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Full Length Research Paper

In-vitro osmotic fragility assessment of *Trigonella foenum graecum* linn. seed extract on sheep erythrocytes

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The search for new hypoglycaemic compounds for the treatment of diabetes or any condition that leads to hyperglycemia. Several evidences suggest that oxidative stress plays a major role in pathogenesis of disease. Damage to RBC membrane lipid and protein is caused by high levels of free radicals which will finally result in hemolysis. An extract from seeds of Trigonella foenum graecum was evaluated for its protective action against H_2O_2 induced oxidation and different concentration of NaCl induced hemolysis in normal sheep erythrocytes on treatment with fenugreek extract the oxidative modification in both were found to reduce significantly. The total phenolic content in extract was determined on spectrophotometrically using folin-ciocalfeau procedure and the fenugreek inhibition of lipid peroxidation was concentration dependant upto 160µg/l of extract which contained 1.2mM gallic acid equivalent(GAE) of phenolic compounds these findings demonstrated the sound knowledge regarding patient anti-oxidant property of fenugreek seeds.

Keywords: fenugreek seed extract, osmotic fragility test, sheep red blood cells, lipid peroxidation, hemolysis

INTRODUCTION

Trigonella foenum graecum (Fenugreek) commonly known as Methi, annual herb belongs to the family leguminosae. The genus Trigonella means 'little triangle', owing to the triangular shape of flowers and the species foenum graecum means 'greek hay'. Seeds of fenugreek are rich in proteins, mucilaginous fibre and chemical constituents such as trigonelline, flavanoids, saponins, vitamins, sapogenins (Flammang et al., 2004). It is widely used in India due its strong aroma as a spice in food preparations. The seeds of fenugreek are reported to have anti-oxidant, anti-radical, anti-infective, anti-tumor, anti-bacterial, anti-fungal, anti-viral, anti-diabetic, anti-helmntic, antiatherosclerotic (Cowan, 1999). It is also used for the treatment of galactagogue, bronchitis, fever, congestion, migraine, diarrhoea, hypotensive, anemia, flatulence, irregular menstrual cycles, analgesic, arthritis (Shetty, 1997).

Fenugreek posses potent anti-oxidant activity due to the presence of polyphenols and thus inhibits haemolysis and oxidative damage induced by reactive oxygen species on lipids, proteins and nucleic acids. Studies have reported that lipid peroxidation induce coronary heart disease and cancer.

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MATERIALS AND METHODS

Chemicals and Reagents

Folin-Ciocalteu Reagent, Ethanol, Hydrogen Peroxide, Disodium ethylenediaminetetraacetate (Na2EDTA) were purchased from (E. Merck). All other chemicals and solvents were of the highest analytical grade.

Fenugreek seeds collected from local market were ground into small pieces using blender and passed through sieve no-80. Extract was prepared with ethanol by soxhelt extraction method for eight hours. It was evaporated to dryness under reduced pressure at 60^0C by rotary evaporator. The extract was stored in a dark coloured bottle in a freezer.

Collection of blood sample

Sheep blood was collected from slaughter house and red blood cells were isolated by centrifugation.

Preparation of stock solutions

Sodium chloride solution

Various concentrations of sodium chloride were prepared by dissolving 0.1,0.2,0.3,0.4,0.5,0.6,0.7,0.8,0.9g of sodium chloride in 100ml of water to yield 0.1%,0.2%,0.3%,0.4%,0.5%,0.6%,0.7%,0.8%,0.9% solutions.

Hydrogen peroxide solutions

10%, 20%, 30% H_2O_2solutions were prepared by dissolving 10,20,30 ml of H_2O_2 in 100ml of water.

Fenugreek solutions

0.1mg of fenugreek extract powder was dissolved in small quantity of water and made up to 100ml in a 100ml volumetric flask (1000µg/ml). From this 10ml is taken into another 100ml volumetric flask made up to 100ml (100µg/ml). From this 1,2,3,4,5,6,7,8,9 ml were transferred to 10ml volumetric flask and made up to 10ml using water to yield 10,20,30,40,50,60,70,80,90 µg/ml solutions respectively. Their respective absorbances were measured using UV-visible spectrophotometry and tabulated in table 2 and calibration curve is drawn in figure 1.

