ABSTRACT

Trigonella foenum-graecum (TFG) is one of medicinal plants containing some steroidal sapogenins such as diosgenin, yamogenin, gitogenin, tigogenin and trigoneoside, also alkaloid trigonellin, which has many activity as antidiabetic, estrogenic and also as anti cancer. The aim of this research is to investigate the correlation of its cytotoxic activity on breast cancer cell line, MCF-7, with its total steroid level. Samples was prepared by fractioned the methanolic TFG with ethylacetate, n-hexane and n-butanol. Each fraction then was separated by chromatographic coloumn. Cytotoxic activity was investigated using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium) assay on MCF-7 cell, while total steroid level was done using spectrophotometric methods. Results showed that there was no correlation between cytotoxic activity and total steroid level. Thus we can conclude that the cytotoxic effect from TFG is not caused by its steroid compounds.

Key words: Trigonella foenum-graecum L., MTT, MCF-7, total steroid level.
INTRODUCTION

Fenugreek seed or Foenigraeci semen is dried seed from *Trigonella foenum-graecum* L. (TFG), Leguminosae, (MMI, 1979). Geographical distribution of TFG is indigenous to the Mediterranean region, China, India and Indonesia (WHO, 2007). TFG contains 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. Sapogenins are the aglycones or non-saccharide of saponins, one of phytochemical compound family. Sapogenins contain steroid or other triterpene frameworks as their key organic feature. Some steroidal sapogenins can serve as a practical starting point for semisynthesis of particular steroid hormones.

TFG contains diosgenin in base free form 0.8-2.2 % (Wiryowidagdo, 2000). TFG also contains alkaloids (trigonelline, an alkaloid pyridine, gentianin and karpain), flavonoids (vitexin in glycoside or ester form, isovitexin, orientin, vicenin, quercetin and luteolin), fatty oil 20-30%, essential oil, saponine, nicotinamide, choline, bitter compound and mucilage (Evans, 2002).

Empirically TFG was use as aphrodisiac, carminative, diuretic, emmenagogue, emollient, galactogogue and tonic (WHO, 2007). Many researches, preclinical and clinical, showed that TFG have activity for diabetic disease (Annida, 2004). TFG also predicted as Phytoestrogen cause have estrogenic effect on ovariectomized female rat and immature female rat (Agustini, 2007). It can induce uterine contraction, so it should be avoided during pregnancy. Phytoestrogen is used as alternative for Hormone Replacement Therapy (HRT) to help reducing menopause symptoms. It can be used for long term until the body can make...
adaption on the new level hormone (Badziad, 2003).

Extracts of TFG seeds and some of their sapo- nine constituents also have been found to have anticarcinogenic activity in different setting. It has been evaluated in the Ehrlich ascites carcinoma model in BALB/c mice, where it affected 70% inhibition of tumor cell growth compared with controls (Sur, 2001). Hibasami et al., (2003) suggest that growth inhibition of human leukemia HL-60 cells by protodioscin, isolated from TFG seeds, results from the induction of apoptosis. Moalic et al., (2001) reported that diosgenin inhibits cell proliferation in the human osteosarcoma 1547 cell line by induction of apoptosis and G1 phase cell cycle arrest.

Refer to data that showed TFG having estrogenic activity as phytoestrogen, inspiring to study its possibility as Selective Estrogen Receptor Modulators (SERMs) candidate. SERMs such as tamoxifen, which are used clinically for the treatment of breast cancer, act as estrogen agonists in certain tissues but exhibit antiestrogenic effects in others (Rosenbaum, 2000). There are several researches about SERMs candidates for post tumor surgery therapy from phytoestrogen pure compounds, such as dammarane, a sapo- nine steroid from ginseng (Oh, 1999), Genistein (an isoflavone from soybean) and resveratrol, a stilbene from grape (Baht, 2001).

This research was done to investigate the correlation between TFG’s cytotoxicity on human breast cancer cell line MCF7 and its total steroids level in methanolic extract and its fractioned (hexane, buthanol and ethylacetate).

MATERIALS AND METHODS
Samples Preparation

TFG seeds were obtained from Tawangmangu, Central Java, Indonesia. Seeds were dried and grind, then were extracted with methanol. The methanolic extract was fractioned with n-hexane, ethylacetic (EtOAc) and n-buthanol. Further, each fraction then separated by vacuum liquid chromatography using various eluens. 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 60°C for 10 minutes till color was occurred and then cooled in another water bath 25°C 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 MCF-7 (Human Breast Ad- enocarcinoma) was 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 37°C, 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 56°C 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 plated.

