Copyright © 2020 University of Bucharest
Printed in Romania. All rights reserved
ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2020; 25(4): 1832-1842
doi: 10.25083/rbl/25.4/1832.1842

**Received for publication, November, 30, 2019**
**Accepted, February, 3, 2020**

*Review*

# *Experimental in vitro* cytotoxicity evaluation of plant bioactive compounds and phytoagents: a review

**BIANCA-MARIA TIHĂUAN[1], LAVINIA MARIANA BERCA[1], MARIAN ADASCALULUI[1]*, ANGEL MARTINEZ SANMARTIN[2], SILVIA NICA[3], DANUT CIMPONERIU[4]*, DENISA DUȚĂ[5]**

[1]Molecular Biology Laboratory, National Institute of Research & Development for Food Bioresources – IBA Bucharest, 5 Băneasa Ancuța, 020323, Telephone/Fax: +40733229834, Bucharest, Romania
[2]Technology Transfer Office Director, National Technological Centre for the Food and Canning Industry, CTCC/Concordia s/n, 30500 Molina de Segura, Murcia, Spain
[3]Bucharest University Emergency Hospital, 169, Splaiul Independentei, 050098, Bucharest, Romania
[4]Department of Genetics, University of Bucharest, 1-3 Intrarea Portocalelor, 060101, Bucharest, Romania
[5]Physico-Chemistry Laboratory, National Institute of Research & Development for Food Bioresources – IBA Bucharest, 5 Băneasa Ancuța, 020323, Telephone/Fax: +40756069052, Bucharest, Romania

## Abstract

Bioactive compounds derived from plants are becoming more and more sought-after in recent years. Their impressive potential in diverse industries transforms their status from research curiosities into viable solutions for shortcomings in medicine, pharmacology or food biotechnologies. Needless to say, their beneficial potential comes with challenges regarding cytotoxicity management and assessment. Bioactive compounds with less toxic effect to the host are good candidates for developing new therapeutic solutions. Evaluation of cell functions regarding enzymatic activity, adherence, membrane permeability, enzyme production, nucleotide uptake or ATP production, can be performed by an array of assays. The main purpose of this review is to exhibit and evaluate some of the most effective solutions for *in vitro* cytotoxicity assessment of bioactive compounds (such as phenols, flavonoids, alkaloids, terpenes, etc.) and to overview their particularities as well as their intended purposes.

**Keywords** Cytotoxicity assays, plants, cell culture, bioactive compounds, cell viability.

**To cite this article:** TIHĂUAN B-M, BERCA LM, ADASCALULUI M, SANMARTIN AM, NICA S, CIMPONERIU D, DUȚĂ D. Experimental *in vitro* cytotoxicity evaluation of plant bioactive compounds and phytoagents: a review. *Rom Biotechnol Lett*. 2020; 25(4): 1832-1842. DOI: 10.25083/rbl/25.4/1832.1842

✉ *Corresponding author: DANUT CIMPONERIU, Associate Professor Ph.D., Department of Genetics, University of Bucharest, 1-3 Intrarea Portocalelor, 060101, District 6, Bucharest, Romania, E-mail: dancimponeriu@yahoo.com
MARIAN ADASCALULUI, Ph.D., Molecular Biology Laboratory, National Institute of Research & Development for Food Bioresources – IBA Bucharest, 5 Băneasa Ancuța, 020323, Bucharest, Romania, E-mail: marian_adascalului@yahoo.com

# Introduction

Plants lavish us with their impressive and enormous source of bioactive compounds that, due to their intrinsic biological properties, expand their benefits in fields such as nutrition, pharmacognosy, phytochemistry and phytotherapy as well as in other human health-promoting areas (BELLO USMAN, 2014; KAUFMAN, 1989). Natural products of plants (secondary metabolites) (AKINYEMI O, 2018; RASKIN, 2002) possess various biological activities, among the most exploited ones being the antioxidant and anti-inflammatory effect, which play an important role in neutralizing free radicals among other beneficial effects (AKINYEMI O, 2018; RASKIN, 2002). However, the effects of these compounds may vary within wide limits depending on the exposure particularities (e.g. dose, rhythm, route of administration, association with other substances like drugs, toxins, etc.), health status or stage of ontogenetic development.

Numerous studies recommend performing evaluations of safety regarding the use of bioactive compounds derived from plants as potential therapeutic agents. The good candidates in such cases are ones that exhibit a lower therapeutic index, hence, cytotoxic level of bioactive compounds derived from plants must be evaluated *in vitro* thoroughly against appropriate host cells (CALIXTO, 2000; MUN CHAN, 2015; SASIDHARAN, 2011; WHO, 1993).

Cytotoxicity can be expressed in many forms and has a different significance depending on the perspective from which we analyze it. Cytotoxic compounds can lead to accidental cell death (referred to as necrosis) or programmed cell death (referred to as apoptosis) (P. O'BRIEN, 2007). Cell viability and proliferation are key indicators for apprehending the mechanisms of certain genes, proteins, and pathways involved in cell survival or death (apoptosis as well as necrosis) after exposure to potentially harmful agents such as bioactive compounds derived from plants (RISS, 2004) especially because these agents may cause perniciousness effects on cells in the form of cell membranes disruption, interference with protein synthesis, ATP leakage or inhibition of enzymatic reactions (WEYERMANN, 2005).

For a successful experiment, it is crucial to accurately and efficiently assess cell health, whether prior to experimental treatments or as the readout of those treatments goes in order to minimize common pitfalls.

Nowadays, plenty of assays based on the evaluation of cellular functions are available. Testing protocols entail even multiplexing more than one assay in order to grasp a complex picture of cellular enzymatic production and activity, adherence, membrane permeability, nucleotide uptake or ATP production, all essential indicators of viability or its downfall (CHRZANOWSKA C, HUNT SM, MOHAMMED R, 1990; P. O'BRIEN, 2007; RISS, 2004; WEYERMANN, 2005). *In vitro* cytotoxicity assays are widely recommended and used for evaluating bioactive compounds (such as phenols, flavonoids, alkaloids, terpenes, etc.). These assays sparked a lot of interest in recent years due to their fast and inexpensive nature, but mainly because obtained results can provide solid pieces of information much needed when proceeding to animal testing.

This review aims to exhibit some of the most effective solutions for in vitro cytotoxicity assessment of bioactive compounds and to overview their particularities in use.

# Cytotoxicity assays for *in vitro* evaluation of plant bioactive compounds

Assays for evaluation of cytotoxicity can be classified in a plethora of forms, either according to a targeted compound, a mechanism of action or reported to the cell survival rate and regeneration capacity, but in this review, we propose a classification based on various ways endpoints can be measured. For sure, the evaluation of viable cultured cells can be determined through a variety of techniques, but in this review we discussed the dye exclusion or uptake methods, colorimetric assay, as well as fluorescent and luminescent assays.

**Dye exclusion viability assays** are among one of the simplest and widely used. Most popular dyes are erythrosine and trypan blue. The specificity of this procedure depends on the specific ability of viable cells to exclude dyes, and on the ineptitude of dead cells to exclude them. The staining technique is a simple one, the examinations require an optical microscope or a hemocytometer, but the main downside is that a large number of samples are difficult to carry out or require extended periods of time for fulfilment (YIP, 1972). The use of this dye assays is recommended and intended mainly for cells in suspension (KRAUSE, 1984).