Preparation of 7.4pH buffer

2.38g of disodium hydrogen phosphate, 0.19g of potassium dihydrogen phosphate and 8.0 g of sodium chloride were dissolved in a little quantity of water and made up to 1000ml with water.

An aqueous extract was prepared and used for in vitro studies. The polyphenolic content of the extract was assessed by the method of Singleton and Rossi (1965) (Awika et al., 2003) and expressed as mg gallic acid equivalents (GAE) per gram of dry extract, using a standard curve generated with gallic acid.

Determination of Total Phenolic Content (TPC)

TPC in different solvent extracts of fenugreek seeds was determined spectrophotometrically following Folin-Ciocalteu method (Parpart et al., 1947). The appropriate dilution of extract 200 µl oxidized with 1 ml of Folin-Ciocalteu reagent, and then the reaction mixture was neutralized with saturated 2 ml of 7.5 % sodium carbonate (w/v). The final mixture volume was brought up to 7 ml with deionized water. The absorbance of the resulting blue colour was measured at 765 nm on UV-Vis. spectrophotometer with a 1 cm cell after incubation for 2 hours in dark at room temperature. Gallic acid was used as a standard for the calibration curve. The phenolic compound content was determined as gallic acid equivalents using the calibration curve.

Osmotic fragility test

Preparation of sample one volume of the collected sheep blood sample was centrifuged. RBCs that settled in the pellet were then washed and suspended in sterile 1X phosphate buffer saline solution (pH 7.4). The suspended RBCs were then incubated with increasing concentrations of 30% H2O2, namely, 10, 20, and 50 mM for 2 h.

And another set was framed using series (n=14-16) of hypotonic solutions with NaCl content ranging from 0.1 % to 0.9 %, to which a small amount of fresh blood is added. After centrifugation and absorbance reading at 540 nm, the percent hemolysis is calculated (table 1) for each solution and plotted against NaCl concentrations (figure 2). The resulting osmotic fragility curve is then compared with that obtained with normal controls. The result of the OFT may be expressed as the concentration of NaCl causing 50 % hemolysis, i.e. the median corpuscular fragility (MCF) that for fresh normal samples is between 4.0 and 4.45 g/L NaCl.

$$\% \text{ of Hemolysis} = \frac{\text{Absorbance of reading of test Supernant} \times 100}{\text{Absorbance of reading of 100\% Hemolysis}}$$

The other volume of blood was first incubated with 100 µl of Fenugreek Extract for 1 h. The blood was centrifuged. The RBCs that settled in the pellet were then washed and suspended in pH7.4 phosphate buffered saline. The suspended RBCs were incubated with

Table 1. Haemolysis induced by different concentrations of solutions

S.No	Volume of RBC sample	Solution	% of solution	λ_{max}	Optical Density	% of Haemolysis
1	40		0.1		-	-
2	40		0.2		0.89	100
3	40		0.3	540nm	0.82	91.96
4	40		0.4		-	-
5	40	**NaCl**	0.5		0.01	1.635
6	40		0.6		0.001	0.179
7	40		0.7		0	0
8	40		0.8		0	0
9	40		0.9		0	0
10	40	**H_2O_2**	10		0.58	24.2
11	40		20	426nm	0.50	25.6
12	40		30		0.79	32.9
13	40	**Buffer**	7.4pH Buffer	223nm	0	0
14	40	**Fenugreek extract**	160µg/ml	219nm	0	0

Table 2. Calibration Curve of Fenugreek Extract

S.No	Concentration	absorbance
1	10	0.078
2	20	0.133
3	30	0.163
4	40	0.181
5	50	0.188
6	60	0.246
7	70	0.283
8	80	0.372
9	90	0.351
10	100	0.361

