doi.org/10.1002/cbdv.202201098

REVIEW

CHEMISTRY & BIODIVERSITY

Cytotoxic Activity of Herbal Medicines as Assessed *in Vitro*: A Review

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Since time immemorial, human beings have sought natural medications for treatment of various diseases. Weighty evidence demonstrates the use of chemical methodologies for sensitive evaluation of cytotoxic potentials of herbal agents. However, due to the ubiquitous use of cytotoxicity methods, there is a need for providing updated guidance for the design and development of *in vitro* assessment. The aim of this review is to provide practical guidance on common cell-based assays for suitable assessment of cytotoxicity potential of herbal medicines and discussing their advantages and disadvantages Relevant articles in authentic databases, including PubMed, Web of Science, Science Direct, Scopus, Google Scholar and SID, from 1950 to 2022 were collected according to selection criteria of *in vitro* cytotoxicity assays and protocols. In addition, the link between cytotoxicity assay selection and different factors such as the drug solvent, concentration and exposure duration were discussed.

Keywords: cytotoxicity, herbal medicines, *in vitro*, protocol, techniques.

1. Introduction

Recently, there has been growing worry about the unwanted consequences of synthetic drugs and medicine because of their unpalatable side effects.[1,2] This necessitates the search for new classes of safe and effective medicines with known mechanisms of action.[3,4] Admittedly, natural components have shown more reliable and secure outcomes,[5] unless the toxicity and safety implications have been identified in humans.[6,7] Since time immemorial, human beings have sought medications to relieve pain and treat various disease conditions.[8,9] Weighty evidence demonstrates the use of medicinal plants up to 6,000 years ago.[10] Today, several drugs are extracted from medicinal herbs,[11,12] and the herbs are contributing to most of the medicinal process and daily food preparation in modern society. It is estimated that nearly 35,000 to 70,000 species from various families are valuable for these purposes.[13] Surprisingly, about 80% of the people in the African and Asian countries are in need of (wholly or partially) plant-based drugs.[14] In spite of the remarkable growth of traditional medicine in each society, herbal medicines have played a key role in treating disease,[15] and are known for their medicinal properties, including antimicrobial, antioxidant, anti-inflammatory, analgesic, antipyretic, antidiabetic, antihypertensive and anticancer effects.[16] In the past decades, the approaches to discovering new drugs have generally provided important information for determining the biological

activity and deriving structure-activity relationships (SAR), with drug efficacy tests, based on cell-based assays for cytotoxicity testing as a pre-clinical development, which is simple, economical and reliable.[17–20] The clinical trials are expensive; however, monolayer cultures are ideal, simple and controllable for the evaluation and screening of drug potency and give information about cytotoxicity and biological activity of components before execution of *in vivo* experiments.[21,22] *In vitro* cytotoxicity assays (CTAs) are implemented to evaluate the toxic potential of chemical and natural materials in cell culture models to help detect the ability of plant extracts to affect cell viability, cellular growth, and cell damage.[23,24] It can also provide valuable information on genotoxic and carcinogenic dispositions which reflect the complicated effects of compounds in herbal medicines.[25] The ability of herbal extracts and natural compounds to inhibit cellular viability, growth, proliferation and colony formation can be ascertained as an indication of cytotoxicity,[26] which is widely applied in drug discovery and basic research to screen and identify the toxic compounds and provide guidance for the design and development for *in vivo* assessment.[27–29] Natural compounds with cytotoxic effects can prevent cellular attachment, cause obvious morphological alterations, inhibit cell cycle and DNA replication leading to significantly decreased cell viability.[30,31] Toxicity as-

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says at the cellular level can be useful to reveal the potential risks associated with herbal extracts,[32] and also allow identification, analyses and test on a wide range of bioactive natural compounds. These efforts precede drug discovery and development of new anticancer agents, without adverse effects on normal healthy cells which is always limit the use of many anticancer drugs.[33–35] Cytotoxicity assays (CTAs) of herbal medicines are dependent on the concentration, mechanism of cytotoxicity, evaluation methods, cell type, components and exposure over time.[36–39] There are several methods for measuring cell sensitivity to herbal extracts, and cytotoxicity evaluation.[40,41] The use of standard test methods for cytotoxicity assessment could enable determination of concentration that may result in higher and lower toxicity.[42–44] *In vitro* cytotoxicity or cell viability assays provide many advantages and disadvantages, which are summarized in *Table 1*. Therefore, the current review provides practical guidance on common cell-based assays for researchers planning to assess the cytotoxicity potential of herbal medicine based on the standard methods published by the authorities in international organizations and research institutes.

Every year, studies expand on the chemical composition and properties of herbal medicines to assess the various interactions and molecular mechanisms which are associated with use of crude extracts, essential oils or compounds.[63–65] The main objectives of cytotoxicity and cell viability assays are to assess any potential of anticancer agents, and evaluate the deleterious side effects associated with herbal medicines which damage normal cells and organisms.[34,35] The cell-based cytotoxicity assays are widely used and

Table 1. General advantages and disadvantages of *in vitro* cytotoxicity or cell viability assays.

Advantages	Disadvantages
• The cost of *in vitro* testing is much lower than animal or usage testing[45]	• They do not provide enough valuable technical information to replace animal tests[46,47]
• Reduce the number of animal sacrifices during the toxicity assays of various compounds[48,49]	• Unable to account for metabolic responses or reactions[50]
• High precision of the response due to the lowest biological variation than *in vivo* systems[50]	• Cannot monitor the various behavioural responses.
• Can be versatile and well-controlled to address different scientific questions[51]	• The absence of inflammatory and different tissue-protective strategies[52]
• Bio-safety and fewer ethical issues[53]	• Cannot assess the drug pharmacokinetic effects[50]
• Rapid Investigation and screening[54]	• Lack of developed systems to study the interactions among the different cell types[55,56]
• Wide availability of various cell types[57]	• false positive or false negative test results[52,56]
• Well-suited assays that can be standardized for large-scale screening[58,59]	
• Using cell-based assays may be more relevant than *in vivo* experiments[60]	
• Assessment of toxic compounds simultaneously under controlled conditions[61]	
• Repetition[62]	

have several limitations, but the advantages outweigh the disadvantages. These assays are rapid,[54] simple and inexpensive[45] to evaluate the toxicity and cancer cell growth inhibition of herbal extracts, essential oils or purified compounds (*Table 1*).

