IN SILICO STUDY ON ESTROGENIC EFFECT OF BIOACTIVE COMPOUNDS OF *Trigonella oenum-graecum* L. AND ACTIVITY ON T47D CELL LINE

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Abstract

Trigonella foenum-graecum (TFG) is one of medicinal plants contains some steroidal sapogenin such as diosgenin, yamogenin, gitogenin, tigogenin and trigoneoside, also alkaloid trigonellin and some flavonoids such as vitexin, isovitexin, orientin, isoorientin, which are has many activity as antidiabetic, estrogenic and also anti cancer. As phytoestrogen, TFG was predicted having potency as Selective Estrogen Receptor Modulators (SERMs) which is used for dependent hormonal breast cancer treatment. This experiment was carried out to investigate interaction from some sapogenin steroids and flavonoids in TFG to estrogen receptor alpha (ERα) and its activity to breast cancer cell line as confirmation. In silico prediction was carried out to investigate their estrogenic activity by analize their binding affinity to estrogen receptor alpha (ERα) using AutoDock Vina program. In vitro confirmation activity of TFG extract and its fractions were carried out using MTT assay on ERα positive human breast cancer cell line, T47D. Results show that free binding energies of diosgenin and yamogenin are -6.4 kcal/mol, estradiol is -6.0 kcal/mol and tamoxifen is -5.1 kcal/mol. While cytotoxicity assay shows that ethyl acetate fraction gives the lowest IC50, 58.63 ppm, with total steroid contains 20.03 ppm. From this results we can conclude that diosgenin and yamogenin have greater binding affinity to ERα comparing to estradiol and tamoxifen. In vitro assay confirmation showed that ethyl acetate fraction have a cytotoxic effect on T47D cells.

Keywords : *Trigonella foenum-graecum, Sapogenin steroids, T47D, Estrogen Receptor alpha, binding affinity.*

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INTRODUCTION

Fenugreek seed or Foenigraeci semen is dried seed from *Trigonella foenum-graecum* L., (TFG), Leguminosae, (WHO, 2007). Empirically, TFG seed was used for hemorrhoids, asthma, ulcers, muscle pain and often used as a preventative hair loss and skin softener. Many studies showed its activity as antidiabetic, anticancer and for hypercholesterolemia handling (Mills, 2000). TFG has antiandrogen activities, due to beta-sitosterol, palmitic-acid and stearic-acid, and also has the ability to lower total cholesterol, LDL, VLDL cholesterol and triglycerides significantly. The anti-hyperglycemic and anti-inflammatory properties noted in fenugreek are of additional benefit. TFG can induce uterine contraction, so it can not consume during pregnancy (Hoffman, 2004). Agustini's study (2007) showed that ethanolic extract of TFG seed have estrogenic effect on ovariectomized and immature female Wistar rats. Phytoestrogen is used as alternative for Hormone Replacement Therapy (HRT) to help reducing menopause symptoms. It can be used for long term therapy until the body can make adaptation on the new level hormone (Badziad, 2003). Phytoestrogen also have potency to handling depending hormonal breast cancer which is known as natural Selective Estrogen Receptor Modulators (SERMs).

TFG was predicted having estrogenic like effect also because of some sapogenin steroid ingredients, e.g. diosgenin, precursor for sexual hormone (Evans, 2002), its isomer Yamogenin (Dewick, 1997), gitogenin, tigogenin, and trigoneoside (saponine like estrogen) which have effect as phytoestrogen for menopause symptoms therapy (Hoffman, 2004). TFG seed contains diosgenin in base free form 0.8 - 2.2 % (Wiryowidagdo, 2000). TFG also contains fatty oil 20-30%, alkaloids (trigonelline, an alkaloid pyridine, gentianin and karpain), flavonoids e.g. vitexin in glycoside or ester form, isovitexin, orientin, vicenin, quercetin and luteolin (Hoffman, 2004), essential oil, saponine, nicotinamide, choline, bitter compound and mucilage (Evans, 2002).

