Original Article

# Cytotoxicity evaluation of *Clinacanthus nutans* through dimethylthiazol diphenyltetrazolium bromide and neutral red uptake assays

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## ABSTRACT

**Objectives:** The aim of this study was to compare the results of dimethylthiazol diphenyltetrazolium bromide (MTT) and neutral red uptake (NRU) assays of *Clinacanthus nutans* cytotoxicity. **Materials and Methods:** Mouse fibroblast (L929) cells were exposed to 0.01%, 0.1%, 0.25%, and 0.5% (W/V) *C. nutans* in a 96-cluster-well-culture plate for 24 h. The cell viability after exposure to *C. nutans* was determined by MTT and NRU assays in separate tissue culture plates. The two assays were compared through an intra-class correlation coefficient (ICC) analysis. **Results:** No significant differences in cytotoxicity were noted between the two assays ($P > 0.05$). The ICC values for agreement between two assays for the negative and positive control groups and *C. nutans* concentrations of 0.01%, 0.1%, 0.25%, and 0.5% were 0.84, 0.83, 0.77, 0.68, 0.74, and 0.71, respectively. **Conclusion:** In general, the MTT and NRU assays performed similarly, exhibiting moderate to good correlation for the evaluation of the cytotoxicity of *C. nutans*.

**Key words:** *Clinacanthus nutans*, dimethylthiazol diphenyltetrazolium bromide, intra-class correlation coefficient, neutral red uptake

## INTRODUCTION

Herbal ingredients are commonly found in many products, including cosmetics, drugs, foods, and oral hygiene products, such as mouth rinse, toothpaste, and lozenges. Herbal ingredients can exhibit antibacterial effects to maintain health. The essential properties of a given herb should be thoroughly evaluated before its use. For example, the biological properties of herbal substances should be evaluated to exclude the possibility of adverse effects on human tissue. The toxic effects of an herbal substance are of particular concern because the damage caused can not only be acute, such as a degeneration of tissue, but can also be chronic, such as a delay in wound healing.

*Clinacanthus nutans* is widely grown in tropical Asian countries and constitutes an important herbal medicine in Thailand. This plant exerts a potential beneficial effect on wound healing based on evidence provided by practitioners of folk medicine and detailed several scientific reports from Thailand.[1] *C. nutans* or Phaya-Yor in Thai [Figure 1] has been traditionally used in Thailand for the treatment of herpes infections.[2] In addition, this herb also exhibits excellent and rapid-acting anti-inflammatory activity, which makes a topical product of *C. nutans*, a valuable natural product for the relief of minor skin inflammation.[3,4] However, scientists in Thailand found that dysentery and fever can be treated by this plant. Due to its anti-cell lysis property, the plant has been used as an anti-venom agent for snake and scorpion bites and to remove nettle rashes. In addition, this plant has recently received much attention because of its potential application in cancer treatment.[5]



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**Figure 1:** *Clinacanthus nutans* (www.madoorayong.com)

Three levels of biological testing are available for dental materials.[6] The initial test of a material involves screening for *in vitro* toxicity. Most of the new dental materials such as dental adhesives were introduced in the clinical practice to investigate their biocompatibility by *in vitro* screening test before *in vivo* experiment.[7] The second level of testing is performed on animals to evaluate the tissue or bone response of the host. The third test simulates clinical practice and is known as the usage test. Preclinical evaluation is necessary to establish the biocompatibility of medical devices and materials used in dentistry. International Organization for Standardization (ISO) 7405 recommends that high priority has to be given to minimize 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 the *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.[8]

Various methods are available for assessing the cytotoxic effects of materials in cell culture. The dimethylthiazol diphenyltetrazolium bromide (MTT) assay and the neutral red uptake (NRU) assay are well-known and widely used methods. The MTT assay determines the functional state of mitochondria, which indicates cell viability. A mitochondrial dehydrogenase enzyme in living cells reduces the yellow tetrazolium salt MTT to blue MTT formazan, which is precipitated in uninjured cells.[9] The MTT assay is always used for the biocompatibility evaluation of dental materials.[10] The NRU assay is based on the incorporation of the NR dye into lysosomes of viable cells.[11] Both assays are based on colorimetric measurements of viable cells after incubation with test materials.

The aim of this study was to compare the relevance of the MTT and NRU assays for cytotoxicity screening of a Thai herb.

