Original Article

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Antiproliferative effect on breast cancer cells of seed proteins from *Momordica charantia* L.

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Abstract

Thai bitter gourd or Marakhenok (*Momordica charantia* L.), a common vegetable in Thailand, has also been used as traditional medicine. The ribosome-inactivating proteins (RIPs), a group of plant enzymes inhibiting polypeptide chain elongation in the RNA, are found in this plant. These proteins possess several bioactivities such as antiviral, antidiabetic, antibacterial and cytotoxic properties. The RIPs in *Momordica charantia* seed were extracted with normal saline and purified by precipitating with varying concentrations of ammonium sulfate solution; they were 0-30%, 30-60% and 60-90% respectively. The protein fractions of 0-30%, 30-60% and 60-90% ammonium sulfate saturation were obtained and tested for antiproliferative activity on breast cancer cell lines (MCF7 and T47D, both ER+), comparing with MRK29, the major protein isolated from 30-60% protein fraction. The antiproliferative effect of the crude proteins, 0-30%, 30-60%, 60-90% protein fractions and MRK29 had the following ED_{50} values on MCF7 of 221.4±4.89, 175.08±4.75, 255.19±7.12, 237.08±16.92, and 228.49±16.02 μg/ml, respectively and on T47D of 232.56±1.95, 118.78±9.26, 215.96±8.59, 175.31±7.49 and 154.46±30.29 μg/ml, respectively. The proteins affected the fibroblast cell, a normal cell control, with ED_{50} values of 545.74±48.62, 88.10±2.71, 453.74±50.60, and 587.25±29.70 and 358±114.18 μg/ml, respectively. The colony assay on T47D was performed to prove that the antiproliferative effect of *Momordica* seed proteins caused the cell death.

Keyword: *Momordica charantia* L., Marakhenok, ribosome-inactivating proteins (RIPs), antiproliferative effect on breast cancer cell lines, Momordica seed proteins

1. INTRODUCTION

There are usually two types of *Momordic*a charantia fruits in Thailand, the long bright green and the small dark green fruits. The small fruit is of our interest. *Momordica charantia* is a cucurbitaceous plant, distributed in all tropical parts of the world and cultivated throughout South America, Asia and Africa. The plant is a slender climbing vine with long stalk, yellow solitary male and female flowers borne in the leaf axils. The fruit looks like a warty gourd. The young fruit is emerald green, turning to orange-yellow when ripes. It has been traditionally used in gout, rheumatism, constipation and subacute cases of the spleen and liver diseases. The fruit juice or a leaf tea is employed for diabetes, malaria, colic, sore and wound, infection, worm and parasite, measle, hepatitis, fever and also as an emmenagogue. Leaves act as galactogogue and root is astringent[1,2]. The *Momordica charantia* seed contained ribosome-inactivating proteins (RIPs), carbohydrates, triterpenes and lipids[3]. Our study plans to prepare the seed proteins. The *Momordica charantia* seed proteins could be extracted with normal saline and purified by precipitating with varying concentrations of ammonium sulfate solution. Several RIPs inhibit polypeptide chain elongation

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by depurinating a specific nucleotide in the RNA of large ribosomal subunit[4, 5]. There are two types of RIPs; type I and type II. RIPs I or single chain ribosome inactivating proteins (SCRIPs) are single-chain polypeptides consisting of N-glycosidase domain. The structure of RIPs II is constructed from two or more than two polypeptide chains, which are composed of A (active) and B (binding) chains. The lectin binding domain is toxic to cell and makes RIPs II more toxic than RIPs I. The RIPs in *Momordica charantia* seeds are type I[6-10]. Those with molecular weights between 11-30 kDa possess potential medicinal properties such as momorcharin, momordin I, α-momorcharin, momordin II, ß-momorcharin, MAP30, MRK29, ɣ-momorcharin, δ-momorcharin, ε-momorcharin and etc[11,18]. The RIPs I can inhibit the protein synthesis and accompany with p53 protein giving the signaling pathway to bax and bak enzymes into the cell which leads via mitochondria to apoptosome and induces apoptosis of cancer cells[12]. Moreover number of preliminary studies both *in vitro* and *in vivo* with crude extract of *Momordica charantia* have shown anticancer activity against lymphoid leukaemia, lymphoma, choriocarcinoma, melanoma, breast cancer, skin tumors and prostatic cancer[13-17]. There are many *in vivo* and *in vitro* studies of *Momordica charantia* as antidiabetes, anti-HIV, antiflatulence activities and a relief of stomachache, sluggish digestion and intestinal parasites illness[12, 15, 18]. Our previous study on *Momordica charantia* seed proteins from Thai bitter gourd (Marakhenok) showed that the isolated seed protein with the molecular weight of 28.6 kDa, MRK29, *in vitro* inhibited HIV-I[19]. The aim of this study was to prepare *Momordica* seed proteins and investigate their antiproliferative effect on breast cancer cells.