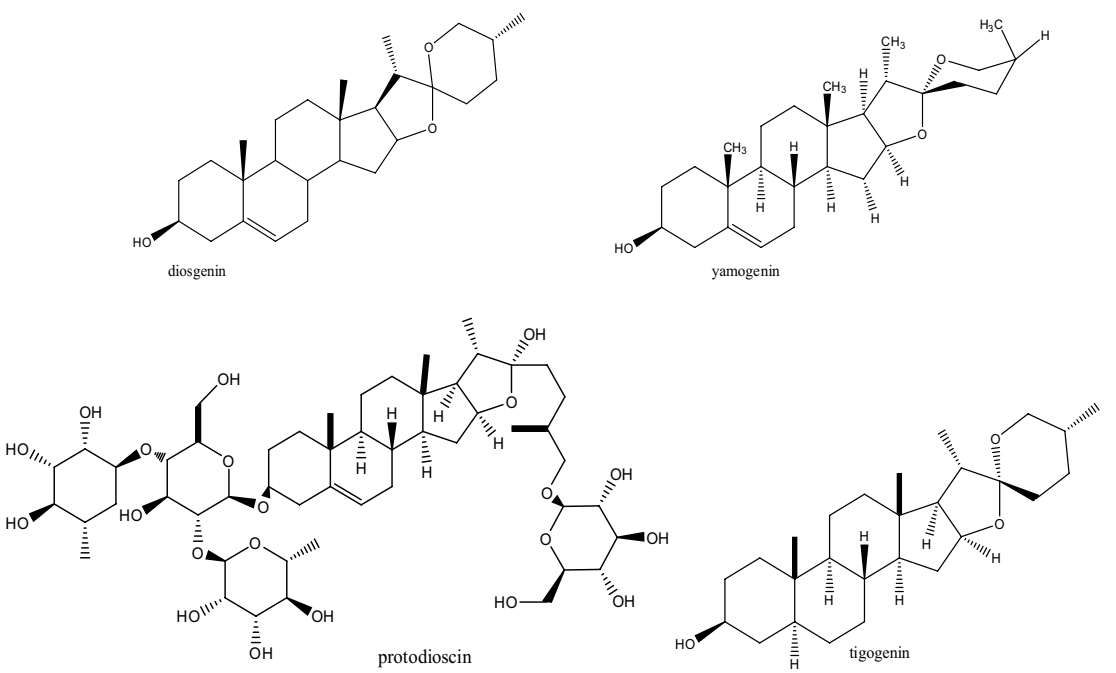

Figure 1.Sapogenin Steroids of *Trigonella foenum-graecum* L.

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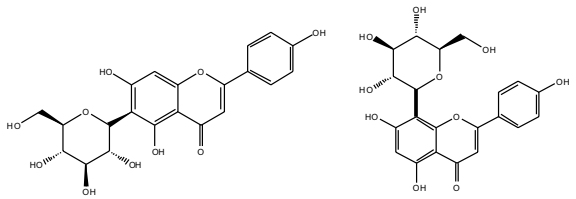

Figure 2.Flavonoids of *Trigonella foenum-graecum* L.

This study was carried out to investigate interaction from some sapogenin steroids and flavonoids in TFG to estrogen receptor alpha (ERα and the cytotoxicity activity of fenugreek seed to breast cancer cell line with estrogen receptor positive as confirmation. In silico prediction was carried out to investigate their estrogenic activity by analize their binding affinity to estrogen receptor alpha (ERα) using AutoDock Vina program. In vitro confirmation activity of TFG extract and its fractions were carried out using MTT assay on ERα positive human breast cancer cell line, T47D

METHODS

Docking Process

Docking process was carried out using protein Estrogen Receptor Alpha (ERα) with PDB ID: 2YAT and four sapogenin steroids and two flavonoids of TFG as ligands. They are Diosgenin, Tigogenin, Gitogenin and Protodioscin, also Vitexin and Isovitexin. We also docked estradiol and tamoksifen, as comparison. The 3D protein structure also needs to be generated for docking. Docking files were prepared by using MGL Tools 1.5.4 software. Docking was carried out to investigate the estrogenic activity by analize their binding affinity to estrogen receptor alpha (ERα using AutoDock Vina program. The size of the docking grid was 40 Å×40 Å×40 Å, which encompassed the entire ERα structure. After the docking simulation, we visualized the result with PyMol program.

