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Original Research Article

In vitro anti-estrogenic potential of FertiZen-R™- a nutraceutical ingredient for estrogen modulation

Mihir Gadani, Ratna Upadhyay*, Supriya Raut, Sneha Badak

Department of Phytochemistry, Zenherbs Lab Pvt. Ltd., Mumbai, Maharashtra, India

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***Correspondence:**
Dr. Ratna Upadhyay,
E-mail: Ratna.upadhyay@fitcircle.in

ABSTRACT

Background: According to CDC, polycystic ovarian syndrome (PCOS) is responsible for infertility in women with 6-12% incidences all over the world. The current treatment options available have several side effects such as amenorrhea and obesity amongst others. Dietary interventions such as non-estrogenic and androgen-suppressing foods along with nutraceutical products is considered for treating PCOS with minimum side effects. One such product of Zenherbs lab called FertiZen-R™ is a combination of three phytochemicals from three plants viz., *Foeniculum vulgare*, *Linum Usitatissimum*, *Glycyrrhiza glabra*, hibiscus extract and is developed as a product to treat and control PCOS.
Methods: The FertiZen-R™ was quantified for the presence of phytochemicals such as glycyrrhizic acid, polyphenols, and saponins. It was further tested for estrogenic/anti-estrogenic potential on estrogen-positive breast cancer cell line (MCF-7) using an E-Screen assay.
Results: The FertiZen-R™ showed presence of 5% glycyrrhizinic acid, 5% saponins, and 2-3% polyphenols. It exhibited a strong anti-estrogenic potential with 40-50% inhibition from concentrations as low as 0.156 mg/ml like tamoxifen (IC_{50} at 0.156 mg/ml), while inositol, a natural growth promoter, showed no effect on the cell viability.
Conclusions: FertiZen-R™ showed anti-estrogenic potential when tested in-vitro and can be used to treat PCOS in women even with ER-positive breast cancer cells. However, clinical studies to determine the dosage are required to warrant the potential of FertiZen-R™.

Keywords: FertiZen-R™, Infertility, MCF-7, Nutraceutical, PCOS, Reproductive health

INTRODUCTION

According to the Centers for Disease Control and Prevention (CDC), polycystic ovarian syndrome (PCOS) is the most common cause of female infertility affecting 6-12% of women of reproductive age. PCOS is a multifactorial disorder with a variety of metabolic, genetic, endocrine, and environmental abnormalities.[1,2] The abnormal function of the female sex hormone estrogen and estrogen receptor is responsible for the progression of PCOS. The estrogen hormone mediates its genomic effects through ERα and ERβ and the non-genomic signaling of estrogen occurs via the G-protein-coupled estrogen receptor (GPER). Changes in these pathways affect cellular activities including cell cycles, proliferation, migration, invasion, and ovulation, coupled with upregulation of luteinizing hormone and testosterone, also hyperinsulinemia all leading to PCOS.[3-5] The clinical manifestation of PCOS include hirsutism, acne, alopecia, and anovulatory cycles, and it may increase the chance of miscarriages, gestational diabetes, and hypertension.[6] The condition of PCOS can potentially increase the risk of endometrial cancers, obesity, and other potential metabolic complications.[2,4]

Primarily in postmenopausal women, estrogen production ceases and the plasma levels drop by 80-90%.[7] During this period, the testosterone produced by the adrenal cortex

rises in the adipose, muscle, and connective tissues expressing the CYP19 gene, and the testosterone is converted to estrogen. Aromatase is the protein product of the gene CYP19 defining the rate-limiting step in the conversion of testosterone to estrogen. And this locally produced estrogen increases the proliferation of breast tumors.[8] Considering the harmful effects of PCOS, it has been important to explore natural alternatives to drug therapy to treat PCOS in women.

The therapeutic approaches for PCOS involve drug treatment and changes in lifestyle to reduce weight. Treatment with drugs has several side effects such as amenorrhoea, an increase in body weight, a reduction in bone mineral density, and obesity.[9] Hence, the use of non-pharmacological treatments like herbal medicine, functional foods, nutraceutical products, and dietary intervention may be considered more significant and appropriate to treat PCOS. For instance, soy isoflavones, which act as phytoestrogens, can bind to the estrogen receptor in cells and render the effect of endogenous estrogen inefficient.[9]

Table 1: Review of pharmacological properties of herbs present in FertiZen-R™ to prevent PCOS and improve reproductive health in women.

