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In vitro estrogenic activities of fenugreek *Trigonella foenum graecum* seeds

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***Background & objectives*: *Trigonella foenum graecum* commonly known as fenugreek, has been widely cultivated in Asia, Africa and Mediterranean countries for the edible and medicinal values of its seeds. Earlier reports show that fenugreek seeds provide a mastogenic effect resulting in enhanced breast size. However, very little is known about its estrogenic effect. The present study investigated the effect of chloroform extracts of fenugreek seeds (FCE) in breast cancer cells for its estrogenic effect, and to assess its capacity as an alternative to hormone replacement therapy (HRT).**

***Methods:* The effect of FCE on cell proliferation of estrogen receptor (ER) positive breast cancer cells, MCF-7 was studied by MTT assay at a concentration range of 20 to 320 µg/ml. The competitive ER binding assay (HAP assay) was done to find out the ER binding capacity of the extract. Transfection and reporter assay (DLR assay), and RT- PCR with an estrogen responsive gene *pS2* were done to find out the transcriptional regulatory activity of FCE.**

***Results*: FCE stimulated the proliferation of MCF-7 cells, showed binding to ER (IC_{50} = 185.6 ± 32.8 µg/ml) and acted as an agonist for ER mediated transcription via ERE. It also induced the expression of estrogen responsive gene *pS2* in MCF-7 cells.**

***Interpretation & conclusion*: Our study provided the evidence for estrogenic activities of fenugreek seeds. Further *in vitro* and *in vivo* studies could demonstrate its suitability as an alternative to HRT.**

Key words Estrogen - estrogen receptor - hormone replacement therapy - phytoestrogen - *Trigonella foenum graecum*

Hormones such as estrogen and progesterone play an important role in human growth, and are responsible for regulating the complex cellular events associated with differentiation, function and growth of female reproductive tissues. Women in the menopause suffer from bone density reduction, cardiovascular disease, sweating and anxiety because of a lack of estrogens[1]. Exogenous estrogens or hormone replacement therapy (HRT) was introduced to improve the menopausal symptoms, which quickly took effect but caused undesirable side effects such as irregular bleeding and increased risk of breast cancer.

Efforts are on worldwide to discover an alternative HRT with minimal risks. It was found that natural compounds from certain plants called phytoestrogens

could be used for management of menopausal symptoms and have a few side effects[2]. Phytoestrogens appear to have both estrogenic and anti-estrogenic effects[3-5]. Therefore, these have been considered as a part of selective estrogen receptor modulators (SERMs) and studied as an alternative for hormone replacement therapy[6].

In India, *Trigonella foenum graecum* (fenugreek) is commonly consumed as a condiment and used medicinally as a galactagogue by nursing mothers to increase inadequate breast milk supply[7]. Studies have shown that fenugreek use was associated with increases in milk production of as much as 900 per cent[8]. Several studies have also shown the anticancer properties of this herbal plant[9-12]. The chemopreventive aspects and the potential protective effect of fenugreek seeds against 7, 12-dimethylbenz[*a*] anthracene (DMBA) in rats has been reported[13].

This study was aimed to evaluate the estrogenic effects of chloroform extract of fenugreek seeds (FCE) on ER positive breast carcinoma cell line MCF-7. The effects of the extracts on estrogen dependent gene expression, the *pS2* gene was also examined by RT-PCR.

Material & Methods

Chemicals: 17-β estradiol, ICI 182-780, phenol red-free Dulbecco's modified Eagle's medium (PR-free DMEM) and foetal bovine serum (FBS) were purchased from Sigma-Aldrich (St. Louis. MO, USA). Dimethyl sulphoxide (DMSO), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and TLC silica gel (Kieselgel 60 F_{254}) plates were obtained from Merck (Darmstadt, Germany).

Cell culture: MCF-7 cell lines obtained from ATCC (Manassas, VA, USA) were cultured in PR-free DMEM supplemented with 10 per cent heat inactivated FBS, 100 U/ml benzyl penicillin and 100 µg/ml streptomycin. The culture was maintained at 37°C in a humidified atmosphere of 5 per cent CO_2.