Sample Preparation

TFG seed were obtained from Tawangmangu, Central Java, Indonesia. Seeds were dried and grind, then were extracted with methanol and ethanol. The methanolic extract was fractioned with n-hexane, ethylacetic (EtOAc) and n-buthanol. Every extract and fraction was dried with vacuum rotary evaporator.

Total Steroid Analysis (Chapagin, et.al., 2005)

1 mg dried extract/fraction diluted in 2 mL ethylacetate in a tube, then 1 mL reagent A (contains p-anysaldehyde and ethylacetate (0.5 : 99.5)) and 1 mL reagent B (contains sulfuric acid glacial and ethyl acetate (1:1) were added. Tube was put in water bath 600C for 10 minutes untill the color was occurred and then cooled in another water bath 250C for 10 minutes. Color was measured by Spectrophotometer UV Vis 423nm, against ethyl acetate solution as reagent blank. Results were compared with curve standard of Diosgenin (Sigma).

Cell Culture

The cell lines T47D (Human Breast Cancer with Estrogen Receptor Positive) were obtained from Laboratory for Development of Industrial Agro and Biomedical Technology (LAPTIAB-BPPT) Indonesia. Cells were routinely maintained and grown in 75 cm2 flasks at 370C, 5% CO2 and in a 95% humidified atmosphere. The growth medium was prepared as following : RPMI 1640, Gibco life Technologies with phenol red and 2 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml Streptomycin, 1 mM sodium pyruvate and supplemented with 10% Foetal Bovine Serum (FBS, Gibco Life Technologies) which already heat inactivated at 560C for 30 min. Passaging of cells was carried out using 4 ml of trypsin-EDTA at room temperature for 75 cm2

flask, respectively for 3 min. After that, 10 ml media with 10% FBS were used to reduce the action of trypsin on cells. After centrifugation, the obtained cells were platted.

Cytotoxicity test with MTT method

Cells were platted into 96-well plates (10,000 cells/well) in medium RPMI with phenol red containing 10%Fetal Bovine Serum (FBS), 100U/ml penicillin, 0.1 mg/ml streptomycin and 1mM sodium pyruvate, then incubated for 24 hours at 370C, 5% CO2 and in a 95% humidified atmosphere. After 24 hours, medium was changed with samples (extracts and phases of TFG) in growth medium in different concentration and incubated for another 24 hours at 370C, 5% CO2 and in a 95% humidified atmosphere. Assays were done in wide range concentration, from 10 ppm until 500 ppm, divide into six variation concentration.After 24 hours treatment, the cells were washed with Phosphate Buffer Saline (PBS). Then the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetr azolium) solution in medium, was added followed by incubation for 4 hours at 370C, 5% CO2 and in a 95% humidified atmosphere. The crystal of formazan blue will be formed. After that, reaction was stopped by added Sodium Dodecyl Sulphate (SDS) into every well. Leave plate in dark place for 12 hours (overnight). The intensity of the color formed was measured by ELISA reader at 570nm.

RESULTS AND DISCUSSION

Recent studies suggest that TFG and its active compounds may possess anticarcinogenic potential. Raju (2004) showed that diosgenin, a steroid saponin from TFG can inhibit azoxymethane-induced aberrant crypt foci formation in F344 rats and induces apoptosis in HT-29 human colon cancer cells. Also refer from Agustini (2007) that showed TFG have estrogenic effect on ovariectomized and immature rats. Some phytoestrogens are believed to have selective estrogen receptor modulators (SERMs) activity with no action in the uterus but beneficial effects in the hypothalamus/pituitary unit and in the bone and are presently the focus of clinical interest (Wuttke, 2003). According to Stephen RD Johnston (2005), the study to search the ideal profile of a novel SERMs in comparison with tamoxifen, should have greater binding affinity into ER, and also having ability to antagonize estrogen dependent growth of breast cancer cells in vitro (preclinical). Base on this information, it is an interesting phenomena to investigate the interaction between some steroid sapogenin from TFG and its potency on cancer cell line, especially cell line with estrogen receptor positive.

