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Cytotoxicity evaluation of a Thai herb using tetrazolium (MTT) and sulforhodamine B (SRB) assays

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Abstract

Background: Various assays are used to evaluate the cytotoxic effect of chemicals on cultured cells. The sulforhodamine B (SRB) colorimetric assay is based on the ability of the SRB dye to bind basic amino acid residues on proteins. In contrast, the MTT (dimethylthiazol-diphenyltetrazolium bromide) colorimetric assay is based on mitochondrial uptake and succinate dehydrogenase reduction of soluble, yellow, MTT tetrazolium salt to an insoluble blue MTT formazan product. The aim of this study was to evaluate the cytotoxicity of a Thai herb by comparing MTT and SRB assay results.

Methods: Mouse fibroblast (L929) cells were exposed to 0.01, 0.1, 0.25, and 0.5% (*w/v*) of a Thai herb in a 96-cluster well culture plate for 24 h. Cell viability after exposure to the Thai herb was determined by MTT and SRB assays in separate tissue culture plates. The two assays were compared using intra-class correlation coefficient (ICC) analysis.

Results: There were no significant differences between the two cytotoxicity assays ($p > 0.05$). The ICC values showing the agreement of the two assays in the negative and positive control groups and Thai herb concentrations of 0.01, 0.1, 0.25, and 0.5% were 0.93 and 0.99 and 0.53, 0.51, 0.95, and 0.98, respectively.

Conclusions: In general, the MTT and SRB assays performed similarly, exhibiting moderate to excellent correlation in the evaluation of the cytotoxicity of a Thai herb.

Background

The MTT (dimethylthiazol-diphenyltetrazolium bromide) colorimetric assay determines the functional state of mitochondria, indicating cell viability. A mitochondrial dehydrogenase enzyme in living cells reduces yellow tetrazolium MTT salt to blue MTT formazan, which is precipitated in uninjured cells (Edmondson et al. 1998).

The MTT assay is the most widely used cell viability assay, and several modifications of the original method have been described (Mosmann 1983). However, the amount of MTT is not linear with cell number at high cell densities (Ruben and Neubauer 1987; Plumb et al. 1989). Cell lines under a number of conditions have large intra-assay and inter-assay variations. Intra-assay variations refer to variations in results within a dataset obtained from one experiment, while inter-assay variations refer to the precision of results among different assays (Park et al. 1987). The sulforhodamine B (SRB) protein stain is used for in vitro chemosensitivity testing. The SRB assay appears to be more sensitive than the MTT assay, with a better linearity with cell number and higher reproducibility (Skehan et al. 1990; Rubenstein et al. 1990). These assays are relevant to medical devices and materials used in dentistry, as pre-clinical evaluations are necessary to establish the biocompatibility of all devices and materials. The International Organization for Standardization (ISO) 7405 (International Organization for Standardization; ISO 7405 2008) recommends that high priority be given to minimizing the use of animals in the biological testing of materials. As scientific knowledge advances the understanding of basic mechanisms, an in vitro model that simulates an in vivo test or clinical use, which may yield equally relevant information, is advocated. A cell culture assay is one method of choice for toxicity screening. MTT, SRB, and neutral red uptake (NRU) assays are widely used for cytotoxic screening evaluation of dental agents and materials. MTT was used in both

