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Two New Glucosides from Trigonella Foenum Graecum Seeds and Its Anti Oxidant Activity.

Rashmi Yadav
Assistant Professor, Oxford college of Pharmacy, Ghaziabad, U.P.

Abstract: *Trigonella foenum- graecum seeds (fenugreek) commonly known as 'Fenugreek'contains a number of phytoconstituents viz. alkaloids, glycosides, amino acids, proteins, phenolic acids, terpenoids, flavonoids, Vitamins, sugars, minerals, gums & mucilage etc. The objective of the present study was to isolate and characterize phytoconstituents(s) from the methanolic extract of Trigonella foenum-graecum seeds. The methanolic extract was subjected to column chromatography and eluted with solvent mixtures of increasing polarity, composed of petroleum ether, benzene and chloroform, methanol to isolate phytoconstituents. The structures of the isolated compounds were established on the basis of elemental analysis and spectroscopic evidences (IR, UV, 1HNMR, 13CNMR, MS). Four constituents* ***Salicylaldehyde geranilanyl tetraglucoside A, Salicylaldehyde geranilanyl tetraglucoside B, n- Hexatetracontonol and N-Tetratriacont-16, 18 dien-1-ol*** *were isolated from the methanolic extract of fenugreek seeds. The methanolic extract was also screened for the antioxidant activity (in vitro) using 1, 1-diphenyl-2-picryl- hydrazyl (DPPH) method. The results were compared with standard antioxidant Ascorbic acid and it was observed that methanolic extract of fenugreek possess strong antioxidant potential with IC50 value 4.938 µg/ml while the standard Ascorbic acid had 8.10 µg/ml.*

Keywords: *Trigonella foenum-graecum, Column chromatography, Fenugreek, Isolation, Antioxidant activity.*

I. Introduction

Trigonella foenum- graecum (fabaeace). Is an annual herb 50-70 cm high. Seeds (3-6 mm) oblong compressed, smooth, dark yellow to light brown. Fenugreek seed contains carbohydrates, mainly mucilaginous fiber, proteins, fixed oils (lipids), alkaloids, flavonoids, free amino acids, vitamins A, B & C, saponins and steroids. (Annida B. *et al*., 2004. Yashikawa M., *et al.,* 1997). Fenugreek seeds posses as Anti-diabetic activity**,** Antiplasmodic activity**,** Hypolipidemic activity, Immunomodulatory activity, Antibacterial activity, Anthelmintic activity, Anti-inflammatory, Analgesic activity and Antioxidant activity. (Zia, *et al*., 2001. Sharma, *et al*., 1990. Ahmadiani, *et al*., 2001. Pandian, *et al.,* 2002. Ribes, *et al.,* 1987) .In the present study phytoconstituents of Trigonella foenum- graecum seeds were isolated from column chromatography and characterized. The Antioxidant activity of methanolic extract of Trigonella foenum- graecum seeds were performed using DPPH mathod.

II. Materials and Methods

Plant material

The Trigonella foenum-graecum seeds were purchase from the R.R Herbs, Delhi and authenticated by the Dr. H.B Singh Taxonomist, National Institute of Science Communication and Information Resources (NISCAIR) New Delhi. A Voucher herbarium specimen (specimen no.RIT/MP/G/2010/279)

Preparation of extract

Dried coursed powder was taken and successively extraction was done by methanol solvent using soxhlet apparatus unit. Than solvent extract was concentrated under vacuum and stored at 2-8^{0}C in dark place.

