

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

H. J. Barrales Cureño<sup>\*1</sup>, C. Reyes Reyes<sup>1</sup>, J. A. Cortez Ruiz<sup>2</sup>, A. Luna Cruz<sup>3</sup>, P. Andrade Hoyos<sup>4</sup>, E. Zaragoza Ruiz<sup>1</sup>, V. M. Ocaño Higuera<sup>5</sup>, M. C. Calderón Caballero<sup>6</sup>, L. M. Sánchez Herrera<sup>7</sup>, S. Chávez Salinas<sup>1</sup>, L. G. López Valdez<sup>8</sup>

<sup>1</sup>Departamento de Ingeniería en Biotecnología. Universidad Politécnica del Valle de Toluca. Carretera Toluca-Almoloya de Juárez Km. 5.6 Santiaguito Tlalcalcali, Almoloya de Juárez, Estado de México C.P. 50904. Toluca, Estado de México. México.

<sup>2</sup>Instituto Tecnológico de Mazatlán. Corsario 1 No. 203, Colonia Urías. C.P. 82070. Mazatlán, Sinaloa, México.

<sup>3</sup>Catedrático CONACYT. Facultad de Ciencias Agropecuarias. Universidad Michoacana de San Nicolás de Hidalgo. Avenida Francisco J. Múgica S/N. Ciudad Universitaria, CP. 58030. Morelia, Michoacán, México.

<sup>4</sup>Ingeniería en Innovación Agrícola Sustentable. Instituto Tecnológico Superior de la Sierra Norte de Puebla. Av. José Luis Martínez Vázquez No. 2000. Jicolapa, Zacatlán, Puebla.

<sup>5</sup>Tecnología de Alimentos. Blvd. Luis Encinas y Rosales S/N, Col. Centro, Hermosillo, Sonora, México.

<sup>6</sup>Instituto Tecnológico Superior de Uruapan. Carr. Uruapan-Carapan 5555 Col. La Basilia, C.P. 60015. Uruapan, Michoacán. México.

<sup>7</sup>Unidad de Tecnología de Alimentos. Universidad Autónoma de Nayarit. Ciudad de la Cultura, Amado Nervo S/N, Los Fresnos, C.P. 63155. Tepic, Nayarit.

<sup>8</sup>Preparatoria Agrícola. Universidad Autónoma Chapingo, Carretera México-Texcoco Km. 38.5, Chapingo, 56230 Texcoco de Mora, México.

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<sub>50</sub>) 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, pharmaceuticals, 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<sub>2</sub> (-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:

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

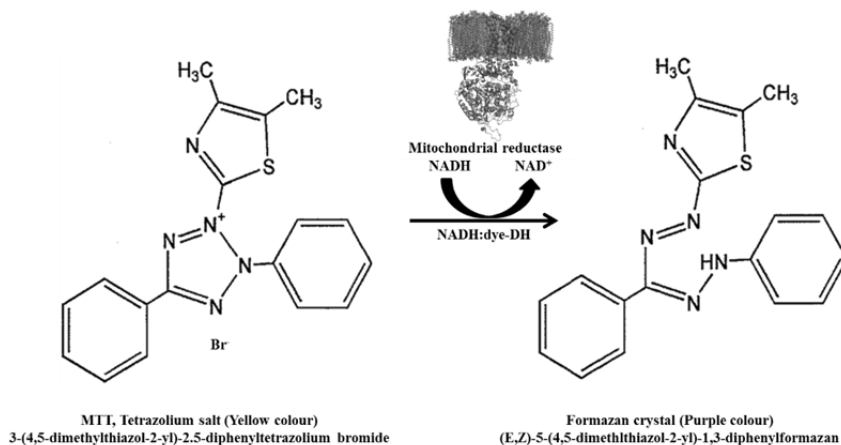
The dose response curve is calculated by taking into account both the concentration range used and the percentage of cell growth reduction. Subsequently, the concentration that produces the reduction of cell viability is calculated by 50%.