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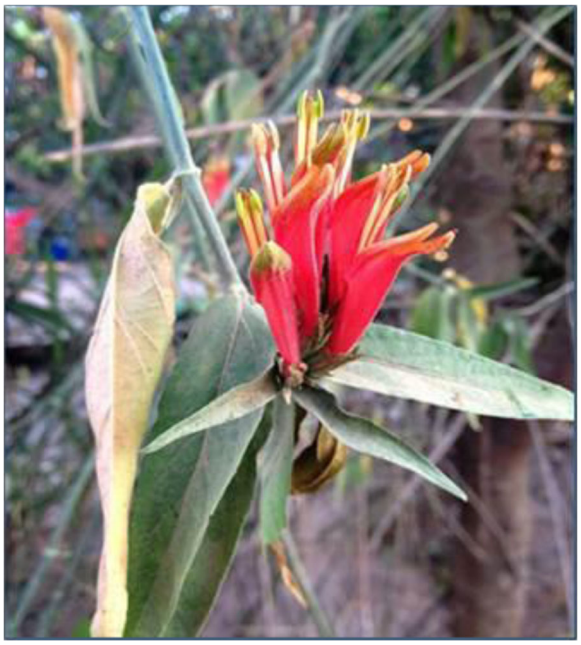

Fig. 1 *Clinacanthus nutans* leaves and flowers. From Twin Lotus Co.,Ltd., Bangkok, Thailand

static and dynamic (perfusion condition) statuses of biocompatible assessment of various dentine-bonding agents (Vajrabhaya et al. 2003a; Vajrabhaya et al. 2009); NRU was used to assess the optimal time of conditioned medium storage for the cell survivability of an avulsed tooth (Vajrabhaya et al. 2003b); and SRB was used for biological screening of six endodontic sealers (Vajrabhaya et al. 1997). In general, the MTT and NRU assays perform similarly in the evaluation of the cytotoxicity of herbal plants, exhibiting a moderate to good correlation (Vajrabhaya and Korsuwannawong 2016).

Thus, the aim of this study was to evaluate the cytotoxicity of a Thai herb by comparing MTT and SRB assay results.

Methods

Test materials

We used a powder formulation of the Thai herb Phaya-Yor in Fig. 1, also known as *Clinacanthus nutans* (Twin Lotus Co., Ltd., Bangkok, Thailand). The color of the Thai herb powder is brown, with a maximum moisture of 8% and pH range of 6.00 to 8.00. The minimum powder fineness can pass a No. 80 sieve with 75% efficiency. *Clinacanthus nutans* was dried in a hot air oven at 60 °C for 72 h, ground into a fine powder using a Blender Panasonic MX-898 NC (Sripiboon Electric Co. Ltd., Bangkok, Thailand), and then filtered through a No. 80 sieve. A total of 0.2 g *Clinacanthus nutans* was weighed and mixed into 1 ml Dulbecco's modified Eagle's medium (DMEM; Invitrogen, CA, USA) for a 20% (*w*/*v*) solution. Then, the solution was incubated at 37 °C under 5% CO_2 for 24 h. The stock solution of *Clinacanthus nutans* was centrifuged at 3500 rpm for 10 min, and the supernatant was diluted into 0.5, 0.25, 0.1, and 0.01% (*w*/*v*) solutions.

As a positive control, the ISO 10993-5 (International Standard Organization; ISO 10993-5 2009) recommends using polyvinyl chloride (PVC; Hatano Research Institute, Food and Drug Safety Center, Kanagawa, Japan) films with a size of 3 cm^2/2 ml media. The films were soaked in 70% alcohol for 1 min for sterilization,