***Trypan blue assay***

The exclusion assay of Trypan Blue, an acid azo dye, relies on the capacity of living cells with undamaged cell membranes to exclude this dye (viable cells remain unstained), whereas dead cells do not, as a result, they are stained blue (RISS, 2004), (STROBER, 2001). This method is utilized as an indicator of membrane integrity, the cost per assay is low and the protocol itself is simple (STONE, 2009). Evaluation is performed using a hemocytometer, keeping in mind that counting errors (~10%) could occur (P. O'BRIEN, 2007; TIWARY, 2015). Processing numerous samples concurrently is one of the shortcomings of this assay, especially in cases where precise time frames of step-by-step cytotoxic effects are required (YIP, 1972). As it goes for most of the dye exclusion tests, distinguishing healthy cells from cells that are alive but losing cell functions is not possible. On that account, it is not sufficiently sensitive to use for *in vitro* cytotoxicity testing, it can be used as a rapid, preliminary assay in evaluations of cellular viability (KIM, 2016a). In this manner, this assay is used widely for toxicity assessment of bioactive compounds derived from plants such as coumarins, flavonoids, saponins, phenols, alkaloids (KUMAR, 2014; SUNARPI, 2018; TIWARY, 2015).

M. Kumar Roy et al., 2007, utilized the TB assay to evaluate the viability of HL-60 cells after treatment with a

flavonoid extracted from a methanolic extract of *Oroxylum indicum*. The main active, baicalein decreased with 50% the viability of cells, as a consequence the number of stained cells increased, therefore the induction of cells apoptosis was observed [21].

***Erythrosine B assay***

This assay has certain advantages over other traditional colorimetric membrane-exclusion dyes, but its use as a vital dye in eukaryotes is not as widespread as other dyes with a comparable mechanism of action (e.g., trypan blue) (KIM, 2016a). The EB assay has its limitations mainly by not being a method suitable for automatic counters, therefore numerous errors can occur due to human errors and reusable cell counting chambers (KIM, 2016a; KRAUSE, 1984). Without a doubt, non-invasive and unharmful assays for cell viability are required for research and innovation in the regenerative medicine sector, drug development studies, genetic engineering, single-cell analysis, microbial food culture or other biotechnologies, therefore lots of studies report using the Erythrosine B dye exclusion assay (ARIANTARI, 2020; CHORMOVA, 2015; YAMASHITA, 2019).

Using this method as reference, Yamashita K. et al., 2019, analyzed several natural food pigments in order to find if monascus pigment (MP) or anthocyanin pigment (AP) work as a good viability indicators of dye exclusion test for *Euglena gracilis* (YAMASHITA, 2019). Soo In Kim et al, 2016, demonstrated that EB can be used for vital staining of three cell lines into an automatic counting in order to encourage development of alternatives and to avoid the toxic side effect of trypan blue or similar dyes (KIM, 2016b). Josef D Franke et al, 2019, demonstrated EB`s versatility and usefulness not only on eukaryotic cells but as a broadly applicable colorimetric vital dye for assaying viability in both Gram-positive and Gram-negative bacteria (FRANKE, 2020).

**Colorimetric assays** are definitely the preferred ones due to their easy use character, rapidity and good correlation of bioactive compound action on metabolic activity of cells. Cytotoxicity assays are widely performed because the test compounds, including plant-derived extracts and purified compounds, may be intended for use as pharmaceuticals or nutraceuticals, in which case minimal to no toxicity is important (MCGAW, 2014). With regard to *in vitro* cell culture systems, a test substance is considered to be cytotoxic if it interferes with attached or suspended cells, significantly alters their morphology, adversely affects the rate of cell growth, or causes cells to die (NILES, 2008). Hereinafter we present an array of methods for assessing cytotoxicity using colorimetric approaches.

*MTT assay*

Assays that measure metabolic activity are suitable for analyzing proliferation, viability, and cytotoxicity. Reduction of tetrazolium salts, such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium tetrazolium bromide (MTT), 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) and water-soluble tetrazolium salts (WST-1), to colored formazan compounds, or the bio-reduction of resazurin by mitochondrial enzyme succinate dehydrogenase with the requirement of cellular nicotinamide adenine dinucleotide occurs only in metabolically active cells (RISS, 2004; STONE, 2009).

MTT assay, is a very popular and widely used colorimetric assay in *in vitro* evaluation of cytotoxicity (PRÄBST, 2017). This protocol is easy to follow and apply, quantification of obtained results is performed using a spectrophotometer at a specific wavelength (SCHANDA, 2007). In comparison with dye exclusion methods the MTT assay has specific characteristics that provides leverage. It has high reproducibility, and it complies with both cell viability and cytotoxicity tests, and for safety reasons is more user-friendly (KOYANAGI, 2016; PRÄBST, 2017; STONE, 2009).

Since MTT formazan is insoluble in water and it forms in the cells purple needle-shaped crystals solubilization using a solvent (DMSO or isopropanol) is required. It should be kept in mind that the cytotoxicity of MTT formazan might lead to false-positive or false-negative results (ASLANTÜRK, 2017; BOPP, 2008A; STONE, 2009).

Compared to MTT other tetrazolium salt reductants such as XTT produces a water-soluble dye. The orange-colored formazan is water-soluble and its intensity can be measured with a spectrophotometer (GAVARIĆ, 2015; HAWSER, 2001; KNIGHT, 2006; LEE, 2019).

On the other hand, the formazan produced by WST-1 is more soluble than that of XTT and MTS, which leads to a wider linear range and higher sensitivity (YIN, 2012). Studies showed that the MTS assay has numerous advantages in use (rapidity, precision, high specificity) [13], [39], being very competitive to other toxicological tests, especially to MTT. This assay provides ideal properties for cytotoxicity measurement due to its reliability and flexibility for onsite toxicological assessments [40]-[42].

Although the MTS assay is a generally good alternative to MTT assay, Piwen W. et al., 2010, results demonstrate that MTT and MTS based assays provide an underestimation of the anti-proliferative effect of EGCG, and suggest the importance of careful evaluation of the method for *in vitro* assessment of cell viability and proliferation depending on the chemical nature of botanical supplements [42].

Another approach in assessing metabolic activity using tetrazolium salts is the WST-8 assay. WST-8 is not cell-permeable, it produces the water-soluble formazan upon cellular reduction which results in low cytotoxicity, aspect that provides the possibility of further experiments using the same cells [13]. Although it should be considered that the reduction of substrates is impacted by changes in intracellular metabolic activity. WST-8 assay can be further applied for the high-throughput screening of dehydrogenases [53].

In accordance with our current interest, numerous studies utilize the MTT assay in evaluation of cytotoxicity

of bioactive compounds, such as flavonoids and terpenoids (*Prema, n.d.*), alkaloids, phenols, flavonoids, terpenoids, glycosides, saponins, steroids and tannins (THAKKAR, 2014), vanillin and curcumin (FORT, 2018). Recently Cristiane F.C. et al, 2018, conducted in their study two *in vitro* cytotoxicity assays, MTT and NRU, and observed that over a wide range of concentration, ethanolic extract of goldenberry (containing phenolic compounds important for human health, like chlorogenic and caffeic acid, and rutin) did not show *in vitro* cytotoxicity, demonstrating the possibility of use in food (CODEVILLA, 2018).

S.P. Hawser et al., 2001, presented in their study that the XTT-based assay in combination with the MEC assay might allow one to fully appreciate the anti-*Aspergillus* activities of antifungal agents such as mulundocandin. Their conclusion was that further studies should be undertaken to evaluate the clinical usefulness of the XTT-based method for determination of the susceptibilities of these pathogens to echinocandin-like compounds (HAWSER, 2001).