2. Methods

In this review, the most relevant published articles and books were collected through a meticulous search on the electronic databases consisting of Web of Science, Scopus, Science Direct, PubMed, and Google Scholar. The search with different combinations of keywords was cytotoxicity assays, biological activity, and toxicity of herbal medicine, phytochemistry and *in vitro* assays. The articles and books published from 1950 to date were only searched and the inclusion was based on two sets of general and specific criteria. In general criteria, the books and articles were selected according to the *in vitro* cytotoxicity assays and protocols and the second set of criteria was used for selecting specific articles with cytotoxicity tests of herbal medicine whose advantages or disadvantages are discussed in details.

2.1. Parameters to Measure Cellular Response in Cytotoxicity Assays of Herbal Medicine

In vitro toxicity tests are an attempt in non-human experimental models[50,66] which are designed to assess the potential toxicity of compounds, herbal extracts, essential oils and other materials.[67,68] Cell-based assays are commonly used as platforms for evaluating cell viability, proliferation and cytotoxicity.[69] Various assessment methods have been used to measure viability or toxicity, and identifying the most appropriate cell health assay method can be a challenging task.[70,71] Therefore, in an attempt to improve the study quality and efficacy, choice of a suitable assay method for cytotoxicity assessment significantly contributes to determining the efficacy of a natural compound.[72–74] Different measurable parameters which are based on the determination of the total protein content, cell number or growth, reduction of vital dye, specific enzymatic release, vital dye release and other measurable parameters, which may be quantified as an indicator for cytotoxicity.[75,76] Different parameters can be effective to determine the cellular response to herbal extracts or natural compounds. When designing and performing cytotoxicity and cell viability assays, various factors including drug solvents, concentration of herbal extracts or natural compounds, drug exposure duration, optimization of cell-seeding density and assay timing have crucial roles in the reliability and reproducibility of results. In conducting cytotoxicity assays, solvent compatibility is extremely important in cell culture systems, where cells are exposed to different test compound concentrations.[77,78] Numerous organic solvents, without toxic effects on cells, are widely used for dissolving the hydrophobic compounds in different types of cell culture assays.[79–81] Therefore, in a preliminary assay, it is important to determine the maximum or minimum tolerable concentration for solvent on cell toxicity that can be used to prepare solutions of herbal extracts or other compounds.[82–85] Dimethyl sulfoxide (DMSO), ethanol and methanol are suitable solvents widely used as vehicles for drug delivery,[86] crude extraction, and dissolving various plant extracts for enhanced solubility in biological studies.[87] Many studies have reported that compounds in plant extracts have different polarities and can be prepared as solutions in polar and non-polar solvents.[88–90] There are several reports and evaluations that DMSO, ethanol, methanol and other organic solvents can be toxic at certain concentrations,[90–93] so, it is necessary to determine the concentration with little or no toxicity. In cytotoxicity evaluation, concentration and exposure time of tested herbal extracts, essential oils or natural compounds with different patterns must be considered by an experimental *in vitro* model. Drug exposure duration in testing for cytotoxic effects of herbal medicines will reveal the changes in cell responses over time, so in order to determine the cytotoxicity effect of compounds, the cultured cells should be treated with different concentrations of compounds at various times. The following parameters for a concentration of agent that reduces cells' vitality to half is observed as (CC_{50}), agent concentration which reduces cells' growth to half (GI_{50}), total growth inhibition (TGI), plating efficiency (PE), which is defined as the ratio of the number of colonies formed to the number of cells seeded of each condition; the surviving fraction (SF) is the number of colonies under treatment conditions expressed in terms of PE that must be determined in a reliable research.

Standardization plays a crucial role in cell-based assays and choosing the appropriate cell lines for cytotoxicity assay can influence the test performance to have an accurate assessment. The cell lines are selected according to the functional properties, available equipment, desired administration route, cytotox-

icity assay and applicable exposure conditions.[94,95] These parameters should be considered prior to initiating a cytotoxicity assay.[96] Three types of cells include primary cells, self-renewing cells and transformed cells (continuously grown cells or cell lines) are widely used in *in vitro* assays for biological assessments. Primary cells are cells isolated directly from human or animal tissues and have been used in translational and biomedical investigations.[95] Transformed cells or continuously grown cells are generated by genetic modification and have stable characteristics in the course of time, and can be used in drug development and cytotoxicity assay.[51] Other cell lines are self-renewing cells, which can be used for long term maintenance planning of *in vitro* assay.[97] Numerous cell lines including lung fibroblasts, Chinese hamster ovary (CHO) cells, canine renal cells, corneal epithelial cells, HeLa cells, and MCF-7 cells which have been widely used in cytotoxicity assays[98] should be chosen according to an assay protocols to have a good and well-conducted experiment design.

2.2. Methods for Viability and Cytotoxicity Evaluation of Herbal Medicine

There are a variety of methods for evaluation or assessment of cell sensitivity for herbal extracts, essential oils and compounds (*Figure 1*). The assays used to measure cell viability or cytotoxicity include the dye exclusion test, colorimetric assays, fluorometric assays and luminometric assays, and each assay has its own advantages and disadvantages (*Table 2*).

2.2.1. Dye Exclusion Assays

Dye exclusion assays are very common *in vitro* methods widely used to measure and estimate the number of viable and dead cells. In these assays, the plasma membranes of viable cells exclude the mentioned dye (unstained with the dye), while dead cells do not exclude them (stained with the dye), thus the proportion of live cells can be determined by these procedures.[135] Numerous staining techniques, including trypan blue, congo red, eosin-nigrosin and erythrosine B are used to perform these experiments