## MATERIALS AND METHODS

### Test materials

We used a powder formulation of the Thai herb Phaya-Yor, also known as *C. nutans* (Twin Lotus Co., Ltd., Bangkok, Thailand). The color of the Thai herb powder is brown, with a maximum of moisture 8% and pH range of 6.00–8.00. The minimum powder fineness can pass a No. 80 sieve with 75% efficiency. *C. 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. *C. nutans* was weighed at 0.2 g and mixed into 1 ml of (Dulbecco's modified Eagle's medium [DMEM], Invitrogen, CA, USA) for a 20 g% (w/v) solution. Then, the solution was incubated at 37°C under 5% $CO_2$ for 24 h. The stock solution of *C. nutans* was centrifuged at 3500 rpm for 10 min, and the supernatant was diluted into 0.5 g%, 0.25 g%, 0.1 g%, and 0.01 g% (w/v) solutions.

As a positive control, the ISO 10993-5[12] recommended using polyvinyl chloride (Hatano Research Institute, Food and Drug Safety Center, Kanagawa, Japan) with the size of 3 $cm^2$/2 ml of media. The films were sterilized by soaking in 70% alcohol for 1 min, washed in normal saline solution for 1 min, and left 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[12] recommended using Thermanox plastics cover slips (NUNC™ Naperville, IL, USA) with a size of 6 $cm^2$/2 ml of media. Thermanox plastics cover slips were cut into small pieces, soaked in DMEM, and incubated in 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 (ATCC, cell line, ECACC No. 2869501, NCTC clone 929). Cell were maintained at 37°C under 5% $CO_2$ and 100% humidity in DMEM and supplement with 10% fetal calf serum and antibiotics (200 µg/ml penicillin G, 200 µg/ml streptomycin, 2 µg/ml fungizone). The medium was changed every other day. When cells reached confluency, 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 $1 \times 10^4$ 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 media was removed from each well. Then, 100 µl of elute from the *C. nutans* solution at concentrations of 0.01%, 0.1%, 0.25%, and 0.5%; the positive control or negative control was inserted into 96-cluster-well-culture plates (8 wells/test material). Two 96-cluster-well-culture plates were separately prepared to evaluate cell viability using the MTT and NRU assays. The experiments were repeated in triplicate. Following a 24-h incubation period at 37°C under 5% $CO_2$, cell viability of both plates was assessed.

**Dimethylthiazol diphenyltetrazolium bromide 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. MTT solution was then discarded, and 100 µl of isopropanol was added. The plates were swayed 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 the cell viability percentage against extract concentrations.

**Neutral red uptake assay**

The test material was removed from each well of the second plate. Then, 150 µl of neutral red dye (100 µg/ml) dissolved in the serum of free medium (pH 6.4) was added to the culture medium and incubated for 3 h at 37°C. Cells were washed with phosphate-buffered saline, and 150 µl of elution medium (EtOH/AcCOOH, 50%/1%) was added followed by gentle shaking for 60 min for complete dissolution. Absorbance was recorded at 540 nm using a microtiter plate reader.

Cell viability was expressed as a percentage of the control values. The intra-class correlation coefficient (ICC) and the limits of agreement statistic[13-15] were used to compare the scores.[16] The limits of agreement statistic were also used as a descriptive measure of agreement.[17]

## RESULTS

The cytotoxic effect of the plant extracts on the viability of the L929 cell lines are presented as percent cell viability in Figure 2. Values obtained from both the NRU and MTT assays were comparable. Extracts from the *C. nutans* were noncytotoxic, whereas the positive control exhibited the highest toxicity.

The ICC values for agreement of the negative and positive control reactions of the MTT and NRU assay were 0.84 and 0.83, respectively. The results indicate that the strength of agreement was good. The ICC values for agreement of extracts at varying w/v concentrations of 0.01, 0.1, 0.25, and 0.5% were 0.77, 0.68, 0.74, and 0.71, respectively. The results revealed that the strength of agreement ranged from moderate to good [Tables 1 and 2, Figure 3].

## DISCUSSION

The aim of this study was to assess whether the MTT and NRU assays reveal comparable cytotoxicity levels for an herbal plant. This study revealed that the correlations between the MTT and NRU assays in the cytotoxicity screening of *C. nutans* at the concentrations of 0.01%, 0.1%, 0.25%, and 0.5% ranged between good and moderate agreement. The negative and positive controls exhibited good agreement between both assays.

