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SELECTIVITY OF ETHYL ACETATE FRACTION OF *Gynura procumbens* ON COLON CANCER AND BREAST CANCER

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Abstract

Gynura procumbens is widely used as traditional remedy in South-East Asia. *Gynura procumbens* exhibites anti inflammatory, antioxidant, and reduced blood pressure activity. The aim of this study was to determine chromatographic profile of ethyl acetate fraction of *Gynura procumbens* (FEG) and to investigate its cytotoxic properties and selectivity to colon cancerand breast cancer cancer cells.

The chromatographic profile of FEG was determined using HPTLC densitometric and HPLC. MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to determine the growth inhibitory effect of FEG on the growth of WiDr, MCF-7, and T47D cells. NIH3T3, a normal cells was used to determine the selectivity of FEG, which contained small amount of quercetin as identified from chromatographic profile both HPTLC and HPLC. FEG inhibited cell growth of WiDr, of MCF-7 and of T47D cells in time dependent manner. Quercetin affected cell growth inhibition approximately two fold higher at WiDr and MCF-7, whereas FEG had lower effect on T47D cell. Quercetin did not seem as the main active compound of FEG. At this study, FEG caused less inhibition on the growth of NIH3T3 cells than that of on all cell lines. Selectivity index (SI) of FEG on WiDr, MCF-7 and T47D were 4.97, 2.77 and 7.79 respectively.

According to the datas obtained, FEG possesses moderate to high cytotoxicity properties on WiDr, MCF-7 and T47D cells. FEG demonstrates selective effect against cancer cells and reveals prospective properties as cancer chemoprevention agent.

Keywords: *Gynura procumbens*, colon cancer, breast cancer, cytotoxicity, selectivity.

Abbreviations: **FEG**, ethyl acetate fraction of *G. procumbens*, **DMSO,** dimethyl sulfoxide**, FBS,** fetal bovine serum**, MTT,** 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide**, SI,** selectivity index.

Introduction

Breast cancer and colon cancer are the two of the most five deadly cancer diseases together with lung cancer, stomach cancer and hepatocarcinoma. Approximately 1,2 million cases of colorectal cancer are expected to occur in 2007. While 1,3 million new cases of invasive breast cancer are expected in 2007 (Garcia *et al*., 2007). Breast cancer caused 519.000 death per year, while colon cancer causes 639.000 patient death per year (WHO, 2006). In Indonesia, breast cancer prevalence is the second range after cervix cancer and become the major caused on women mortality .

Combination therapy using surgery, chemotherapy and radotherapy resulted dissatisfied achievement. Many cancer chemotherapeutic drugs present relatively poor selectivity for neoplastic cells. The failure of cancer chemotherapy partly is resulted from the low selectivity of anticancer drugs and the unclear of molecular target on cancer. Drugs with low selectivity affect several side effect on patient. Research efforts have been carried on to overcome these problems by developing more effective and selective anticancer drugs through consideration of the molecular target and mechanisms. Plant bioactive compounds are normally cheaper and produce lower side effects expected to chemotherapy. Thus, the searching for active compounds extracted from plant is an important path of research in the discovery of novel anticancer candidates.

Gynura procumbens (*G. procumbens*) is a medicinal plant with several biological activity related with anticancer. *G. procumbens* extract and its fractions exhibit high antioxidant properties shown by xantine oxidase inhibition and radical scavenging activity. The uncontrolled production of radical oxygen species (ROS) and unbalance antioxidant protective system have an important part on cancer initiation and development (Rosidah *et al*., 2008). These natural antioxidants may become a beneficial agent to be developed as chemoprevention substance for cancer disease. *G. procumbens* inhibited carcinogenesis process of lung cancer (Sugiyanto *et al*., 2003) and breast cancer (Meiyanto *et al*., 2007a; Meiyanto *et al*., 2007b) and has antimutagenic properties on benzo(a)pyrene-induced mice lung cancer (Meiyanto, 1996). The phenolic compounds of *G. procumbens* suppressed cells proliferation and induced apoptosis at HeLa (Meiyanto dan Septisetyani, 2005), and T74D cells (Maryati, 2006), and also has antiangiogenic properties (Jenie *et al*., 2007). Phenolic compounds of *G. procumbens* decreased Cox-2 expression (*in vivo*) and elevated *p53* and *Bax* expression (*in vitro*) (Maryati *et al*., 2006).