Plant material and extract preparation: Fenugreek seeds were purchased from commercial sources. Dry seeds (10 g) were ground in an electric grinder (Krups, Germany) and were extracted with chloroform (250 ml) in a soxhlet apparatus for 20 h. The extract was concentrated using rotary vacuum to get the solid mass. The yield obtained was 5 per cent (w/w). The concentrate was dissolved in DMSO for further use.

Phytochemical analysis: 10µl of FCE (1mg/ml in chloroform) was spotted on the TLC plate along with a standard solution of 17-β estradiol (1mg/ml). Stationary phase consisted of TLC aluminium sheets pre-coated with silica gel 60 F_{254}, mobile phase consisted of methanol: chloroform: glacial acetic acid (7:4:1 v/v). The spots were detected under (short-wave) UV light and Rf value of the extract was calculated with the standard.

Cell proliferation assay: To evaluate the effect of various solvent extracts on cell proliferation, MTT assay was performed[14]. Cells were seeded in 96-well plates at a density of 5 x 10^3 cells per well and treated with 0, 20, 40, 80, 160 and 320 µg/ml of FCE or 10 nM 17-β estradiol (E_2) or 10 nM ICI 182-780. After incubating for 48 h, the drug containing medium was aspirated, 100 µl of MTT reagent (2mg/ml) added and incubated for 2 h at 37°C. The viable cell number is directly proportional to the production of formazan following solubilisation with MTT lysis buffer (20% sodium dodecyl sulphate in 50% dimethyl formamide), which can be measured spectrophotometrically at 570 nm. The cell proliferation data (RPE, relative proliferation effect) were calculated as RPE = [(S-1)/(E-1)] x 100 where S = proliferation of samples and E= proliferation of positive control (10 nM E_2).

Competitive estrogen receptor binding assay (HAP assay): HAP assay was carried out to confirm the competitive binding of the extract to the estrogen receptor[15]. MCF-7 cell cytosolic extracts was used for competition-binding studies. Cytosol was prepared from cells grown for 3 days in estrogen depleted medium. The protein content was measured spectrophotometrically at 570 nm using Bradfords reagent (Sigma Aldrich, St. Louis, MO, USA). About 40 µg of the total protein (ER preparations) was incubated overnight at 4°C with the varying concentration of extract (range 20-320 µg/ml), 20 nM ^{3}[H] estradiol ± 100 fold molar excess of diethyl stilbesterol (DES) in a final volume of 250 µl. A 60 per cent hydroxyapatite (HAP) suspension in TEM buffer was added and the mixture was incubated at 4°C for 15 min. The HAP bound receptor-[^{3}H] E_2 complex was separated by centrifugation at 200 x *g* for 15 min. After washing twice with Tris buffer (10 mM) the HAP pellet was extracted with 1 ml absolute ethanol. These extracts were added to 4 ml scintillation cocktail and the radioactivity was measured in Wallac 1409 liquid scintillation counter (Wallac Oy, Turku, Finland). Data were expressed as the ratio of bound [^{3}H] E2 in the presence of a competitor to the bound [^{3}H] E2 in control x 100.

Transient transfection and reporter gene assays: For transfection experiment 10^5 cells were seeded per well in 12 well dishes in PR free DMEM with 10 per cent FBS charcoal DCC treated serum. After 24 h, MCF-7 cells were transfected with 2.5 µg ER (pHEG0- ER) 2.5 µg pG5-ERE-luc expression vectors with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 24 h cells were washed once with phosphate buffered saline (PBS) and 2 ml PR free DMEM with 10 per cent FBS DCC treated was added containing final concentrations of FCE (100 µg/ml) or 17β-estradiol (10 nM) or tamoxifen (500 nM). Protein was extracted 48 h later and firefly and *Renilla* luciferase activity were measured on a TD20/20 luminometer (Turner designs, Sunnyvale, CA, USA) using a Dual luciferase assay kit (Promega, USA) according to the manufacturer's instructions. Firefly luciferase activity was normalized to Renilla luciferase expression. Expression vectors were kindly provided by Dr Paul Murugan, (Molecular Imaging Program, Stanford University, CA) and Bert W. O'Malley, (Baylor College of Medicine, Housten, Texas).

