towards MCF-7 cancer cells (Figure 2). The data also revealed that chloroform fraction showed highest anticancer activity (IC_{50}2.93 ± 5.5) followed by ethyl acetate and butanol fractions (Table 4). Previously, the whole plant methanolic extract of fenugreek has been shown to have growth inhibition and apoptotic effects on MCF-7 cells.[25]

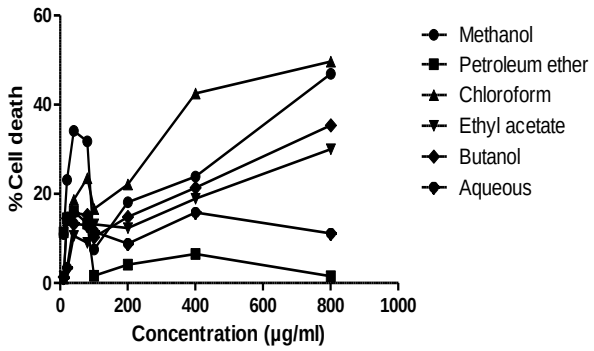

Figure 2: Anticancer activity of *T. foenum graecum* leaves extract and fractions on MCF-7 cancer cell line treated for 48 hrs.

GC-MS analysis of phytoconstituents present in bioactive fractions

As the chloroform fraction of fenugreek leaves displayed highest anticancer activity; therefore GC-MS was performed to analyze the phytochemical content of the chloroform fraction.

Table 4: IC_{50} values of extract/ fractions of *T. foenum graecum* leaves against MCF-7 cancer cell line.

S. No.	Extract/Fractions	LogIC_{50}±SE
1	Methanolic	4.02±4.5
2	Petroleum ether	Not converged
3	Chloroform	2.93±5.5
4	Ethyl acetate	3.59±3.2
5	Butanol	3.68± 3.4
6	Aqueous	6.37±1.7

The GC-MS data revealed that the chloroform fraction of *T. foenum graecum* leaves was found to contain 20 compounds representing 81.72% of the total fraction (Table 5).The major compound identified in chloroform fraction was di-(2-ethylhexyl) phthalate (DEHP) (34.9%) (Fig. 3). Similarly, DEHP isolated from *Aloe Vera Linne* has shown cytotoxic effects towards K562, HL60 and U937 cancer cell lines.[26] Additionally, DEHP isolated from *Calotropis gigantea* flowers displayed potent dose dependent anti-tumor effects in mice bearing Ehrlich ascites carcinomas.[27] Taken together, these results specify that DEHP exhibit potent anticancer properties.

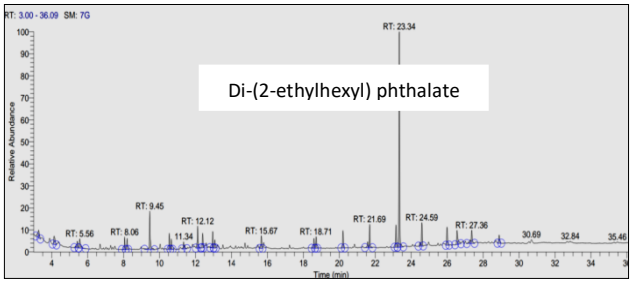

Figure 3: Chromatogram showing the GC-MS analysis of chloroform fraction of fenugreek leaves.

CONCLUSION

The aim of our study was to investigate the antioxidant and anticancer potential of *T. foenum graecum* methanolic leaves extract and fractions. The antioxidant activity and total phenolic content, was found to be highest in butanol fraction. As compared to other fractions the chloroform fraction of fenugreek leaves displayed highest *in vitro* cytotoxic effects on breast cancer cells. The major compound present in chloroform fraction was found to be di-(2-ethylhexyl) phthalate which suggest that DEHP might be responsible for the bioactivity. Further, isolation and characterization of the phytocomponents present in fenugreek leaves has to be done to confirm these bioactivities.

Table 5: List of compound identified by GC-MS in Chloroform fraction of *T. foenum-graecum.*

S.No	Compounds Name	Molecular formula	Retention time (RT)	Area%
1.	Decane	$C_{10}H_{22}$	3.27	2.72
2.	Aziridine 1,2,3 trimethyl trans	$C_5H_{11}N$	4.14	2.30
3.	Cyclopropane nonyl	$C_{12}H_{24}$	5.42	1.60
4.	2,6-Dimethydecane	$C_{12}H_{26}$	5.56	2.13
5.	1-Hexadecanol	$C_{16}H_{34}O$	8.06	1.69
6.	Tetradecane	$C_{14}H_{30}$	8.19	1.41
7.	Phenol,2,6-bis(1-1 dimethylethyl)	$C_{14}H_{22}O$	9.45	6.68
8.	2-Hexadecanol	$C_{16}H_{34}O$	10.55	1.94
9.	Eicosane	$C_{20}H_{42}$	10.65	1.24
10.	1,6,6 trimethyl-7-3(oxobut-1-enyl)-3-8-dioxatricyclo octan-5-1	$C_{13}H_{16}O_4$	11.34	1.69
11.	Ether (2-ethyl-1cyclodecan-1yl) methyl methyl	$C_{13}H_{16}O_4$	12.12	3.83
12.	4-hydroxy-3,5,6-trimethyl-4-(3-Oxo-1-butenyl) - Phenol, 3-isopropoxy-5-methyl	$C_{10}H_{14}O_2$	12.40	1.86
13.	1-Octadecene	$C_{18}H_{36}O$	12.96	2.03
14.	Eicosane	$C_{20}H_{42}$	13.07	1.05
15.	1-Heneicosanol	$C_{21}H_{44}O$	15.67	2.04
16.	Docosyl pentafluoropropionate	$C_{25}H_{45}F_5O_2$	18.59	1.71
17.	Tetracosane	$C_{24}H_{50}$	20.21	2.56
18.	Diisooctylphthalate/di-(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	23.34	34.98
19.	Tetratriacontane	$C_{34}H_{70}$	24.59	4.30
20.	13-Docosenamide	$C_{22}H_{43}N_O$	26.55	3.96

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