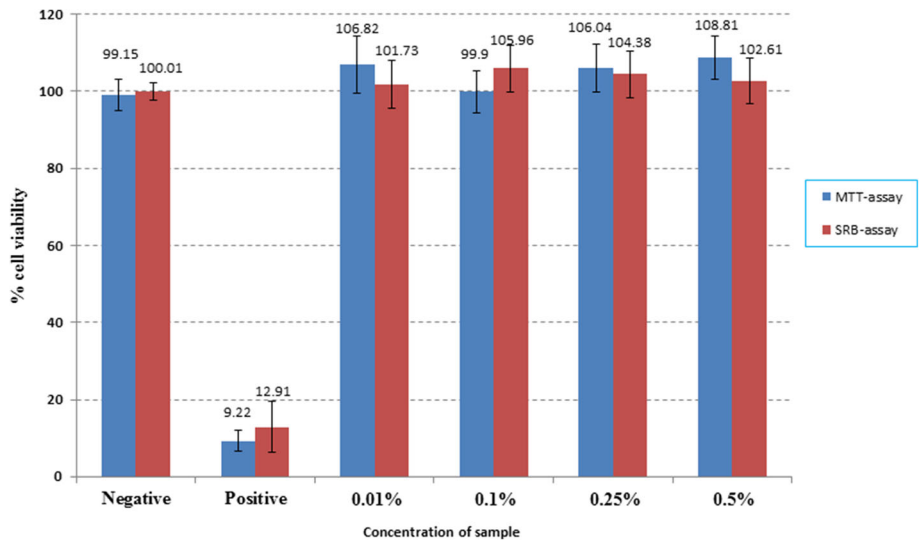

Fig. 2 Cell viability of L929 cells after exposure to the negative control, positive control, or different concentrations of a Thai herb

washed in normal saline solution for 1 min, and left to dry. Then, the films were inserted into DMEM and incubated at 37 °C under 5% CO_2 for 24 h before testing.

As a negative control, the ISO 10993-5 (International Standard Organization; ISO 10993-5 2009) recommends using Thermanox plastic cover slips (NUNC™, Naperville, IL, USA) with a size of 6 cm^2/2 ml media. The Thermanox plastic cover slips were cut into small pieces, soaked in DMEM, and incubated under 5% CO_2 at 37 °C for 24 h before testing.

Cell culture procedure

The target cells used in this experiment were a continuous line of mouse fibroblast L929 cells (NCTC clone 929; American Type Culture Collection (ATCC, VA, USA; ECACC No. 2869501)). The cells were maintained at 37 °C under 5% CO_2 and 100% humidity in DMEM and supplemented with 10% fetal calf serum and antibiotics (200 μl/ml penicillin G, 200 μg/ml streptomycin, and 2 μg/ml fungizone). The medium was changed every other day. When the cells reached confluence, they were detached using 0.2% (*w*/*v*) trypsin and transferred to new culture flasks.

After sufficient growth for experimentation, the cells were trypsinized and plated in 96-cluster well culture plates at a concentration of 1×10^4 cells/well. Each well contained 100 μl of cell suspension, and the plates were incubated for 24 h at 37 °C under 5% CO_2 to obtain a monolayer culture. After 24 h of incubation, the old medium was removed from each well. Then, a 100-μl eluted volume from the *Clinacanthus nutans* solution at concentrations of 0.01, 0.1, 0.25, and 0.5%; the positive control; or negative control was inserted into a 96-cluster well culture plate (8 wells/test material). Two 96-cluster well culture plates were separately prepared to evaluate cell viability using MTT and SRB assays. The experiments were repeated in triplicate. Following a 24-h incubation period at 37 °C under 5% CO_2, the cell viability of both plates was assessed.

MTT assay

The test materials were removed from each well of the first plate. Then, 50 μl of MTT reagent (5 mg/ml) was added and incubated for 2 h at 37 °C in the CO_2 incubator. The MTT solution was then discarded, and 100 μl of isopropanol was added. The plates were placed on a shaker to solubilize the formations of purple crystal formazan. The absorbance was measured using a microplate reader at a wavelength of 570 nm. The results were used to construct a graph of cell viability percentage against extract concentrations.

SRB assay

The SRB assay is used for cell density determination, based on the measurement of cellular protein content. The method described here has been optimized for the toxicity screening of compounds to adherent cells in a 96-well format. After an incubation period, cell monolayers were fixed with 10% (wt/vol) trichloroacetic acid and stained for 30 min; then, excess dye was removed by washing the cells repeatedly with 1% (vol/vol) acetic acid. The protein-bound dye was dissolved in 10 mM Tris base solution for optical density (OD) determination at 510 nm using a microplate reader.