To our knowledge, no specific study had been reported addressing the selective cytotoxic effects of FEG on colon cancer WiDr and breast cancer MCF-7 and T47D and normal NIH3T3 cell lines. Thus, in this present study, we have evaluated the *in vitro* cytoxicity of FEG on those cells. Results from both cancerous and normal cell were then compared to determine the selective activity.

Material and Methods

Chemicals and Reagents

Material were used in this study: DMEM (Nacalay, Japan), Fetal Bovine Serum (FBS) (PAA), Dimethyl sulfoxide (DMSO) (Sigma, Aldrich, Germany), penicillin and streptomycin (Gibco), tripsin (Sigma), 3-(4,5-dimethyltiazol-2-il)-2,5-diphenyltetrazolium

bromide (MTT), sodium dodecyl sulphate (SDS). Tris–HCl, ethanol, n-hexane, and ethyl acetate and fraction of ethyl acetate *G. procumbens* (FEG). The fractions was dissolved in DMSO with 0,1 % as maximum concentration. All of the chemicals were on the higher degree of quality.

Human cell lin

Human colon cancer cell WiDr and breast cancer cell MCF-7 and T47D was provided from Cancer Chemoprevention Research Centre (CCRC) collection. NIH3T3 cell was kindly provided from Prof. Masashi Kawaichi, Laboratorium of Gene Function in Animal, Nara Institute of Science and Technology. WiDr cells were cultured in *Rosewell Park Memorial Institute* (RPMI) containing 10% *heat-inactivated fetal bovine serum* (FBS) 1 % Penicillin-streptomycin (v/v), and L-glutamine (1 mM) at 37 ºC and 5% CO2. MCF-7 and T47D cells were routinely grown in Dulbecco's modified eagle's medium (DMEM) and added the same supplement as that to RPMI medium. NIH3T3 cells were routinely grown in DMEM supplemented with 10% heat-inactivated *fetal calf serum* (FCS) (PAA Labortories), 1 % Penicillin-streptomycin (v/v), and L-glutamine (1 mM) at 37 ºC and 5% CO2.

Plant material

The leaves of *G. Procumbens* were obtained from the collection of Balai Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional (BP2TO2T) Indonesia, and was determined at Laboratorium of Pharmacognocy, Faculty of Pharmacy Gadjah Mada University, Indonesia. Dry powder of *Gynura procumbent* was re-macerated with 96 % ethanol for three time with three days maceration for each process. All filtrate then evaporated using rotary evaporator until a thick liquid extract was obtained. The ethanolic extract was dissolved in hot aquadest then fractionated using n-hexane. The water fraction then continued to fractionate with ethyl acetate. The ethyl acetate fraction was evaporated using rotary evaporator. The ethyl acetate extract was concentrated by evaporation under reduced pressure and the temperature was kept not more than 40ºC. The extract was stored at 4ºC prior to use.

Determination of chromatographic profile of FEG

Chromatography profile of FEG was determined using *high performance thin layer chromatography* (HPTLC) dan *high performance liquid chromatography* (HPLC). Mobile phase that used for HPTLC was toluene:chloroform:aceton:acetic acid (4:4:4:1) and using silica gel 60 F_{254} (Merck) as stationary phase. *ultraviolet* (UV) detection at 254 nm in wave length was used for spot visualization. The all spots were scanned using TLC *Scanner* (CAMAG), deuterium and wolfram (D_2 & W) lamp, at 380 nm. Coloumn silica Symmetry C18 (lenght x internal diameter: 150 mm x 4.6 mm, particle size 15 mm) was used as stationery phase for HPLC. The mobile phase was acetonitrile : formic acid (25:75). The eluent was detected by *ultraviolet-visible* (UV-VIS) detector at 360 visualization of the spot. Quercetin (QUER) (Sigma-Aldrich, ≥98% HPLC, CAS No. 117-39-5)) and kaempferol (KAEMP) (Sigma-Aldrich, ≥90% HPLC, CAS No. 520-18-3) were used as marker.

