# Current applications of MTT assays conducted under microscopic cytotoxicity analysis in cancerous cells by using therapeutic agents of plant origin

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Cellular cytotoxicity is defined as a change in basic cellular functions leading to damage that can be detected to microscopic level. Moreover, the bioassay test is based on the metabolic reduction of 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (or MTT), which is a simple method of determining the cellular viability derived from the quantity of cells in a culture. Therefore, MTT is absorbed by cells and then reduced to an insoluble form (formazan) by mitochondrial succinic-semialdehyde dehydrogenase. The reaction produces formazan, which is retained in the cells and can be released by solubilizing them. The amount of live cells is proportional to that of formazan produced. In this way, the amount of MTT reduced is quantified by a colorimetric method where, because of the reaction, MTT is transformed - from a yellowish, hydrophilic compound to a bluish, hydrophobic material. This chapter describes the ranges of concentrations ($IC_{50}$) of cytotoxic/anti-proliferative effects through the MTT. These are derived from potential therapeutic agents that are present in various plant extracts, whereas indication of cellular viability in cancer therapeutics occurs.

**Keywords**: Cell morphology; Cytotoxicity; Growth inhibitory; MTT

## 1. Introduction

Medicinal plants are vegetal species that contain secondary metabolites in their organs, which are used in the treatment of diverse diseases such as the case of cancer [1]. The application of secondary metabolites is varied. For example, it may have multiple purposes in medicine, pharmaceutics, food and biotechnology, among others. The concentration level of secondary metabolites within a plant varies according to geographic and climatic factors, as well as the soil quality, that is, it may produce changes within the ecosystem [2]. Nowadays, cancer is one of the diseases that cause a substantial death rate in the world. Particularly, there are more than 200 types of cancer, which must be treated urgently [3].

Assays relating to cytotoxicity measure survival or proliferation of cells among mammals. Such assays are widely accepted and used in numerous biological studies and preclinical research, which is feasible because of both its simplicity and reliability. Cellular cytotoxicity reflects an alteration of the basic cellular functions, which leads to damage detection. In this regard, different authors have developed a great array of *in vitro* tests to predict the toxic effects that certain drugs and chemical compounds can produce. For this purpose, there is an application of experimental models, primary *in vitro* cultures, and isolated organs such as established cell lines.

The most widely used experimental models in cancer research are four: immortalized cancer cell lines, tumor-derived cells, cultured cells, and the ones maintained *in vitro*. In particular, cell lines retain many characteristics of tumors and allow the advancement in understanding the biology of cancer. On the other hand, cytotoxicity tests of plant extracts can be conducted on deadly cell lines.

It has been determined that an effective quantitative colorimetric method is the MTT assay. This test is used to determine cell viability as a function of the number of cells present in the *in vitro* culture. Such ratio is obtained through the formation of a colored compound derived from the reaction of mitochondria within viable cells [4]. In this way, the amount of MTT reduced by this colorimetric method is quantified [5]. The MTT assay is employed in a variety of

studies, which include the quantification of lymphokines [6] and cytotoxicity [7-9], along with those related to cell proliferation and activation [10, 11].

This successful method, initially developed by Mosmann in 1983 and subsequently amended in 1986 by Francois Denizot and Rita Lang, is used in monitoring the sensitivity of human tumor cells to chemotherapeutic agents [12].

## 2. MTT Method

The MTT method is a colorimetric assay for viable cell quantification, also referred to as mitochondrial reduction assay. It is used to determine the possible cytotoxic effect of an active substance on tumor cell lines or in primary cultures of normal cells. The essence of this assay is based on the metabolic reduction of 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide or methyl thiazole tetrazolium (MTT) bromide, which is carried out by the enzyme Mitochondrial succinate dehydrogenase from metabolically active mitochondria cells. This enzyme transforms MTT from a yellow hydrophilic soluble compound to a blue hydrophobic insoluble compound (formazan) by cleavage of the tetrazolium ring by dehydrogenase enzymes. Consequently, that transformation enables the mitochondrial function of the treated cells to be determined.