Sulistiyani S. Falah et al, 2014, treated cells with flavonoids extract at various concentrations and observed a reduced amount of water-soluble formazan that can be produced through reaction of reagents of WST-8 kit assay. The amount of formazan dye produced is directly proportional to the number of metabolically active cells and indicates the reducing potential of the cell. Thus, these observations were consistent with the reduced number of living cells in this experiment and the presence of the cytotoxic effect of flavonoid extract of *S. polyanthum* to the cell [54].

***LDH assay***

The lactate dehydrogenase (LDH) is a cytosolic enzyme with specificity for a variety of conditions involving cellular damage. LDH assay may be one of the widely used colorimetric methods for the assessment of cellular cytotoxicity. When semipermeable cellular membranes are damaged as a consequence of exposure to an agent, the LDH released into the medium becomes an indicator of death (FOTAKIS, 2006).

LDH (normally found within the cell cytoplasm) released from cells upon damage is measured with a coupled enzymatic reaction that convert a tetrazolium salt (iodonitrotetrazolium (INT)) into a red color formazan by diaphorase. LDH catalyze conversion of lactate to pyruvate and thus NAD is reduced to NADH/H+. Afterwards, a catalyst transfers H/H+ from NADH/H+ to the tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT), which is reduced to red formazan (DECKER, 1988; GEORGE, 2015), (*"Cytotoxicity Detection Kit (LDH) From Roche Applied Science | Biocompare Product Review," n.d.*), (SAAD, 2006).

On Caco-2 cells after the treatment with a *Rubus fairholmianus* extract (primarily containing tannins and anthocyanins) (RFRA), the semipermeable membrane damage was measured by correlation with the LDH discharge. The RFRA treatments provoked the release of LDH. Authors discovered that there was a dose-dependent increase within the LDH release observed at higher concentrations of RFRA. The intracellular LDH release is a measure of irreversible death thanks to semipermeable membrane damage, whereas Xia et al, 2007, reported the direct involvement of LDH upregulation and subsequent induction of apoptosis (GEORGE, 2015; XIA, 2007).

***Sulforhodamine B assay***

Sulforhodamine B (SRB) assay is suitable for measuring induced cytotoxicity on suspended or attached cell cultures. As one of the widely used methods for cytotoxicity screening, SRB is a bright pink aminoxanthene dye with two sulfonic groups, it binds to protein basic amino acid residues under mild acidic conditions. The amount of dye extracted from stained cells is directly proportional to the cell mass (SKEHAN, 1990). The SRB assay is simple, sensitiveness to environmental fluctuations are minimal, is reproducible and more rapid than the formazan-based assays and gives better linearity. It provides a good signal-to-noise ratio and a steadfast endpoint that isn't a time-dependent measurement, as do the MTT or XTT assays (HOUGHTON, 2007). This assay does not rely on intermediary metabolism, as it is independent of it (SKEHAN, 1990), reproducibility of this assay is high if protocol warnings are respected (HOUGHTON, 2007).

In their study, La-ongthong Vajrabhaya et al, 2018, evaluated the performance and accuracy of MTT and SRB. The assays were compared to determine their correlation in the cytotoxicity evaluation of *Clinacanthus nutans.* Moderate to excellent agreement was noted for the evaluation of this Thai herbal plant (VAJRABHAYA, 2018).

***NRU assay***

The neutral red uptake (NRU) assay is based on the ability of viable cells to take up the weakly cationic dye neutral red, being a method suitable for *in vitro* evaluation of cytotoxicity, cell viability, proliferation and adhesion (BORENFREUND, 1985). This dye penetrates cell membranes and concentrates in the lysosomes of viable cells, and it is excluded by dead cells. Using an acidified ethanol solution the dye is extracted and the absorbance is measured using a spectrophotometer (RISS, 2013). The uptake of neutral red by viable cells can be modified by alterations in cell surface or lysosomal membranes. Thus, it is possible to distinguish between viable, damaged, or dead cells (BORENFREUND, 1985). The assay is a quantitative one and is capable of measuring cell replication, cytostatic effects depending on the seeding density – keeping in mind that this particularity is cell line specific (REPETTO, 2008). For example, spontaneous apoptosis in the course of proliferation when the cell density became higher than $1 \times 10^6$/ml has been reported on HL60 cells (SAEKI, 1997), but, on the other hand resting T cells die rapidly by apoptosis when cultured under diluted conditions but survive for extended periods when cultured at high cell density (MA, 2010). Researchers should consider the effect of seeding density before running any

experiments. NRU assay is a good marker of lysosomal damage. Also, speed and simple evaluation are some advantages of this assay. It has been reported that the NRU assay is either minimally or not at all affected by natural factors, such as variations of temperature and salinity levels, but is mainly influenced by pollutants (DAILIANIS, 2003).

Taking into consideration that some bioactive compounds, such as phenols can interact with the reaction substrate of almost every cytotoxicity assay, Merve Bacanli et al., 2017, evaluated the cytotoxicity profiles of different phytochemicals (e.g. galangin, curcumin, puerarin, ursolic acid) focusing on comparing the performance of NRU and MTT assay. Their results concluded that there is no difference between the results from NRU and MTT assays (BACANLI, 2017).

***Clonogenic assay***

The colony formation assay is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony. Biologists consider the clonogenic assay a sophisticated tool that has the ability to differentiate between cytotoxic (cell kill) and cytostatic (decreased growth rate) effects of tested compounds. This type of assay allows continuous exposure to the selected agent and essentially tests every cell in the population for its ability to undergo "unlimited" division. Clonogenic assay is the method of choice for assessing radiosensitivity, but can also be used to determine the effectiveness of other cytotoxic agents. The clonogenic integrity post-irradiation is examined by the ability to divide and form colonies of at least 50 cells (FRANKEN, 2006; MRID, 2019). The assays detect all cells that have retained the capacity of reproduction after treatments that can cause cell death as a result of damage to chromosomes, apoptosis, etc. This assay is mainly used in cancer research biology and usually is associated with other cytotoxicity methods.

The clonogenic assay was performed to confirm the potential of ethanol extracts of needles and berries of *Juniperus oxycedrus* subsp. *oxycedrus* (Joo) species to suppress the growth of MDA-MB-468 and MCF-7 cell lines. R.B. Mrid et al., 2019, demonstrated in their study that Joo extracts showed promising results by reducing the clonogenic ability of both cell lines (MRID, 2019).

**Fluorometric assays** are known and selected due to their accurate assessments of adherent or suspended cell lines (BOPP, 2008b; RISS, 2013), such techniques are easy to use, are more sensitive than colorimetric assays and commercial kits are available from several companies with experimental procedures available in kit packages.

Cytotoxicity plays an important role in a number of pathological processes, including carcinogenesis and inflammation. It may also modulate the activity of other agents, including free radicals, irritants, and genotoxins (PUTNAM, 2002). In this light, the time of cell exposure to bioactive compounds should be carefully considered as well (ELLWOOD, 2014). Cytotoxicity methods that evaluate a spectrum of metabolic responses to specific agents/ bioactives, are the fluorometric assays. This type of assays analyze cell viability and cytotoxicity with the use of a fluorescence microscope, fluorometer, fluorescence microplate reader or flow cytometer, and they offer many advantages over traditional dye exclusion and colorimetric assays (RISS, 2013).