Cytotoxicity assessments

Cells (10^4 cells/well) were cultured at 96-well plate. After 24 hours growth, cells culture medium was replaced with 5-FU, CISP, and/or FEG-contained medium. After incubated for 24 hours, the medium was replaced with MTT-containing medium (0,5 mg/mL) and incubated for 4 hours at 37°C, 5 % CO_2. The reaction was stopped with 10% SDS in 0,1 N HCl solution and was incubated for overnight in light protected chamber, to dissolve formasan salt. The absorbance of each well was measured with ELISA reader at 595 nm. The ratio between treated and control cells absorbance refer to percentage (%) of viable cells.

Selectivity index (SI).

In the present study, the degree of selectivity of FEG refered to the previous report (Badisa *et al.*, 2006), was calculated using formula: SI=LC50 in normal cells /LC50 in cancer cell lines, where LC50 is the concentration required to kill 50% of the cell population.

Results

Chromatography profile of ethyl acetate fraction of *Gynura procumbens*

Ethyl acetate fraction of *Gynura procumbens* was fractionated twice separately, produced two different batch of ethyl acetate fraction (FEG). This FEG was obtained from liquid-liquid fractionation of ethanolic extract using n-heksane-hot water and continued with ethyl acetate-water. From the two fractionations were obtained percentages of FEG 1,6 % and 1,7 %, respectively. FEG was analized its chromatographic profile using *High Performance Thin Layer Chromatography* (HPTLC) and *High Performance Liquid Chromatography* (HPLC) as shown on Figure 1 and 2 respectively. Quercetin (QUER) and kaempferol (KAEMP) were used as marker compound. Both FEG from different batch had similar chromatographic profile as result of HPTLC and HPLC. Most of chromatogram refered to flavonoid compound. From the scanning of maksimum wave lenght we concluded FEG may contained quercetin, but not kaempferol. However the amount of quercetin was not dominated the active compound of FEG.

Inhibitory effect of FEG on WiDr, MCF-7 and T47D cells growth.

To determine the potency of FEG as anticancer agent, first the cytotoxicity properties were examined in WiDr colon cancer cell, MCF-7 and T47D breast cancer cells. Cell viability was examined using MTT assay method after 24 hours incubation. FEG caused cell growth inhibition on WiDr, MCF-7 and T47D cells in dose dependent manner with IC_{50} value 119 µg/mL, 214 µg/mL and 76 µg/mL, respectively (Table 1). FEG had moderate to high potency as cytotoxic agent. The highest potency was shown on T47D cell. Quercetin also caused cell growth inhibition on three cells in dose dependent manner with IC_{50} value 56 µg/mL, 169 µg/mL, 127 µg/mL, respectively. These results demonstrated that quercetin was not the main active compound of FEG.

FEG shows lower cell growth inhibition in normal cells than that of cancer cells

Cytotoxicity results showed moderate to high potency of FEG to be developed as anticancer agent. In order to determine the selectivity of FEG, we performed cytotoxic properties of FEG on normal cells, NIH3T3 (Figure 3). FEG gave no significant effect up to 400 µg/mL on NIH3T3 cell growth after 24 hours incubation. While after 72 hours, FEG caused cell

growth inhibition at minimal concentration 600 µg/mL. IC_{50} value of FEG was measured after 24 and 72 hours of FEG treatment, resulted 592 µg/mL and 538 µg/mL, respectively. FEG shows lower cell growth inhibition in NIH3T3 than that in WiDr, MCF-7 and T47D cells. FEG shows lower inhibition on the growth of normal cells than that of cancer cells.

Discussion

Many researchers are now interested in examining the use of herbal medicines as a health care method. Herbal medicines continue to be accepted forms of treatment in the Orient, and the plant derived drugs based on traditional practices represent a huge proportion of the pharmaceutical production in modern Western countries (Newall *et al*., 1996 Schulz *et al*., 1998). Development of biologically targeted agents that exploit differences between cancerous and normal cells and permit greater specificity for cancer cells with less damage to normal cells is still the ultimate goal in the field of antineoplastic drug discovery (Adam, 2001). Until now, no ideal cytotoxicity assay has been developed; hence, it is always advisable to support results with more assays where possible. Besides, it is important that of compare the cytotoxicity of a novel compound against several cell lines and even with other commercial cytotoxic agents. In this study, we showed the profile of bioactive compounds of ethyl acetate fraction of *G. procumbens* (FEG). We also confirmed the cytotoxicity properties of FEG and its selectivity directed human colon cancer and breast cancer cells.

