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The MTT viability assay yields strikingly false-positive viabilities although the cells are killed by some plant extracts

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Abstract: The MTT assay is one of the often used cell viability/cytotoxicity assays. However, when the methanol extracts of plants are used to test their cytotoxic potential, interference may occur, resulting in false-positive viability results. Therefore, in this study, the reliability of the MTT assay was investigated in the case of plant use. The methanol extracts of three different plants (*Hypericum adenotrichum*, *Salvia kronenburgii*, and *Pelargonium quercetorum*) were tested in breast cancer cell lines (MCF-7 and MDA-MB-231) using the MTT assay and the results were compared to the ATP assay, which is a much more sensitive and reliable assay due to its interference-free feature. Additionally, decreased cell density was confirmed with phase-contrast microscopy and fluorescence staining (Hoechst 33342 dye). Although both of the viability/cytotoxicity assays are considered as metabolic assays, viabilities (in %) in the MTT assay were found to be strikingly higher when compared to the results with the ATP assay. Even in the case of total death, the MTT assay still produced artificial/false increases in viability. The morphology-based evaluation of viability/cytotoxicity by phase-contrast microscopy and Hoechst 33342 staining were greatly compatible with the ATP assay results. Overestimated (false) viabilities in the MTT assay suggests a serious interference between the MTT assay itself and the extracts used. Some ingredients of plants may have reducing activity (like the dehydrogenase activity of the cells) that converts the MTT compound into the colored formazan that is the principle of the assay. Therefore, the MTT assay may not be a suitable assay for some plant extracts, urging great caution when plants are used.

Key words: MTT assay, ATP assay, interference, cytotoxicity, plant extract, breast cancer

1. Introduction

In the past years, several methods have been developed to determine cell viability in cell culture (Cook and Mitchell, 1989). Among these methods tetrazolium salt-based assays are widely used in order to measure cytotoxicity or cell proliferation (Mosmann, 1983; Berridge et al., 1996).

The principle of the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay is the reduction of water-soluble yellow tetrazolium salt by the dehydrogenase system of metabolically active/live cells into water-insoluble blue/magenta (MTT) formazan crystals (Morgan et al., 1998). In this way, the concentration of dissolved formazan crystals can be quantified using a spectrophotometer and it is in direct correlation to the number of metabolically active cells (Gabrielson et al., 2002; Tunney et al., 2004; Wang et al., 2010). The MTT assay represents a simple and rapid colorimetric assay and yields quantitative data (Alley et al., 1988). This assay is carried out entirely in 96-well microtiter plates; thus, large numbers of experiments examining a number of variables can be readily performed (Cole, 1986).

On the other hand, the MTT assay has some disadvantages that are dependent on the cell ability to overcome cell death. One remarkable disadvantage is that damaged mitochondria may be still able to reduce MTT to formazan crystals (Mosmann, 1983; Page et al., 1988; Sieuwerts et al., 1995). Loveland et al. (1992) showed that cells with inactivated mitochondria were also able to produce formazan crystals as well as cells with active mitochondria. Furthermore, many nonmitochondrial dehydrogenases and flavin oxidases are able to reduce MTT (Altman, 1976; Burdon et al., 1993). Besides, different conditions and some chemicals/phytochemicals can also lead to changes in metabolic activity (Plumb et al., 1989; Hsu et al., 2003). The MTT compound may interact with some chemicals/phytochemicals, resulting in false results in viability (Hsu et al., 2003; Ulukaya et al., 2004; Devika and Stanely Mainzen Prince, 2008).

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More importantly, investigated agents may interfere with mitochondrial dehydrogenase activity, resulting in activation or inhibition of mitochondrial dehydrogenases and thus over/underestimation of the MTT assay results (Jaszczyszyn and Gąsiorowski, 2008).