Table 3. % of Oxidation induced by different Concentrations of Hydrogen peroxide

S.No	H_2O_2		
	10%	20%	30%
1	9.8	13.2	13.3
2	16.7	22.5	22.6
3	20.5	27.7	27.8
4	22.7	30.7	30.8
5	23.6	31.9	32
6	30.9	41.8	41.9
7	35.6	48.1	48.2
8	46.8	52.3	51.8
9	44.2	59.7	63.4
10	45.4	60.1	69.7

increasing concentrations of H2O2, namely, 10, 20, and 50 mM for 2 h. The percentage hemolysis was evaluated by performing osmotic fragility test as described by Dacie et al., (1964) and repoported in table 3.

RESULTS

The yield of the extract obtained by the soxhelt method was calculated as present by weight of the fenugreek seed. According to the chemical composition and polar nature of phenolic compounds fenugreek contain a relatively high percentage yield in ethanol. The phenolic compound may contribute directly to the anti-oxidant action. Therefore it is necessary to investigate total phenolic content. The total phenolic content was determined by following a modified folin-ciocalteu reagent method. The results were expressed as gallic acid equivalent. TPC was in the range of 1.35-6.85mg/g of the fenugreek extract. The amounts of total phenolic compounds were higher in ethanol extract 6.85mg/g. the effect of fenugreek extract on RBC membrane integrity was plotted in figure 3.