Table I.Predicted ligand free binding energy and residue contact in docking simulation

No.	Compounds	Predicted ΔG (kcal/mol)	Binding site
1.	Estradiol	-6.0	Arg 548, Arg 515
2.	Tamoksifen	-5.1	Ser 381, Ala 546
3.	Vitexin	-7,5	Ser 518, Arg 515, Arg 548, Ala 546, His 377
4.	Isovitexin	-6.8	Arg 515, His 377
5.	Diosgenin	-6.4	Arg 515
6.	Protodioscin	-5.4	Glu 380, Asn 519, Arg 548, Asp 545
7.	Tigogenin	-6.0	-
8.	Yamogenin	-6.4	-

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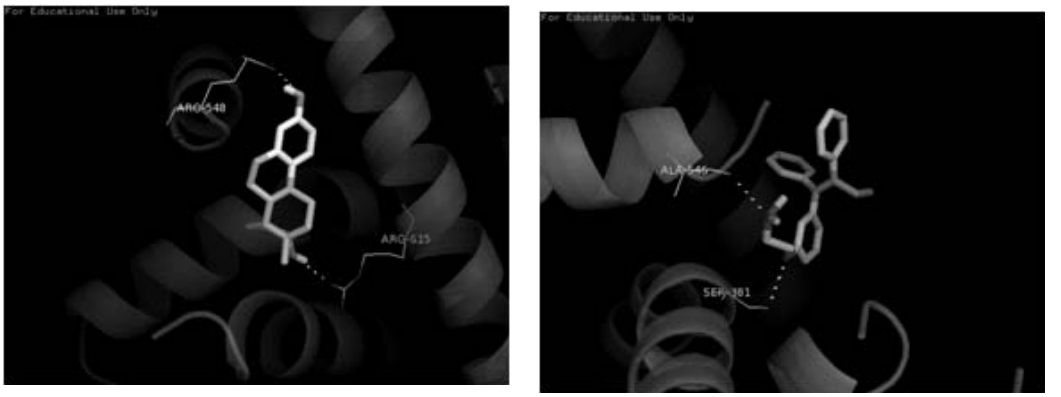
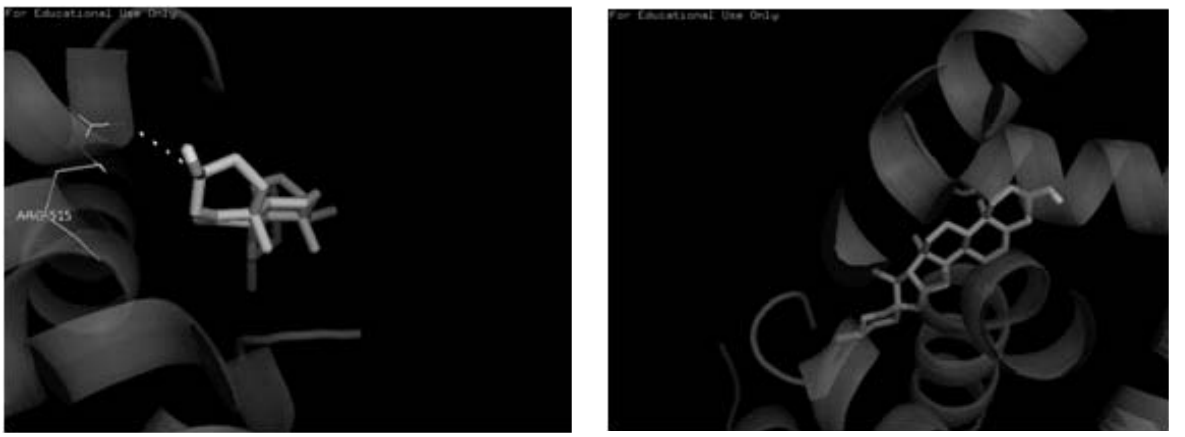
Interaction with ER was predicted using in silico method, which was carried out using AutoDockVina program with protein ERα, PDB ID: 2YAT. Free binding energy of interaction between some sapogenin steroids and flavonoids to ER? is showed in table I.