***AlamarBlue assay***

Working similarly to MTT, the phenoxazin-3-one dye reduction assay is based on the conversion of the resazurin (blue nonfluorescent dye) to resorufin (pink fluorescent) by mitochondrial and other enzymes such as diaphorases (GONZALEZ, 2001; J. O'BRIEN, 2000). Resazurin is a cell-permeable indicator that can be used to monitor cellular viability (ANSAR AHMED, 1994; J. O'BRIEN, 2000). The quantity of produced resofurin is related to the number of viable cells. The proportion of viable cells can be quantified using a fluorometer (MCGAW, 2014). Compared to the tetrazolium assays, the AlamarBlue is more sensitive and costs per test are lower. It can be multiplexed with other methods, in addition, AB shows no cytotoxic effects and the tested cells do not need to be destroyed, thus enabling to perform several tests or kinetic measurements on the same set of cells (BOPP, 2008b).

A more recently available resazurin-based colorimetric agent is PrestoBlue. One of the advantages of using this agent are that the results of an assay can be measured visually, using absorbance, or by fluorescence reading of the reduced resorufin product. PrestoBlue is a fast, flexible, live assay, it is also sensitive, being able to detect as few as 12 cells per well (BONCLER, 2014).

Olaf Tyc et al., 2016, evaluated in their study several botanical extracts (high in ellagitannins and flavones) for their antimicrobial activity against pathogenic model microorganisms (*Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*). Besides being a reliable method for cytotoxicity evaluation, authors demonstrated that AlamarBlue assay can be used in HTS methods to screen for antimicrobial activity of botanical extracts (TYC, 2016).

***Protease activity assay***

The proteases are involved in a multitude of physiological reactions being in direct connection to apoptosis mechanisms. Several biomarkers have been described and employed for appraisal of bioactive compounds implications in generating cytotoxicity in cell culture. Quite a few approaches can be utilized in determining the optimal functions of this enzymes, among most popular ones are the fluorogenic protease substrates for evaluation of the matrix metalloproteinase activity, caspase activity or lysosomal cathepsin markers.

The widely used fluorogenic protease substrate (glycylphenylalanyl-aminofluorocoumarin; GF-AFC) evaluates the protease activity on intact viable cells and cells that have lost membrane integrity. The GF-AFC substrate is linked to selective detection of proper protease activity of viable cells (NILES, 2007; RISS, 2013). An optimal activity rate of proteases is an indicator of the viable cell population, on the opposite side a decrease in protease

activity is a clear indicator of dead cells. A correlation between the generated signal of GF-AFC and other established methods for determining cell viability has been documented (RISS, 2013). The substrate non-toxic nature makes it quite advantageous compared to the effect of tetrazolium salts on cells, its minimal vulnerability upon exposure of the GF-AFC substrate to cells results in minor alterations of viability. Therefore, this assay qualifies for multiplexing with other assays, because the cell population that remains viable can be evaluated from different points of view with separate assays, hence GF-AFC does not interfere with cells viability (RISS, 2013).

For this type of approach (selective detection of protease activity using a GF-AFC substrate) the most popular products are the CellTiter-Fluor™ Cell Viability Assay kit and the ONE-Glo™ + Tox Luciferase Cell Viability Assay kit from Promega Corporation. Its versatility in use (single-well, sequential, multiplex) constitutes key factors in selection (*Promega Corporation, 2017*).

The caspase-mediated cleavage cascade is one of the mechanisms involved in apoptosis. But, substitutes in evaluating cell death represented by the lysosomal cathepsin proteases who may initiate or propagate pro-apoptotic signals are potential sensitive markers in various toxicological investigations. The Cathepsin-H Activity Assay kit (produced by BioVision or SinoBiological) is a fluorescence-based assay that uses a specific cathepsin-H substrate tagged with AFC (amino-4-trifluoromethyl coumarin). Samples that contain cathepsin-H will cleave the synthetic substrate R-AFC to release free AFC. As for GF-AFC, in this case the fluorescent signal released from AFC can measured using a fluorometer or fluorescence plate reader.

***Fluorometric microculture assay***

The fluorometric microculture cytotoxicity assay (FMCA) is a non-clonogenic cell viability assay used for measurement of the cytotoxic effect of different compounds *in vitro*, therefore it measures the total living cell density, after a short incubation time. The assay is based on hydrolysis of fluorescein diacetate (FDA) by esterases in cells with intact plasma membranes. The assay is available in a semi-automated plate setup and a version fully adaptable to robotics (LINDHAGEN, 2008).

Applications of FMCA are comparable to those of the commonly used colorimetric MTT assay (BLUMENTHAL, 2005). The two assays have slightly different endpoints: the MTT assay measures cell metabolism, whereas the FMCA measures the esterase activity of cells with intact plasma membranes by measuring the fluorescence generated when the non-fluorescent probe, fluorescein diacetate (FDA), is hydrolyzed (BLUMENTHAL, 2005; LINDHAGEN, 2008). The assays have been shown to give similar results, but the fluorescence detection makes the FMCA a more sensitive assay. In addition, preparation of plates together with a staining procedure without organic solvents are features that make the FMCA easy to work with (LINDHAGEN, 2008).

**Luminometric assays** are methods for determining cell viability or cell cytotoxicity in response to exposure to test agents or conditions being very important to pharmaceutical and environmental testing, pesticide and herbicide testing and drug discovery. Methods for determining cell proliferation are of widespread use in basic research, as well as food safety testing and bio-production of regulated biologics. Therefore, methods that reliably and accurately measure cell viability and/or toxicity after exposure to a test agent are important in determining whether a particular agent/bioactive compound presents a real or potential risk when exposed to a given cell type (MATTA, 2018; RISS, 2013; WAKURI, 2017). Luminometric assays that are capable of monitoring transcriptional activity, intracellular ATP level and protein-protein interaction provide fast results and are relatively easy to use as generally experimental procedures are available in kit packages (RISS, 2013).

***ATP assay***

Luminescent assays stir a lot of interest due to their wide range of applications, to their sensitivity and broad linearity being advantageous choices for assessing apoptosis and cytotoxicity (FAN, 2007). As we know, the adenosine tri-phosphate is formed exclusively in the mitochondria, is the energy source in cells, and considered to be a highly sensitive end point in measuring cell viability (MAEHARA, 1987). Cells lose their membrane integrity after exposure to cytotoxic compounds and lose the ability to synthetize ATP. Critical depletion of the cellular level of ATP indicates loss of basic metabolic functions (GARCÍA, 2003; RISS, 2013).

The ATP assay is based on the reaction of luciferin to oxyluciferin. The luciferase catalyzes this reaction in the presence of Mg2+ ions and ATP yielding a luminescent signal. There is a linear relationship between the intensity of luminescent signal and ATP concentration (MUELLER H, 2004) or cell number (RISS, 2013). As users report, the ATP assay is the fastest cell viability assay to use, and less prone to artifacts than other viability assays (RISS, 2013).

The ATP assay can be performed using various testing kits such as the CellTiter-Glo® from Promega Corporation or the ATP Assay Kit from Abcam, Sigma-Aldrich or BioVision. The kit provides a specific and accurate method for detect the number of viable cells in culture by quantitating the amount of ATP present, which indicates the presence of cells with properly functioning metabolic functions (PROMEGA, 2017). The effect of the treatment should be evaluated after 24 hours by microscope visualization (phase contrast photo) and by staining the samples with fluorescein diacetate (FDA) and propidium iodide (PI).