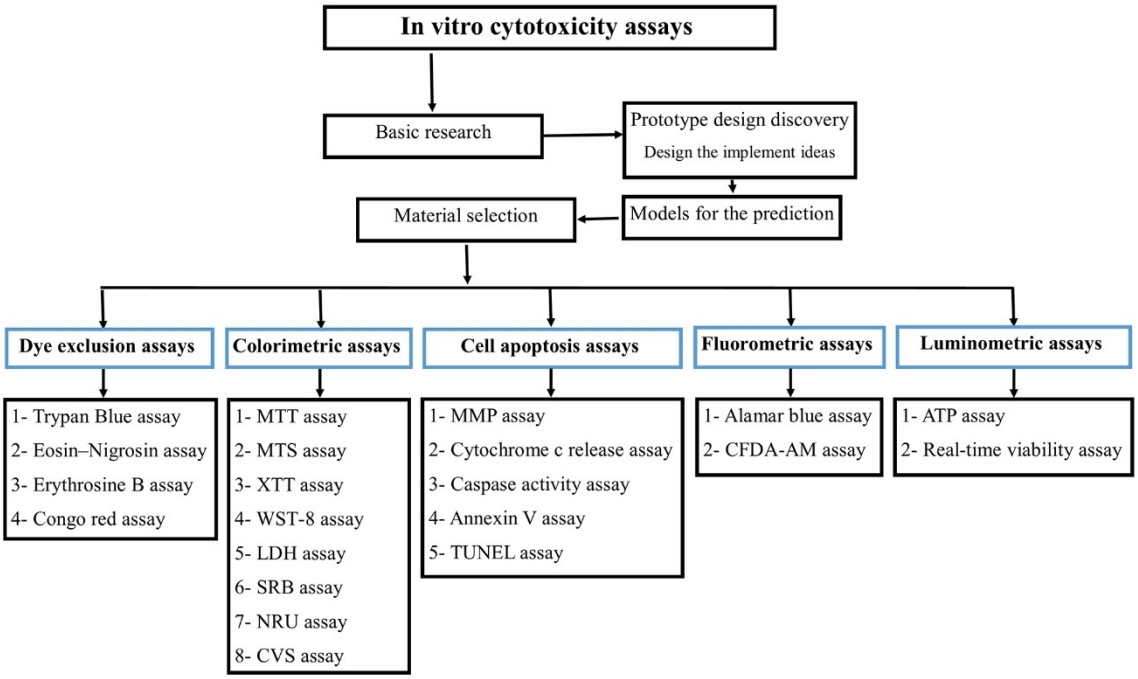

Figure 1. Methods for viability and cytotoxicity evaluation.

Table 2. The advantages and disadvantages of cell cytotoxicity and viability methods.

Methods	Advantages	Disadvantages
Dye exclusion assays		
Trypan blue	• Inexpensive and simple • Reliable indicators of membrane integrity[99] • Nonviable cells will appear within seconds after exposure[100]	• Errors may occur during cell counting[100] • Difficult to handle the large number of samples when cytotoxic effect over time is required (Time-consuming). • Differentiating between viable cells that lose their ability and functions, and healthy cells is impossible. • Toxic effect on cells[101]
Eosin-Nigrosin	• Easy[102] • cost effective[102]	• Time-consuming • Chemical staining components will affect the morphometric dimensions of sperm in long exposure time[103]
Erythrosine B, Congo red	• Affordability[101] • Adaptability[101] • Biosafety[101]	• Its process requires a lot of time and effort[101] • Contamination of the reusable cell counting chamber • Different hemocytometer fill rates[101]
Colorimetric assays		
MTT	• Safe and easy to use[104] • High reproducibility[104] • One of the best standard assay for cytotoxicity evaluation • Suitable for HTS (high-throughput screening)[105]	• Formazan crystal conversion depends on cellular metabolic activity and mitochondrial interference[106,107] • Require several washing steps[108,109] • Insoluble in water. • Organic solvent such as isopropanol or DMSO to solubilize the crystals is needed. • Because of needle-shaped MTT formazan crystals in the cell culture, removing the cell culture medium is difficult[100,110]
MTS	• Rapid Investigation and screening[111,112] • Sensitive and economic[111,112] • High precision[111,112] • Reliable[111,112] • Aqueous soluble formazan	• MTS reagents influence the absorbance level[113,114] • Several factors such as time, incubation, and cell type can influence the absorbance measurement[113,114]
Colorimetric assays		
XTT	• Easy to handle • Biosafety • High sensitivity and accuracy • Very fast	• Reductive capacity in viable cells including enzymatic regulation, variations of cell cycle, and other cellular and environmental factors affect the absorbance measurement[115]
WST-1	• Safe and easy to use[69] • High reproducibility • Produced dye is water-soluble and it does not need solvent	• Additional time, more than standard incubation will affect the measurement
WST-8	• Low cytotoxicity[111] • Formazan dye is water-soluble[111] • Easy to handle[69]	• The changes of intracellular metabolic activity effect on reduction[116] • Indirect effects on viable cells can affect the measurements
LDH	• Rapid and simple screening[117,118] • More relevant[117,118] • Distinguishing of growth inhibition and cell death is detectable[119]	• There are limitations to using compounds and serum since they have inherent LDH activity • The cell death occurrence is not detectable.

Table 2. (cont.)

Methods	Advantages	Disadvantages
Sulforhodamine B (SRB)	• Easy to use and sensitive[120] • Fast • High reproducibility	• There is low sensitivity through nonadherent cells[121]
Neutral Red Uptake (NRU)	• Widely used marker for lysosomal damage • Rapid screening The cellular enumeration is an independent process of enzymatic conversion of dye[118] • Easy to handle	• The assay is affected by pollutants and the interference of test compounds is reported[122] • There is not deference between cytotoxicity and cytostasis.
Crystal violet staining (CVS)	• Rapid • Versatile	• It is not suitable to assess the effect of compounds on cellular metabolic activity and this is appropriate technique to evaluate the effect of chemotherapeutics and other agents on cell viability[123]
Cell apoptosis assays		
Mitochondrial membrane potential	• Rapid Investigation and screening • Numerous potentiometric dyes are available. • Specific interaction which provides important information regarding cellular energy status[124]	• Isolation of adult and primary viable cells is impossible[125] • Various reagents and substrates are needed[125] • Unquantifiable detection[126] • Lack of clinical application[126]
Cytochrome c	• Help to identify the changes in the early stage of the intrinsic pathway of apoptosis[127]	• (Cc) is unstable when it is released into the cytoplasm[128] • The protein-protein interaction(between native proteins) may occur and make a change in fluorescent protein tag[127]
Caspase activity	• Reliable, simple and rapid screening[129] • Numerous antibodies are available. • It is possible to analyze the individual caspases signaling of apoptotic processes[130] • Efficient and economical[130] • Multiplatform assessment including laser scanning cytometer (LSC) and flow[131]	• Caspases involve in a nonlethal alternative functions. • Low specificity[129] • The caspases activation is not always defined as an apoptosis[129] • Substrate overlapping will affect the specificity of the assay[127] • It is not specific to a particular caspase[131]
TUNEL	• Fast and simple to perform[131] • It can be used to distinguish early apoptosis and necrotic[132] • High sensitivity[129] • Useful assay for assessment of high numbers of apoptotic cells[127]	• There is a risk of false-positives[131] • The results of this test can be confusing, since necrotic cells and homogenate preparation will produce DNA fragments • Required large samples to achieve accurate results • Expensive and time-consuming technique[129,131] • It is not a suitable assay with low apoptotic cell numbers[127]
Fluorometric assays		
Alamar blue	• Non-radioactive, simple assay for rapid assessment[133] • Non-toxic[133]	• Does not give information about cell counting like hemacytometer
CFDA-AM	• Appropriate for parallel assay on the same cell lines • Non-toxic[134]	• Fluorescent compounds may interfere in assay