Cell viability was expressed as a percentage of the control values. The intra-class correlation coefficient (ICC) and limits of agreement statistics (Bland and Altman 1986; Bland and Altman 1990; Bland and Altman 1995) were used to compare the scores (Deyo et al. 1991). The limits of agreement statistics were also used as a descriptive measure of agreement.

Table 1 Criteria for grading the strength of assay agreement

Intra-class correlation coefficient	Strength of agreement
< 0.25	Poor
0.25–0.50	Fair
0.50–0.75	Moderate
0.75–0.90	Good
> 0.90	Excellent

Results

The cytotoxic effects of the plant extracts on the viability of the L929 cell lines are presented as percent cell viability in Fig. 2. Values obtained from both SRB and MTT assays were comparable. *Clinacanthus nutans* extracts were non-cytotoxic, whereas the positive control exhibited the highest toxicity.

The ICC values for the agreement of the negative and positive control reactions of MTT and SRB assays were 0.930 and 0.991, respectively, indicating an excellent strength of agreement. The ICC values for the agreement of the extracts at varying *w*/*v* concentrations of 0.01, 0.1, 0.25, and 0.5% were 0.526, 0.513, 0.947, and 0.975, respectively. The strength of agreement ranged from moderate to excellent (Indrayan 2012) (Tables 1 and 2 and Figs. 3, 4, and 5).

Table 2 Intra-class correlation coefficient of the negative and positive controls and various concentrations of *Clinacanthus nutans* for comparative analysis of dimethylthiazol bromide and sulforhodamine B protein

Intra-class correlation coefficient		Strength of agreement
Negative	.930	Excellent agreement
Positive	.991	Excellent agreement
0.01	.526	Moderate agreement
0.1	.513	Moderate agreement
0.25	.947	Excellent agreement
0.5	.975	Excellent agreement

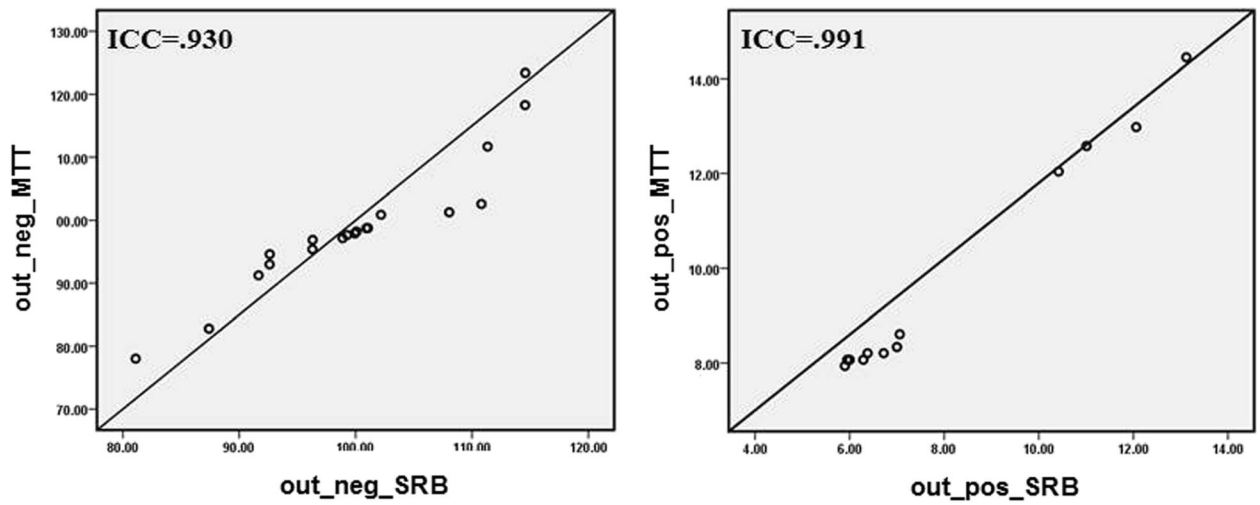

Fig. 3 Intra-class correlation coefficient values for agreement between the MTT and SRB assays in negative and positive group