The product of the reaction, the formazan, is retained in the cells and can be released by the solution thereof. The ability of cells to reduce MTT is an indicator of the integrity of mitochondria. The functional activity thereof is interpreted as a measure of cellular viability. It is important to mention that mitochondria belonging to dead cells are not able to respire. Determination of the ability of cells to reduce MTT to formazan after exposure to a compound allows obtaining information about the toxicity of the compound being evaluated. Cells should be stored under sterile conditions in liquid $N_2$ (-190 °C). The exposure period of the test substance varies; it may be for short periods (one to 2 hours) or long periods (from 24 to 72 hours) of treatment. The optical density should be measured at the end of the incubation period at 550 nm, which is done by a 620 nm filter as reference. In general, at least eight replicas of each evaluated concentration are advised to be conducted. Up to six concentrations of the compound need to be evaluated, reaching a concentration of 1000 μg/ mL or up to the maximum solubility limit of the product.

If this concentration is reached and no toxicity is observed, then it is necessary to increase the concentration range up to 100,000 μg / mL or up to the maximum soluble concentration of the compound in the medium. It is necessary to take into account that if the product that is evaluated precipitates in the culture, these results must be discarded. A medium control, a solvent control and a positive control should be employed in the test. This method is used to measure cell survival and proliferation. Normally, the number of living cells is proportional to the amount of formazan produced and therefore, this method allows measurement of cell survival and proliferation, as is useful in determining the cytotoxicity of potential therapeutic agents.

### 2.1 Analysis of results

The results are expressed as percentage of living cells, according to the following relation:

$$\% = \text{O.D. of the treated cells / O.D. of the control cells x 100.}$$

The dose response curve is calculated by taking into account both the concentration range used and the percentage of cell growth reduction. Subsequently, the concentration that produces the reduction of cell viability is calculated by 50%.

Finally, the cells are examined under fluorescence microscopy. Fluorescent microscopy effect of the extracts plant for the detection of morphological changes characteristic to the apoptotic cells i.e. cell pyknosis, chromatin condensation and nuclear fragmentation.

## 3. Mechanism of action

The MTT (methyl thiazole tetrazolium) dye is used as an index of the integrity of the internal mitochondrial membrane in living cells. The substance is a yellow tetrazolium salt which is used to measure cell activity and viability due to the breakdown caused by the reduction (acceptance of a $H^+$) of the ring of the tetrazolium salt MTT by the action of mitochondrial enzyme dehydrogenases (Succinate dehydrogenases). Thus, blue formazan crystals are formed, wherein the absorbance can be determined spectrophotometrically within a wavelength range of 540-570 nm [7]. Such crystals, which are water-insoluble, are soluble in dimethyl sulfoxide (DMSO), isopropanol or other solvents. On the other hand, cell viability is proportional to the absorbance, evidenced by the presence of formazan crystals in the solution.

Figure 1 shows metabolization of chemical structures of yellow MTT and purple formazan product in living cells.