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<sup>+</sup>) 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  $\leq 20$   $\mu\text{g/ml}$ , while this value was deemed at  $\leq 4$   $\mu\text{g/ml}$  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 <i>Elaeis guineensis</i> vs. MCF-7 and Vero cell (cells were derived from kidney and breast cancer, respectively)	22.00 $\mu\text{g/ml}$ and 15 $\mu\text{g/ml}$ $IC_{50}$ : 15 $\mu\text{g/ml}$	Phase contrast microscope	15
Methanol leaf extract of <i>Hibiscus rosa sinensis</i> (Malvaceae) vs. K-562 (leukaemic cancer cell line)	20-100 $\mu\text{g/ml}$ $IC_{50}$ : 30.9 $\mu\text{g/ml}$	Microscope slides	16
Methanol extract of <i>Ononis hirta</i> vs. MCF-7, Hep-2 and Vero	200, 100, 150, 100, 50, 25, 15, 5 $\mu\text{g/ml}$ $IC_{50}$ : 27.96, 54.22 and 41.87 $\mu\text{g/ml}$ , respectively	Light microscope (Novex, Holland).	17
Methanol crude extracts and dichloromethane extracts of seven <i>Piper</i> 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 $\mu\text{g/ml}$ $IC_{50}$ : Methanolic crude extracts, <i>P. nigrum</i> and <i>P. retrofractum</i> in MDA-MB-468 cells: 9.04 $\mu\text{g/ml}$ and 12.27 $\mu\text{g/ml}$ respectively. Dichloromethane extracts, <i>P. nigrum</i> and <i>P. betle</i> , MDA-MB-468: 7.94 $\mu\text{g/ml}$ and 11.26 $\mu\text{g/ml}$ , respectively	N.D.	18
Ethanol extracts of leaves of <i>Annona muricata</i> vs. T47D cancer cells	31.25, 62.5, 125, 250 and 500 $\mu\text{g/ml}$ $IC_{50}$ : Ethanol extracts of leaves 17.149 $\mu\text{g/ml}$ and can induce apoptosis. Ethyl acetate fraction: $IC_{50}$ : 31.268 $\mu\text{g/ml}$	Fluorescence microscope	19
Ethanol extracts of <i>Rosa damascena</i> vs. HeLa cell line	30 to 1000 $\mu\text{g/ml}$ for 24, 48 and 72 h. $IC_{50}$ : 24 h: 2135 $\mu\text{g/ml}$ , 48 h: 1540 $\mu\text{g/ml}$ , 72 h: 305.1 $\mu\text{g/ml}$	N.D.	20
Methanol extracts of <i>Stephania</i>	0.01, 0.1, 1, 10 and 100 $\mu\text{g/ml}$	Inverted microscope	21

<i>wightii</i> vs. Breast adenocarcinoma (MCF-7) and cervical cancer (HeLa)	IC <sub>50</sub> : MCF-7: 11.56 µg/ml HeLa : 63.4 µg/ml		
n-hexane extract from male flowers of <i>Alnus sieboldiana</i> 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 IC <sub>50</sub> : VERO: 145, 73, 44 and 48 µg/ml HEK293: 154, 46, 23 and 40 µg/ml	N.D.	22
<i>Parthenium hysterophorus</i> vs. A549 tumoral lung cell line	3.9, 7.81, 15.6, 31.25, 62.5, 125, 250 µg/ml IC <sub>50</sub> : 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 IC <sub>50</sub> : for TCC cells was 300 µg/mL and for L929 cells was 379 µg/ml	Light microscope	24
Methanol extract of <i>Caralluma acutangula</i> vs. MCF7 and HEPG2	0-50 µg/ml IC <sub>50</sub> : MCF7: 7.06 µg/ml and for HEPG2: 6.16 µg/ml	Inverted microscope	25
Methanol extracts of <i>Echinophora platyloba</i>	50, 100, 200, 300, 400, 500, 600 and 800 µg/ml IC <sub>50</sub> : 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 <i>Cystoseira</i> species ( <i>C. humilis</i> , <i>C. tamariscifolia</i> and <i>C. usneoides</i> )	125-3.9 µg/mL for 72 h IC <sub>50</sub> : <i>C. humilis</i> Hexane: >10 µg/mL Diethyl ether: 8.28 µg/mL Ethyl acetate: 5.04 µg/mL Methanol: >10 µg/mL <i>C. tamariscifolia</i> Hexane: 0.63 µg/mL, Diethyl ether: 0.30 µg/mL, Ethyl acetate: 0.17 µg/mL Methanol: 1.08 µg/mL <i>C. usneoides</i> 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 <i>Brucea javanica</i> vs. HTB-43	500, 250, 125, 62.5, 31.25, 15.625, 7.8125 y 3.9 µg/ml IC <sub>50</sub> : Fruits Hexane: >500 µg/ml, Chloroform: 15.86 µg/ml, Methanol: 8.52 µg/ml, Water: 47.25 µg/ml Leaves 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 <i>Mikania cordata</i> vs. MCF-7 breast cancer cells	50, 25, 12.5, 6.25 µg/ml IC <sub>50</sub> : 7.506 µg/ml	Axio Observer inverted microscope (Carl Zeiss)	29
Hydroalcohol of <i>Punica granatum</i> L. vs. influenza A and B in MDCK y Vero cell lines	25, 50, 100, 200, 250, 500, 700 and 1000 µg/ml IC <sub>50</sub> : MDCK: 400 µg/ml Vero: 137.3 µg/ml	Light microscope	30
Ethanol of <i>Consolida orientalis</i> L. vs. HeLa	0.0312, 0.0625, 0.125, 0.25, 0.5, 1, 2.5, 5, 7.5 and 10 mg/ml IC <sub>50</sub> : 1.6 mg/ml	N.D.	31
Methanol extracts of <i>Artocarpus heterophyllus</i> vs. A549 cell line	IC <sub>50</sub> : 35.26 µg/ml	Light microscope	32
Methanol extracts of <i>Triticum aestivum</i> vs. HeLa (human cervical	0.05 µg/ml a 1000 µg/ml	N.D.	33