Reverse transcriptase - PCR: MCF-7 cells were seeded onto 100 mm plates and were incubated with PR free DMEM supplemented with 10 per cent charcoal treated foetal bovine serum for 48 h before treatment. After 48 h, test compounds, FCE (50,100 µg/ml), E2 (10 nM) or ICI 182-780 (10 nM) were added to this medium. After incubating for 48 h total RNA was purified from the cells using Tri reagent (Sigma Chemical Co., St. Louis, MO, USA) according to the manufacturer's protocol. cDNA was prepared from 5 µg of total RNA using AccuScript™ 1st strand cDNA synthesis kit (Stratagene, USA) as per manufacturer's instruction. The *pS2* cDNA fragments were amplified using the primer pairs (Sigma-Aldrich, MO, USA), forward-5'TTTGGAGCAGAGAGGAGGCAATGG3', reverse-5'TGGTATTAGG ATAGAAGCACCAGGG3'(product size 240 bp)[16]. The reaction mixture containing 10 mM Tris HCl (*p*H 8.5), 50 mM KCl, 2 mM MgCl, 200 µM dNTPs, primers (2.5 pM each), 2.5 units of *Taq* polymerase were subjected to amplification cycles of 94°C for 4 min, followed by 30 cycles of 94 °C for 1 min, 59 °C for 30 sec, 72 °C for 1min in an Eppendorf thermal cycler (Eppendorf, Germany). Aliquots (5 µl) of each PCR mixture were analysed by electrophoresis in 1.2 per cent agarose gel and fragments were visualized by ethidium bromide staining. The intensity of bands was quantified in a Flour-S multi imager (BioRad, Hercules, USA) by using Quantity one densitometry software (BioRad, Hercules, USA). The transcripts were normalized with *gapdh* expression level. The gene expression was shown as ratio of densitometric value of target mRNA to that of *gapdh*.

Statistical analysis: All experiments were done thrice Values were expressed as mean ± SE and Tukey's post hoc test was done to analyze significance of difference between different groups using the statistical analysis software package SPSS (Version 16.0, IBM, USA).

Results

Phytochemical analysis: The extracts were screened for the presence of phytoestrogenic compounds. The TLC chromatograph gave similar R_f values of 0.69 and 0.74 respectively for FCE and 17-β estradiol standard indicating that phytoestrogenic compounds were present in FCE.

Stimulation of MCF-7 cell proliferation: The proliferative effect of FCE relative to that of 17-β estradiol (10 nM, 100%) was expressed as relative proliferative effect (RPE). FCE (40 µg/ml to 320 µg/ml) significantly (P< 0.05) stimulated the proliferation of MCF-7 cells in a dose dependent manner as compared with that of unexposed control. At higher concentrations of 160 and 320 µg/ml the proliferartive effect was almost similar to that of 10 nM 17-β estradiol (RPE = 91.74 ± 4.63 %). The proliferative effect was found to be inhibited by ER antagonist ICI 182-780. The RPE after treatment with ICI 182-780 decreased to 47.5 ± 3.5 per cent Fig. 1.

Competitive binding to ER: The ability of FCE to bind to ER was studied by competition binding studies using cytosol of MCF-7 cells. FCE inhibited the binding of labelled estradiol to ER (Fig. 2). The degree of inhibition was found to be dependent on the concentrations of FCE and the labelled ligand. The IC_{50} value of binding, the concentration of the extract required to reduce the

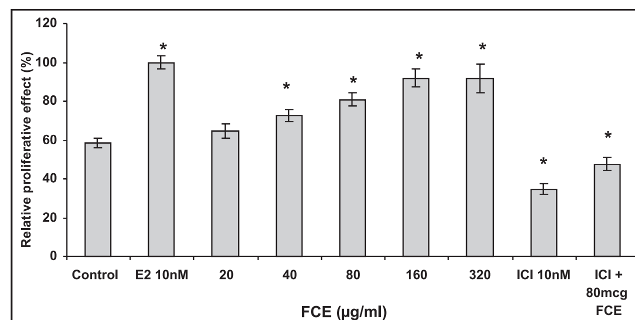


Fig. 1. Estrogenic activity of chloroform extract of fenugreek seeds (FCE) in MCF-7 cells. Results from three independent experiments are expressed as mean ± SE. $^*P$<0.05 compared to control.

specific radioligand binding by 50 per cent was 185.6 ± 32.8 µg/ml.