Isolation of phytoconstituents

Glass column (3ft) was packed with silica gel using wet packing method in Pet. Ether (60^{o}C-80^{o}C). Air bubbles were allowed to escape by the running the column blank thrice with the Pet. Ether (60^{o}C-80^{o}C). The methanolic extract was packed and column was eluted with solvents in increasing grade of polarity. The elution of the column was carried out with successive series of solvents in various combinations, viz. , petroleum ether, chloroform in petroleum ether (0.5%, 1%, 2%, 5%, 10%, 15%, 20%, 25% ,……95%), chloroform, methanol in chloroform(0.5%, 1%, 2%, 5%, 7%). The elutes were collected in volume of20 ml , elutes which showing similar Rf value were combined and concentrated. Than the preparative layer chromatography were performed with these fractions and the spots were detected under Uv light, Iodine chamber. The spots were collected using spatula and added in the solvent.The spectral analysis were done by UV, IR, ^{1}HNMR, ^{13}C NMR, Mass spectroscopic data. (Stahal E. 1965)

Evaluation of antioxidant activity

Antioxidants or inhibitors of oxidation are the compounds which retard or prevent the oxidation in general and prolong the life of oxidizable matter (Kalia, 2005). Antioxidants can interfere with the oxidation process by reacting with free radicals, chelating catalytic metals and also by acting as reactive species scavenger. Polyphenolic compounds like flavonoids and phenolic acids, commonly found in plants, have been reported to have multiple biological effects, including antioxidant activity (Paramapojn and Gritsanapan, 2009).The antioxidant activities of the individual compounds may depend on structural factors, such as number of phenolic, hydroxyl or methoxyl groups and other structural features (Patt and Hudson, 1990). Among the antioxidative compounds vitamin A, C, E, selenium, carotenoids, ascorbic acid show very strong intensity of antioxidative activities (Dekkers *et al.*, 1996).

The molecule of 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that molecules do not dimerise, as would be the case with most other free radicals. The delocalisation also gives rise to the deep violet colour, characterised by an absorption band in ethanol solution centred at about 517 nm. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet colour (although there would be expected to be a residual pale yellow colour from the picryl group still present). Representing the DPPH radical by Z□□ and the donor molecule by AH, the primary reaction is

Z*□ □ □ □ +AH =□ ZH+ □ □ A*□

Where, ZH is the reduced form and A□□ is free radical produced in this first step. This latter radical will then undergo further reactions which control the overall stoichiometry, that is, the number of molecules of DPPH reduced (decolorised) by one molecule of the reductant. The reaction [1] is therefore intended to provide the link with the reactions taking place in an oxidising system, such as the auto-oxidation of a lipid or other unsaturated substance; the DPPH molecule Z□□ is thus intended to represent the free radicals formed in the system whose activity is suppressed by the substance AH (Molyneux, 2004).

Preparation of Reagents and Dilutions

The 500µM Solution of DPPH was made by using 23 mg of DPPH (Assay 85%) of Hi Media Laboratories Pvt. Ltd. CAS No. 1898-66-4. 23 mg DPPH was dissolved in 100 ml methanol in a 100 ml volumetric flask and stored in dark.TRIS [2-amino-2 (hydroxy methyl)propane1-3di-ol] Buffer pH 7.4 was made by adding 0.605gm of TRIS Buffer 7.4 of Qualigens Fine Chemicals in 30 ml of water and adding 0.33 ml of concentrated HCl, diluted to 100 ml with distilled water. The use of TRIS buffer was to prevent the sudden pH change during the preparation of test dilutions (Anonymous, 2006).A series of 10 dilutions for Ascorbic acid (Standard antioxidant) in the range of 2.0 µg/ml to 20 µg/ml was prepared using Ascorbic acid. All dilutions were prepared in methanol. 2 ml solution from the above dilutions with concentrations 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 µg/ml were further diluted by adding 2 ml DPPH, 0.5 ml TRIS, 0.5 ml methanol each so that the final concentration of the dilutions for measurement of absorbance by UV Visible spectrophotometer was in the range of 0.4 µg/ml to 4.0 µg/ml. For the preparation of various dilutions of methanolic extract of fenugreek seeds a series of 10 dilutions were prepared for methanolic extract in the concentration range of 4 mg/ml to 40 mg/ml in methanol. 1 ml from the above dilutions with the concentrations 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 mg/ml were further diluted by adding 2 ml DPPH, 0.5 ml TRIS, 0.5 ml methanol each so that the final concentration of the dilutions for measurement of absorbance by UV Visible spectrophotometer was in the range of 0.4 mg/ml to 4 mg/ml. The 500µM solution of DPPH was prepared by dissolving 23 mg of DPPH in 100 ml of methanol.