HPTLC analysis of FEG shown three main spots, similar with previous results from study of Sugiyanto *et al*., (2003). Previous study showed 3 (three) main spots that had been identified as flavon/flavonol. The first flavonoid was identified as flavonol derivate with 5 –OH and 3,7 $–OCH_3$. He second flavonoid was flavonol derivate with 5,7 –OH and 3-Oglucosil. The third flavonoid was similar with the second flavonoid. Flavonol derivative that has 7 -alkoksi, 3' and 4' di-OH, and 3,5 di-OH (Sugiyanto *et al*., 2003). The third flavonoid was quercetin derivative with alkoksi substitution on 7 –OH. Previous results also found quercetin, kaempferol and its derivate in *G. procumbens* (Akowuah *et al*., 2002). Methanolic extract may contain kaempferol-3-O-rutinosida dan astragalin (Rosidah *et al*., 2009), and its ethanolic extract has been identified as kaempferol-3-O-rutinosida, quercetin-3-O-rutinosida, dan isobioquercetin compounds (Kim *et al*., 2011). HPLC results of FEG showed chromatogram peak with retention time (tR) value of 41.7 similar to the tR value of quercetin 41.6. Therefore FEG may contain quercetin. However there are others compound also contained in the chromatogram peak.

FEG shows moderate to high cytotoxicity properties on colon cancer and breast cancer as indicated on the IC_{50} value at WiDr, MCF-7 and T47D cells. While quercetin presented higher potency (almost 3 fold) on cytotoxicity compared to FEG on WiDr and MCF-7 cells. Conversely quercetin demonstrated lower potency on cytotoxic properties on T47D cells. This phenomenon may cause by different cells characteristic or different target of action. The results of cytotoxicity assay showed that quercetin does not seem as the main active compound of FEG. There are other compounds contained in FEG that have pivotal portion on its cytotoxicity properties.

Since FEG had high potency of cytotoxicity, its impotant to reveal the selectivity of this fraction. FEG produced no significance effect on cell growth inhibition up to 400 µg/mL

concentration at NIH3T3 cells. Selectivity of FEG on WiDr and T47D cells higher than that on T47D. FEG exhibited very high selectivity on WiDr and MCF-7 cells as shown by selectivity index (SI), that is > 3. Even as on MCF-7, FEG demonstated moderate selectivity properties.

Conclusion

The present *in vitro* studies FEG possesses moderate to high cytotoxicity properties on WiDr, MCF-7 and T47D cells. FEG shows selective effect against cancer cells. Further FEG reveals prospective properties to be developed as cancer chemoprevention agent.

Author's Contribution

NAN have made conception and design of this study, acquisition of data, analysis and interpretation of statistical of data, and drafted the manuscript. EM and S interpreted data and reviewed the manuscript. All authors have already read and approved the final manuscript.