although with inherent advantages and disadvantages (*Figure 2*).[136,137] Dye exclusion tests are simple and economical but require many difficult and time-consuming steps in their experimental procedures to cover a large number of samples.[135] Several factors which play a significant role in the determination of cell viability should be considered to have an accurate and valid results. In dye exclusion assays, cellular responses to cytotoxic agents would require more time for loss of membrane integrity in damaged cells, so dyes do not interact with injured cells unless the cell membrane is disrupted. Also, proliferation of surviving cells may continue through the test. Furthermore, culture period plays a key role in dye exclusion tests, and the lethally injured cells do not appear in cellular disintegration phase.[138–140] Dye exclusion assays have a valuable role in *in vitro* chemosensitivity testing and these assays are capable of recognizing the kill target cells in non-dividing cell populations.[140]

Trypan blue, as an inexpensive and simple method, is reliable indicators of membrane integrity, but is not suitable for large number of samples when cytotoxic effect over time is required because it will be time-consuming and has toxic effect on cells. Also, this method is not suitable for experiments where the number of cells with normal activity is important because differences between viable cells that lose their ability and functions, and healthy cells is impossible.[99–101] Although eosin-nigrosin is an easy and cheap method, it is a time-consuming method and its chemical compounds affect morphometric dimensions of the cell in long exposure time. Therefore, when the morphometric dimensions of the cell are important, the cells should be exposed to eosin-nigrosin in the least possible time.[102,103] However,

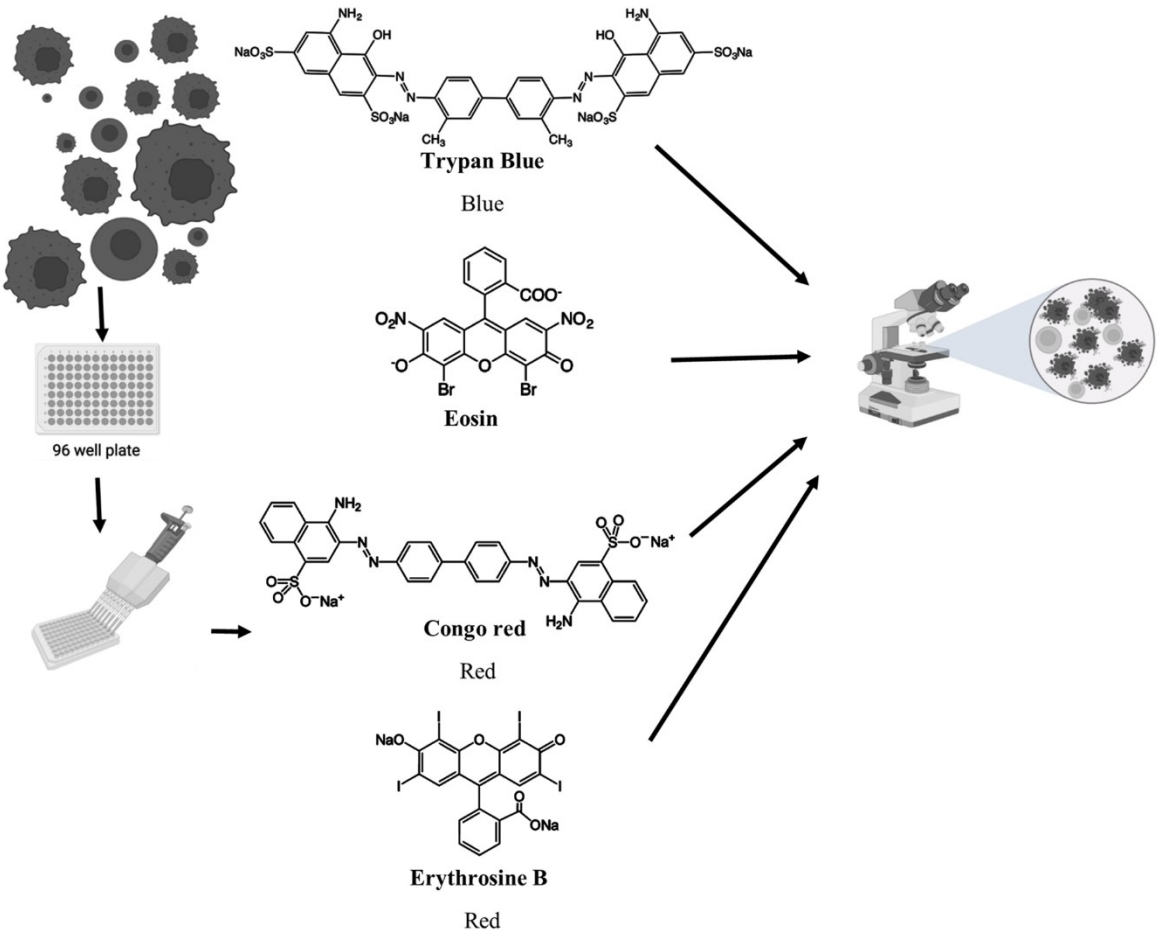

Figure 2. Dye exclusion assays to measure the number of viable and dead cells, the picture was created with BioRender (https://biorender.io).

erythrosine B and congo red are time-consuming and laborious process, but they are safe for cells.[101]

2.2.1.1. Trypan Blue Assay

Trypan blue stain assay was developed in 1975 to assess the cell viability in suspension culture, which has been widely used to determine potential toxic effects of agents and compounds for identifying dead cells.[141] Trypan blue is a dye with a highly negatively charged nature which only stains the non-viable cells with damaged membranes.[100] In this assay, the viable cells with intact cell membranes exclude the trypan dye stain, whereas dead (non-viable) cells with damaged membrane do not exclude the trypan blue stain. There are five main general steps to completing this assessment, and each one must be done carefully. To perform this assay, suspension (non-adherent cells) or adherent cells are incubated with serial dilutions of each tested agent for various times, and to assess cell viability, the phosphate-buffered saline (PBS) or serum-free complete medium and 0.4% trypan blue solution are prepared, then the cell suspension in different tested groups is centrifuged at 100 × g for 5 min to discard the supernatant. Each pellet obtained is dissolved in 1 ml PBS. In the next step, mix the one-part cell suspension and one part of 0.4% trypan blue stain and allow to stay for 3 min at room temperature. Counting cells should be done within 3–5 min after mixing trypan blue with cell suspension to encourage accurate result. Longer exposure time to trypan blue will induce cell death. To count the number of viable and dead cells, place a drop of the mentioned mixture on a hemocytometer under a binocular microscope.[142] Because of losing membrane protein selectivity, trypan blue is absorbed into the cytoplasm of dead cells and makes them blue, while live cells have a clear cytoplasm since the viable cells exclude the dye.[142,143] To calculate the cell viability percentage of the trypan blue assay, divide the total number of unstained or viable cells per milliliter to the total number of viable and nonviable cells, and multiply by 100, with the following equation:

$$\text{Cell viability } (\%) = [(\text{Total number of viable cells})/(\text{Total number of viable and nonviable cells})] \times 100 \quad (1)$$