Discussion

In our study, MTT and SRB assays performed similarly in the cytotoxicity evaluation of *C. nutans*. The assays had moderate correlations at the low concentrations of 0.01 and 0.1% but excellent agreement at the high concentrations of 0.25 and 0.5%. The negative and positive controls exhibited excellent agreement between both assays. Our study is similar to Patel and Patel's (2011) study in which the cytotoxicity activity of a methanolic extract of *Artocarpus heterophyllus* was investigated using two in vitro cytotoxicity assays—MTT vs. SRB. The IC_{50} values of this methanolic extract were 35.26 and 35.27 μg/ml for MTT and SRB, respectively, in the A549 cell line ($p > 0.05$). Patel et al. (2009) also used both assays to evaluate the cytotoxicity of a *Solanum nigrum* methanolic extract on HeLa cells; the inhibitory concentration range for *Solanum nigrum* was between 10 and 0.0196 mg/ml in both assays. This study and the two studies mentioned above (Patel and Patel 2011; Patel et al. 2009) confirmed the same correlation of two assays in other cell lines besides L929 cells. Moreover, mouse fibroblast L929 is a normal cell line which is recommended by International Standard for the testing of medical devices (International Standard Organization; ISO 10993-5 2009) and responds more sensitively than primary cells (Schedle et al. 1995).

By contrast, Keepers et al. (1991) compared two assays for in vitro chemosensitivity testing of drugs with different mechanisms of action on two human tumor

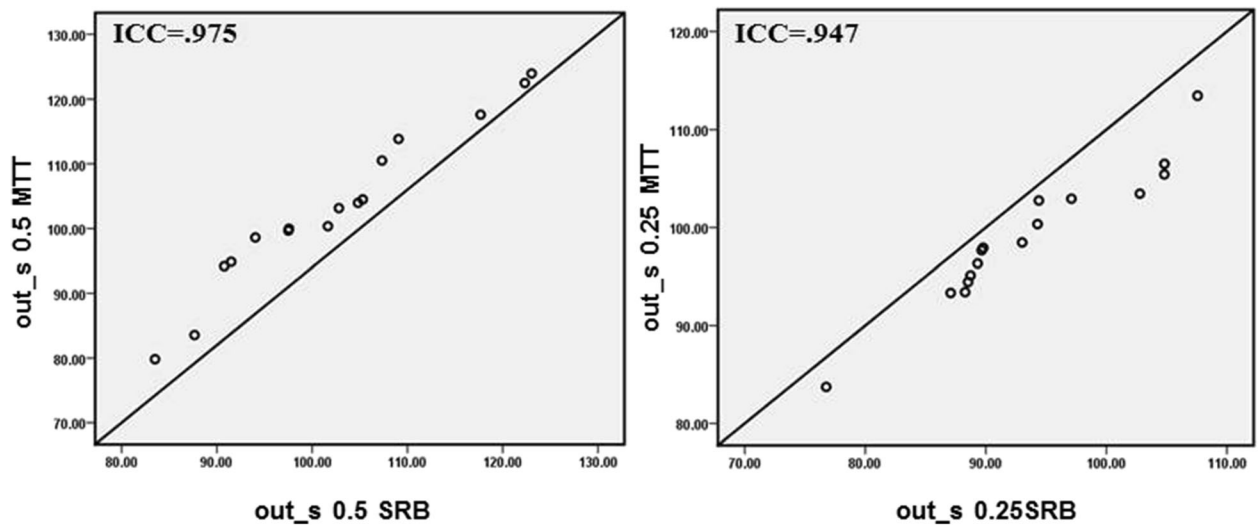

Fig. 4 Intra-class correlation coefficient values for agreement between the MTT and SRB assays at concentration of 0.5 and 0.25%