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FIGURE

Figure 1

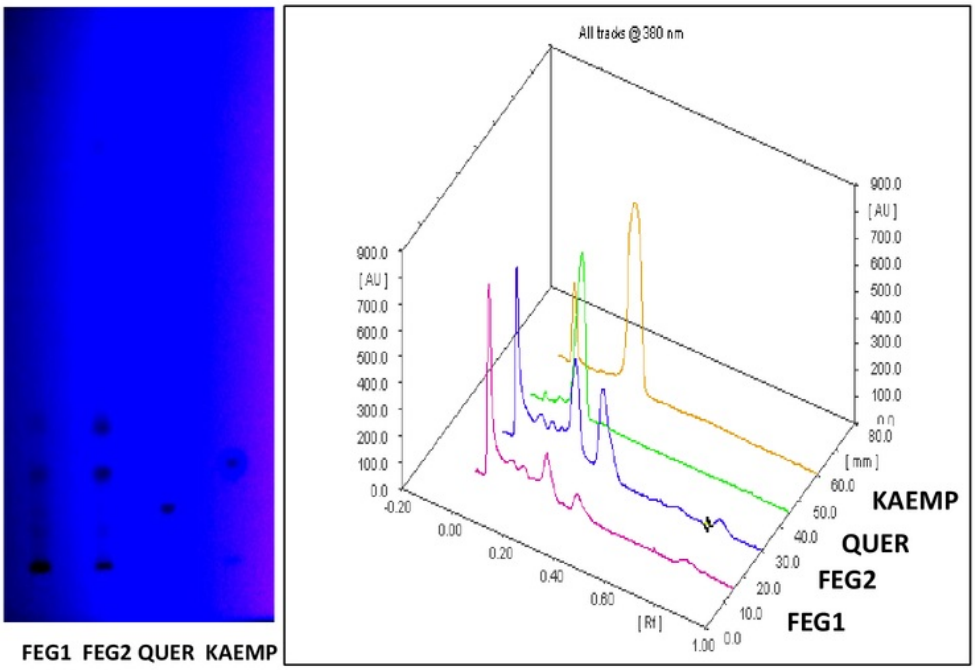

Figure 1. Chromatogram profile of FEG was compared with Quercetin (QUER) and Kaempferol (KAEMP) as marke[12]using HPTLC. FEG, QUER and KAEMP wre analized using HPTLC with silica gel 60 F254 as stationary phase and toluene:kloroform:aseton:asam asetat (4:4:4:1) as mobile phase (Lalla *et al.*, 2003). Silica plate was visualized under UV light 254 nm then scanned using densitometer, 380 nm.