2. MATERIALS AND METHODS

Chemicals

Chemicals for protein extraction and purification; sodium chloride, 1N hydrochloric acid, ammonium sulfate, 50mM sodium phosphate buffer pH 6.3, 20 mM sodium phosphate buffer pH 6.3, 1N sodium hydroxide. Chemicalsfor SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) for determination of protein and the molecular weight. Detecting reagent; Coomassie Brilliant Blue R-250 and Bio-Rad kit for total proteins.

Plant Materials

The plant was identified by **Forest Herbarium-BKF** and the voucher number was BKF No. 1372266. *Momordica charantia* (MC) fresh seeds were collected from the farm in the Supanburi province, Thailand. They were washed, and the seeds were taken out from the seed coats were removed. The kernel was kept in the freezer.

Plant protein extraction

The seed coats were removed from 100 g fresh seeds and resulting 50 g kernel, which was homogenized with sodium chloride solution (8.7 g/1 L) in blender at 4ºC for 2-3 min until a white emulsion occurred. The pH was adjusted to 3.6 with 2N HCl and stirred on the magnetic stirrer at 4ºC for 15 min. The emulsion was filtered through the sheath cloth to remove the cell debris and transfered to refrigerated centrifuge at 24,000 rpm for 15 min. The supernatant was filtered through 0.45 µm millipore for removal of lipids. The filtrate was dialyzed, lyophilized and the powdered crude extract was obtained[19].

The preparation of protein fractions

The proteins in filtrate were precipitated with varying concentrations of ammonium sulfate solution. They were 0-30% (16.6 g/100 ml), 30-60% (18.4 g/100 ml), 60-90% (20.4 g/100 ml) and 90-100% (7.6 g/100 ml) of ammonium sulfate saturation. The precipitated protein were separated and dissolved in 20 mM phosphate buffer pH 6.3. They were put in the dialysis bag [(Cellu·Sep®), Cellu-Sep T2/Nominal MWCO: 6,000-8,000] and transferred to the beaker containing 20 mM phosphate buffer pH 6.3. The buffer was changed every 3 h for 3 times. The protein fractions were lyophilized to obtain the powdered protein fractions. The isolation of MRK29 was isolated from 30-60% protein fraction using the method from the previous study[19].

The SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis) with fluorescence gel scanner

To determine the molecular weights of the seed proteins SDS-PAGE was performed. Twelve percent acrylamide solution was poured in between the glass plates. After the gel had set (about 20-30 min) the overlay was removed and the top of the separating gel was washed several times with distilled water. The stacking gel was prepared and poured directly onto the acrylamide separating gel. The appropriate comb was placed into the gel, which was put in a vertical position at room temperature, for approximately 30 min. 700 ml Electrode buffer was added to the tank. Protein sample and the buffer gel (in the ratio of 1:2), 100 µl, was boiled for 5 min and mixed on vortex. 5 µl Sample was applied onto the gel. The development took about 60-90 min. Then the current was switched off and the glass plate was removed from the tank. The gel was immersed in Comassie blue solution, left overnight and then destained with the buffer until the blue band was clearly seen. The molecular weight of the seed proteins was determined by comparing with the molecular weight marker (Amersham Low Molecular Weight Calibration Kit for SDS Electrophoresis).