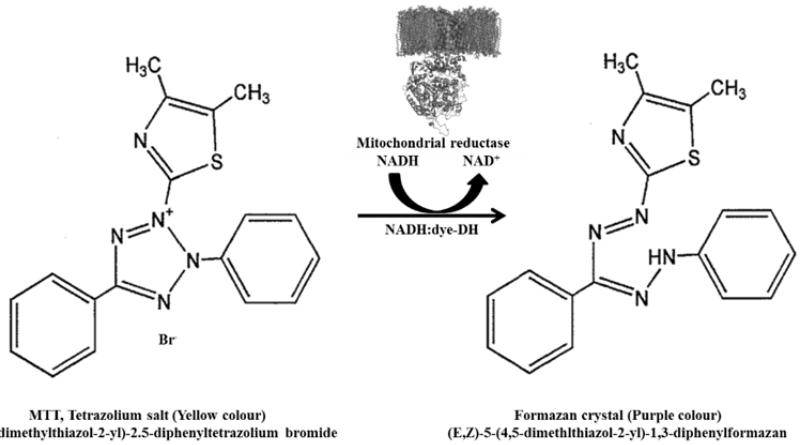


**Fig. 1** Metabolization of chemical structures of yellow MTT (molecular formula: $C_{18}H_{16}BrN_5S$; molecular weight: 414.32 g/mol) and purple formazan (molecular formula: $C_{18}H_{17}BrN_5S$; molecular weight: 335.42 g/mol) product in living cells.

The cells capture the MTT by means of endocytosis [13], reducing it to the level of mitochondria. Other cell compartments produce formazan, whereas a decrease in formation of formazan is connected to the loss of cellular viability. According to US NCI (United States National Cancer Institute) plant screening program, a crude extract is generally considered to have *in vitro* cytotoxic activity with $IC_{50}$ value ≤ 20 µg/ml, while this value was deemed at ≤ 4 µg/ml for a pure compound [14]. The parameters obtained from the cytotoxic test were $IC_{50}$ values, ie values that produce inhibitory concentrations of cancer cells by 50%. Table 1 shows the plant extract type, cancer cell line, treatments, $IC_{50}$ and microscopy morphological analysis method.

**Table 1.** Plant extract type, cancer cell line, treatments, $IC_{50}$ and microscopy morphological analysis method.

| Plant extract and cancer cell line | Treatments (concentration range) and $IC_{50}$ (cytotoxic concentration) | Microscopy morphological analysis method | Reference |
|---|---|---|---|
| Methanol extract of *Elaeis guineensis* vs. MCF-7 and Vero cell (cells were derived from kidney and breast cancer, respectively) | 22.00 µg/ml and 15 µg/ml<br>$IC_{50}$: 15 µg/ml | Phase contrast microscope | 15 |
| Methanol leaf extract of *Hibiscus rosa sinensis* (Malvaceae) vs. K-562 (leukaemic cancer cell line) | 20-100 µg/ml<br>$IC_{50}$: 30.9 µg/ml | Microscope slides | 16 |
| Methanol extract of *Ononis hirta* vs. MCF-7, Hep-2 and Vero | 200, 100, 150, 100, 50, 25, 15, 5 µg/ml<br>$IC_{50}$: 27.96, 54.22 and 41.87 µg/ml, respectively | Light microscope (Novex, Holland). | 17 |
| Methanol crude extracts and dichloromethane extracts of seven *Piper* genus plants vs. Three breast cancer (MCF-7, MDA-MB-231, MDA-MB-468) and one normal breast (MCF- 12A) cell lines at 72 h | 0-80 µg/ml<br>$IC_{50}$: Methanolic crude extracts, *P. nigrum* and *P. retrofractum* in MDA-MB-468 cells: 9.04 µg/ml and 12.27 µg/ml respectively.<br>Dichloromethane extracts, *P. nigrum* and *P. betle*, MDA-MB-468: 7.94 µg/ml and 11.26 µg/ml, respectively | N.D. | 18 |
| Ethanol extracts of leaves of *Annona muricata* vs. T47D cancer cells | 31.25, 62.5, 125, 250 and 500 µg/ml<br>$IC_{50}$: Ethanol extracts of leaves 17.149 µg/ml and can induce apoptosis.<br>Ethyl acetate fraction: $IC_{50}$: 31.268 µg/ml | Fluorescence microscope | 19 |
| Ethanol extracts of *Rosa damascena* vs. HeLa cell line | 30 to 1000 µg/ml for 24, 48 and 72 h.<br>$IC_{50}$: 24 h: 2135 µg/ml,<br>48 h: 1540 µg/ml,<br>72 h: 305.1 µg/ml | N.D. | 20 |
| Methanol extracts of *Stephania* | 0.01, 0.1, 1, 10 and 100 µg/ml | Inverted microscope | 21 |