The protein structure was prepared for docking as described previously. This included the addition of missing hydrogens. During docking, series of poses (ligand-protein complexes) were generated for each molecule. Docking simulations carried out on the Active Binding Site, which is composed of Ser 381, Cys 447, Ser 530. Table I shows the calculated free binding energy (ΔGbind) and residue contact of flexible-ligand docking simulation. The negative and low value of ΔGbind indicated the strong favorable bond between enzyme and ligand. Based on docking simulation result, diosgenin and yamogenin,vitexin and isovitexin could be proposed as a potential ligand that protein Estrogen Receptor α (ERα) drug.



Estradiol Tamoksifen

Figure 3. Interaction and active binding site of Estradiol (left) and Tamoksifen (right)) into ERα



Diosgenin Yamogeninin

Figure 4. Interaction and active binding site of Diosgenin (left) and Yamogenin (right)) into ERα

Total steroids level analyses showed that buthanolic fraction contains the highest total steroid (25.321 ppm) while IC50 on T47D ethylacetic fraction gives the lowest IC50 (58.63 ppm). Total steroids analyses results and IC50

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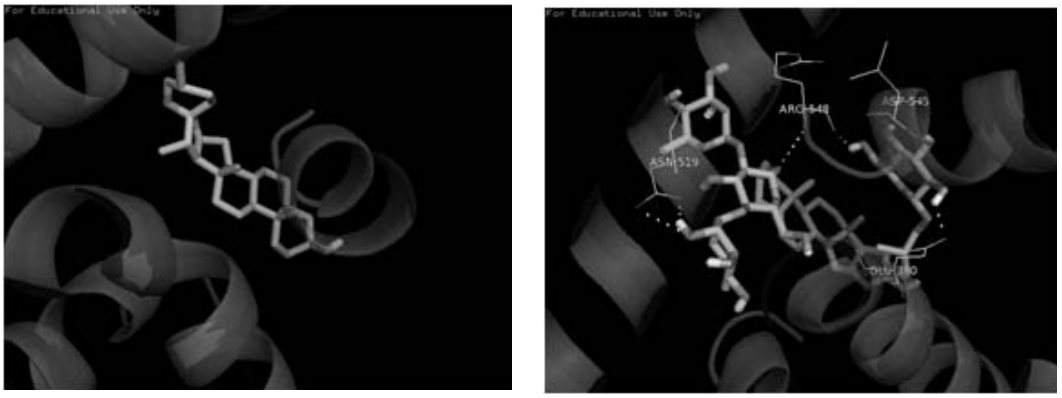
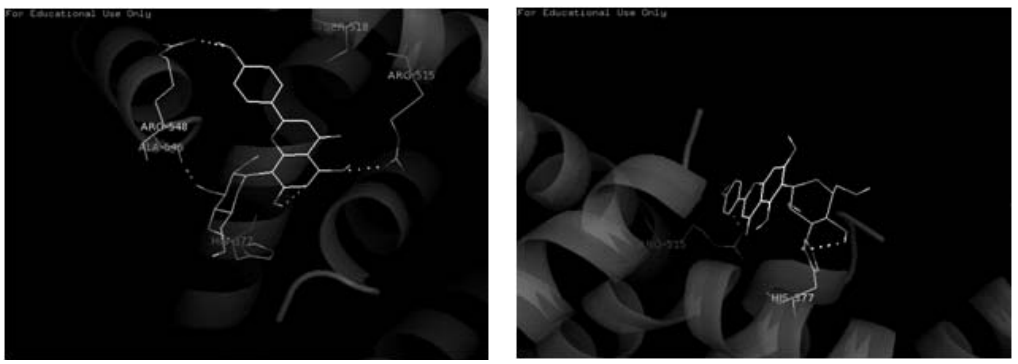