The determination of total proteins (Bradford assay)

The concentration of total proteins in sample was determined by Bradford assay using Coomassie Brilliant Blue R-250 plus protein assay reagent (Bio-Rad) in microtiter plate. The method is based on the change of the maximum absorbance from 465 nm to 595 nm when the dye binds with proteins in acidic solution of Brilliant Blue R-250. The microprotein assay procedure is determined to define proteins in solution with microgram level. The bovine serum albumin (BSA) stock solution was diluted with double distilled water to 5 concentrations; they were 2, 4, 6, 8 and 10 µg/ml as standard solutions. Prepared dilutions, 80 µl, were transferred to the well; one well was used as a blank. Then 20 µl of dye reagent was added to each well. They were mixed on vortex for 30 sec and incubated for 8-10 min. The absorbance was measured at 595 nm by microplate reader. The standard curve was established. To determined total proteins in the samples, 100 µl sample solution (5 mg/ml) was pipetted into the tubes. Bradford reagent solution was added to the tubes to make 5 ml solution. They were mixed on vortex and incubated at room temperature for at least 5 min. The protein absorbance was measured at the wavelength of 595 nm.

Cell viability assay

The yellow tetrazolium salt or MTT, 3-(4, 5-dimethylthiazolyl-2)-2, 5 diphenyltetrazolium bromide (Sigma, USA), is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. In brief, cells were seeded into each well of 96 well plate at the density of $1x10^4$ cells per well for 24 hours. The various conentrations of the extracts ranging from 1-200 µg/ml were added into each well. After 48 hours incubation at 37ºC, the 50 µl MTT solution at 1 mg/ml in phosphate buffer saline were added and cells were incubated for 4 hours in the incubator before discarding the supernatant. Then, 100 µl of 100% dimethyl sulfoxide (DMSO, sigma, USA) were added and cells were incubated for 10 min on the rotator plate at room temperature. Doxorubicn was used as the positive control. The measurement was done and analysed using EL808 Ultra Microplate Reader (Biotek Laboratories, USA). The assay was performed in triplicate with three independent experiments. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometry, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. The number of assay steps has been minimized as much as possible to expedite sample processing. The MTT reagent yields low background absorbance values in the absence of cells. For each cell type the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation.

In experiment MCF-7 and T47D breast cancer cells were obtained from Tanawan Kummalue, Department of Clinical Pathology, Faculty of Medicine Siriraj Hospital, Mahidol university[20].

Colony assay

T47D human breast cancer cells were seeded at $2x10^4$ cells/well in 60 mm tissue culture dish. After 24 hours incubation, the crude extract and the 60-90% fraction were added at the ED_{50} concentration. Cells were cultured with the extracts for 48 h before changing into the regular medium. After culturing for 7 and 10 days, the colony count was done and photographs were taken. Cells deprived of the extract were used as negative control and doxorubicin was used as positive control[20].

Statistical analysis

The data were shown as mean ± SD. The ED_{50} value which means the 50% inhibition of cell growth was calculated by using the R2 equation. A p value < 0.05 was considered significant.

3. RESULTS AND DISCUSSION

1. Extraction and purification

We repeated the methods of extraction and purification from previous study[19]. Fresh seeds, 600 g, were decorticated resulting 300 g kernel. They were extracted with sodium chloride solution or normal saline solution and purified by ammonium sulfate precipitation. The protein fractions were obtained and total proteins were determined as shown in Table1.

The total proteins in each fraction were determined by Bradford assay. The crude extract had the highest total proteins and 0-30% protein fraction the lowest. 90-100% Protein fraction was discarded because it did not contain proteins.

Table 1. Total proteins in crude extract and different protein fractions

Samples* (g)	Total proteins
Crude extract (5.6896 g)	71.12 mg
0-30% (0.4223 g)	4.02 mg
30-60% (4.4722 g)	63.06 mg
60-90% (0.9951 g)	10.75 mg

*Values in percentage were concentrations of ammonium sulfate solution

2. The determination of molecular weight of proteins by sodium dodecyl sulfate-polyacry lamide gel electrophoresis (SDS-PAGE) with fluorescence gel scanner

The molecular weights of proteins in crude extract and in protein fractions were determined on SDS-PAGE with fluorescence gel scanner comparing with marker (Amersham Low Molecular Weight Calibration Kit for SDS Electrophoresis) as shown in Tables 2 and 3 and Figures 1a and 1b.

RIPs with molecular weights of 14-30 kDa in MC seed and their pharmacological activities were shown in Table 3.

Ammonium sulfate solution caused the protein precipitation (salting out). The high molecular weight proteins were found in 0-30% fraction. The increasing ammonium sulfate concentration precipitated the lower molecular weight proteins.