***Real-time viability assay***

Real-time continuous monitoring of cellular behavior offers numerous advantages in contract with traditional endpoint assays due to the possibility of continuous monitoring of cell adhesion, morphology, and rate of proliferation (DUELLMAN, 2015). Although by now we mainly presented methods with specific endpoints, assays that allow evaluation in real time and on site visualization of cell processes under treatment with bioactive compounds, are new exciting approaches that must be explored. More than that, conventional assays such as alamarBlue, or MTT do not allow real-time detection of cell death in the same experiment.

This assay utilizes a luciferase and a small molecule pro-substrate for detecting cellular viability. The pro-substrate and luciferase are added directly to the cell culture medium where the viable cells with an active metabolism reduce the pro-substrate into a substrate and generates a luminescent signal. The real-time viability assay can be performed in continuous read and endpoint measurement, also one of the advantages is the possibility of multiplexing this assay with other luminescent assays (DUELLMAN, 2015; RISS, 2013).

A limitation of the real-time assay results from the eventual depletion of pro-substrate by metabolically active cells (DUELLMAN, 2015; RISS, 2013). Commercially available kits such as the RealTime-Glo™ MT provide a nonlytic, homogeneous, bioluminescent method to measure cell viability in real time using a simple, plate-based method (PROMEGA, 2016).

An important upgrade in real-time viability monitorization are impedance-based technologies. For example, the xCelligence Real-Time Cell Analyzer (RTCA) are able to measure an entire experiment through numerous time points and create curves that reflect cellular viability in all of its stages. This is a significant advantage over the classic tetrazolium salt assays where the optimization of whole experiment at various time points to choose the best point for final reading it is not possible (FAYEMI, 2019; STEFANOWICZ-HAJDUK, 2020).

The latest MTS, XTT, and WST assays seem to be more appropriate choices over MTT, but false-positive results generated due to interactions between the formazan and tested compound are possible, especially for colored ones as different bioactive compounds are. As we know by now, in all the end-point assays absorbance is dependent on tetrazolium salt concentration as well as cell density, a direct impact on the amount of produced formazan has been observed. Analyzers such as the xCelligence system does not have these disadvantages as they do not require to manipulate the medium during the experiment or addition of any labels, which may alter cell function (FAYEMI, 2019; STEFANOWICZ-HAJDUK, 2020).

# Conclusions

The hunt for an ideal *in vitro* cytotoxicity assay is meticulous and requires taking into consideration various factors. On one hand, it should resemble characteristics such as efficiency, safe use, reliability, rapidity, time- and cost-effectiveness, also minor to zero interferences with the tested compound.

On the other hand, on a more specific note, a crucial role is played primarily by the evaluated analyte. As we mentioned previously, bioactive compounds can interact with the reaction substrate of almost every cytotoxicity assay. On top of that, the cell line used, how it interacts with the substrate, its degree of toxicity and the environmental conditions, just to name a few, influence the accountability of results and their veridicality in correlation with expectations.

All things considered, as a guideline for obtaining reliable results, the scientific literature recommends assessing cytotoxicity with at least two types of assays (for say, analysis of membrane integrity and activity of a metabolic function) especially while evaluating bioactive compounds.

# Acknowledgments

This work was supported by a grant of the Ministry of Research and Innovation through Program 1 – Development of the National R&D System, Subprogram 1.2 – Institutional Performance – Projects for Excellence Financing in RDI, project no. 26 PFE/17.10.2018.

# References

1. AKINYEMI O, OYEWOLE SO, & JIMOH KA. "Medicinal plants and sustainable human health: a review." *Horticulture International Journal*. 2018, 2(4), 194-195. https://doi.org/10.15406/hij.2018.02.00051
2. ANSAR AHMED, S., GOGAL, R.M., & WALSH, J.E. "A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H] thymidine incorporation assay." *Journal of Immunological Methods*. 1994, 170(2), 211-224. https://doi.org/10.1016/0022-1759(94)90396-4
3. ARIANTARI, N. P., ANCHEEVA, E., FRANK, M., STUHLDREIER, F., MEIER, D., GRÖNER, Y., … PROKSCH, P. "Didymellanosine, a new decahydrofluorene analogue, and ascolactone C from *Didymella* sp. IEA-3B.1, an endophyte of *Terminalia catappa*." *RSC Advances*. 2020, 10(12), 7232-7240. https://doi.org/10.1039/C9RA10685E

4. ASLANTÜRK, Ö., ÇELIK, T., KARABEY, B., & KARABEY, F. "Active Phytochemical Detecting, Antioxidant, Cytotoxic, Apoptotic Activities of Ethyl Acetate and Methanol Extracts of Galium aparine L." *British Journal of Pharmaceutical Research*. 2017, 15(6), 1-16. https://doi.org/10.9734/bjpr/2017/32762
5. BACANLI, M., ANLAR, H.G., BAŞARAN, A.A., & BAŞARAN, N. "Assessment of Cytotoxicity Profiles of Different Phytochemicals: Comparison of Neutral Red and MTT Assays in Different Cells in Different Time Periods." *Turkish Journal of Pharmaceutical Sciences*. 2017, 14(2), 95-107. https://doi.org/10.4274/tjps.07078
6. BELLO USMAN, A., ABUBAKAR, S., ALAKU, C., & NNADI, O. "Plant: a Necessity of Life." *International Letters of Natural Sciences*. 2014, 20, 151-159. https://doi.org/10.18052/www.scipress.com/ILNS.20.151
7. BLUMENTHAL, R.D. "An overview of chemosensitivity testing." *Methods in Molecular Medicine*. 2005, 110, 3-18. https://doi.org/10.1385/1-59259-869-2:003
8. BONCLER, M., RÓZALSKI, M., KRAJEWSKA, U., PODSWDEK, A., & WATALA, C. "Comparison of PrestoBlue and MTT assays of cellular viability in the assessment of anti-proliferative effects of plant extracts on human endothelial cells." *Journal of Pharmacological and Toxicological Methods*. 2014, 69(1), 9-16. https://doi.org/10.1016/j.vascn.2013.09.003
9. BOPP, S.K., & LETTIERI, T. (a). "Comparison of four different colorimetric and fluorometric cytotoxicity assays in a zebrafish liver cell line." *BMC Pharmacology*. 2008, 8, 8. https://doi.org/10.1186/1471-2210-8-8
10. BOPP, S.K., & LETTIERI, T. (b). "Comparison of four different colorimetric and fluorometric cytotoxicity assays in a zebrafish liver cell line." *BMC Pharmacology*. 2008, 8, 8. https://doi.org/10.1186/1471-2210-8-8
11. BORENFREUND, E., & PUERNER, J.A. "A simple quantitative procedure using monolayer cultures for cytotoxicity assays (HTD/NR-90)." *Journal of Tissue Culture Methods*. 1985, 9(1), 7-9. https://doi.org/10.1007/BF01666038
12. CALIXTO, J.B. "Efficacy, safety, quality control, marketing and regulatory guidelines for herbal medicines (phytotherapeutic agents)." *Brazilian Journal of Medical and Biological Research*. 2000. Associacao Brasileira de Divulgacao Cientifica. https://doi.org/10.1590/S0100-879X2000000200004
13. CHORMOVA, D., FRANKOVÁ, L., DEFRIES, A., CUTLER, S. R., & FRY, S.C. "Discovery of small molecule inhibitors of xyloglucan endotransglucosylase (XET) activity by high-throughput screening." *Phytochemistry*. 2015, 117, 220-236. https://doi.org/10.1016/j.phytochem.2015.06.016
14. CHRZANOWSKA C, HUNT SM, MOHAMMED R, T.P. The use of cytotoxicity assays for the assessment of toxicity. EHT 9329, Final Report to the Department of the Environment.
15. CODEVILLA, C.F., TISCHER, B., GINDRI, A.L., NOGUEIRA-LIBRELOTTO, D.R., BARIN, J.S., SILVA, C. DE B. DA, … MENEZES, C.R. DE. "Cytotoxicity and antioxidant activity of goldenberry extracts obtained with high intensity ultrasound." *Ciência Rural*. 2018, 48(2). https://doi.org/10.1590/0103-8478cr20170362
16. "Cytotoxicity Detection Kit (LDH) From Roche Applied Science | Biocompare Product Review." (n.d.). Retrieved February 28, 2020, from https://www.biocompare.com/Product-Reviews/40927-Cytotoxicity-Detection-Kit-LDH-From-Roche-Applied-Science/
17. DAILIANIS, S., DOMOUHTSIDOU, G.P., RAFTOPOULOU, E., KALOYIANNI, M., & DIMITRIADIS, V.K. "Evaluation of neutral red retention assay, micronucleus test, acetylcholinesterase activity and a signal transduction molecule (cAMP) in tissues of Mytilus galloprovincialis (L.), in pollution monitoring." *Marine Environmental Research*. 2003, 56(4), 443-470. https://doi.org/10.1016/S0141-1136(03)00005-9
18. DECKER, T., & LOHMANN-MATTHES, M.L. "A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity." *Journal of Immunological Methods*. 1988, 115(1), 61–69. https://doi.org/10.1016/0022-1759(88)90310-9
19. DUELLMAN, S.J., ZHOU, W., MEISENHEIMER, P., VIDUGIRIS, G., CALI, J.J., GAUTAM, P., … VIDUGIRIENE, J. "Bioluminescent, Nonlytic, Real-Time Cell Viability Assay and Use in Inhibitor Screening." *Assay and Drug Development Technologies*. 2015, 13(8), 456-465. https://doi.org/10.1089/adt.2015.669
20. ELLWOOD, K., BALENTINE, D. A., DWYER, J. T., ERDMAN, J. W., COURTNEY GAINE, P., & KWIK-URIBE, C. L. "Considerations on an Approach for Establishing a Framework for Bioactive Food Components 1,2." *Adv Nutr*. 2014, 5, 693-701. https://doi.org/10.3945/an.114.006312
21. FAN, F., & WOOD, K.V. (, February 13). "Bioluminescent assays for high-throughput screening." *Assay and Drug Development Technologies*. 2007. Mary Ann Liebert, Inc. 2 Madison Avenue Larchmont, NY 10538 USA. https://doi.org/10.1089/adt.2006.053
22. FAYEMI, P. O., OZTURK, I., KAAN, D., ÖZCAN, S., YERER, M. B., DOKUMACI, A. H., … YETIM, H. "Bioactivities of phytochemicals in *Callistemon*