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|---|---|---|---|
| *wightii* vs. Breast adenocarcinoma (MCF-7) and cervical cancer (HeLa) | $IC_{50}$: MCF-7: 11.56 µg/ml<br>HeLa : 63.4 µg/ml | | |
| n-hexane extract from male flowers of *Alnus sieboldiana* vs. Vero (ECACC No. 84113001, monkey, African green, kidney) and HEK293 (ATCC No. CRL-1573, human, embryonic kidney) cell lines | 1.03– 2,100 µg/ml<br>$IC_{50}$: VERO: 145, 73, 44 and 48 µg/ml<br>HEK293: 154, 46, 23 and 40 µg/ml | N.D. | 22 |
| *Parthenium hysterophorus* vs. A549 tumoral lung cell line | 3.9, 7.81, 15.6, 31.25, 62.5, 125, 250 µg/ml<br>$IC_{50}$: 50.45 µg/ml | N.D. | 23 |
| Aqueous extract of fresh red garlic vs. Human transitional cell carcinoma (TCC) and normal cells (L929) | 0, 50, 100, 200, 250, 400, 800 and 1600 µg/ml<br>$IC_{50}$: for TCC cells was 300 µg/mL and for L929 cells was 379 µg/ml | Light microscope | 24 |
| Methanol extract of *Caralluma acutangula* vs. MCF7 and HEPG2 | 0-50 µg/ml<br>$IC_{50}$: MCF7: 7.06<br>µg/ml and for HEPG2: 6.16 µg/ml | Inverted microscope | 25 |
| Methanol extracts of *Echinophora platyloba* | 50, 100, 200, 300, 400, 500, 600 and 800 µg/ml<br>$IC_{50}$: 24 h: 236.13, 36 h: 143.40, 48 h: 69.38 | Phase-contrast inverse microscopy | 26 |
| Organic extracts (hexane, diethyl ether, ethyl acetate and methanol extracts) from three *Cystoseira species* (*C. humilis, C. tamariscifolia and C. usneoides*) | 125-3.9 µg/mL for 72 h<br>$IC_{50}$: *C. humilis*<br>Hexane: >10 µg/mL<br>Diethyl ether: 8.28 µg/mL<br>Ethyl acetate: 5.04 µg/mL<br>Methanol: >10 µg/mL<br>*C. tamariscifolia*<br>Hexane: 0.63 µg/mL, Diethyl ether: 0.30 µg/mL, Ethyl acetate: 0.17 µg/mL Methanol: 1.08 µg/mL<br>*C. usneoides*<br>Hexane: 4.37 µg/mL, Diethyl ether: 0.65 µg/mL, Ethyl acetate: 7.37 µg/mL, Methanol 7.16 µg/mL | LEICA DM2000 microscope and Olympus SZX7 microscope | 27 |
| Crude extracts of the leaves and fruits of hexane, chloroform, methanol and water of *Brucea javanica* vs. HTB-43 | 500, 250, 125, 62.5, 31.25, 15.625, 7.8125 y 3.9 µg/ml<br>$IC_{50}$: Fruits<br>Hexane: >500 µg/ml, Chloroform: 15.86 µg/ml, Methanol: 8.52 µg/ml, Water: 47.25 µg/ml<br>Leaves<br>Hexane: 103.95 µg/ml, Chloroform: 8.46 µg/ml, Methanol: 26.48 µg/ml, Water: 195.68 µg/ml | Microscope | 28 |
| Ethyl acetate of *Mikania cordata* vs. MCF-7 breast cancer cells | 50, 25, 12.5, 6.25 µg/ml<br>$IC_{50}$: 7.506 µg/ml | Axio Observer inverted microscope (Carl Zeiss) | 29 |
| Hydroalcohol of *Punica granatum* L. vs. influenza A and B in MDCK y Vero cell lines | 25, 50, 100, 200, 250, 500, 700 and 1000 µg/ml<br>$IC_{50}$: MDCK: 400 µg/ml<br>Vero: 137.3 µg/ml | Light microscope | 30 |
| Ethanol of *Consolida orientalis* L. vs. HeLa | 0.0312, 0.0625, 0.125, 0.25, 0.5, 1, 2.5, 5, 7.5 and 10 mg/ml<br>$IC_{50}$: 1.6 mg/ml | N.D. | 31 |
| Methanol extracts of *Artocarpus heterophyllus* vs. A549 cell line | $IC_{50}$: 35.26 µg/ml | Light microscope | 32 |
| Methanol extracts of *Triticum aestivum* vs. HeLa (human cervical | 0.05 µg/ml<br>a 1000 µg/ml | N.D. | 33 |