3. Antiproliferative effect on breast cancer cells

The breast cancer cell was selected for the study because of the highest incidence of breast cancer in Thai women[2]. The ER+ cancer cell lines, MCF7 and T47D, were used to test the antiproliferative effect of MC seed proteins.

Estrogen receptor (ER) positive has receptors for estrogen. This suggests that the cancer cells, like normal breast cells, may receive signals from estrogen that can promote their growth[21]. MTT assay was applied and the result was shown in Table 4.

Table 2. Molecular weights of MC proteins

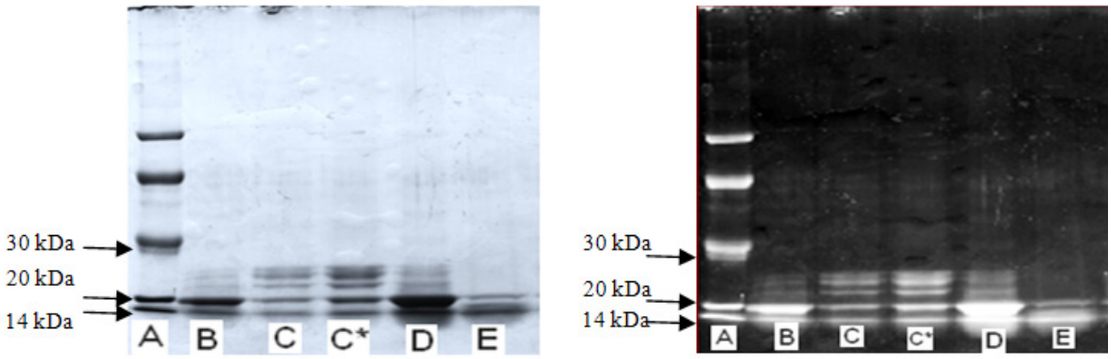
Samples	Molecular weights (kDa) of MC major proteins
Crude extract	14-29 kDa
0-30% fraction	20-28 kDa
30-60% fraction	19-29 kDa
60-90% fraction	20-21 kDa

Table 3. Some bioactive MC seed proteins

Proteins	Activity	Mr* (kDa)	pI**	Reference
MAP30	Anti-HIV, anti-HSV, anti-tumor	30	>9	22,4
MRK29	Anti-HIV (reverse transcriptase)	29	>9	19
α-momorcharin	Abortifacient, antitumor, immunosuppressive, Anti-HIV, N-glycosidase, Ribonuclease, Deoxyribonuclease	30	>9	4
ß-momorcharin	Abortifacient, antitumor, immunosuppressive	29	>9	23,24,25,26,29
Momordin I	Protein synthesis inhibitor, cytostatic, antiviral	29	>9	26,27
Momordin II	Protein synthesis inhibitor, immunotoxin	29	>9	28
Ribonuclease	N-glycosidase	22	-	4
Hispin	tRNA ribonuclease, N-glycosidase activity, antifungal	21	-	30
Momorcharin I	Ribosome-inactivating activity, protein	26	-	31
Momorcharin II	synthesis inhibitor in cell free system	28	-	
Momordica charantia inhibitor	Anti-HSV I, ribosome inactivating activity, protein synthesis inhibitor in cell free system	23		32,33,34,35

* Mr relative mars or molecular weight

** The isoelectric point or isoionic point (pI) is the pH at which the amino acid does not migrate in an electric field. It is the pH, at which the amino acid is neutral. The pI is given by the average of the pKa that involves the zwitterion. The pI can be calculated from pI = 1/2 (pKa1 + pKa2).



1a 1b

Figure 1. SDS-PAGE with fluorescence gel scanner

(1a) before the background adjustment
A molecular weight marker (14-74 kDa)
B The crude protein (17-29 kDa)
C 0-30% protein fraction (20-27 kDa)
(1b) after the background adjustment.
C* C left overnight
D 30-60% protein fraction (19-27 kDa)
E 60-90% protein fraction (20 kDa)

Table 4. Antiproliferative effect on breast cancer cells of *Momordica* seed proteins.