Effect of FCE on luciferase activity in MCF-7 cells: Dual luciferase assay was carried out by using a ERE driven reporter plasmid, [PG5 ERE luc], which has a reporter gene that contains a single copy of an ERE. Fig. 3 demonstrates the effect of vehicle control, FCE (80 µg/ml), 17- β estradiol and tamoxifen on luciferase activity of MCF-7 cells transfected with ERα and EREs. 17- β estradiol was used as control for agonistic activity whereas tamoxifen was used as a control for antagonistic activity. In MCF-7 cells transfected with ERα and ERE-luc there was an upregulation of luciferase transcription after treatment with FCE and was comparable with the response of luciferase transcription of 17- β estradiol treatment. The antiestrogen tamoxifen significantly decreased the luciferase activity at concentration of 500 nM ($P<0.05$) (Fig. 3).

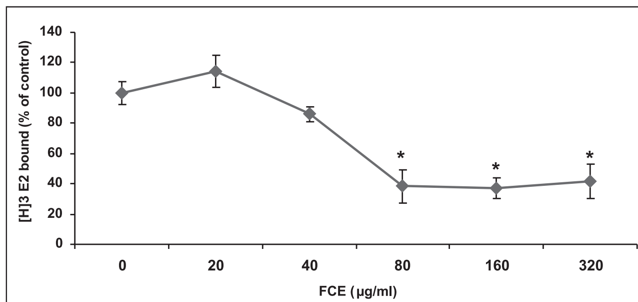


Fig. 2. Estrogen receptor affinity assay of FCE. Binding of 20 nM [^{3}H] E2 to cytosolic estrogen receptor in the presence of varying concentrations (20-320 µg/ml) of FCE. Results expressed as mean ± SE of three separate experiments for each data point. *$P<0.05$ compared to control.

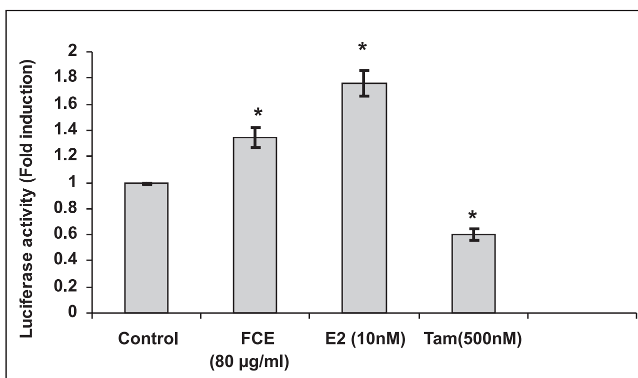
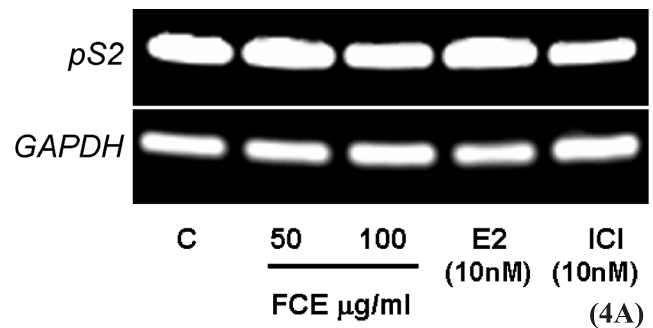


Fig. 3. Dual luciferase assay carried out using a ERE driven reporter plasmid [PG5 ERE luc]. The ERE promter luciferase activity of control was defined as 100 per cent. Results were obtained from three independent experiments and expressed as mean ± SE. Tam, tamoxifen; E2-17-B-estradiol. *$P<0.05$ compared untreated control.