2.2.1.2. Eosin-Nigrosin Assay

A staining technique to assess sperm vitality is the eosin-nigrosin assay, which was developed in 1975,[144] and also this fluorescent red dye can be used to stain the muscle fiber, red blood cells, collagen and cytoplasm,[145] and facilitate the visualization under optical microscopes to determine the cell viability.[146] Eosin is used to measure the live and dead cells, and penetrate through the unviable cells with damaged membranes and change the color of dead cells to dark pink, while viable cells exclude the eosin and are seen clearly and white in color. This assay has been widely used to determine sperm vitality when sperm motility percentage is lower than 25%.[147,148] The nigrosin component stains the dead sperm to a dark pink color and make them visualized. Also, it can be used to analyse and evaluate the sperm size and shape. Eosin-nigrosin assay should be done consistently after the sperm motility assessment, on the same semen sample. To perform these assays, prepare the reagents including Eosin Y 1% (add 0.5 g of Eosin Y to 50 ml of deionized water) and 10% nigrosin (add 5 g of nigrosin to 50 ml of deionized water), then mix 1 drop of collected semen and 2 part of eosin 1% on a Boerner slide well and mix it by wooden stirrer for 15 seconds at room temperature. In the next step, add 2 drops of 10% nigrosin to the mentioned mixture in the Boerner slide well and mix it again with wooden stirrer.[149] Then, put a drop of this mixture on a hemocytometer under a light microscope and count the live and dead sperm (cells) and calculate the cell viability percentage with the following equation:

$$\text{Cell viability } (\%) = [(\text{Total number of viable cells})/(\text{Total number of viable and nonviable cells})] \times 100 \quad (2)$$

2.2.1.3. Erythrosine B and Congo Red Assays

Erythrosin B stain is a non-toxic vital dye which is widely used to measure cell viability and it can be used as a food-coloring additive.[150] Similarly, congo red is a direct diazo dye which was discovered by Paul Bottiger in 1883 and developed in 1885.[151] Congo red assay has been used to assess cell viability and stain cytoplasm of the non-viable cells. Membrane integrity plays a crucial role in these assays to assess the cell viability, so that erythrosin B and congo red assays are dependent on cell membrane integrity, similar to

trypan blue assay and stain the cells with damaged membrane. To perform these assays, prepare the reagents which include 1% erythrosine B and 1% congo red and PBS. Then the cell suspension in different test groups is centrifuged at 100 × g for 5 min, then discard supernatant and each pellet is dissolved in 1 ml PBS. In the next step, mix one-part cell of suspension and one part of 1% erythrosine B or 1% congo red and allow to stay for 3 min at room temperature. To get the most accurate result, counting cells should be done within 3–5 min after mixing the mentioned dyes with cell suspension since longer exposure time will induce cell death and reduce accuracy. To count the number of viable and dead cells, place a drop of the mentioned mixture on a hemocytometer under a binocular microscope, and calculate the cell viability percentage of these assays, according to the following equation:

$$\text{Cell viability } (\%) = [(\text{Total number of viable cells})/(\text{Total number of viable and nonviable cells})] \times 100 \quad (3)$$

Furthermore, congo red binds to amyloid and we can use this assay in antiglycative activity assessment. To perform this assay, prepare the reagents and mix the five parts of glycated samples and one part of congo red and allow them to stay for 20 min at room temperature and measure the absorbance at 530 nm.[152] Congo red binding assay is commonly used to determine the levels of modification in secondary protein structure. Congo red has a particular affinity for hydrophobic clefts of β-sheet structure, and after binding, has a strong absorbance at 530 nm. Thus, the assay assesses the protective property of a compound in the secondary structure of protein.

2.2.2. Colorimetric Assays

Colorimetric assays are widely used to evaluate cellular viability and to identify the effective or toxic substances in drug development process.[153] NADH and NADPH ratio illustrate the cellular metabolic activity. The reaction of biochemical markers including MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide), MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), XTT (2,3-bis (2-methoxy-4-nitro-5-sulphophenyl) −5-carboxanilide-2H- tetrazolium monosodium salt), WST1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium monosodium salt), WST8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt), LDH (lactate dehydrogenase), SRB (sulforhodamine B), NRU (Neutral Red Uptake) and CVS (crystal violet staining) as the enzymatic reduction compounds can be used in cytotoxicity examinations (*Figure 3*). The reduction of these mentioned compounds in colorimetric assays will result in color change which can be determined by spectroscopic methods.[154]

MTT, MTS, XTT, WST-1 and −8, LDH, SRB, NRU and CVS are easy to use with high reproducibility. While the cytotoxic MTT has to be dissolved in DMSO, the other above assays are water-soluble. MTT is one of the best standard assays for cytotoxicity evaluation and is suitable for HTS high-throughput screening, but because of needle-shaped MTT formazan crystals in the cell culture, removing the cell culture medium is difficult.[100,110] In LDH assay, distinguishing of growth inhibition and cell death is detectable. But there are limitations to using this method for compounds and serum since they have inherent LDH activity.[119] SRB has low sensitivity through nonadherent cells; thus, this method is not suitable for evaluation of these cells.[121] NRU widely used marker for lysosomal damage. However, CVS is not suitable to assess the effect of compounds on cellular metabolic activity, it is appropriate technique to evaluate the effect of chemotherapeutics and other agents on cell viability.[123]