Samples	Fibroblast ED_{50}(µg/ml)	MCF7 ED_{50}(µg/ml)	T47D ED_{50}(µg/ml)
MRK-29	358.89±114.18	228.49±16.02	154.46±30.29
Crude extract	545.74±48.62	221.4±4.89	232.56±1.95
0-30%	88.10±2.71	175.08±4.75	118.78±9.26
30-60%	453.74±50.60	255.19±7.12	215.96±8.59
60-90%	587.25±29.70	237.08±16.92	175.31±7.49

The crude extract inhibited moderately both breast cancer cells. 0-30% Protein fraction inhibited the breast cancer cells strongly but also the normal cell. Its action was not specific and might produce the toxicity. 30-60% Protein fraction produced moderately cytotoxic effect and was safe. 60-90% Protein fraction was very interested because it inhibited the breast cancer cells better and safer.

From our previous study, MRK29 was the major protein in 30-60% protein fraction. It inhibited HIV-I, Our study discovered that it also inhibited the breast cancer cells.

4. The colony assay

The colony assay[25] on T47D was performed to prove that the antiproliferative effect of Momordica seed proteins caused the cell death (Table 5.). The colony pictures were shown in Figures 2 and 3.

The result of colony assay showed that 60-90% protein fraction inhibited T47D. The activity on Day 7 and Day 10 of this fraction was better than the crude extract when compared with the positive control (1 µg/ml doxorubicin) and negative control (Figures 2 and 3.). In addition, the amount of 60-90% protein fraction was used less than the amount of the crude extract in the inhibited T47D cell. Colonies could be seen as dots at the bottom of the well after staining with crystal violet in this experiment the count was performed by using the software.

60-90% Protein fraction was very interesting and could be developed further to the herbal product. We reported here for the first time the finding of Momordica seed protein with 20-21 kDa. Previously there was a report of RIPs I (hispin) with the molecular weight of 21 kDa in *Benin hispada*, a cucurbitaceous plant. Hispin had the properties of tRNA ribonuclease, N-glycosidase and antifungal activities[33].

Table 5. Colony assay of treated T47D on Day 7 and Day 10

Condition	Colony number				Average	
	Day7	Day10	Day7	Day10	Day7	Day10
Negative	1,378	CC	1,327	CC	1,352.5	CC
Positive (1 µg/ml Doxorubicin)	0	0	0	0	0	0
Crude extract (250 µg/ml)	2	44	0	31	1	37.5
60-90% (180 µg/ml)	0	23	0	29	0	26