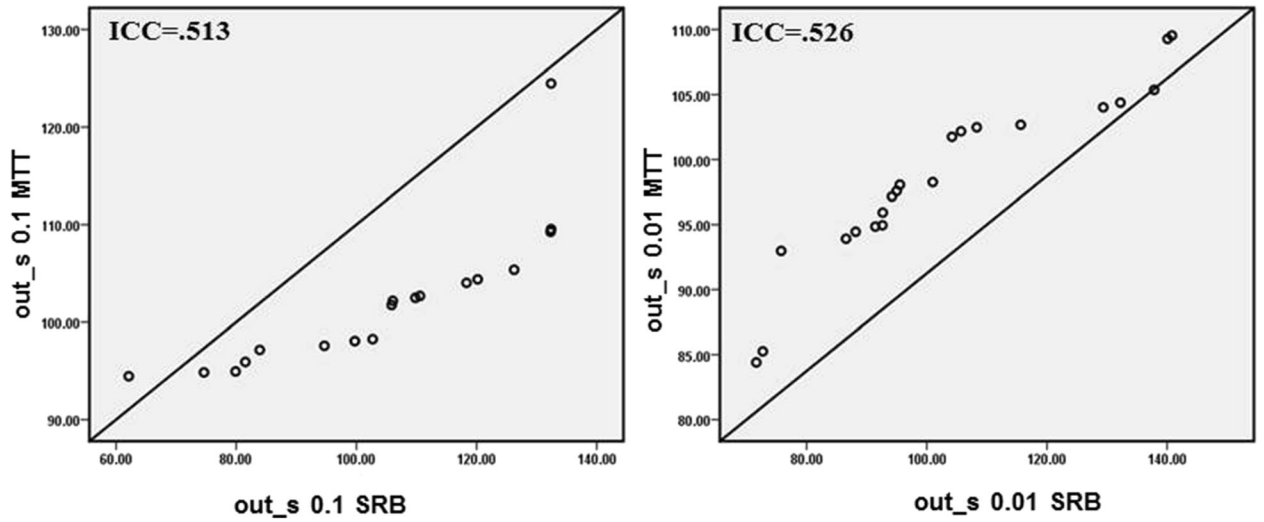

Fig. 5 Intra-class correlation coefficient values for agreement between the MTT and SRB assays at concentration of 0.1 and 0.01%

cell lines—MT29 and 11B. In that study, the SRB assay had better linearity with cell number and higher sensitivity, and its staining was not cell line dependent. In contrast to the MTT assay, the SRB assay stained recently lysed cells. However, cell debris was not stained by SRB; therefore, drug sensitivity was not affected. The ^{51}Cr-release method is a more sensitive cytotoxicity assay and has been modified for application to dental materials. Vajrabhaya et al. (1997) compared the SRB staining assay against ^{51}Cr-release in cytotoxicity tests of six endodontic sealers. In their study, Apexit was the least toxic sealer in both assays. Since SRB staining is an easily conducted test of viability, it may become the method of choice over the ^{51}Cr-release assay in the evaluation of endodontic sealer cytotoxicity.

Both assays can be used to evaluate herbal plant cytotoxicity, but each assay has some limitations. For example, MTT is categorized as a carcinogen. Thus, MTT waste must be appropriately eliminated after testing by environmental pollution control agencies. Light contamination must be prevented during SRB staining because SRB can be degraded by light exposure. Additionally, the staining process should be carefully performed within a specific time according to the manufacturer's instructions; otherwise, the protein will be washed out and result in underestimated OD.

Conclusions

Two in vitro cytotoxicity assays, MTT and SRB, were compared to determine their correlation in the cytotoxicity evaluation of a Thai herbal plant. Moderate to excellent agreement was noted for the evaluation of *Clinacanthus nutans* cytotoxicity. Thus, both MTT and SRB assays can be used for cytotoxic screening of *Clinacanthus nutans*.

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Availability of data and materials

The spectral datasets supporting the conclusions of this article are included within the article in the "Results" section.

Authors' contributions

Both authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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