**Cytotoxicity test with MTT method**

Cells were plated 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 1 mM sodium pyruvate, then incubated for 24 hours at 37°C, 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 37°C, 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-Diphenyltetrazolium) solution in medium, was added followed by incubation for 4 hours at 37°C, 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**

TFG was extracted with methanol to obtain more chemical phyto compound in it. After that, methanolic extract was fractioned by vacuum liquid chromatography and divided into three fractions based on its polarity, they are non-polar fraction (n-hexane fraction), semi-polar fraction (ethyl acetic fraction) and polar fraction (Buthanolic fraction). Methanolic extract and other three fractions then were analyzed for cytotoxicity activity on human breast cancer cell-line, MCF-7, and its total steroidal level. Result of these analyze are shown by Figure 2. Ethylacetic fraction gives the lowest IC50 (41.48 ppm) and the highest level of total steroid (20.033ppm) was given by n-buthanolic fraction, while the lowest total steroid level was given by n-hexane fraction (3.693ppm) and less cytotoxicity activity was given by methanolic extract (186.007 ppm). From this data we can see that three fraction are more active than its methanolic extract. We can predict that the characteristic of active compound on MCF-7 cell is semi-polar.
Figure 2. Comparative of cytotoxicity activity on MCF7 (IC\textsubscript{50}) and its total steroid level from methanolic extract and its first fractionation.

Figure 3. Comparative of cytotoxicity activity on MCF7 (IC\textsubscript{50}) and its total steroid level from column chromatographic fractions of Ethylacetic fraction.

Screening then continued by separated the ethylacetic fraction using vacuum column chromatographic with gradient elucidation using mixed dichloromethane and ethanol. All fractions were grouped based on their spots in thin layer chromatography. Results of cytotoxic activity and its steroid level analyze were showed in Figure 3.

The most active fraction is EA2 with IC\textsubscript{50} = 3.812 ppm and the less active fraction is EA4 with IC\textsubscript{50} = 2662 ppm, while the highest level of total steroid was given by EA3 fraction (27.533 ppm) and the lowest level of total steroid was given by EA1 fraction (13.417 ppm). Also in these results of ethyl acetate fraction, the same pattern of total steroid level and its cytotoxic activity are not found.
To investigate more about the correlation of these two parameters, the screening to n-Hexane fraction also was done. After separated by column chromatography using also gradient elucidation of mixed dichloromethane and ethanol, each fraction then screened by MTT assay and their level of Steroids. Results are showed in Figure 4. The most active fraction is HX4 with $IC_{50} = 35.57$ ppm and the less active fraction is HX3 with $IC_{50} = 396.19$ ppm, while the highest level of total steroid was given by HX4 fraction (25.055 ppm) and the lowest level of total steroid was given by HX2 fraction (3.92 ppm).

This research showed that there was no correlation between total steroid level and its cytotoxicity activity on breast cancer cell line, MCF-7. As we know, the major 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 cell line by induction of apoptosis and G1 phase cell cycle arrest. Raju et al. (2004) also reported that diosgenin, a steroid saponin of *Trigonella foenum-graecum* (fenugreek), inhibits azoxymethane-induced aberrant crypt foci formation in F344 rats and induces apoptosis in HT-29 colon cancer cells. Yoshihiro et al. (2001) studied about cytotoxicity activities on human promyelocytic 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 (25R)-Spirost-5-en-3b-ol (Diosgenin) glycoside derivatives Diosgenin b-D-glucoside showed no cytotoxic activity against HL-60 cells ($IC_{50} > 20$ ppm)

**CONCLUSIONS**

The most active TFG fraction on MCF-7 cell-line is ethylacetic fraction, with $IC_{50} = 41.48$ ppm and its fraction EA2 ($IC_{50} = 3.812$). The highest level
of total steroid in first fractionation is buthanolic fraction (25.31 ppm). While from column chromatographic results of ethylacetic fraction, the highest total steroid level given by EA3 (27.533 ppm). The cytotoxic activity on MCF-7 of TFG maybe caused by another group compound except its sapogenin steroid. The isolation of active compound should be continue. So we can conclude that there was no correlation between cytotoxic activity on MCF-7 cell and total steroid level. The cytotoxic effect on MCF-7 cell from TFG is not caused by its steroid compounds.

ACKNOWLEDGEMENT

We would like to thank Rahma Micho Widyanto from cell culture laboratory of LAPTIAB who has done the maintenance of MCF-7 cells. We also would like to thank Siti Aisyah and Purnama Dwi Tistianto, bachelor students of Departement of Pharmacy, Faculty of Medicine and Health Science, State Islamic University Syarif Hidayatullah Jakarta, who have helped in extraction and fractionation work in laboratorium of phytochemistry, LAPTIAB, Serpong.

REFERENCES