Name of the botanical extract	Known reproductive benefits	References
FertiZen-R™		
Foeniculum vulgare	It can reduce serum estrogen levels, and increase progesterone levels and endometrial thickness. Can reduce insulin resistance and has anti-inflammatory properties required in the management of PCOS.	10,11
Linum usitatissimum	Reducing effect on ovarian volume and cyst numbers, regulating menstrual cycles, and hormonal index such as insulin. It improves lipid profiles and metabolic dysfunction	12
Glycyrrhiza glabra	Agonist of mineralocorticoid receptors, an inhibitor of androgen synthesis, and exhibits estrogenic activity	13
Hibiscus rosasinensis	Reported to treat amenorrhoea and dysmenorrhoea	14

FertiZen-R™, is a nutraceutical product of Zenherb Labs Pvt. Ltd., is a proprietary blend of *Foeniculum vulgare, Linum Usitatissimum, Glycyrrhiza glabra,* hibiscus extract. The known anti-estrogenic and reproductive health benefits of the herbs used to formulate FertiZen-R™ are given in Table 1.[10-14] In the current study, FertiZen-R™ was tested for anti-estrogenic ability in breast cancer cell lines (MCF-7). As mentioned earlier, abnormal estrogen activity increases the proliferation of breast cancer in postmenopausal women. This natural blend with a mixture of phytochemicals such as saponins, polyphenols, and glycyrrhizic acid exhibited potential anti-estrogenic activity in MCF-7 cells when tested *in-vitro* using the estrogen screening assay or E-Screen.[15,16]

METHODS

Materials

MCF-7 HTB-22™ cells were procured from ATCC. Dulbecco's modified eagle medium (DMEM), phenol red free-DMEM, and fetal bovine serum (FBS) were procured from Himedia laboratories, India. Dulbecco's phosphate-buffered saline (DPBS), dimethyl sulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) reagent was procured from Sigma. Trypsin-EDTA, antibiotic penicillin-streptomycin (10,000 units/ml of penicillin and 10,000 µg/ml of streptomycin), and charcoal-stripped FBS was obtained from Gibco, thermofischer. The positive control used for cell proliferation in the E-screen assay was 17β-estradiol procured from Progynova® and the standard anti-estrogenic or inhibitor of cell proliferation tamoxifen was obtained from Cytotam. All the other chemicals used for chemical assay were of analytical grade.

Quantitative phytochemical analysis

Estimation of saponins

The saponin content was estimated as per Deshmukh et al, with slight modifications.[17] The sample was weighed (2 gm) and transferred to a round bottom flask and it was refluxed for 30 minutes with 25 ml of 90% v/v ethanol and followed by filtration through Whatmann's filter paper number 1. The biomass was further refluxed with 25 ml of petroleum ether, 25 ml of ethyl acetate, and 25 ml of chloroform for 30 minutes each and decant the supernatant and dissolve the biomass in methanol, further precipitated with acetone and kept for 5 minutes in ice. The extract was filtered through Whatmann's filter paper number 4 and the filtrate was dried at 105ºC. The filter was then concentrated to half of its original volume, and precipitation with acetone was repeated till no further precipitate was obtained. The percentage of saponin content was calculated as follows:

% of saponins = (total weight of precipitate/weight of sample) x 100

Estimation of glycyrrhizic acid

The content of glycyrrhizic acid was mentioned as percentage content in the FertiZen-R™ blend and was

determined using the gravimetric method.[18] To 2 gm of the blend, 15 ml of hot water was added and was heated for completely dissolve the samples. After cooing, 25 ml of 80% ethanol was added and after shaking, 95% ethanol was added. The solution was filtered and washed with 80% ethanol till the solution was colourless. The filtrate was evaporated to make it viscous and to this solution 3 ml of 10% sulfuric acid was added with 30 ml of distilled water and was incubated overnight in refrigerator for settling the residue which was then separated from the supernatant by filtering. The residue left on the filter paper was solubilized with 50% ethanol containing 1-2 drops of ammonia. The solution was further filtered and the filtrate and paper washing was evaporated in a pre-weighed beaker and the weight of the residue was determined. The total glycyrrhizic acid content was determined using the formula as follows:

% glycyrrhizinic acid = (weight of the residue/weight of the sample)*100

Estimation of polyphenol

The polyphenol content was identified using the Folin Ciocalteu method.[19] The FertiZen-R™ blend were prepared in water along with gallic acid as the standard. To 2 ml of the test or standard solutions, 2 ml of 1N FC reagent was added followed by 10 ml of sodium carbonate (15 gm in 50 ml distilled water). The volume of the system was 25 ml. All the tubes were incubated in the water bath at 60ºC±50ºC for 5 minutes and were then incubated at room temperature for 30 minutes. The absorbance was measured at 700nm using water as the blank.