The ATP assay is also used to determine cell viability. It is the fastest and the most sensitive cell viability assay, and it is less prone to artifacts compared to other viability assay methods (Riss et al., 2013). Therefore, it may be considered as a kind of reference assay for cell viability measurement. The ATP assay was first developed by Lundin et al. as a somatic cell viability assay (Lundin et al., 1986). The principle of this method is conversion of luciferin to oxyluciferin by the luciferase enzyme in the presence of Mg^{2+} ions and ATP. The ATP assay yields a luminescent signal and a linear relationship exists between the intensity of the luminescent signal and the ATP concentration, and therefore the number of cells (Mueller et al., 2004).

Plant extracts contain diverse chemicals and some of them could reduce the activity of the MTT compound, giving false results. If this occurs, then the MTT assay has to be expected to result in false-positive results for viability, regardless of any dehydrogenase activity in the cell. Therefore, in this study, we aimed to investigate how reliable the MTT assay is in the testing of the cytotoxic activity of plant extracts (*Hypericum adenotrichum*, *Salvia kronenburgii*, and *Pelargonium quercetorum*) at 7 different concentrations (1.56–100 µg/mL) on the MCF-7 and MDA-MB-231 breast cancer cell lines in comparison with the ATP assay as a reference assay. Results of our study indicate that the MTT assay gave false-positive results on cell viability. Due to this finding, the MTT assay has limited application for plant extract testing on different cell lines.

2. Materials and methods

2.1. Collection and identification of plants

P. quercetorum was collected from Hakkari, Turkey, in June 2006 and identified with the aid of flora books (Davis et al., 1988). *S. kronenburgii* was collected from Van, Turkey, and *H. adenotrichum* was collected from the Kırkpınar region at Uludağ Mountain (Bursa, Turkey) in June 2010. These specimens were identified by Prof Dr Gürcan Güleryüz, Faculty of Science, Uludağ University, Bursa, Turkey.

2.2. Extraction of *H. adenotrichum*, *S. kronenburgii*, and *P. quercetorum* samples

Fifteen grams of the aboveground parts of the plant samples was extracted by adding 150 mL of solvent methanol (Merck) in a Soxhlet extractor for 24 h. The crude extracts were concentrated using a rotary evaporator at 40 °C. The residues were then lyophilized for 42 h and stored at –20 °C prior to the testing.

2.3. Cell culture and chemicals

Breast cancer cell lines MCF-7 and MDA-MB-231 were cultured in RPMI-1640 supplemented with penicillin G (100 U/mL), streptomycin (100 µg/mL), L-glutamine, and 10% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO_2.

Lyophilized plant extracts were dissolved in DMSO at a concentration of 0.05 g/0.5 mL as a stock solution. Further dilutions were made in culture medium. The ATP assay (adenosine 5′-triphosphate (ATP) bioluminescent somatic cell assay kit), the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) substance, and all the other reagents and substances were obtained commercially (Sigma Aldrich, USA).

2.4. Experimental design

MCF-7 and MDA-MB-231 cells were seeded at a density of 1×10^4 cells per well of 96-well plates in 200 µL of culture medium. The untreated cells received only medium and solvent (0.1% DMSO as final concentration). All of the cells were treated with three different plant extracts (*H. adenotrichum*, *S. kronenburgii*, and *P. quercetorum*) in the range of 1.56–100 µg/mL for a period of 48 h.

2.5. The MTT viability assay

The MTT viability assay was performed with slight modifications as previously described (Mosmann, 1983). MTT was first prepared as a stock solution of 5 mg/mL in phosphate-buffered saline (PBS, pH 7.2) and filtered. At the end of the treatment period (48 h), 20 µL of MTT solution was added to each well. After incubation for 4 h at 37 °C, 100 µL: of solubilizing buffer (10% sodium dodecyl sulfate dissolved in 0.01 N HCl) was added to each well. After overnight incubation, the 96-well plate was read by an enzyme-linked immunosorbent assay (ELISA) reader at 570 nm for absorbance density values to determine the cell viability. The viable cells produced a dark blue formazan product, whereas no such staining was formed in the dead cells. The percentage of the viable cells was calculated using the following formula: (%) = [100 × (sample abs)/(control abs)].