Measurement of Antioxidant Activity

The absorbance of final dilutions of Ascorbic acid prepared above after 30 minutes of addition of DPPH were measured at λ max 517 nm using methanol as blank and the readings were recorded.

Measurement of Antioxidant activity of Methanolic extract was done by taken 1 ml from each dilutions of Methanolic extract (concentration range of 4 mg/ml to 40 mg/ml) 2 ml DPPH, 0.5 TRIS and 1.5 ml methanol were combined in a 5 ml volumetric flask and the absorbance of the final dilutions were measured after 30 minutes of DPPH addition at λ max 517 nm using methanol as blank and the readings were recorded along with the calculated % inhibition.

Statistical analysis
The percentage inhibition was calculated using:

$$\text{Percent Inhibition} = \frac{Ac-As}{Ac} \times 100$$

Where, Ac is absorbance of control, As is the absorbance of sample.

IC_{50} value (a concentration at 50% inhibition) was determined from the curve between percentage inhibition and concentration. All determinations were done in triplicate and the IC_{50} value was calculated by using the equation of line (Papuc *et al.,* 2008).

III. Observation and Results

The extract was dissolved in minimum amount of methanol and absorbed on silicagel. The dried slurry was chromatographed over silica gel column prepared in petroleum ether. The column was eluted with petroleum ether, chloroform and methanol in increasing order of polarity to isolate the following compounds.

Compound Ry- 1
IUPAC name: 2- hydroxybenzaldehyde-2α-D-glucopyranosyl (2 α-1b)-α-D-glucopyranosyl (2b-1c)-α-D-glucopyranosyl- (2c-1d)-α-d- (2d-geranilanyl)-glucopyranoside. (Salicylaldehyde geranilanyl tetraglucoside A).Elution of the column with chloroform: methanol (97: 3) afforded light brown crystals of **Salicylaldehyde geranilanyl tetraglucoside A** and recrystalized from methanol.
Rf value: , Melting Point: 157-158^{0}C, **Uv λ max** (MeoH): 205, 276 nm, (Log E- 5.1, 3.6), **IR υ max (KBr)**: 3522, 3418, 3360, 2928, 2845, 1701, 1650, 1525, 1049, 826 cm^{-1}.
+ve FAB m/z (rel-int): 895 (M+H)+ ($C_{41}H_{67}O_{21}$). (10.1)
^{1}H NMR: The NMR data are present in Table 1.

Compound Ry- 2
$CH_3(CH_2)_{44}CH_2OH$ (n- Hexatetracontonol)
Elution of the column with pet ether-chloroform (2:3) gave colourless crystals of **n- Hexatetracontonol a**nd recrystallized with $CHCl_3$:MeOH (1:1).**Rf :, Melting Point:** 71-72^{o}C, **Uv λ max** (MeoH): 345nm, 359.4nm, **IR υ max (KBr):** 3424, 2924, 2847,1655,1026, 825, 763,cm^{-1}.
^{1}H NMR (DMSO):
3.32 (2H,brs,CH_2-1), 2.36 (2H,m,CH_2), 1.94 (2H,m,CH_2), 1.66 (2H,m,CH_2), 1.44 (2H, m, CH_2), 1.27 (4H,brs,2×CH_2), 1.24 (8H,brs, 4×CH_2), 1.21 (68H, brs,34× CH_2), 0.83 (3H, t, j= 6.2 hz, Me-46)
To FAB m/z rel-int: 663,[M] + ($C_{46}H_{95}O$] (21.6)