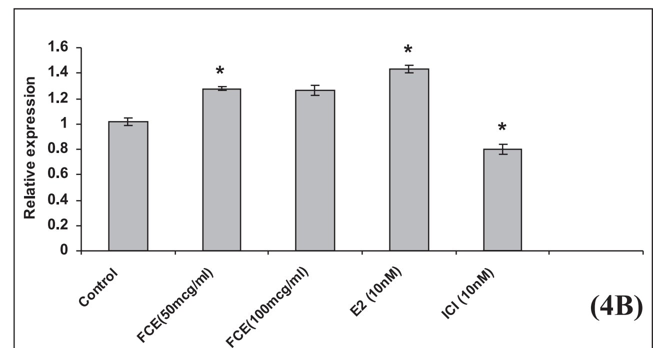


Fig. 4 Effects of the extract on *pS2* gene expression in MCF-7 cells. 5 µg of total RNA were extracted from MCF -7 treated with vehicle control, FCE (50, 100 µg/ml), 17 β-estradiol (10 nM) or ICI 182-780 (10nM) and *pS2* mRNA levels were measured by RT-PCR and normalized using *gapdh* mRNA as an internal standard (**4A**).The graph (**4B**) shows the ratio of density of *pS2* to that of endogenous control *gapdh* and it represents mean ± SE of three replicates. *$P<0.05$ compared with untreated control.

FCE stimulates pS2 expression in MCF-7 cells: MCF-7 cells treated with FCE (50,100 µg/ml) for 48 h showed an upregulation of Trefoil factor 1 (*pS2*) gene expression as compared with that of the untreated control (Fig. 4A). The relative expression of *pS2* levels was plotted after normalization with *gapdh* in comparison with the control (Fig. 4B). The *pS2* gene expression levels were found to be upregulated by 17- β estradiol, E_2 (10 nM) and downregulated by ER antagonist ICI 182-780 (10 nM).

Discussion

The excess of estrogen can cause breast, endometrial, ovarian, and prostate cancer and its deficiency can result in menopausal symptoms, cardiovascular disease, and osteoporosis[17-19]. The major causes of estrogen deficiency in females are menopause and ovariectomy.

The aim of this study was to investigate the potencies of fenugreek seed extract for estrogenic effect, to assess its capacity as alternative to HRT for postmenopausal women. *In vitro* assays accepted

world wide for their ability to reflect the estrogenecity/antiestrogenecity of compounds, *i.e*., cell viability assays, competitive binding studies, reporter gene analysis as well as analysis of endogenous estrogen sensitive markers were used.

FCE co-migrated with 17β - estradiol in TLC separations indicating that FCE contains estrogen like compounds. In MCF-7 cells FCE mimics the effects of estradiol and induces cell proliferation and the effect was found to be inhibited by ER antagonist ICI 182-780. In this study we confirmed that FCE bound ER and inhibited the binding of labelled estradiol to ER. The degree of inhibition was dependent on the concentrations of FCE and the labelled ligand. At higher concentrations, inhibition by the extract appeared to be a plateau. This may be due to the presence of multiple ligand binding proteins in the MCF-7 cytosolic extracts. The classical estrogen receptor α primarily localized in the nucleus of a cell has 595 aminoacids and a molecular mass of 66 kDa. There is an alternative membrane localized form of the classical estrogen receptor that is primarily released in to the cytosol when membranes are exposed to estradiol[20-22]. The ability of FCE to bind ER raised the possibility that it might function as an agonist or antagonist.

DLR assay measures estrogen activity through binding of estrogen receptor (ER α) in MCF-7 cells transiently transfected with a plasmid coding estrogen response elements with a reporter gene (*ERE-luc*) and a plasmid coding for ERα. FCE activated the expression of estrogen responsive reporter genes in MCF-7 cell lines while estrogen antagonists like tamoxifen downregulated the effects. In MCF-7 cells transfected with ERα and *ERE-luc* there was an upregulation of luciferase transcription after treatment with FCE and this was comparable with that of 17- β estradiol treatment. In the presence of an agonist, the ER initiates transcriptional activation by binding to specific EREs in the promoters of target genes[23].

The *pS2* gene was originally identified as an estrogen inducible transcript in MCF-7 cells and encoded for a secretary protein from these cells[24]. *pS2* gene expression is frequently used as a marker for assessing the estrogenicity of various compounds[25]. Since FCE induced *pS2* transcription, it was confirmed that this could affect the transcription of estrogen responsive genes causing estrogenic effects.

In conclusion, our study provided evidence for the potential estrogenic activities of chloroform extract of fenugreek seeds. Further studies including *in vivo* experiments need to be done to assess the physiological significance of fenugreek as an alternative to HRT and its importance as a dietary ingredient in the treatment of post menopausal health ailments in women.

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