The study of the effect of *C. nutans* on a human gingival fibroblast cell line revealed no toxicity. Moreover, the extracts at concentrations of 0.01, 0.05, and 0.01%

**Table 1: Criteria for grading the strength of assay agreement**

| ICC | Strength of agreement |
|---|---|
| <0.25 | Poor |
| 0.25-0.50 | Fair |
| 0.50-0.75 | Moderate |
| 0.75-0.90 | Good |
| >0.90 | Excellent |

ICC: Intra-class correlation coefficient

**Table 2: Intra-class correlation coefficient of the negative and positive controls and various concentrations of *Clinacanthus nutans* for the comparative analysis of dimethylthiazol diphenyltetrazolium bromide and neutral red uptake assays**

| Samples (%) | ICC value | Interference |
|---|---|---|
| Negative | 0.829 | Good agreement |
| Positive | 0.835 | Good agreement |
| 0.01 | 0.768 | Good agreement |
| 0.10 | 0.675 | Moderate agreement |
| 0.25 | 0.741 | Moderate agreement |
| 0.50 | 0.714 | Moderate agreement |

ICC: Intra-class correlation coefficient

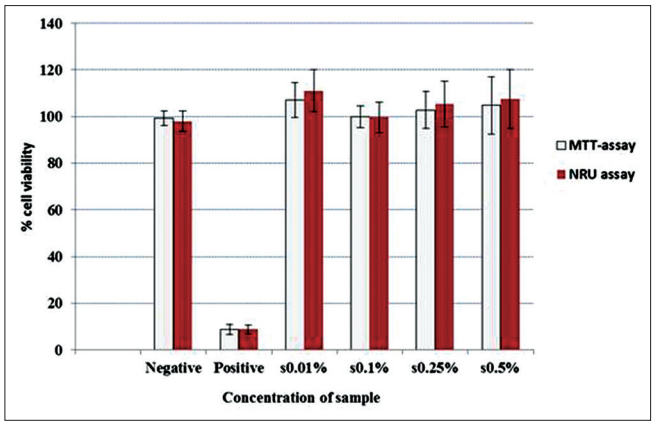


**Figure 2:** Cell viability of L929 cells after exposure to the negative control, positive control, or different concentrations of a Thai herb

promoted cell proliferation and cell migration, suggesting an alternate adjunctive regimen for oral wound healing.[18] Thus, the biocompatibility of *C. nutans* in the new regimen product should be evaluated before the product is launched into the market.

Lysosome enlargement can affect NRU results. For example, Olivier *et al*.[19] reported that polyol substances, which are normally found in drugs, food, and cosmetic products, may increase the osmotic pressure within lysosome. This increase promotes the entry of water and the subsequent swelling and fusion of small lysosomes to form a lysosome that is significantly larger than the original size. Rorig