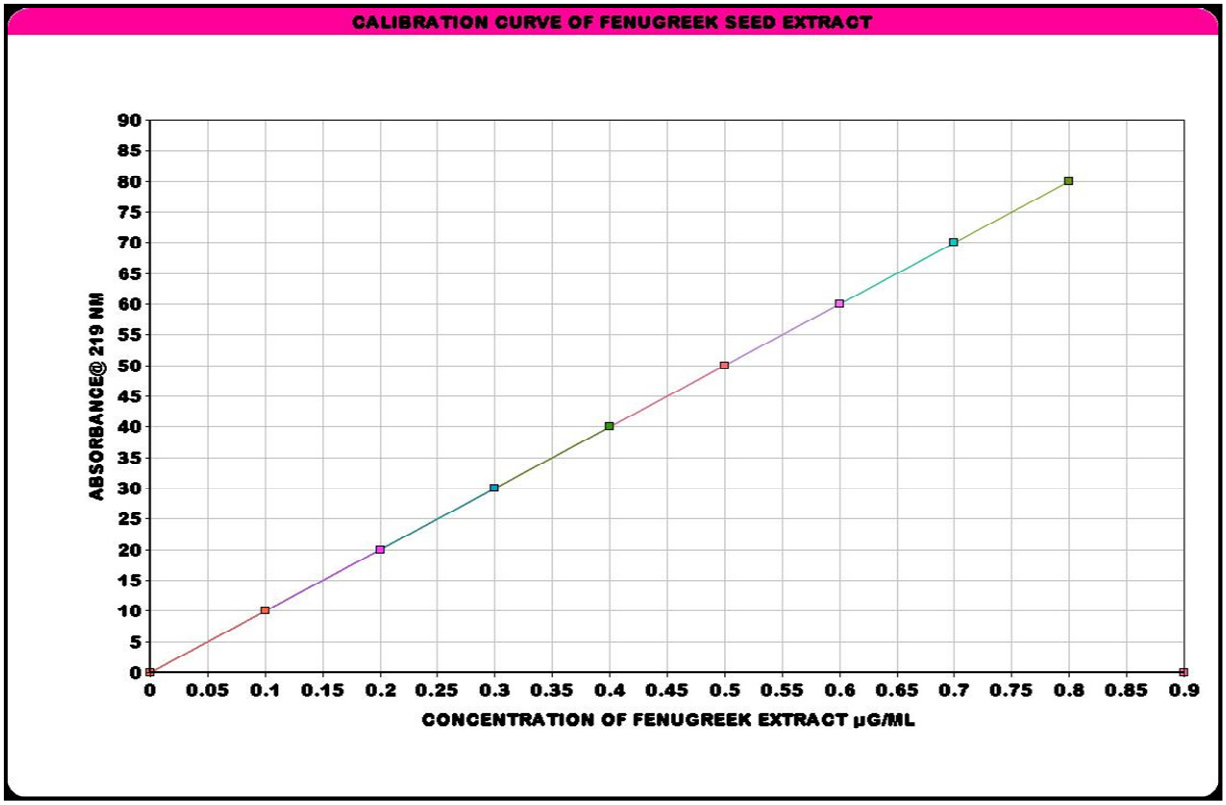

Figure 1. Calibration Curve of Fenugreek Seed Extract

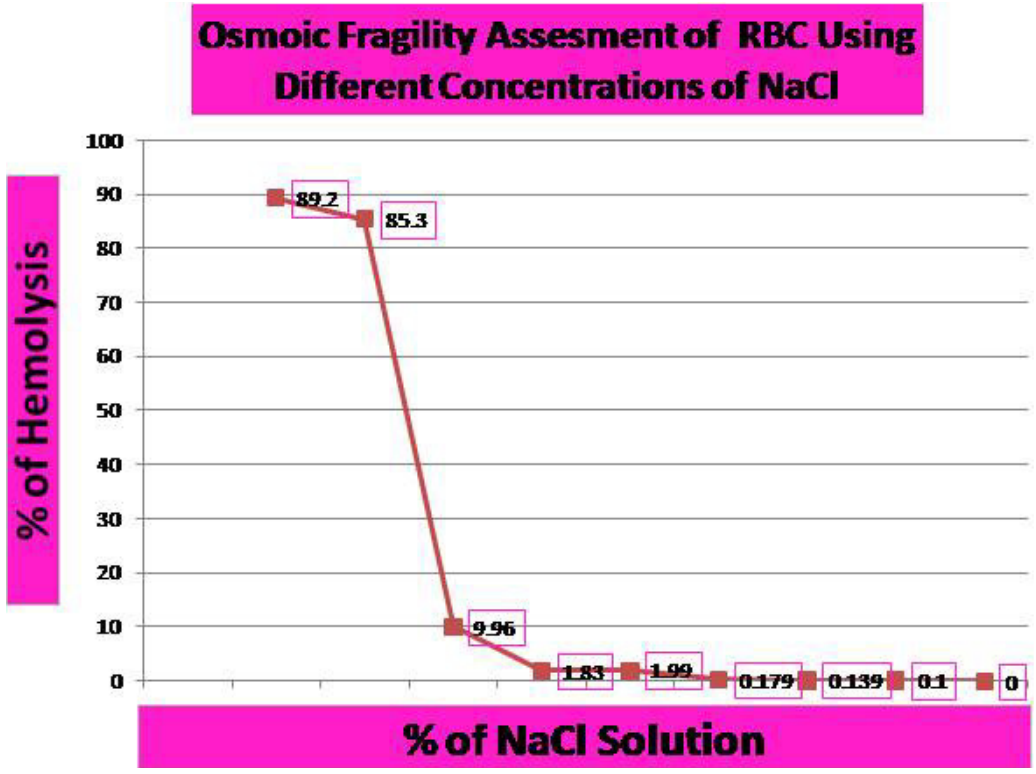

Figure 2. Osmotic Fragility Assesment of RBC using Different Concentrations of NaCl