cancer)	IC <sub>50</sub> : 133.6 µg/ml		
Methanol extracts of <i>Catharanthus roseus</i> y <i>Emblica officinalis</i> vs. HCT 116 (Human colorectal carcinoma)	0, 2, 4, 8, 16, 32, 64 y 128 µg/ml IC <sub>50</sub> : 46.21 µg/ml and 35.21 µg/ml respectively	N.D.	34
Hydroalcoholic extract of the flowers of <i>T. divaricata</i> possessed vs. human cancer cell line (HeLa)	18.75, 37.5, 75, 150, 300 µg/ml and Control IC <sub>50</sub> : 150 µg/ml and 300 µg/ml	N.D.	35
Methanol root extracts of <i>Sophora pachycarpa</i> vs. A549, HeLa, HL-60, MCF-7, and PC3 cell lines and leukocytes as non-malignant cells	0-250 µg/ml IC <sub>50</sub> : HL-60 CH <sub>2</sub> Cl <sub>2</sub> : 10.52, EtOAc: 6.651, n-butanol: 35.29 and H <sub>2</sub> O: >250	N.D.	36
Methanol extracts of <i>Argemone Mexicana</i> vs. HeLa and MCF-7 cells	0.5, 0.45, 0.4, 0.35, 0.3, 0.25, 0.2 and 0.15%. IC <sub>50</sub> : 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 IC <sub>50</sub> : 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 <i>Averrhoa Bilimbi</i> vs. MCF-7 human breast cancer cell lines	2.8, 2.9 and 3 µg/ml IC <sub>50</sub> : 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 <i>Cassia occidentalis</i> vs. HeLa	1, 10 µg/ml IC <sub>50</sub> : 130 µg/ml	Fluorescence microscope	40
Methanol extracts of <i>Mentha piperita</i> , <i>M. spicata</i> , <i>M. aquatica</i> , <i>M. crispa</i> , <i>M. pulegium</i> and <i>M. longifolia</i> vs. HeLa and Hep2	0.5 mg/ml IC <sub>50</sub> : 28.1-166.2 µg/ml	N.D.	41
Petroleum ether, dichloromethane, ethanol, and water extracts of the genus <i>Euphorbia</i> 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 IC <sub>50</sub> : 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 <i>Gaultheria trichophylla</i> vs. MCF-7 and MDAMB-468	10,25,50,100,200,300,400 and 500 µg/mL IC <sub>50</sub> : 100 and 500 µg/ml, respectively.	Confocal microscope	43
Methanol extracts of <i>Indigofera cassioides</i> 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 IC <sub>50</sub> : 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 <i>Clitoria ternatea</i> vs. Aspirated tumor cells from peritoneal cavity of mice	10,25,50,10,200,300,400,500 µg/ml and control IC <sub>50</sub> : Petroleum ether extract of 36 µg/ml and ethanolic extract of 57 µg/ml		45
non-fractionated aqueous extract of <i>A. sylvaticus</i> vs. cultures of non-tumor cells (NIH3T3) and tumor cells (OSCC-3)	10,20,40,80,160,320 µg/ml IC <sub>50</sub> : 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 °C. Prepare fresh each month [47]. Extraction buffer 20% (w.v) dissolved at 37 °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.