2.2.2.1. MTT Assay

MTT test is the most commonly used assay developed in 1983.[109] It was adapted for chemosensitivity assessment in 1986.[155] This assay relies on the conversion of tetrazolium component (MTT) into formazan crystals by some specific enzymes in the mitochondria of viable cells thus exhibiting the mitochondrial function or dysfunction in cells. Also, MTT assay has been used to evaluate toxicity and proliferation in drug development studies. Tetrazolium salts are commonly soluble and colorless compounds which are reduced by mitochondrial dehydrogenase enzymes in viable cells and change into insoluble formazan crystals. Solubilization of the formazan crystals plays a significant role in MTT assay; various solubilization solutions such as dimethyl sulfoxide (DMSO), acidified isopropanol, sodium dodecyl sulfate (SDS), acidified ethanol solution, dimethylformamide (DMF) are used to dissolve the insoluble formazan crystals into a soluble colored product followed by absorbance determination for cell viability.[156–158] MTT solution is prepared by dissolving

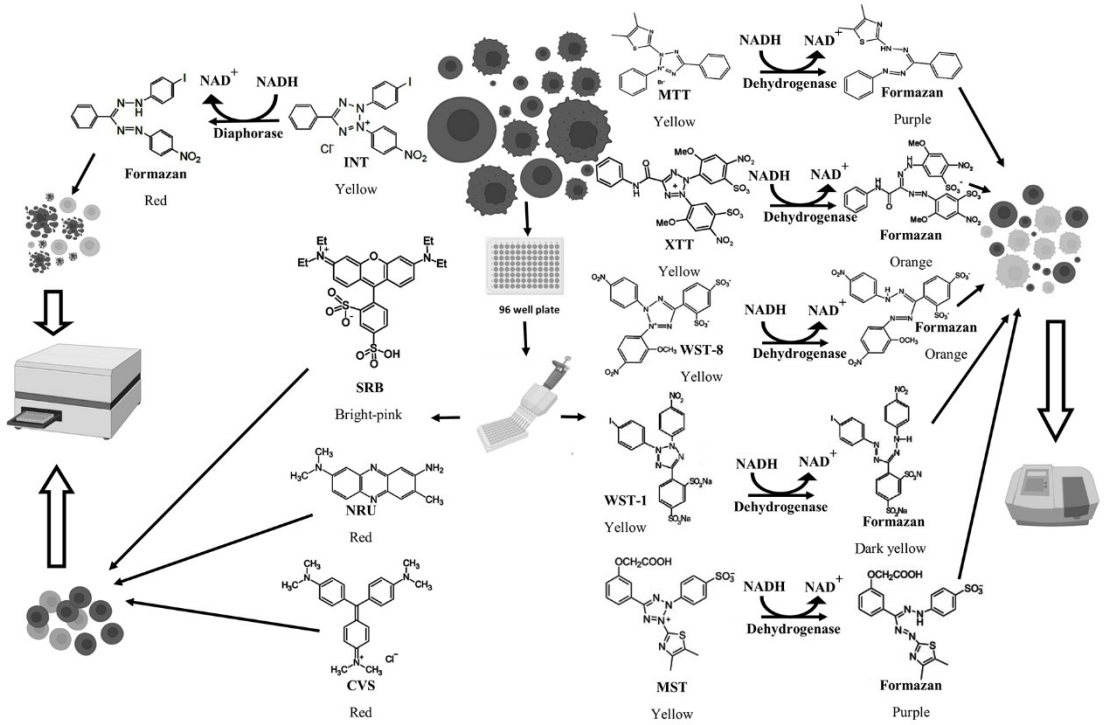

Figure 3. Colorimetric assays to measure the number of viable and dead cells, the picture was created with BioRender (https://biorender.io).

5 mg of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-tetrazolium bromide) in 1 ml of Dulbecco's phosphate-buffered saline (DPBS) and pass the solution through a 0.2 μm filter into a sterile and light-resistant container. The MTT solution is stored in light-protected place at −20 °C until analysis (stable for at least 6 months) or for immediate use it is stored at 4 °C (stable for one or two days). In the next step, under the fume hood, prepare 40% (v/v) DMF to 2% (v/v) glacial acetic acid, then add 16% (w/v) SDS and adjust pH to 4.7 to use as a solubilization solution. To prevent or reduce SDS precipitation, solubilization solution is stored at room temperature. To perform this assay, use seeded cell suspensions into 96-well plates (at a concentration of 5×10^4 cells/100 μl/well) in different tested groups which are incubated in a humidified atmosphere with 5% CO_2 at 37 °C. In the next step, add 10 μl of prepared MTT solution to each well (5 mg/ml concentration in each well) and incubate for 3–4 h at 37 °C, and solubilize the formazan crystals in 100 μl of solubilization solution and measure the absorbance by microplate reader at 570 nm. To calculate the cell viability percentage of the MTT assay, use the following equation:

$$\text{Cell viability } (\%) = \left[\frac{\text{Mean OD}_{\text{sample}}}{\text{Mean OD}_{\text{blank}}} \right] \times 100 \qquad (4)$$

2.2.2.2. MTS Assay

MTS, a colorimetric assay, is very simple and widely used in response to compounds and agents from various sources to evaluate cell cytotoxicity and viability. Reduction of MTS tetrazolium compound or 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymeth-oxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium to soluble purple formazan dye in the presence of phenazine methosulfate (PMS), is done by NAD(P)H-dependent oxidoreductase enzymes in mitochondria of viable cells.[159] In this assay, a group of tetrazolium reagents which include PMS (phenazine methyl sulfate) or PES (phenazine ethyl sulfate) have been used to eliminate solubilization step. These compounds can penetrate

through the cell membrane and convert tetrazolium to a soluble formazan product.[160] To prepare the MTS solution, add 2 mg/ml of MTS powder to dulbecco's phosphate-buffered saline (DPBS) and dissolve it to have a clear yellow solution, then dissolve the 0.21 mg/ml of polyethersulfone (PES) in MTS solution and add 1 N HCL to adjust PH on 6.0–6.5, in the next step, filter the solution by a 0.2-μm filter and transfer them into a sterile and light-resistant container, then store the MTS solution in the light-protected place, at −20 °C until analysis (stable for at least 6 months) or for immediate use store it at 4 °C (stable for one or two days). To perform this assay, use seeded cell suspensions into 96-well plates (at a concentration of 5×10^4 cells/100 μl/well) in different tested groups which are incubated in a humidified atmosphere with 5% CO_2 at 37 °C. In the next step, add 20 μl of prepared MTS solution to each well (0.33 mg/ml concentration in each well) and incubate for 1–4 h at 37 °C, and measure the absorbance by microplate reader at 570 nm. To calculate the cell viability percentage of the MTS assay, use the following equation:

$$\text{Cell viability } (\%) = \left[\frac{\text{Mean OD}_{\text{sample}}}{\text{Mean OD}_{\text{blank}}} \right] \times 100 \quad (5)$$