*citrinus* against multi-resistant foodborne pathogens, alpha glucosidase inhibition and MCF-7 cancer cell line." *Biotechnology & Biotechnological Equipment*. 2019, 33(1), 764-778. https://doi.org/10.1080/13102818.2019.1616615

23. FORT, R.S., TRINIDAD BARNECH, J.M., DOURRON, J., COLAZZO, M., AGUIRRE-CRESPO, F.J., DUHAGON, M. A., & ÁLVAREZ, G. "Isolation and structural characterization of bioactive molecules on prostate cancer from mayan traditional medicinal plants." *Pharmaceuticals*. 2018, 11(3). https://doi.org/10.3390/ph11030078
24. FOTAKIS, G., & TIMBRELL, J.A. "In vitro cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride." *Toxicology Letters*. 2006, 160(2), 171-177. https://doi.org/10.1016/j.toxlet.2005.07.001
25. FRANKE, J.D., BRAVERMAN, A.L., CUNNINGHAM, A.M., EBERHARD, E.E., & PERRY, G.A. "Erythrosin B: a versatile colorimetric and fluorescent vital dye for bacteria." *BioTechniques*. 2020, 68(1), 7-13. https://doi.org/10.2144/btn-2019-0066
26. FRANKEN, N.A.P., RODERMOND, H.M., STAP, J., HAVEMAN, J., & VAN BREE, C. "Clonogenic assay of cells in vitro." *Nature Protocols*. 2006, 1(5), 2315-2319. https://doi.org/10.1038/nprot.2006.339
27. GARCÍA, O., & MASSIEU, L. "Glutamate Uptake Inhibitor L-Trans-Pyrrolidine 2,4-Dicarboxylate Becomes Neurotoxic in the Presence of Subthreshold Concentrations of Mitochondrial Toxin 3-Nitropropionate: Involvement of Mitochondrial Reducing Activity and ATP Production." *Journal of Neuroscience Research*. 2003, 74(6), 956-966. https://doi.org/10.1002/jnr.10825
28. GAVARIĆ, N., KOVAČ, J., KRETSCHMER, N., KLADAR, N., SMOLE MOŽINA, S., BUCAR, F., … BOŽIN, B. "Natural Products as Antibacterial Agents – Antibacterial Potential and Safety of Post-distillation and Waste Material from Thymus vulgaris L., Lamiaceae." *Concepts, Compounds and the Alternatives of Antibacterials*. 2015, (June 2016). https://doi.org/10.5772/60869
29. GEORGE, B.P.A., TYNGA, I. M., & ABRAHAMSE, H. "In vitro antiproliferative effect of the acetone extract of rubus fairholmianus gard. Root on human colorectal cancer cells." *BioMed Research International*. 2015, 2015. https://doi.org/10.1155/2015/165037
30. GONZALEZ, R.J., & TARLOFF, J.B. "Evaluation of hepatic subcellular fractions for Alamar blue and MTT reductase activity." *Toxicology in vitro*. 2001, 15(3), 257-259. https://doi.org/10.1016/S0887-2333(01)00014-5
31. HAWSER, S.P., JESSUP, C., VITULLO, J., & GHANNOUM, M.A. "Utility of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino) carbonyl]-2H-tetrazolium hydroxide (XTT) and minimum effective concentration assays in the determination of antifungal susceptibility of Aspergillus fumigatus to the lipopeptide class o." *Journal of Clinical Microbiology*. 2001, 39(7), 2738-2741. https://doi.org/10.1128/JCM.39.7.2738-2741.2001
32. HOUGHTON, P., FANG, R., TECHATANAWAT, I., STEVENTON, G., HYLANDS, P.J., & LEE, C.C. "The sulphorhodamine (SRB) assay and other approaches to testing plant extracts and derived compounds for activities related to reputed anticancer activity." *Methods*. 2007, 42(4), 377-387. https://doi.org/10.1016/j.ymeth.2007.01.003
33. KAUFMAN, P.B. Plants: their biology and importance. Harper & Row.
34. KIM, S.I., KIM, H. J., LEE, H.J., LEE, K., HONG, D., LIM, H., … YI, Y. W. (a). "Application of a non-hazardous vital dye for cell counting with automated cell counters." *Analytical Biochemistry*. 2016, 492, 8-12. https://doi.org/10.1016/j.ab.2015.09.010
35. KIM, S. I., KIM, H. J., LEE, H. J., LEE, K., HONG, D., LIM, H., … YI, Y. W. (b). "Application of a non-hazardous vital dye for cell counting with automated cell counters." *Analytical Biochemistry*. 2016, 492, 8–12. https://doi.org/10.1016/j.ab.2015.09.010
36. KNIGHT, S.A.B., & DANCIS, A. "Reduction of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT) is dependent on CaFRE10 ferric reductase for Candida albicans grown in unbuffered media." *Microbiology*. 2006, 152(8), 2301-2308. https://doi.org/10.1099/mic.0.28843-0
37. KOYANAGI, M., KAWAKABE, S., & ARIMURA, Y. "A comparative study of colorimetric cell proliferation assays in immune cells." *Cytotechnology*. 2016, 68(4), 1489-1498. https://doi.org/10.1007/s10616-015-9909-2
38. KRAUSE, A.W., CARLEY, W.W., & WEBB, W.W. "Fluorescent erythrosin B is preferable to trypan blue as a vital exclusion dye for mammalian cells in monolayer culture." *Journal of Histochemistry and Cytochemistry*. 1984, 32(10), 1084-1090. https://doi.org/10.1177/32.10.6090533
39. KUMAR, N.N., RAMAKRISHNAIAH, H., KRISHNA, V., & RADHIKA, M. "Cytotoxic activity of Broussonetia papyrifera(l.) vent on MCF-7, HeLa and HepG2 cell lines." *International Journal of Pharmacy and Pharmaceutical Sciences*. 2014, 6(5), 339-342.
40. LEE, S., OH, D.-G., SINGH, D., LEE, H.J., KIM, G. R., LEE, S., … LEE, C.H. "Untargeted Metabolomics Toward Systematic Characterization of Antioxidant Compounds in Betulaceae Family Plant Extracts."