2.6. The ATP viability assay

The cells were seeded, grown, and treated as for the MTT assay (see above). The ATP assay determines the level of cellular ATP as an indirect measure of the number of viable cells (Andreotti et al., 1995). The experiment was performed for luminometric measurement of cell growth (viability) according to the standard protocol of the manufacturer (ATP Bioluminescence Assay, Sigma Aldrich, USA), using a count integration time of 1 s and a luminometer (FL×800 Microplate Fluorescent Reader, Bio-Tek, USA). The results are given in relative light units (RLU).

2.7. Fluorescence imaging

Using a fluorescent microscope, cell density was detected on the basis of the nuclear morphology. Hoechst 33342 dye stains all of the alive or dead (primary necrotic or secondary necrotic) cells.

MCF-7 and MDA-MB-231 cells were seeded in a 96-well plate at the density of 1×10^4 cells per well in 200 µL of culture media. After 24 h, cells were treated with plant extracts (100 µg/mL) and then incubated for 48 h. At the end of the treatment, 2 µL of Hoechst 33342 dye was added to each well (for 5 µg/mL final concentration). Then cells were incubated with the dye and analyzed via fluorescence microscopy.

3. Results and discussion

Several experimental data demonstrated that the MTT assay yields false-positive results for viability (Ulukaya et al., 2004, 2008; Peng et al., 2005), and these false results led to overestimation of viability and thus underestimation of the cytotoxic potency of tested compounds. Therefore, in our study we have tested the reliability of the MTT assay compared to the ATP viability assay using plant extracts and two different cell lines.

Figure 1 represents the viability curves of three different plant extracts. It was found that viability levels of the MTT assay were strikingly higher than those of the ATP assay. Depending on the doses, the viability levels were even higher than in the untreated control cells (100%). Surprisingly, although the cells were totally dead at especially higher concentrations, the MTT assay still produced extremely high viability values. These viability values were false and this finding was confirmed using both phase microscope evaluation and fluorescence imaging that confirmed cell death (Figures 2 and 3). In Figure 2, it is clearly shown that cell densities decreased when both cell lines were treated with 100 µg/mL plant extracts. However, at the same concentration, the MTT assay did not present any cytotoxic activity. In contrast, it even produced an abnormally high viability value. The results of microscopic imaging were more compatible with the ATP assay results. In fact, the ATP assay reliably showed these cell deaths (cytotoxic effects). According to these data, it should be thought that these plant extracts could interfere (reduce) with the MTT compound, leading to false-positive results for viability.

Decreased cell viability (cytotoxic effect) was also shown via fluorescent microscopy (Figure 3). Therefore, the nuclei of living cells were examined after staining with Hoechst 33342 dye. Based on fluorescent imaging, all plant extracts caused a significant decrease in cell viability at 100 µg/mL concentration. In addition, the cell death by apoptosis was evident. The arrows in the figure show pyknotic nuclei, a well-known feature of apoptosis. These results are found to be compatible with the ATP viability assay. Taken together, the phase-contrast and fluorescent images confirm the ATP assay, not the MTT assay results.

Taking the false increments in viability into account, we suspected an interfering effect of the plants with the MTT compound. To determine interference of the plant extracts with the MTT compound, the plant extracts were tested in a cell-free culture system. For this purpose, the MTT compound was added to the extract solution and then incubated for 4 h. Table 1 shows the absorbance values of plant extracts without cells. According to the interference analysis results, the absorbance values of plant extracts were higher than the blank (with usual cell culture medium only) at the higher concentrations of extracts. This clearly demonstrates the existence of interference of plant extracts with the MTT compound.

To demonstrate the differences between the MTT and ATP assays, IC_{50} values (half-maximal inhibitory concentration) were also calculated (Table 2). Based on the MTT results, IC_{50} values of all plant extracts were found higher than 100 µg/mL in both cell lines. However, when IC_{50} values were calculated using the ATP assay, these values were found to be lower than the MTT assay results. These results also demonstrate that the IC_{50} values should be interpreted with caution if the MTT assay is employed when plants are concerned for cytotoxicity.