Percent polyphenol content: (sample absorbance × standard weight × purity of standard)/(standard absorbance × sample weight)

E-Screen assay

The E-screen assay was performed as per the methodology given by Villalobos et al, with slight modifications.[20] MCF-7 cells were grown in DMEM, supplemented with 10% FBS, 1% penicillin-streptomycin and passaged when ~70-80% confluence was reached. Cells were washed with DPBS and trypsinized. Followed by, the cells were centrifuged at 1200 rpm for 5 minutes at 25°C. The supernatant was discarded and cells were suspended in the desired volume of the growing medium. A volume of 100 µl of cell suspension with 1 x 104 cells/well in a 96-well plate was added and incubated at 37°C with 5% CO_2 for 16-24 hours. The supernatant was discarded and 100 µl of test medium was added to each well. The cells were treated with seven concentrations of the stimulator (17β estradiol) or inhibitor (tamoxifen) or the test sample (FertiZen-R™) with two replicates for each dilution. The plate was incubated at 37°C with 5% CO_2 for 72 hours and processed to assess the cell proliferation levels and cell viability was calculated using MTT assay. The media was removed and 50 µl/well of 1 mg/ml of MTT solution was added to the plates. The plate was further incubated at 37°C with 5% CO2 for 3 hours. After the incubation period, the MTT solution was discarded and 100 µl/well of DMSO was added followed by, the plate was kept on a plate shaker for 20 minutes and read at 570 nm. The instrument readouts were directly corresponding to the rate of cell proliferation in comparison to the untreated cell control.

Statistical analysis

For all the measurements average and standard deviation was measured.

RESULTS

Quantitative analysis

The FertiZen-R™ product showed the present of saponins, glycyrrhizic acid, and polyphenols and the percentage of these phytochemicals present is given in Table 2.

Table 2: Standardization of PCOS FertiZen-R™.

Phytochemicals	FertiZen-R™
Glycyrrhizic acid	5%
Polyphenols	2-3%
Saponins	5%
Tannic acid	-

FertiZen-R™ showed antiproliferative activity in the MCF-7 cell line

Assessment of the anti-estrogenic activity (Figure 1) of test items was performed using human breast cancer estrogen-positive MCF-7 cells. Cells were incubated with different concentrations of the stimulator (17β-estradiol), inhibitor (tamoxifen), and test samples (FertiZen-R™, and inositol).

The positive control tamoxifen showed anti-proliferative activity from concentrations as low as 3.125 µg/ml where the cell viability was found to be 76.90% after which the cell viability dropped at 47.64% at 100 µg/ml and 43.82% at 200 µg/ml. The IC50 value for Tamoxifen was between 50-100 µg/ml. For FertiZen-R™, anti-proliferative activity was observed but was at higher concentrations as compared to Tamoxifen. It showed better anti-estrogenic ability by inhibition of MCF-7 cells starting from 0.156 mg/ml where the cell viability was found to be 51.56%. As in the case of tamoxifen, FertiZen-R™ did not show any concentration-dependent cytotoxicity in MCF cells, where the cell viability altered between 50-60% from concentrations 0.156 mg/ml to 2.5 mg/ml. However, at a higher concentration of FertiZen-R™ i.e., 5 mg/ml, the cell viability dropped to 34.83% and at 10 mg/ml it further decreased to 25.13%. As seen in Figure 1, inositol showed no changes in cell viability as compared to the control. Microscopic images of treated and untreated groups were captured on the day of the MTT Assay (Figure 2).