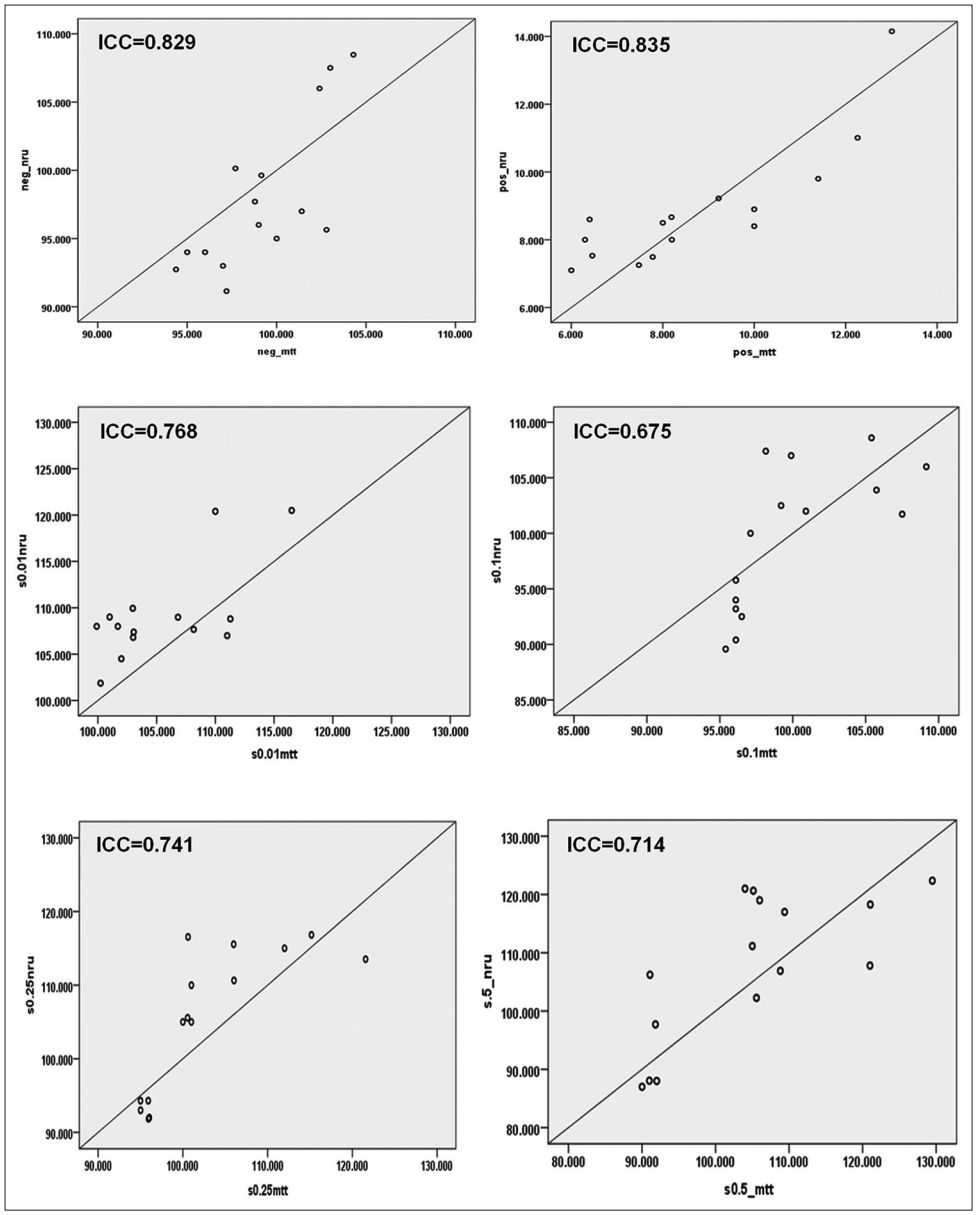


**Figure 3:** Intra-class correlation coefficient values for agreement between the MTT and NRU assays

*et al*.[20] also revealed that weakly basic substances introduced into the cytoplasm also contribute to lysosome swelling, resulting in increased NRU values. Therefore, substances that cause lysosome swelling may result in an underestimation of cytotoxicity when the NRU assay is used for cell viability measurements. Therefore, the NRU assay should be used cautiously with compounds that cause lysosome swelling.

Vian *et al*.[21] compared the correlation of three *in vitro* cytotoxicity assays (NRU, MTT, and [total protein cell content]) for surfactant evaluation using three different cell lines (SIRC, Balb/c 3T3, and L929 cells). A Kappa analysis of the results revealed moderate agreement among the three assays. Lönnroth and Dahl[22] evaluated the effects of both light-cured and chemical-cured dental glass ionomer on cell function and viability using both MTT and NRU assays. These researchers found that the MTT and the NRU assay results were similar for most of the tested materials in L929 cells, but the parallel measurements of the NRU assay varied more widely.

The present study revealed that MTT and NRU assays exhibited good correlation at a 0.01% concentration of *C. nutans*, but moderate correlations were noted for higher concentrations. Both assays can be used to evaluate herbal plant cytotoxicity, but each assay has some limitations. For the NRU assay, the prevention of light contamination during the NR staining procedure is important. Precipitation of NR occurs at the bottom of the tissue culture plate, thus light may be exposed to this area. Although the MTT assay is one of the most sensitive assays for cytotoxicity screening, MTT is categorized as a carcinogen. Thus, MTT waste after testing should be eliminated appropriately by the appropriate environmental pollution control agencies.

## CONCLUSION

Two *in vitro* cytotoxicity assays, MTT and NRU, were compared to determine their correlation in Thai herbal plant evaluation. Good to moderate agreement was noted for the evaluation of *C. nutans* cytotoxicity. Thus, both MTT and NRU assays can be used for cytotoxic screening of this herbal species.

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**Conflicts of interest**
There are no conflicts of interest.

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