Taken together, the MTT assay can suffer from interference with the extracts, resulting in false-positive results for viability. Even though in the case of total cell death, it may still give rise to increases in viability due to the interfering (reducing) activity of the extracts with the MTT compound.

In conclusion, any data in the literature obtained from the MTT assay should be interpreted with great caution to avoid false-positive results for viability if plants are tested for their cytotoxic activity.

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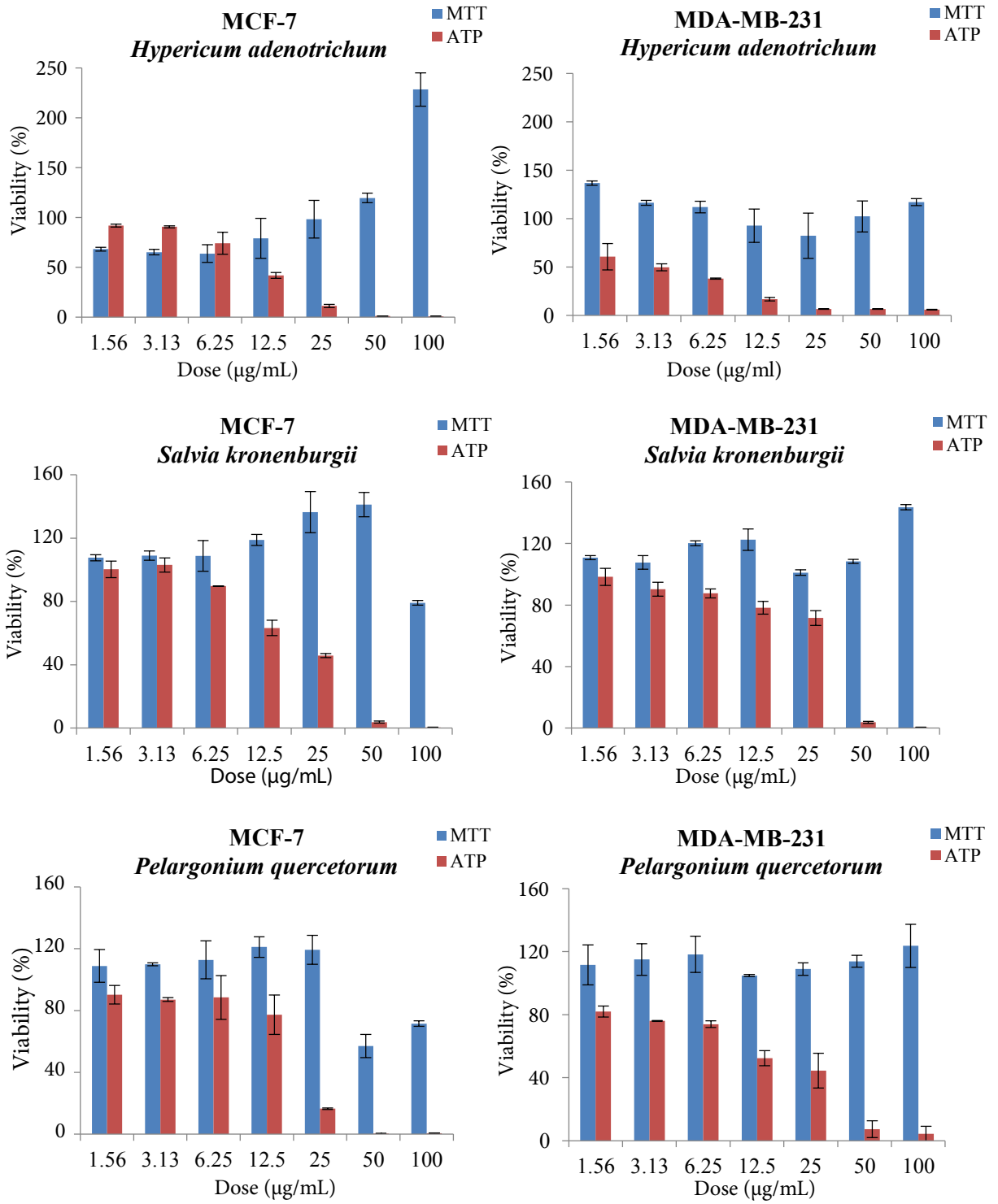

Figure 1. MTT and ATP assays were used to determine the viability after treatment with three different plant extracts at different doses. Note that the MTT assay produces abnormally high viability results in comparison to the ATP assay.