Compound Ry- 3
$CH_3(CH_2)_{14}CH = CH – CH = CH (CH_2)_{14}$ -CH_2OH (n-Tetratriacont-16,18 dien-1-ol) Elution of the column with pet. ether: chloroform (3:7) afforded colour less crystalline mass of n-Tetratriacont-16,18 dien-1-ol recrystallized from acetone methanol (1;1), **Rf value:, Melting point:**-104-105^o C, **Uv λ max** (MeoH): 276nm, 279nm , **IR υ max (KBr):** 344, 2253, 2127, 1654,1026,825,763,cm^{-1}.
^{1}H-NMR (DMSO):δ 5.31 (1H, m,H-19), 5.18 (2H, m,H-17,H-18), 5.08 (1H,m,H-16), 3.25 (2H,brs,H_2-1), 2.72 (2H, m, H_2-15), 2.27 (2H,m,H_2-20), 1.89 (4H, m, 2× CH_2), 1.45(2H, m,CH_2), 1.22 (30H, brs, 15×CH_2), 1.15(16H, brs, 8×CH_2), 0.84 (3H, t, j=6.5hz, Me-34)
+ve FAB ms m/z (rel-int): 491$[M+H]^+$ ($C_{34}H_6O$).

Compound Ry- 4
IUPAC name: 2-hydroxybenzaldehyde- 2β – D - glucopyrrnoryl (2a-1b)-β – D – glucopyranoryl - (2b-1c) – β - D – glucopyranoryl - (2c-1d) - β- D - (2d-geranilanyl) – glucopyranoside. (Salicylaldehyde geranilanyl tetraglucoside B)
Elution of the column with chloroform: methanol (1:19) gave colourless crystal of Salicylaldehyde geranilanyl tetraglucoside (B)
Rf…, Melting Point: 196-197^o C, **Uv max (MeoH):** 273 nm, (log E- 5.6), **IR υ max (KBr) :** 3475,3421,3365,3289, 2928,1701, 1649, 1525, 1425, 1374, 1026, 825, cm^{-1}.
+ve FAB ms m/z [rel-int]: 911$[M+H]^+$ [$C_{41}H_{67}O_{22}$] [5.2].

^{1}H NMR: The NMR data are present in Table 2.

Anti oxidant activity

In the present study it has been showed that fenugreek seeds had moderate antioxidant activity as compared to that of standard antioxidant ascorbic acid. Absorbance of blank was found to be 2.036 at 517 nm, Absorbance of Sample (fenugreek extract and Standard ascorbic acid were taken and IC50, % inhibition were calculated. The results are present in the **Table 3,4** and **Figs 1, 2.**

Calculations of IC_{50}

a) Equation for the Standard curve for Ascorbic acid

$y = 5.010x + 9.410$

$R^2 = 0.979$

When Y=50, the value of X = 8.10

So **IC_{50}** = 8.10 µg/ml.

b) Equation for curve of methanolic extract.

$y = 7.025x + 15.31$

$R^2 = 0.966$

When Y=50, the value of X = 4.938

So **IC_{50}** = 4.938 µg/ml.

IV. Discussion

The methanolic extract was used in column chromatography for isolation of compound. The various components were separated in various elute fractions. The four compounds Salicylaldehyde geranilanyl tetraglucoside A, Salicylaldehyde geranilanyl tetraglucoside B, n- Hexatetracontonol, n- Tetratriacont-16, 18 dien-1-ol were isolated from the fraction of column with the solvent systems chloroform: Methanol (97:3), chloroform: Methanol (1:1), Pet. Ether: Chloroform (3:7). Chloroform: Methanol (1:19). The fractions have shown a dense spot in the PTLC with Rf value……. (Chloroform: Methanol, 4:0.5). The spots were visualized by derivatization with iodine vapors.

The spectral data obtained from UV, IR, 1HNMR, 13CNMR and Mass and on the basis of spectral data the isolated compounds were elucidated as Salicylaldehyde geranilanyl tetraglucoside A, Salicylaldehyde geranilanyl tetraglucoside B, n- Hexatetracontonol, n- Tetratriacont-16, 18 dien-1-ol.

The antioxidant activity (in vitro) was performed by 1, 1-diphenyl-2-picryl- hydrazyl (DPPH) method. The methanolic extract of fenugreek seed had 4.938 µg/ml IC50 value while the standard Ascorbic acid had 8.10 µg/ml. This result showed fenugreek extract have potent antioxidant activity.