*Metabolites*. 2019, 9(9), 186. https://doi.org/10.3390/metabo9090186

41. LINDHAGEN, E., NYGREN, P., & LARSSON, R. "The fluorometric microculture cytotoxicity assay." *Nature Protocols*. 2008, 3(8), 1364-1369. https://doi.org/10.1038/nprot.2008.114
42. MA, Q., WANG, Y., SHUK, A., LO, Y., GOMES, E.M., & JUNGHANS, R. P. "Cell Density Plays a Critical Role in Ex Vivo Expansion of T Cells for Adoptive Immunotherapy." *Journal of Biomedicine and Biotechnology*. 2010, 2010, 13. https://doi.org/10.1155/2010/386545
43. MAEHARA, Y., ANAI, H., TAMADA, R., & SUGIMACHI, K. "The ATP assay is more sensitive than the succinate dehydrogenase inhibition test for predicting cell viability." *European Journal of Cancer and Clinical Oncology*. 1987, 23(3), 273-276. https://doi.org/10.1016/0277-5379(87)90070-8
44. MATTA, H., GOPALAKRISHNAN, R., CHOI, S., PRAKASH, R., NATARAJAN, V., PRINS, R., … CHAUDHARY, P.M. "Development and characterization of a novel luciferase based cytotoxicity assay." *Scientific Reports*. 2018, 8(1), 1-14. https://doi.org/10.1038/s41598-017-18606-1
45. MCGAW, L.J., ELGORASHI, E.E., & ELOFF, J.N. "Cytotoxicity of African Medicinal Plants Against Normal Animal and Human Cells." In Toxicological Survey of African Medicinal Plants (pp. 181-233). Elsevier Inc. https://doi.org/10.1016/B978-0-12-800018-2.00008-X
46. MRID, R. BEN, BOUCHMAA, N., BOUARGALNE, Y., RAMDAN, B., KARROUCHI, K., KABACH, I., … NHIRI, M. "Phytochemical Characterization, Antioxidant and In Vitro Cytotoxic Activity Evaluation of Juniperus oxycedrus Subsp. Oxycedrus Needles and Berries." *Molecules*. 2019, 24(3). https://doi.org/10.3390/molecules24030502
47. MUELLER H, KASSACK MU, WIESE M. "Comparison of the usefulness of the MTT, ATP, and calcein assays to predict the potency of cytotoxic agents in various human cancer cell lines. - PubMed - NCBI." *J Biomol Screen*. 2004, 9(6), 506-515. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/15452337
48. MUN CHAN, S., SOO KHOO, K., & WENG SIT, N. "Interactions between Plant Extracts and Cell Viability Indicators during Cytotoxicity Testing: Implications for Ethnopharmacological Studies." *Tropical Journal of Pharmaceutical Research*. 2015, 14(11), 1991-1998. https://doi.org/10.4314/tjpr.v14i11.6
49. NILES, A.L., MORAVEC, R.A., ERIC HESSELBERTH, P., SCURRIA, M. A., DAILY, W. J., & RISS, T. L. "A homogeneous assay to measure live and dead cells in the same sample by detecting different protease markers." *Analytical Biochemistry*. 2007, 366(2), 197-206. https://doi.org/10.1016/j.ab.2007.04.007
50. NILES, A.L., MORAVEC, R.A., & RISS, T.L. (June). "Update on in vitro cytotoxicity assays for drug development." *Expert Opinion on Drug Discovery*. 2008. Taylor & Francis. https://doi.org/10.1517/17460441.3.6.655
51. O'BRIEN, J., WILSON, I., ORTON, T., & POGNAN, F. "Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity." *European Journal of Biochemistry*. 2000, 267(17), 5421-5426. https://doi.org/10.1046/j.1432-1327.2000.01606.x
52. O'BRIEN, P., & HASKINS, J.R. "In vitro cytotoxicity assessment." In Methods in molecular biology (Clifton, N.J.) (Vol. 356, pp. 415-425). Humana Press. https://doi.org/10.1385/1-59745-217-3:415
53. PRÄBST, K., ENGELHARDT, H., RINGGELER, S., & HÜBNER, H. "Basic colorimetric proliferation assays: MTT, WST, and resazurin." In Methods in Molecular Biology (Vol. 1601, pp. 1-17). Humana Press Inc. https://doi.org/10.1007/978-1-4939-6960-9_1
54. PREMA, R., SEKARAN, D., SEKAR, S., BANNOTH, K., SEKHAR, C., & JEEVANANDHAM, S. (n.d.). Invitro cytotoxicity study on combined plants extracts (Cissus quadrangularis and Aegle marmelos). Pelagia Research Library European Journal of Experimental Biology (Vol. 2012). Retrieved from www.pelagia-researchlibrary.com
55. Promega. "RealTime-Glo ™ MT Cell Viability Assay." . 2016, 1-20. Retrieved from https://www.promega.co.uk/~/media/files/resources/protocols/technical manuals/101/realtimeglo mt cell viability assay protocol.pdf
56. Promega. "CellTiter-Glo 2.0 Assay." *Promega*. 2017, 17.
57. Promega Corporation. CellTiter-Fluor™ Cell Viability Assay . Retrieved from www.promega.com
58. PUTNAM, K.P., BOMBICK, D.W., & DOOLITTLE, D.J. "Evaluation of eight in vitro assays for assessing the cytotoxicity of cigarette smoke condensate." *Toxicology in vitro*. 2002, 16(5), 599-607. https://doi.org/10.1016/S0887-2333(02)00050-4
59. RASKIN, I., RIBNICKY, D. M., KOMARNYTSKY, S., ILIC, N., POULEV, A., BORISJUK, N., … FRIDLENDER, B. (, December 1). "Plants and human health in the twenty-first century." *Trends in Biotechnology*. 2002. Elsevier Current Trends. https://doi.org/ 10.1016/S0167-7799(02)02080-2
60. REPETTO, G., DEL PESO, A., & ZURITA, J.L. "Neutral red uptake assay for the estimation of cell viability/ cytotoxicity." *Nature Protocols*. 2008, 3(7), 1125-1131. https://doi.org/10.1038/nprot.2008.75
61. RISS, T.L., MORAVEC, R.A., NILES, A.L., DUELLMAN, S., BENINK, H.A., WORZELLA, T.J., & MINOR, L. Cell Viability Assays. Assay Guidance Manual. Eli Lilly & Company and the National Center for Advancing Translational Sciences. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/23805433