*CC = cannot be counted

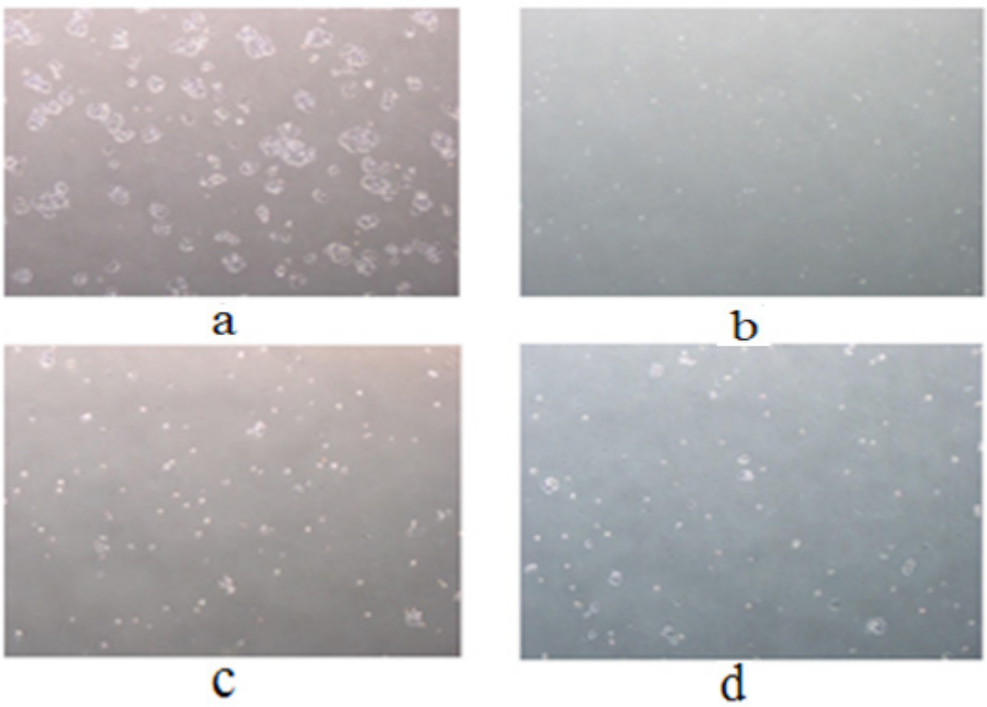


Figure 2. Illustration of colonies detected in the well on Day 7.
a T47D without MC seed proteins as a negative control.
b T47D with doxorubicin as a positive control.
c T47D with the crude extract at 250 µg/ml.
d T47D with 60-90% protein fraction at 180µg/ml.

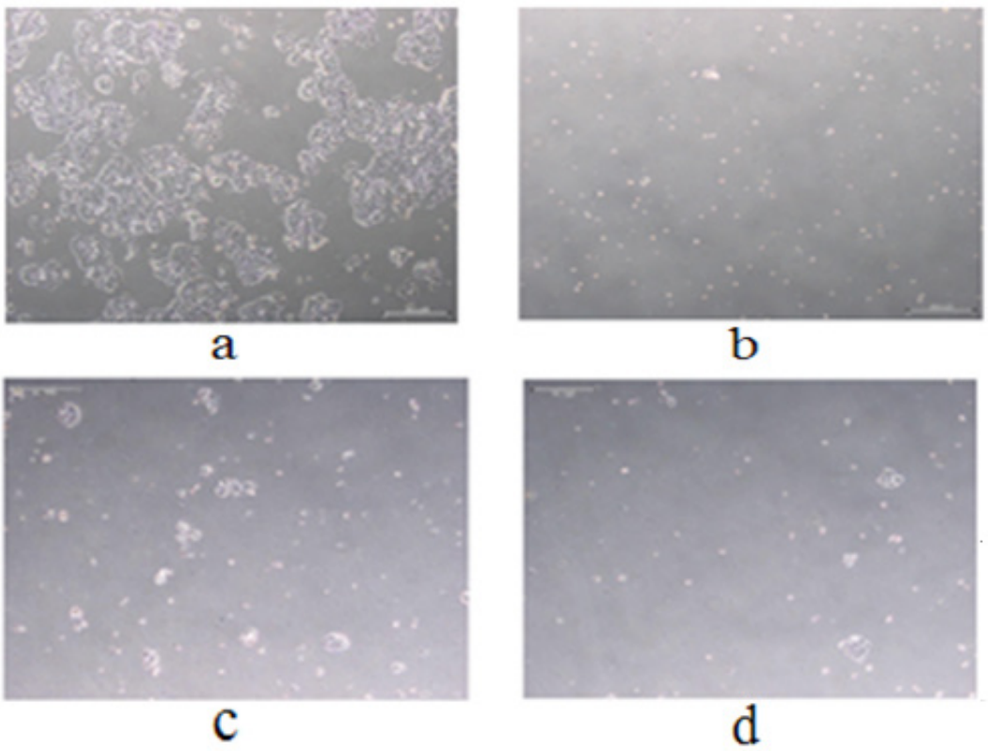


Figure 3. Illustration of colonies detected in the well on Day 10.
a T47D without MC seed proteins as a negative control.
b doxorubicin as a positive control.
c T47D with the crude extract at 250 µg/ml.
d T47D with 60-90% protein fraction at 180 µg/ml.

4. CONCLUSION

The MC seed proteins were extracted with normal saline and purified by precipitating with varying concentrations of ammonium sulfate solutions; they were 0-30%, 30-60% and 60-90%. The protein fractions were tested for antiproliferative activity on breast cancer cells (MCF7 and T47D, both ER+). The crude extract inhibited moderately MCF7 and T47D cell lines. 0-30% Protein fraction inhibited both breast cancer cell lines but it was also toxic to the fibroblast (normal cells). The action was not specific. 30-60% Protein fraction inhibited both breast cancer cells less than 0-30% protein fraction but was safer. 60-90% Protein fraction was the most interesting because it inhibited T47D cell better and was safer. MRK29, the major protein in 30-60 % protein fraction, inhibited both breast cancer cells better than 30-60% and 60-90% protein fractions and was rather safe.

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