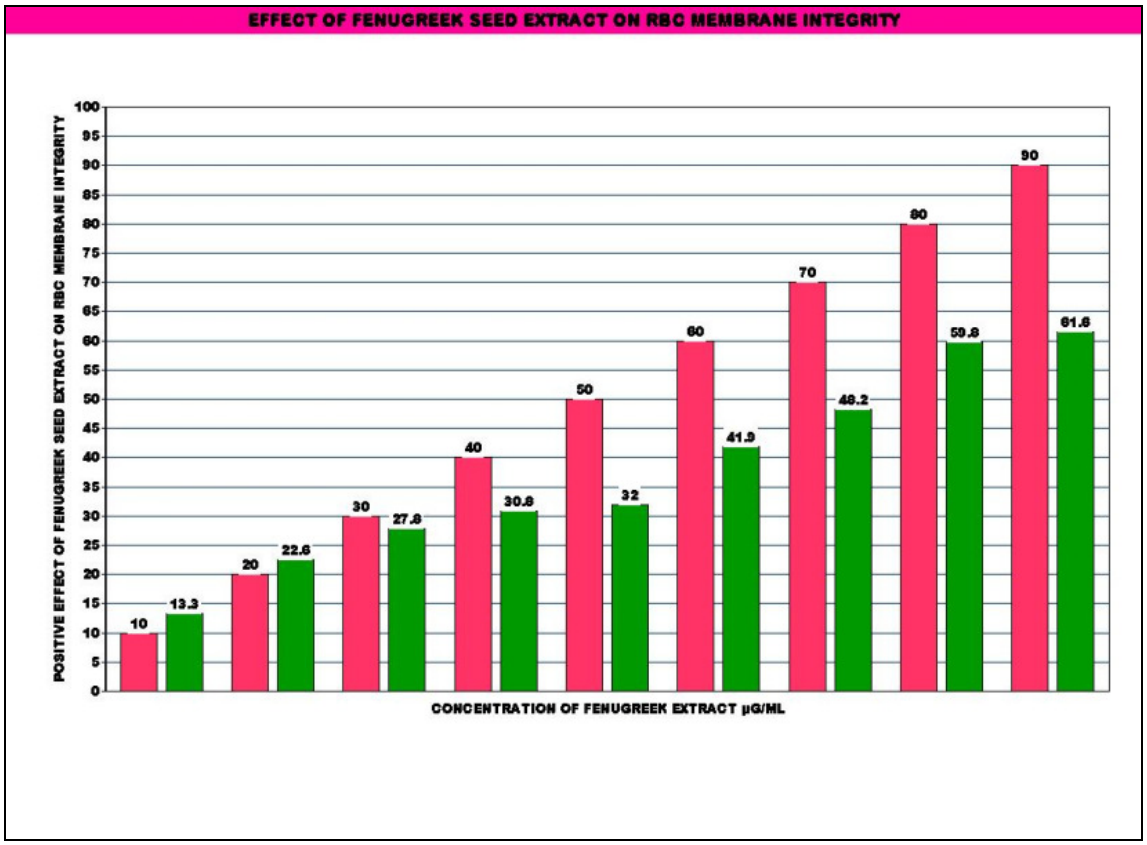

Figure 3. Effect of Fenugreek seed Extract on RBC Memberane Integrity

DISCUSSION

In this study, osmotic fragility test is used to compare whether the erythrocytes collected from the sheep was normal or any disorder of sporocytes such as hereditary spherocytosis or autoimmune haemolytic anemia is present in the collected blood sample. Results obtained from osmotic fragility test shows that there was no significant increase in the rate of hemolysis in 10mM H_2O_2 treated blood sample compared to the control suggesting that there was no significant membrane damage at the concentration. With 20 and 50mM H_2O2 concentration the rate of hemolysis was found to increase as revealed from osmofragility test analysis. Maximum hemolysis was found in 50mM H_2O_2 concentration. Hemolysis was found to decrease in blood incubated with FSE along with H_2O_2 suggesting that fenugreek has some protective maintaining the RBC membranes integrity.

In this study fenugreek proved to be an effective anti-oxidant and an anti-inflammatory agent in protecting RBC from membrane damage due to high glucose or increasing H_2O_2 concentration induced oxidative stress. Fenugreek was found to reduce the damage to the RBC membrane fragility due to oxidative stress imposed by H_2O_2. This may be due to the presence of Phenolic Content in Fenugreek Seed Extract. These findings demonstrated the sound knowledge regarding anti-oxidant property of fenugreek seeds.

CONCLUSION

The evidence that fenugreek can prevent oxidative stress needs to be explored further at the clinical level to determine whether supplementation can lowe the levels of oxidative stress and thereby reduce the incidence of vascular disease in the diabetic patient population.

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