Tigogenin Protodioscin

Figure 5. Interaction and active binding site Tigogenin (left) and andProtodioscin (right) into ERα.



Vitexin Isovitexin

Figure 6. Interaction and active binding site of vitexin (left) and isovitexin (right) into ERα

on T47D can be seen on table II. While the growth percentage of T47D treated with TFG was showed in table III.

Total steroidal contain in Buthanolic fraction (25.321 ppm) nearly the same with ethylacetic fraction (20.033 ppm).

The mayor sapogenin steroid from TFG, diosgenin, has been investigated in many researches for its cytotoxicity activity. Moalic et.al (2001) reported that diosgenin inhibits cell proliferation in the human osteosarcoma 1547

Table II.Total steroid level and IC50 of some fraction of TFG on T47D cells

	TOTAL STEROID LEVEL (ppm)	IC50 on T47D (ppm)
Ext.MetOH	19.769	140.31
Ext. EtOH		128.37
Fr. EtOAc	20.033	58.63
Fr. N-Hex	3.693	206.60
Fr. N-BuOH	25.321	

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Table III. Percentage growth of *Trigonella foenum-graecum.* L on T47D cells

Concentration (ppm)	%Growth T47D cells			
	MeOH extr.	EtOH extr.	EtOAc Phase	Hexane Phase
10	102.28	112.25	88.42	96.75
20	68.69	68.27	90.92	98.94
50	92.78	78.58	33.50	111.20
100	55.81	54.25	17.28	30.21
250	16.69	16.73	16.65	16.35
500	15.72	16.05	16.86	16.69

cell line by induction of apoptosis and G1 phase cell cycle arrest.

Yoshihiro et.al (2001) studied about cytotoxicity activities on human promyelocytic

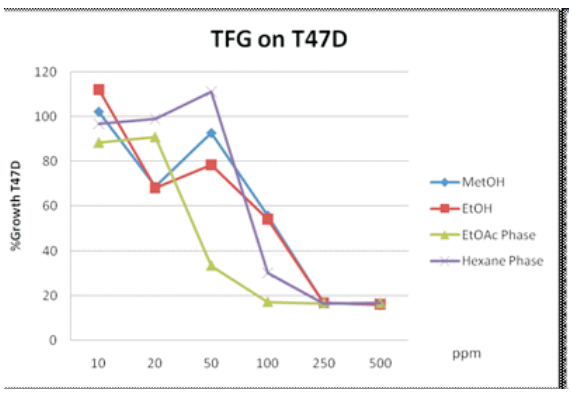

Figure 7. Effect of TFG on growth of T47D cells. The results were plotted as percent of T47D growth (% relative cell growth) versus concentration part permillion (ppm)

leukemia cells, HL-6 and structure-cytotoxic relationships of steroidal saponins. They found that the activities of some sapogenin steroids were sensitive to the monosaccharides constituting the sugar moieties and their sequences, as well as to the structures of the aglycons. They also concluded that structure–activity relationships of (25*R*)-Spirost-5-en-3b-ol (Diosgenin) glycoside derivatives Diosgenin b -D-glucoside showed no cytotoxic activity against HL-60 cells (IC50>20ppm)

CONCLUSION

Diosgenin, yamogenin, vitexin and isovitexin have greater binding affinity to ERá comparing to estradiol and tamoxifen. In vitro assay confirmation showed that ethyl acetate fraction have a cytotoxic effect on T47D cell (IC50 58.63 ppm) and contains steroid compounds (20.03 ppm).

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