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| cancer) | $IC_{50}$: 133.6 µg/ml | | |
| Methanol extracts of *Catharanthus roseus y Emblica officinalis* vs. HCT 116 (Human colorectal carcinoma) | 0, 2, 4, 8, 16, 32, 64 y 128 µg/ml<br>$IC_{50}$: 46.21 µg/ml and 35.21 µg/ml respectively | N.D. | 34 |
| Hydroalcoholic extract of the flowers of *T. divaricata possessed* vs. human cancer cell line (HeLa) | 18.75, 37.5, 75, 150, 300 µg/ml and Control<br>$IC_{50}$: 150 µg/ml<br>and 300 µg/ml | N.D. | 35 |
| Methanol root extracts of *Sophora pachycarpa* vs. A549, HeLa, HL-60, MCF-7, and PC3 cell lines and leukocytes as non-malignant cells | 0-250 µg/ml<br>$IC_{50}$: HL-60<br>CH2Cl2: 10.52, EtOAc: 6.651, n-butanol: 35.29 and H2O: >250 | N.D. | 36 |
| Methanol extracts of *Argemone Mexicana* vs. HeLa and MCF-7 cells | 0.5, 0.45, 0.4, 0.35, 0.3, 0.25, 0.2 and 0.15%.<br>$IC_{50}$: 1.35µg/ml to 1.2µg/ml | Fluorescence microscope | 37 |
| Hexane, water, ethanol, chloroform and ethyl acetate extracts vs. Human cervical cancer (HeLa), lung cancer (A549), breast cancer (MCF7) and liver cancer (HepG2) cell lines | 500 – 31.25 µg/ml<br>$IC_{50}$: 151 µg/ml for A549 of ethanol extract, 145.7 µg/ml for HeLa of ethanol extract, 153 µg/ml for MCF7 of ethanol extract, and 128 µg/ml for HepG2 of ethanol extract | N.D. | 38 |
| Methanolic fruit extract and ethanolic leaf extract of *Averrhoa Bilimbi* vs. MCF-7 human breast cancer cell lines | 2.8, 2.9 and 3 µg/ml<br>$IC_{50}$: 154.9 µg/ml for methanolic fruit extract of and 668 µg/ml for ethanolic leaf extract. | Fluorescence microscope | 39 |
| Ethanol and water extracts of *Cassia occidentalis* vs. HeLa | 1, 10 µg/ml<br>$IC_{50}$: 130 µg/ml | Fluorescence microscope | 40 |
| Methanol extracts of *Mentha piperita, M. spicata, M. aquatica, M. crispa, M. pulegium and M. longifolia* vs. Hela and Hep2 | 0.5 mg/ml<br>$IC_{50}$: 28.1-166.2 µg/ml | N.D. | 41 |
| Petroleum ether, dichloromethane, ethanol, and water extracts of the genus *Euphorbia* vs. Cricetulus griseus Chinese hamster ovary cells (CHO cell line ATCC CCL-61) and human larynx epidermoid carcinoma cells (HEp-2 cell line ATCC CCL23) | 50 µg/ml<br>$IC_{50}$: extract obtained from E. cotinifolia leaves: HEp-2 and CHO, were 35.1 and 18.1 µg/ml, respectively. | N.D. | 42 |
| Methanol extracts of *Gaultheria trichophylla* vs. MCF-7 and MDAMB-468 | 10,25,50,100,200,300,400 and 500 µg/mL<br>$IC_{50}$: 100 and 500 µg/ml, respectively. | Confocal microscope | 43 |
| Methanol extracts of *Indigofera cassioides* vs. HeLa, HEp-2, HEpG-2, MCF-7, HT-29, Vero (Normal African green monkey kidney cells) and NIH 3T3(Normal mouse embryonic fibroblast) cells | 10,25,50,100,200 y 300 µg/ml<br>$IC_{50}$: NIH 3T3 292.40 µg/ml, Vero 280.30 µg/ml, HeLa 55.67 µg/ml, HEp-2 (Human laryngeal epithelial carcinoma) 68.45 µg/ml, HEpG2 (Human liver cancer) 46.88 µg/ml, MCF-7 87.50 µg/mL and HT-29 (Human colon cancer) 50.84 µg/ml | Light microscope | 44 |
| Petroleum ether and ethanolic *Clitoria ternatea* vs. Aspirated tumor cells from peritoneal cavity of mice | 10,25,50,10,200,300,400,500 µg/ml and control<br>$IC_{50}$: Petroleum ether extract of 36 µg/ml and ethanolic extract of 57 µg/ml | | 45 |
| non-fractionated aqueous extract of *A. sylvaticus* vs. cultures of non-tumor cells (NIH3T3) and tumor cells (OSCC-3) | 10,20,40,80,160,320 µg/ml<br>$IC_{50}$: cultures of non-tumor cells (NIH3T3): 64.68 µg/ml and tumor cells (OSCC-3): 61.94 µg/ml | N.D. | 46 |