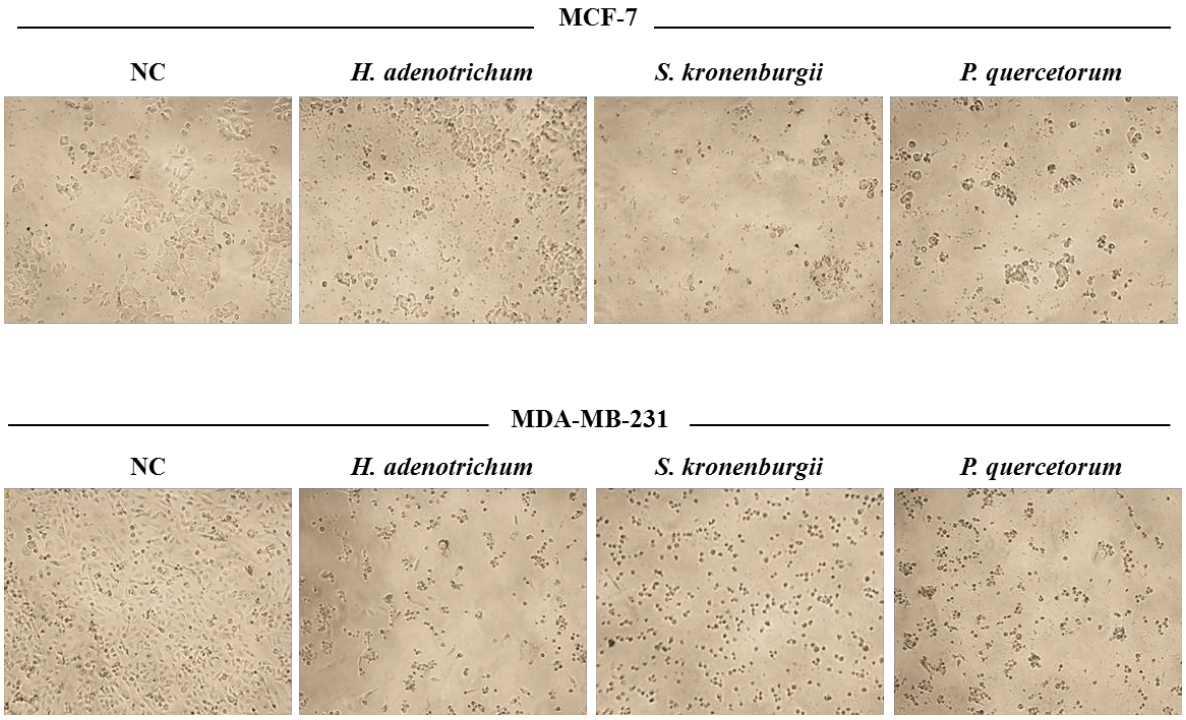

Figure 2. Phase-contrast imaging for demonstration of decrease in cell density. The cells were treated with 100 µg/mL plant extracts for 48 h, and then cells were imaged before the addition of the MTT salt. Magnification 100×.