V. Conclusion

On the basis of above results it conformed that four new components Salicylaldehyde geranilanyl tetraglucoside A, Salicylaldehyde geranilanyl tetraglucoside B, n- Hexatetracontonol, n- Tetratriacont-16, 18 dien-1-ol. were isolated from the methanolic extract of fenugreek seeds. The antioxidant effects of methanolic extract of fenugreek seeds are may be due to the presence of these components in the methanolic extract.

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Table 1: ^{1}HNMR (DMSO- d6) ^{13}C NMR data of compound Salicylaldehyde geranilanyl tetraglucoside A.

Position	^{1}H NMR	^{13}C NMR
1	----	142.54
2	----	162.87
3	8.88d(7.5)	139.13
4	8.02m	125.92
5	6.85m	135.75
6	8.74d(7.8)	140.68
7	9.20brs	205.01
1’	0.75t(6.5)	12.16
2’	0.95m	42.62
3’	1.64m	53.19
4’	1.08m	46.41
5’	1.10m	45.07
6’	1.22m	22.80
7’	1.46m	52.23
8’	0.81d(6.1)	18.51
9’	0.84d(6.3)	19.46
10’	3.40brs	68.95
1a	5.19d(3.3)	104.49
2a	3.86m	82.81
3a	3.45m	71.87
4a	3.53m	75.06
5a	4.35m	76.51
6a	3.23d(8.7), 3.21d(8.6)	62.63
1b	5.05d(6.5)	92.23
2b	3.84m	82.91
3b	3.48m	71.55
4b	3.59m	73.25
5b	4.41	77.39
6b	3.17d(90, 3.20d(9.0)	60.61
1c	4.69d(3.9)	99.21
2c	3.82m	82.79
3c	3.50m	70.07
4c	3.57m	73.28
5c	4.50	77.79
6c	3.09d,(12.3), 3.14d(11.2)	62.60
1d	4.64d(3.7)	92.16
2d	3.79m	79.63
3d	3.52m	77.83
4d	3.55m	74.64
5d	4.11m	77.83
6d	3.03d(13.3) 3.06d(12.1)	60.92

Coupling constants in hertz are given in parentheses

Table 2: ^{1}HNMR (DMSO) ^{13}C NMR data of compound Salicylaldehyde geranilanyl tetraglucoside B.

Position	^{1}H NMR	C^{13}NMR
1	---	146.41
2	---	163.34
3	8.86d[7.5]	145.30
4	8.01m	127.25
5	6.85m	139.88
6	8.72d[7.8]	144.50
7	9.16brs	204.53
1’	0.73t[6.2]	12.83
2’	0.92m	42.03
3’	1.62m	54.86
4’	1.06	49.60
5’	1.08m	48.09
6’	1.20m	22.69
7’	1.44m	53.41
8’	0.82d[6.9]	18.19
9’	0.80d[6.5]	19.78
10’	3.39brs	62.58
1a	5.30d[7.2]	104.43
2a	4.07dd[7.2,6.2]	83.36

3a	3.68m	69.16
4a	3.53m	68.13
5a	4.69m	74.63
6a	3.18d[10.5]3.20d[10.5]	62.58
1b	5.16d[7.1]	103.15
2b	3.85dd[7.1,6.6]	82.05
3b	3.66m	68.38
4b	3.57m	68.08
5b	4.67m	73.88
6b	3.12d[9.3],3.15d[9.5]	62.60
1c	5.02d[7.2]	100.78
2c	4.22dd[7.2,6.5]	77.22
3c	3.70m	70.25
4c	3.62m	67.59
5c	4.63m	73.26
6c	3.33d[11.4]3.35d[11.4]	60.81
1d	4.98d[7.1]	92.19
2d	3.83m	71.58
3d	3.68m	70.53
4d	3.58m	67.57
5d	4.70m	73.23
6d	3.01d[81],3.03d[8.1]	60.91

Coupling constants in hertz are given in parentheses

Table 3: Values of absorbance and percentage inhibition with increase in concentration of methanolic solution of Ascorbic acid (standard antioxidant).