2.2.2.3. XTT Assay

XTT assay is used to assess the cellular viability and cytotoxicity in toxicological evaluations. XTT was synthesized in 1988 and introduced as a colorimetric assay which is based on the bio reduction of yellow tetrazolium salt XTT, in the active mitochondria of viable cells, to orange-colored formazan, which is a soluble product in aqueous solutions, in comparison with other tetrazolium salts.[115] There is a direct link between mitochondrial dehydrogenases activity and number of living cells which can be measure by the absorbance of formazan product in XTT test.[161] To prepare the XTT solution, for immediate use, add l mg/ml of (2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-carboxanilide-2H-tetrazolium, monosodium salt) or XTT in 5 μl/ml PMS (phenazine methyl sulfate). To perform this assay, use seeded cell suspensions into 96-well plates (at a concentration of 5×10^4 cells/100 μl/well) in different tested groups which are incubated in a humidified atmosphere with 5% CO_2 at 37 °C. In the next step, add 100 μl of prepared XTT solution to each well (0.3 mg/ml concentration in each well) and incubate for 4 h at 37 °C, and measure the absorbance by microplate reader at 450 nm and a reference wavelength at 650 nm. To calculate the cell viability percentage of the XTT assay, use the following equation:

$$\text{Cell viability } (\%) = \left[\frac{(A_{450} - A_{650}) \text{ of test cells}}{(A_{450} - A_{650}) \text{ of control cells}} \right] \times 100 \quad (6)$$

2.2.2.4. WST-1 Assay

WST-1 is a highly simple and fast assay developed in 1993 and used to evaluate cell viability and cytotoxicity. This is based on bioreaction of tetrazolium salt WST-1 to colored formazan, by the action of mitochondrial dehydrogenase enzymes. Formazan in this assay is a highly water-soluble and less toxic product, in comparison to XTT salt.[111] To prepare the reagent, mix 5 mM of WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium monosodium salt) to 0.2 mM 1-methoxy phenazine methosulfate (PMS), then add 12.5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer and adjust PH to 7.0. Keep it in a light-protected place. In the next step use seeded cell suspensions into 96-well plates (5×10^4 cells/100 μl/well) in different test groups and incubate in a humidified atmosphere with 5% CO_2 at 37 °C. In the next step, add 10 μl of prepared WST-1 solution to each well and incubate for 2–4 h at 37 °C, and measure the absorbance by microplate reader at 450 nm. Cell viability percentage of WST-1 assay is calculated following the equation:

$$\text{Cell viability } (\%) = \left[\frac{\text{Mean OD}_{\text{sample}}}{\text{Mean OD}_{\text{blank}}} \right] \times 100 \quad (7)$$

2.2.2.5. WST-8 Assay

The synthesis of WST-8, as a second-generation of tetrazolium was done in 1999. This assay is widely used to evaluate cell proliferation and cytotoxicity in many research projects. The principle involves the reduction of WST-8 compound or (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt) to orange-colored formazan which is a soluble product in aqueous solution.[111] The necessary electron carriers of WST tetrazolium extracellular reduction are prepared by electron acceptors including phenazine ethyl sulfate

(PES) and 5-methyl-phenazinium methyl sulfate (PMS).[114] To prepare the WST-8 solution for immediate use, add 5 mM of WST-8 in 0.2 mM 1-methoxy phenazine methosulfate (PMS) and mix it, then add 150 mM NaCl. Use seeded cell suspension in 96-well plates (5×10^4 cells/100 µl/well) in different test groups. Incubate in a humidified atmosphere with 5% CO_2 at 37 °C. Then, add 10 µl of prepared WST-8 solution to each well and incubate for 2–4 h at 37 °C, and measure the absorbance by microplate reader at 450 nm. Cell viability percentage of the WST-8 assay is calculated using the following equation:

$$\text{Cell viability } (\%) = \left[\frac{\text{Mean OD}_{\text{sample}}}{\text{Mean OD}_{\text{blank}}} \right] \times 100 \quad (8)$$

2.2.2.6. LDH Assay

The Lactate dehydrogenase (LDH) release test was developed in 1980.[117] It is a rapid and simple colorimetric technique to assess cytotoxicity in different cell types with plasma membrane damage. Losing of membrane integrity in damaged cells release LDH cytoplasmic enzyme that is used as a catalyst and causes the oxidation-reduction by nicotinamide adenine dinucleotide (NADH) to produce NAD^+ (LDH oxidizes lactate to pyruvate), which reduces the yellow tetrazolium salt (INT) to a red-color formazan. The formazan is a soluble product in aqueous solution.[162] In the first step of this assay, prepare lysis solution (9% (v/v) of Triton X-100), substrate solution (mix 0.28 M of 1-methoxy phenazine methosulfate (PMS) and 0.054 M of L-(+)-lactic acid and add 1.3 mM of β-NAD^+ to solution then add 0.66 mM of INT (iodonitrotetrazolium or 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium) to this mixture and dissolve them in 0.2 M Tris-HCl buffer (pH 8.2), and stop solution (50% DMF and 20% SDS and adjusting the pH to 4.7 or use 1 N HCl as a stop reaction). These solutions must be prepared for immediate use. To perform this assay, use seeded cell suspensions into 96-well plates (5×10^4 cells/100 µl/well) in different test groups. Incubate in a humidified atmosphere with 5% CO_2 at 37 °C. Add 15 µl cell lysis solution to each well and centrifuge the plate at 250 × g for 4 min. In the next step, transfer 50 µl of supernatant to optically clear 96-well plate and add 50 µl substrate solution. Incubate the plate in a humidified atmosphere with 5% CO_2 at 37 °C for 20–30 min. After that, add 100 µl of stop solution to each well and incubate it in a sterile and light-resistant box for up to 30 to 60 min at room temperature and measure the absorbance at 490 nm, with a plate reader. To calculate the cell cytotoxicity percentage of the LDH assay, use the following equation:

$$\text{Cell cytotoxicity } (\%) = \left[\frac{\text{Experimental LDH release (OD490)}}{\text{Maximum LDH release (OD490)}} \right] \times 100 \quad (9)$$