**Acknowledgements** The support by Consejo Nacional de Ciencia y Tecnología (CONACYT) and to Dr. Luis Carlos Barros González, Rector of the Universidad Politécnica del Valle de Toluca is gratefully acknowledged.

## References

- [1] Graham JG, Quinn ML, Fabricant DS, Farnsworth NR. Plants used against cancer - an extension of the work of Jonathan Hartwell. *Journal of Ethnopharmacology*. 2000; 73:347-377.
- [2] Castañeda B, Castro de la Mata R, Manrique R, Ibáñez L. Evaluación de la acción citotóxica del extracto metanólico de *Notholaena nivea* "cuti-cuti". *Cultura*. 2006; 20(20):189-202.
- [3] Barrales CHJ, Ramírez SMF. 2013. Una revisión sobre la producción de taxoides anticancerígenos en cultivos *in vitro* de callos y células de *Taxus* spp. *Revista Colombiana de Biotecnología*. 2013; 15(2):167-177.
- [4] Shayne G.C. Alternatives to *in vivo* studies in toxicology. In: Balantyne B, Marrs T, Syversen T. *General and applied toxicology*, vol 1. USA: Grove's dictionaries Inc; 1999. p.178-182.
- [5] Eisenbrand G, Pool-Zobel B, Baker V, Balls M, Blauboer BJ, Boobis A. *Methods of in vitro toxicology*. Food Chemical Toxicology. 2002; 40(2):193-236.
- [6] Green LM, Reade JL, Ware CF. Rapid colorimetric assay for cell viability: application to the quantitation of cytotoxic and growth lymphokines. *Journal of Immunological Methods*. 1984; 70:257-268.
- [7] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*. 1983; 65:55-63.
- [8] Ferrari M, Fornasiero MC, Isetta AM. MTT colorimetric assay for testing macrophage cytotoxic activity *in vitro*. *Journal of Immunological Methods*. 1990; 131:165-172.
- [9] Visconti A, Minervini F, Lucivero G, Gambatesa V. Cytotoxic and immunotoxic effects of *Fusarium* mycotoxins using a rapid colorimetric bioassay. *Mycopathologia*. 1991; 113:181-186.
- [10] Gerkier D, Thomasset N. Use of MTT colorimetric assay to measure cell activation. *Journal of Immunological Methods*. 1986; 94:57-63.
- [11] Minervini F, Lucivero G, Viscoti A, Bottalico C. Immunomodulatory effects of fusarochromanones TDP-1 and TDP-2. *Natural Toxins*. 1992; 1:15-18.
- [12] Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival, Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *Journal of Immunological Methods*. 1986; 89:271-277.
- [13] Liu Y, Peterson DA, Kimura H, Schubert D. Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. *Journal of Neurochemistry*. 1997; 69(2):581-593.
- [14] Geran RI, Greenberg NH, McDonald MM, Scumaker AM, Abbot BJ. Protocol for screening chemical agents and natural products against animal tumours and other biological systems. *Cancer Chemotherapy Reports*. 1972; 3:1-61.
- [15] Vijayarathna S, Sasidharan S. Cytotoxicity of methanol extracts of *Elaeis guineensis* on MCF-7 and Vero cell lines. *Asian Pacific Journal of Tropical Biomedicine*. 2012; 2(10):826-829.
- [16] Arullappan S, Muhamad S, Zakaria Z. Cytotoxic activity of the leaf and stem extracts of *Hibiscus rosa sinensis* (Malvaceae) against leukaemic cell line (K-562). *Tropical Journal of Pharmaceutical Research*. 2013; 12(5):743-746.