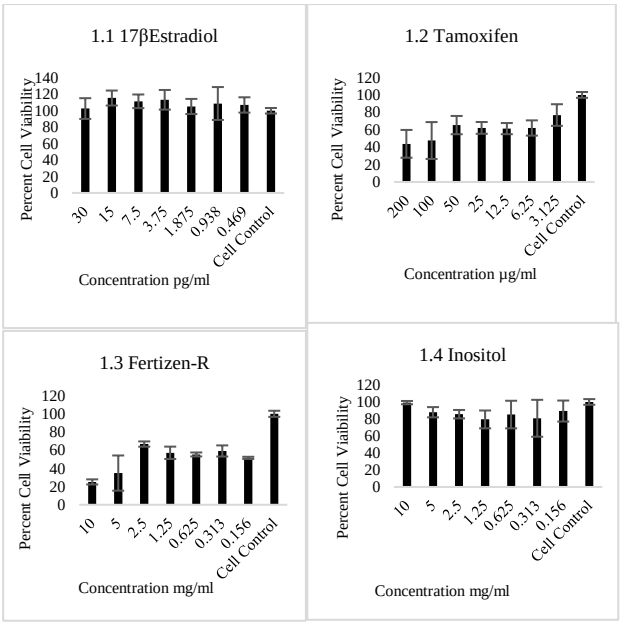

Figure 1: Estrogen activity of 17β estradiol (stimulator of proliferation), tamoxifen (inhibitor) FertiZen-R™, and inositol.

DISCUSSION

It has been proven that cell proliferation is a hallmark of estrogen action. Therefore, cell proliferation induction and inhibition on breast cancer cell line (MCF-7) in presence of estrogen or termed an E-screen assay can be used to determine the estrogenic activity of extracts and drugs.[20] In the current study the estrogenic activity of FertiZen-R™, a nutraceutical product of Zenherb Labs Pvt. Ltd., is a combination *Foeniculum vulgare, Linum Usitatissimum, Glycyrrhiza glabra,* hibiscus extract herbs known in the treatment of PCOS was tested using the E-screen method. The blend showed no proliferative activity in the MCF-7 cell suggesting their role was not as similar was 17β-estradiol, moreover, they inhibited the cell growth of MCF-7 cells proving them as anti-estrogenic in nature. This data corroborated with existing literature (Table 1).

In a study conducted by Ahmad et al, the antiproliferative activity of peel, flesh, and seed extracts of *G. dulcis, G. parvifolia, G. nitida, G. mangostana var. mangosta*, and *G. cambogia* was evaluated on MCF-7 cell lines. Of the tested samples, *G. dulcis* showed the highest anti-proliferative activity with an IC50 value of 2.5±0.0 µg/ml followed by *G. dulcis* flesh, *G. mangostana var. mangostana* peel and *G. dulcis* peel with IC_{50} values of 9.33±3.21, 11.17±1.04 and 17.67±2.08 µg/ml.[21] In yet another study conducted by Resende et al, antiestrogenic activity was exhibited by flavonoids such as 10^{-5} M of quercetin, 10^{-7} M of chrysin, 10^{-5} M of 3-hydroxyflavone in MCF-7 cell line using the E-screen assay and these compounds were anti-proliferative in nature.[22] In the current study, FertiZen-R™ was found to be anti-proliferative against the proliferation of ER-positive MCF-7 human breast cancer cells.

Alvimil, a dietary supplement used to ameliorate female sexual dysfunction is a mixture of 11 herbs including certain polyphenol-contributing plant extracts such as black cohosh, licorice, red raspberry, red clover, and kudzu. These compounds may exhibit estrogenic properties and hence it was tested and it was found that concentrations up to 0.1-50 µg/ml exhibited cell proliferation in the ER-positive breast cancer cells whereas 100 µg/ml inhibited the growth. The dietary intake in humans was further evaluated using ovariectomized mice implanted with MCF-7 and it was found that lower concentration of 500 ppm was capable of promoting breast cancer in the mice models.[23] Hence, it is recommended that women with ER-positive breast cancer cells should avoid taking this dietary supplement for sexual dysfunction.[23] Hence it is important to understand if the phytochemicals present in the nutraceutical product are safe and do not promote the proliferation of MCF-7 cells. FertiZen-R™ did not induce cell proliferation of MCF-7 cells suggesting its non-estrogenic character.

The results were submitted as per experiments conducted *in vitro* and clinical studies are required to prove the efficacy of the product in actual human volunteers.

CONCLUSION

The product FertiZen-R™ can serve as a good health supplement that can provide health benefits via antioxidative and anti-inflammatory ability due to the presence of tannins, saponins, polyphenols, and glycyrrhizic acid. In the current study, the role of FertiZen-R™ as a product useful in the treatment of PCOS was validated using an in-vitro E-screen method. It was found that FertiZen-R™ anti-proliferative against human breast cancer cell line MCF-7 and can be concluded to be anti-estrogenic in nature. The Fertizen- R blend can be used as a potent nutraceutical agent for patients experiencing PCOS.

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