N.D. Not determined.

The mitochondrial enzyme affects the cells, which translates into the metabolization of tetrazolium salts. This action conditions the termination of the tetrazolium ring due to the effect of dehydrogenase enzymes. Such structures lead to formazan, a compound that becomes insoluble in water but soluble through 10% SDS, which is purple in color and proportional to the number of living cells. The cells that die become yellow; an effect that results from the lack of cell respiration, which in turn affects the mitochondria. Similarly, the lack of formation of the tetrazolium ring provokes MTT reagent cannot be reduced to formazan, so that the yellow color remains.

### 3.1 Reagent preparation

MTT is dissolved at a concentration of 5 mg/ml in sterile phosphate buffered saline (PBS) at room temperature. Filter, sterilise and store in a dark container at 4 $^{o}$C. Prepare fresh each month [47]. Extraction buffer 20% (w.v) dissolved at 37 $^{o}$C in a solution of 50% DMF (N,N-dimethyl formamide) and 50% SDW. Adjust pH to 4.7 by adding 2.5% of 80% acetic acid and 2.5% 1N HCl [48].

### 3.1 Data interpretation

Absorbance values indicate a decrease in the rate of cell proliferation, which are lower than those related to control cells. While a greater rate of absorbance indicates a higher speed of growth in cells, such increase could be occasionally offset by cell death. In particular, this condition may be the product of the presence of morphological changes, which can be identified by different types of instruments, such as the phase-contrast, optical, fluorescence, inverted, and confocal microscopes, among other [49]. Finally, a variant of this technique is the MTS assay, which is used in suspension cells. Unlike MTT, its reading occurs at 490 nm.

Cytotoxicity research on tumor cells and plant extracts are carried out by analyzing the cell morphology with the help of the different types of microscopes. These studies yield experimental evidence by the search for new antitumor compounds in medicinal plants distributed throughout the planet. In addition, these methods of screening natural extracts and pure compounds, which are routinely used, bear the potential to contribute to cancer therapy.

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