Concentration (µg)	Absorbance (nm)	% Inhibition
0.4	1.681	16.40
0.8	1.642	18.34
1.2	1.507	25.06
1.6	1.411	29.83
2.0	1.328	33.96
2.4	1.243	38.19
2.8	1.124	44.10
3.2	1.059	47.33
3.6	0.967	51.91
4.0	0.713	64.54

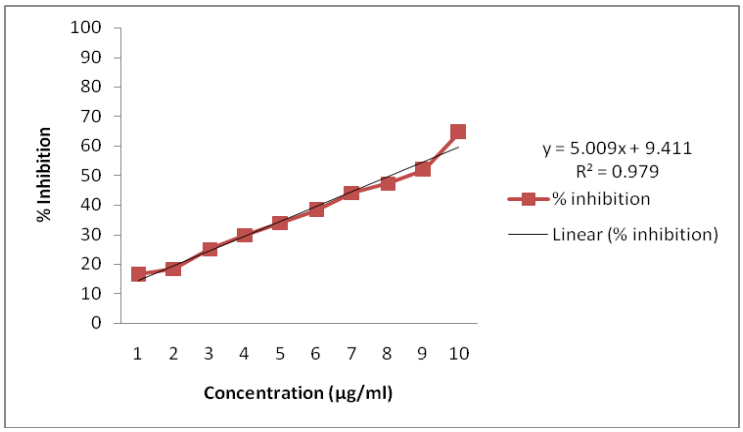
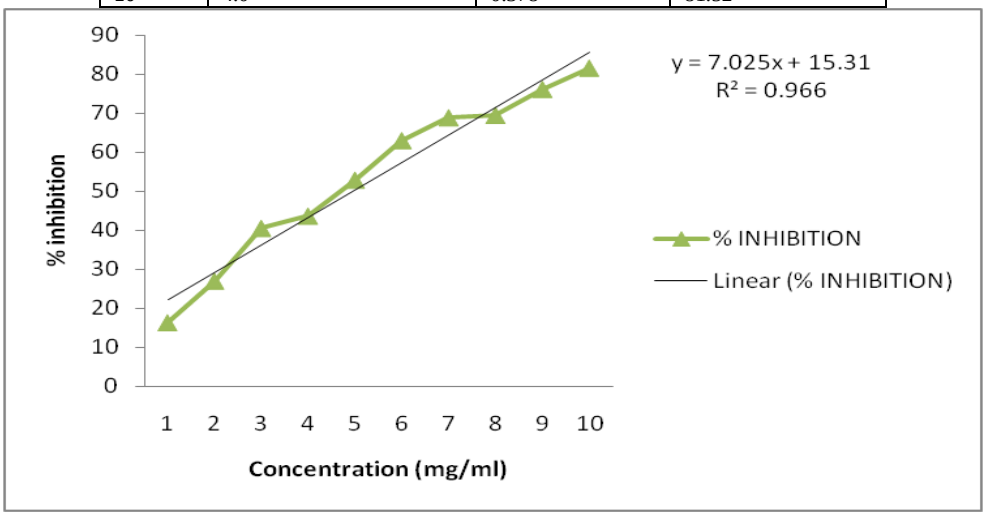


Fig.1: Graphical representation of concentration (µg) vs percentage inhibition of methanolic solution of Ascorbic acid (standard antioxidant)

Table 4: Values of absorbance and percentage inhibition with increase in concentration of methanolic extract.

S. no	Concentration (µgm)	Absorbance(nm)	% Inhibition
1	0.4	1.710	16.42
2	0.8	1.492	27.07
3	1.2	1.216	40.56
4	1.6	1.152	43.69
5	2.0	0.964	52.88
6	2.4	0.757	63.00
7	2.8	0.637	68.86
8	3.2	0.625	69.45
9	3.6	0.489	76.09

10	4.0	0.378	81.52



Figs.2: Graphical representation of concentration (µg) vs percentage inhibition of methanolic extract.