- [17] Talib WH, Mahasneh AM, Antiproliferative Activity of Plant Extracts Used Against Cancer in Traditional Medicine. *Scientia pharmaceutica*. 2010; 78:33-45.
- [18] Sriwiriyan S, Ninpesh T, Sukpondma Y, Nasomyon T, Graidist P. Cytotoxicity Screening of Plants of Genus *Piper* in Breast Cancer Cell Lines, *Tropical Journal of Pharmaceutical Research*. 2014; 13(6):921-928.
- [19] Eka Prasasti Nur Rachmani, Tuti Sri Suhesti, Retno Widiastuti Aditiyono. The breast of anticancer from leaf extract of *Annona muricata* againts cell line in T47D. *International Journal of Applied Science and Technology*. 2012; 2(1):157-164.
- [20] Zamiri-Akhlaghi A, Rakhshandeh H, Tayarani-Najaran Z, Hadi MS. Study of cytotoxic properties of *Rosa damascena* extract in human cervix carcinoma cell line. *Avicenna Journal of Phytomedicine*. 2011; 1(2):74-77.
- [21] Danya U, Udhayasankar MR, Punitha D, Arumugasamy K. Anti-proliferative property of *Stephania wightii* (Arn.) Dunn (Menispermaceae) on mammalian breast adenocarcinoma cancer and cervical cancer cell lines. *International Journal of Health and Pharmaceutical Sciences*. 2012; 1(3):67-71.
- [22] Świątek Ł, Rajtar B, Pawlak K, Ludwiczuk A, Głowniak K, Polz-Dacewicz M. *In vitro* evaluation of cytotoxicity of n-hexane extract from *Alnus sieboldiana* male flowers on VERO and HEK293 cell lines. *Journal of Pre-Clinical and Clinical Research*. 2013; 7(2):107-110.
- [23] Diaz GA; Rodríguez SH, Scull LR. Citotoxicidad de extractos de plantas medicinales sobre la línea celular de carcinoma de pulmón humano A549. *Revista Cubana de Farmacia*. 2011; 45(1):101-108.
- [24] Talebi S, Tavakkol AJ, Rakhshandeh H, Seifi B, Mohammad-Hossein B. *In vitro* antiproliferative effect of fresh red garlic on human transitional cell carcinoma (TCC-5637 Cell Line). *International Journal of Agriculture & Biology*. 2006; 8(5):609-614.
- [25] Al-Faifi ZIA, Masrahi YS, Sayed AM, Al-Turki TA. *In vitro* Anticancer Activity of *Caralluma acutangula* (Decne.) N.E.Br. Extract. *International Journal of Pharmaceutical Sciences Review and Research*. 2016; 38(2):59-63.
- [26] Zare SF, Baradaran B, Majidi J, Babaloo Z. *Echinophora platyloba* DC (Apiaceae) Crude Extract Induces Apoptosis in Human Prostate Adenocarcinoma Cells (PC 3). *Biomedical Journal*. 2014; 37:298-304.
- [27] Vizetto-Duarte C, Custódio L, Acosta G, Lago JHG, Morais TR, Bruno de Sousa C, Gangadhar KN, Rodrigues MJ, Pereira H, Lima RT, Vasconcelos M. H, Barreira L, Rauter PA, Albericio F, Varela J. Can macroalgae provide promising anti-tumoral compounds? A closer look at *Cystoseira tamariscifolia* as a source for antioxidant and anti-hepatocarcinoma compounds. *PeerJ*. 2016; 4:1-24.
- [28] Hidayat AMN, Zainah A, Khamis S and Mohd SF. Antiproliferative study of *B. javanica* extracts against head and neck cancer cells. *Research and Development Seminar*. 2014; 1-7.
- [29] Uy TGC, Licuanan AM, Angeles GED, Bote MLCC, Macaayag EAB, Hernandez CC, Jacinto SD, Guzman-Genuino RM. Anti-Cancer Effect and Mechanisms of Action of *Mikania cordata* Plant Extract on MCF-7 Human Breast Adenocarcinoma Cells. *International Journal of Cancer Research*. 2015; 11(2):80-92.
- [30] Del valle PIC, Peña BR, Valdés GS, Caballero PO. Un extracto de *Punica granatum* L. (BLBu) sobre la apoptosis inducida por virus influenza A y B. *Revista CENIC Ciencias Biológicas*. 2006; 37(2):111-117.
- [31] Nemati F, Ali DA, Eslami B, Mahdavi V, Mirzanejad S. Cytotoxic properties of some medicinal plant extracts from Mazandaran, Iran. *Iranian Red Crescent Medical Journal*. 2012; 15:1-4.
- [32] Patel RM, Patel SK. Cytotoxic activity of methanolic extract of *Artocarpus heterophyllus* against A549, Hela and MCF-7 cell lines. *Journal of Applied Pharmaceutical Science*. 2011; 1(7):167-171.