Figure 2

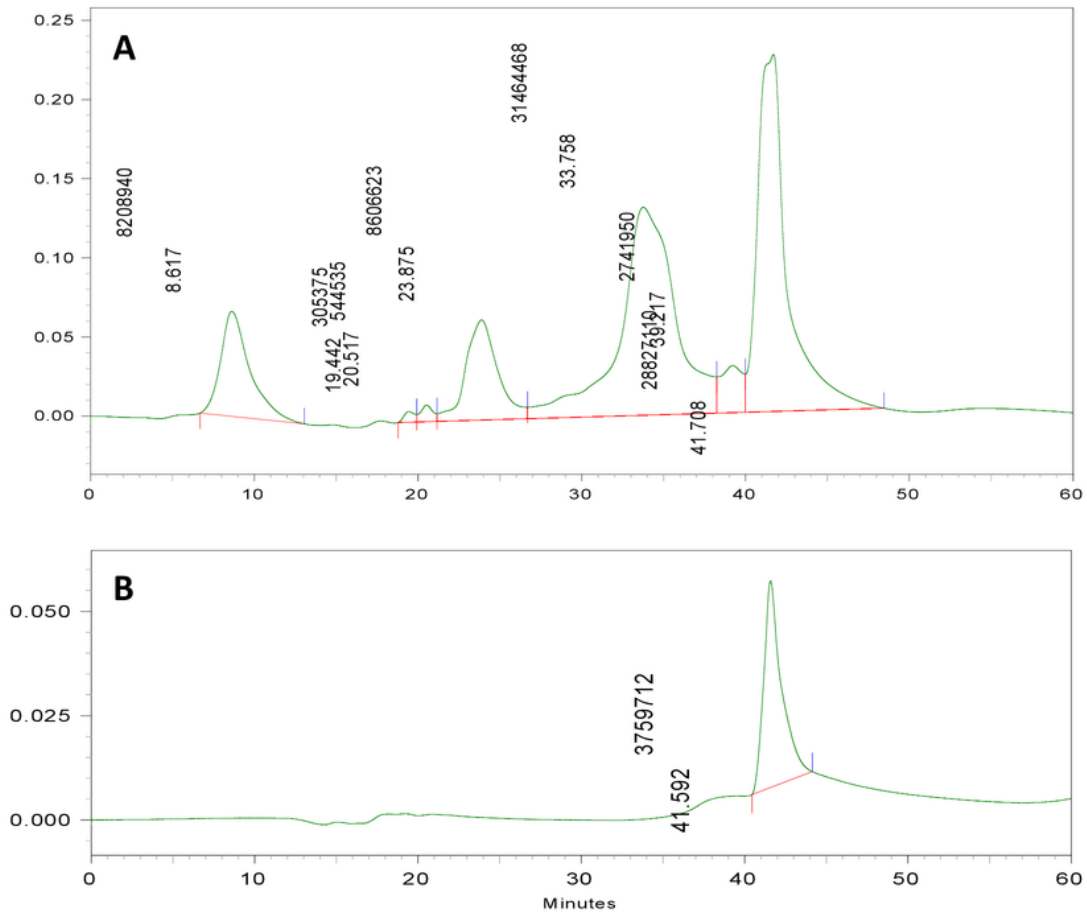

Figure 2. Chromatogram profile of FEG-contained compounds using HPLC. Chromatogram profile of FEG1 (A), FEG2 (B), and QUER (C). FEG and QUER were diluted in ethyl acetate with the indicated concentrations as described at method. FEG and quercetin solution was analyzed using HPC with Asetonitril : asam formiat (25:75) as mobile phase and silica coloumn symmetry C18, detector UV-VIS.

Figure 3

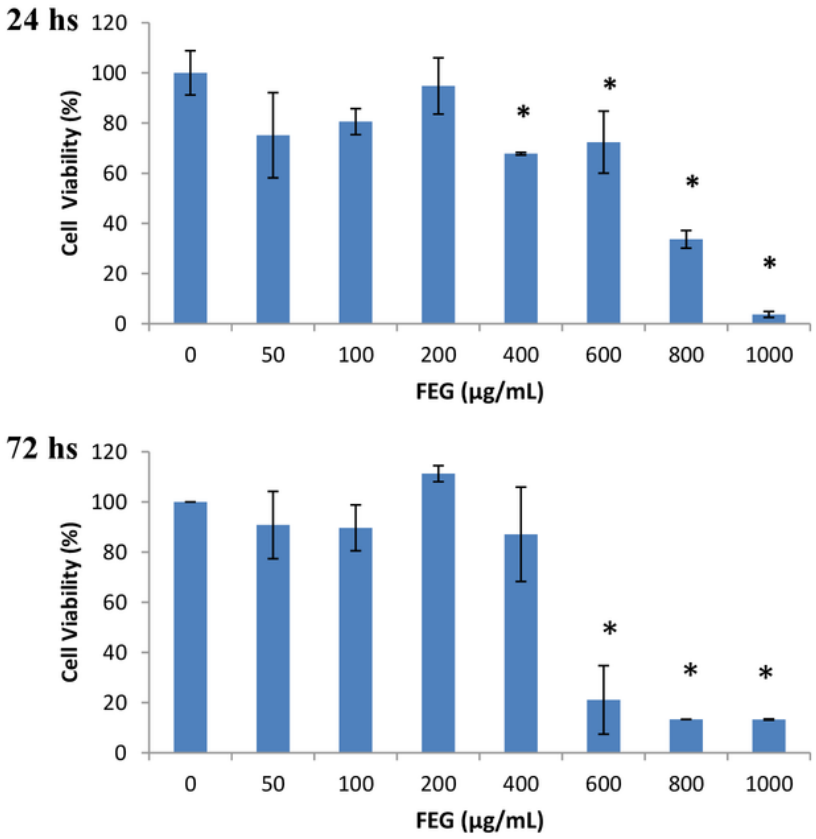

Figure 3. The effect of FEG on NIH3T3 cells growth. FEG treatment up to 400 µg/mL caused no significant cell growth inhibition. Cells (10^4 cells/well) were seeded at 96-well plate. After 24 hours growed, cells were treated with FEG (0-1000 µg/mL) for 24 and 72 hours. Cells viability were determined using MTT assay. T Test analysis, with 95% confidency was used to conclude the significance of cells growth inhibition. *FEG inhibit cell growt significantly (p>0,05).

Table 1. IC_{50} Value of FEG and QUER on WiDr, MCF-7 and T47D cells and The selectivity index (SI) which represents IC_{50} of FEG for normal cell line/IC_{50} for cancerous cell line after 24 hours incubation treatment

Cells	Sample	IC_{50} Value*	Selectivity Index (SI)
WiDr	FEG	119 µg/mL	4.97
	QUER	56 µg/mL	
MCF-7	FEG	214 µg/mL	2.77
	QUER	169 µg/mL	
T47D	FEG	76 µg/mL	7.79
	QUER	127 µg/mL	

***represented from al least 2 (two) independent experiments**

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