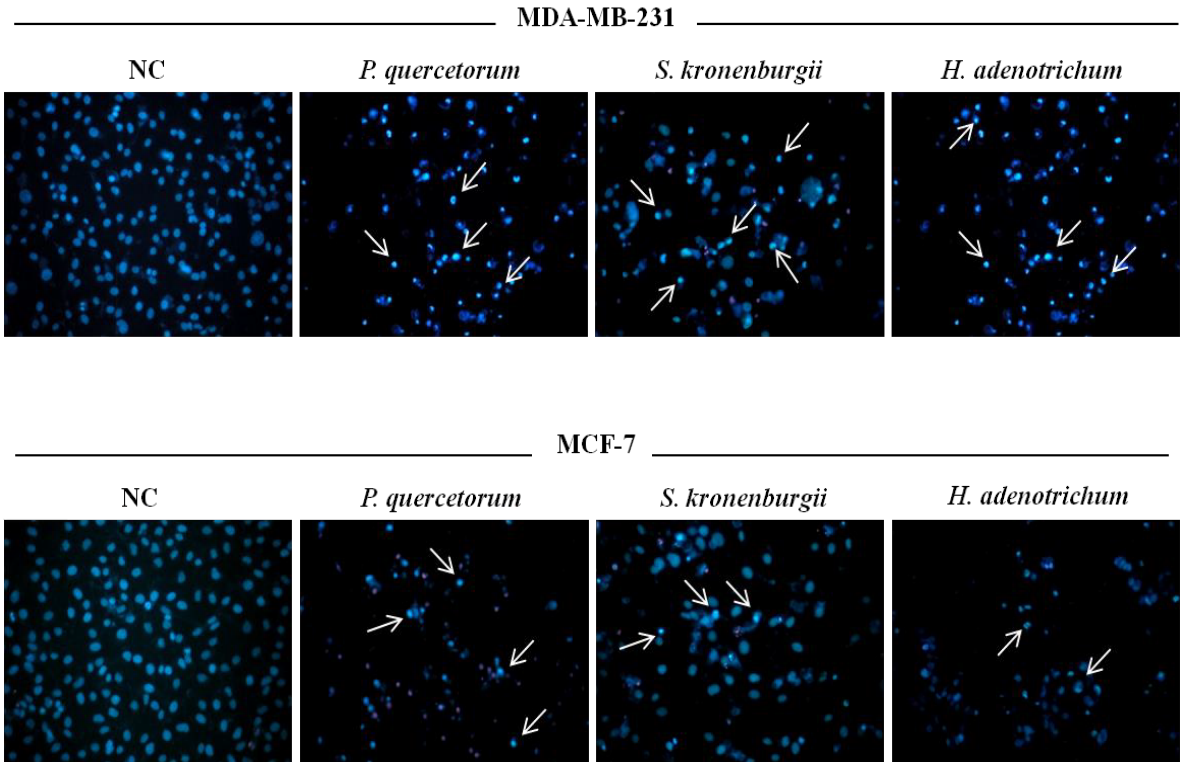

Figure 3. Fluorescence imaging to confirm cell death/apoptosis. The cells were treated with 100 µg/mL plant extracts for 48 h and then stained as explained in Section 2, followed by visualization using fluorescence microscope. Pictures show Hoechst 33342 dye staining. Arrows show the pyknotic nuclei, a well-known feature of apoptosis. Magnification 100×

Table 1. Absorbance values of the extracts alone. *H. adenotrichum*, *S. kronenburgii*, and *P. quercetorum* extracts in usual culture medium were incubated with the MTT salt for 4 h in a cell-free culture system.

Plant extract	Concentrations (µg/mL)							
	Blank	1.56	3.13	6.25	12.5	25	50	100
H. adenotrichum	0.053	0.017	0.034	0.068	0.135	0.271	0.542	1.084
S. kronenburgii	0.051	0.039	0.029	0.033	0.043	0.075	0.139	0.254
P. quercetorum	0.047	0.030	0.040	0.051	0.077	0.113	0.178	0.241

Table 2. IC_{50} values of the extracts determined by using the MTT and ATP assays. Note that great variability occurs.

IC_{50} values	MCF-7		MDA-MB-231	
	MTT assay	ATP assay	MTT assay	ATP assay
H. adenotrichum	>100 µg/mL	10.9 µg/mL	>100 µg/mL	3.1 µg/mL
S. kronenburgii	>100 µg/mL	22.1 µg/mL	>100 µg/mL	33.0 µg/mL
P. quercetorum	>100 µg/mL	18.1 µg/mL	>100 µg/mL	16.3 µg/mL

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