- [33] Janki BP. Anticancer and cytotoxic potential of *Triticum aestivum* extract on Hela cell line. *International Research Journal of Pharmacy*. 2013; 4:103-105.
- [34] Bandopadhyaya S, Ramakrishnan M, Puttalingaiah TR, Shivanna Y. *In-Vitro* evaluation of plant extracts against colorectal cancer using HCT 116 cell line. *International Journal of Plant Science and Ecology*. 2015; 1(3):107-112.
- [35] Sravya DA, Shankarguru P, Devi DR, Hari BNV. Evaluation of *in vitro* anticancer activity of hydroalcoholic extract of *Tabernaemontana divaricate*. *Asian Journal of Pharmaceutical and Clinical Research*. 2012; 5:59-61.
- [36] Vithya T, Kavimani V, Alhasjajiju K, Raj Kapoor B, Savitha BK. An *in vitro* evaluation of cytotoxic activity of *sophora interrupta*. *International Journal of Pharma and Bio sciences*. 2012; 3(2):420-425.
- [37] Gali K, Ramakrishnan G, Kothai R, Jaykar B. *In-vitro* Anti-Cancer activity of methanolic extract of leaves of *Argemone mexicana* Linn. *International Journal of PharmTech Research*. 2011; 3(3):1329-1333.
- [38] Manoharan S, Rangasamy M. Phytochemical analysis and antiproliferative studies of various extracts of *Mollugo cerviana*. *International Journal of Pharmaceutics*. 2016; 6(3):111-115.
- [39] Nair MS, Soren K, Singh V, Boro B. Anticancer activity of fruit and leaf extracts of *Averrhoa Bilimbi* on MCF-7 human breast cancer cell lines: A preliminary study. *Austin Journal of Pharmacology and Therapeutics*. 2016; 4(2):1-5.
- [40] Kilingar NV; Nair S. Comparative Cytotoxic Potentials of the seeds and leaves of *Cassia occidentalis* on HeLa cell Line. *Journal of Pharmacy Research*. 2012; 5(1):261-264.
- [41] Rahimifard N, Hajimehdipour H, Hedayati MH, Bagheri O, Pishehvar H, Ajani Y. Cytotoxic effects of essential oils and extracts of some *Mentha* species on Vero, Hela and Hep2 cell lines. *Journal of Medicinal Plants*. 2016; 9(35):88-92.
- [42] Betancur-Galvis LA, Morales GE, Forero JE, Roldan J. Cytotoxic and antiviral activities of colombian medicinal plant extracts of the *Euphorbia* genus. *Memórias do Instituto Oswaldo Cruz*. 2012; 97(4):541-546.
- [43] Alam F, Saqib QNU, Waheed A. Cytotoxic activity of crude saponins from *Gaultheria trichophylla* against human breast cancer cells MCF-7 and MDAMB-468. *Bangladesh Journal of Pharmacology*. 2015; 10:443-448.
- [44] Kumar RS, Raj Kapoor B, Perumal P. *In vitro* and *in vivo* anticancer activity of *Indigofera cassioides* Rottl. Ex. DC. *Asian Pacific Journal of Tropical Medicine*. 2011; 4(5):379-385.
- [45] Kumar BS, Ishwar BK. *In-vitro* cytotoxic activity studies of *Clitoria ternatea* Linn flower extracts. *International Journal of Pharmaceutical Sciences Review and Research*. 2011; 6(2):120-121.
- [46] Orsine JV, Marques BL, Silva RC, Santos AMF, Novaes MR. Cytotoxicity of *Agaricus sylvaticus* in non-tumor cells (NIH/3T3) and tumor (OSCC-3) using tetrazolium (MTT) assay. *Nutrición Hospitalaria*. 2013; 28(4):1244-1254.
- [47] Pebriana RB, Wardhani BWK, Widayanti E, Wijayanti NLS, Wijayanti TR, Riyanto S, Meiyanto E. Pengaruh ekstrak metanolik daun kenikir (*Cosmos caudatus* Kunth.) terhadap pemecuan apoptosis sel kanker payudara. *Pharmacoon*. 2008; 9(1):21-26.

- [48] Hansen MB, Nielsen SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *Journal of Immunological Methods*. 1989; 119:203-210.
- [49] Cordell G.A. Kinghorn A.D. Pezzuto J.M. Separation, structure elucidation and bioassay of cytotoxic natural products, In: Colegate SM, Molyneux R. J. editors. *Bioactive Natural Products: Detection, Isolation and Structure Determination*. Boca Raton: CRC Press; 1993, p. 99-200.