62. RISS, T.L., MORAVEC, R.A., NILES, A.L., DUELLMAN, S., BENINK, H.A., WORZELLA, T.J., & MINOR, L. Cell Viability Assays. Assay Guidance Manual. Eli Lilly & Company and the National Center for Advancing Translational Sciences. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/23805433
63. SAAD, B., DAKWAR, S., SAID, O., ABU-HIJLEH, G., AL BATTAH, F., KMEEL, A., & AZIAZEH, H. "Evaluation of medicinal plant hepatotoxicity in co-cultures of hepatocytes and monocytes." *Evidence-Based Complementary and Alternative Medicine : ECAM*. 2006, 3(1), 93-8. https://doi.org/10.1093/ecam/nel002
64. SAEKI, K., YUO, A., KATO, M., MIYAZONO, K., YAZAKI, Y., & TAKAKU, F. "Cell density-dependent apoptosis in HL-60 cells, which is mediated by an unknown soluble factor, is inhibited by transforming growth factor β1 and overexpression of Bcl-2." *Journal of Biological Chemistry*. 1997, 272(32), 20003-20010. https://doi.org/10.1074/jbc.272.32.20003
65. SASIDHARAN, S., CHEN, Y., SARAVANAN, D., SUNDRAM, K.M., & YOGA LATHA, L. "Extraction, isolation and characterization of bioactive compounds from plants' extracts." *African Journal of Traditional, Complementary and Alternative Medicines*. 2011, 8(1), 1-10. https://doi.org/10.4314/ajtcam.v8i1.60483
66. SCHANDA, J., & International Commission on Illumination. Colorimetry : understanding the CIE system. CIE/Commission internationale de l'eclairage.
67. SKEHAN, P., STORENG, R., SCUDIERO, D., MONKS, A., MCMAHON, J., VISTICA, D., … BOYD, M.R. "New colorimetric cytotoxicity assay for anticancer-drug screening." *Journal of the National Cancer Institute*. 1990, 82(13), 1107-1112. https://doi.org/10.1093/jnci/82.13.1107
68. STEFANOWICZ-HAJDUK, J., & OCHOCKA, J.R. (, January 1). "Real-time cell analysis system in cytotoxicity applications: Usefulness and comparison with tetrazolium salt assays." *Toxicology Reports*. 2020. Elsevier Inc. https://doi.org/10.1016/j.toxrep.2020.02.002
69. STONE, V., JOHNSTON, H., & SCHINS, R. P. F. "Development of in vitro systems for nanotoxicology: Methodological considerations in vitro methods for nanotoxicology Vicki Stone et al." *Critical Reviews in Toxicology*. 2009. https://doi.org/10.1080/10408440903120975
70. STROBER, W. "Trypan Blue Exclusion Test of Cell Viability." In Current Protocols in Immunology. John Wiley & Sons, Inc. https://doi.org/10.1002/0471142735.ima03bs21
71. SUNARPI, H., SUNARWIDHI, E., ARIYANA, M., NIKMATULLA, A., ZULKIFLI, L., YOSHIE, S., … HAZAMA, A. "Cytotoxicity and Antiproliferative Activity of Indonesian Red Algae Acanthophora muscoides Crude Ethanol Extracts." *Journal of Biological Sciences*. 2018, 18(8), 425-433. https://doi.org/10.3923/jbs.2018.425.433
72. THAKKAR, K.N., NAYAK, J., IYER, S.V, KUMAR, S., & PRASAD, A.K. Antioxidant and in vitro cytotoxic activity of extracts of aerial parts of Cocculus hirsutus (L) using cell line cultures (breast cell line). The Journal of Phytopharmacology (Vol. 3). Retrieved from www.phytopharmajournal.com
73. TIWARY, B.K., BIHANI, S., KUMAR, A., CHAKRABORTY, R., & GHOSH, R. "The in vitro cytotoxic activity of ethno-pharmacological important plants of Darjeeling district of West Bengal against different human cancer cell lines." *BMC Complementary and Alternative Medicine*. 2015, 15(1), 22. https://doi.org/10.1186/s12906-015-0543-5
74. TYC, O., TOMÁS-MENOR, L., GARBEVA, P., BARRAJÓN-CATALÁN, E., & MICOL, V. "Validation of the AlamarBlue® Assay as a Fast Screening Method to Determine the Antimicrobial Activity of Botanical Extracts." *PLOS ONE*. 2016, 11(12), e0169090. https://doi.org/10.1371/journal.pone.0169090
75. VAJRABHAYA, L. ONGTHONG, & KORSUWAN-NAWONG, S. "Cytotoxicity evaluation of a Thai herb using tetrazolium (MTT) and sulforhodamine B (SRB) assays." *Journal of Analytical Science and Technology*. 2018, 9(1), 1-6. https://doi.org/10.1186/s40543-018-0146-0
76. WAKURI, S., YAMAKAGE, K., KAZUKI, Y., KAZUKI, K., OSHIMURA, M., ABURATANI, S., … NAKAJIMA, Y. "Correlation between luminescence intensity and cytotoxicity in cell-based cytotoxicity assay using luciferase." *Analytical Biochemistry*. 2017, 522, 18-29. https://doi.org/10.1016/j.ab.2017.01.015
77. WEYERMANN, J., LOCHMANN, D., & ZIMMER, A. "A practical note on the use of cytotoxicity assays." *International Journal of Pharmaceutics*. 2005, 288(2), 369-376. https://doi.org/10.1016/j.ijpharm.2004.09.018
78. WHO. "Research Guidelines for Evaluating the Safety and Efficacy of Herbal Medicines." *Research Guidelines for Evaluating the Safety and Efficacy of Herbal Medicines*. 1993.
79. XIA, G.-H., CHEN, B.-A., SHAO, Z.-Y., LU, H.-X., KONSTANZE, D., & HARTMUT, D. "Mechanism of 2-methoxyestradiol-induced apoptosis in myelodysplastic syndrome MUTZ-1 cell line." *Zhongguo Shi Yan Xue Ye Xue Za Zhi*. 2007, 15(2), 296-301. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17493335
80. YAMASHITA, K., YAMADA, K., SUZUKI, K., & TOKUNAGA, E. "Noninvasive and safe cell viability assay for *Euglena gracilis* using natural food pigment." *PeerJ*. 2019, 7(4), e6636. https://doi.org/10.7717/peerj.6636
81. YIN, L.M., WEI, Y., WANG, Y., XU, Y.D., & YANG, Y.Q. "Long term and standard incubations of WST-1 reagent reflect the same inhibitory trend of cell viability in rat airway smooth muscle cells." *International Journal of Medical Sciences*. 2012, 10(1), 68-72. https://doi.org/10.7150/ijms.5256
82. YIP, D.K., & AUERSPERG, N. "The dye-exclusion test for cell viability: Persistence of differential staining following fixation." *In Vitro*. 1972, 7(5), 323-329. https://doi.org/10.1007/bf02661722