2.2.2.7. Sulforhodamine B (SRB) Assay

Sulforhodamine B (SRB), a colorimetric assay developed in 1990,[120] is a sensitive and rapid technique to assess cytotoxicity in response to compounds and agents from various sources. SRB is an anionic dye with a bright pink color that has two sulfonic groups which electrostatically bind to the amino-acids of cellular proteins fixed with trichloroacetic acid (TCA) within mild acidic condition. The dissolving step of protein-bound dye is done by the Tris base [tris (hydroxymethyl) aminomethane] and the absorbance can be measured at 510 nm with plate reader.[163,164] Trichloroacetic acid solution (10% concentration), staining solution, 0.057% (wt/vol) of SRB and 1% (v/v) of acetic acid are used to perform this assay. The dissolving solution is prepared with 10 mM unbuffered Tris base solution. In the assay, use seeded cell suspensions into 96-well plates (5×10^4 cells/100 µl/well) and dissolve the dried sample in 10% of DMSO in different test groups, and incubate the tested plates in a humidified atmosphere with 5% CO_2 at 37 °C for 72 h. Afterward, for cell fixation step, add 100 µl of 10% (wt/vol) cold TCA to each well of plates and place them in the incubator for 1 h at 4 °C (it's important to use the exact concentration of TCA and accurate incubation period, since higher or lower concentration will reduce the cell attachment).[164] Use slow-running tap water which is connected to plastic tubing in the faucet, and all test plates should be washed four times. Then use paper towels and blow dryer to remove any excess water. It is noteworthy that direct injection of water into the wells will detach the cells and can increase error rates). After that, add 100 µl of SRB with 0.057% (w/v) concentration and allow it to stay for 30 min at room temperature. Then, remove the unbound dye from the 96-well plates by quickly washing the plates with 1% acetic acid solution, and dry the plates with a blow dryer and allow them to stay at room temperature to dry completely. In the next step, to solubilize the SRB stained cells, add 200 µl of 10 mM unbuffered Tris base solution to 96-well plates

and shaking for 5 min, by gyratory shaker to dissolve protein-bound SRB dye in the solution and measure the absorbance at 510 nm, with plate reader. To calculate the cell growth percentage and growth inhibition percentage of the SRB assay, use the following equations:

$$\text{Cell growth } (\%) = \left[\frac{\text{Mean OD}_{\text{sample}}}{\text{Mean OD}_{\text{blank}}} \right] \times 100 \quad (10)$$

$$\text{Growth inhibition } (\%) = 100 - \text{cell growth} \quad (11)$$

2.2.2.8. The Neutral Red Uptake (NRU) Assay

The Neutral Red Uptake (NRU) assay was developed in 1985 and is widely used to assess cell viability.[59] In this colorimetric assay, the viable cells are able to uptake and bind with neutral red (NR) in the lysosomes.[165] The neutral red (NR) or (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) is a cation dye that has an ability to penetrate the cell membrane of healthy cells through non-ionic diffusion resulting in the dye accumulation in the lysosomes and change the color from orange-red to dark red.[166] Due to the alterations in cell membrane properties of nonviable cells, they cannot be able to uptake the NR. Therefore, this assay will make a clear distinction between living and dead cells, according to different uptake of NR. To prepare the working solution of NR assay, use PBS and make the neutral red staining solution with 40 μg/ml concentration (40 μg neutral red in 10 ml PBS) for storage at light protected place for two months at 20–30 °C. In the next step, prepare neutral red (NR) destain solution (50% ethanol 96%, 1% glacial acetic acid and 49% deionized water). However, it is optional to prepare the 5% of glutaraldehyde for fixation purposes. To perform this assay, use seeded cell suspensions into 96-well plates (5×10^4 cells/100 μl/well) in different test groups which are incubated in a humidified atmosphere with 5% CO_2 at 37 °C. Incubate overnight the neutral red staining solution with 40 μg/ml prepared at the last step (reagent preparation) at the same temperature of cells, and after overnight incubation centrifuge the neutral red staining solution at 600 g for 10 min and remove the precipitated crystals. Afterward, remove the cell culture medium and 100 μl of neutral red staining solution is added to each well of plates. Then, incubate the plate in a humidified atmosphere with 5% CO_2 at 37 °C for 2 h. In the next step, remove the neutral red staining solution and wash the cells with 150 μl PBS by gentle tapping. It is recommended to do the fixation step and add glutaraldehyde with 5% concentration to each well and allow them to stay for 2 min before starting the washing step).[165] Then, to extract the bound dye (NR dye) from the viable cells, add a 150 μl NR destain solution to each well, and shake the plates using the laboratory Microplate Shakers for 10 min to produce a homogeneous mixture. Afterward, measure the absorbance at 540 nm with a plate reader, and to calculate the cell viability percentage of the NRU assay, use the following equation:

$$\text{Cell viability } (\%) = \left[\frac{\text{Mean OD}_{\text{sample}}}{\text{Mean OD}_{\text{blank}}} \right] \times 100 \quad (12)$$

2.2.2.9. Crystal Violet Staining (CVS) Assay

Crystal violet staining (CVS) assay is a colorimetric test developed in 1989 and is used for measuring cell cytotoxicity and investigating the changes of cells in drug development research.[167,168] The crystal violet staining assay can bind to proteins and DNA which specifically interact with nucleo-histones of viable cells so that there is a direct link between nucleoproteins reduction and decreased number of viable cells measured by CVS assay.[123,169] To prepare the 0.5% crystal violet solution, add crystal violet powder to 30% methanol and dissolve it at room temperature.[167,170] Afterward, to perform this assay, use seeded cell suspensions into 96-well plates (5×10^4 cells/100 μl/well) in different test groups which are incubated in a humidified atmosphere with 5% CO_2 at 37 °C, and also in order to prevent nonspecific binding of CVS, wells without cells as negative controls are considered. In the next step, remove the cell culture medium and use slow-running tap water which is connected to plastic tubing in the faucet, and all tested plates should be washed two times, then add 50 μl of 5% CVS solution to each well of plates and incubate at room temperature for 15–20 min. Afterward, the plate is washed with slow-running tap water and remove the unbound CVS from the plate. Use a blow dryer, and allow it to stay at room temperature for 1 h to dry completely. Then add 200 μl of methanol to each dried well, and incubate the plate at room temperature for 15–20 min on a bench rocker with a frequency of 20 oscillations. After that, measure the absorbance at 570 nm with a plate reader and calculate the cell viability percentage of the CVS assay, with the following equation:

$$\text{Cell viability } (\%) = \left[\frac{\text{Mean OD}_{\text{sample}}}{\text{Mean OD}_{\text{blank}}} \right] \times 100 \qquad (13)$$

2.2.3. Cell Apoptosis Assays

Apoptosis is a genetically programmed process of cell death known as physiological cell suicide introduced in 1988.[171] Numerous cell changes associated with complex pathways play a crucial role in apoptosis and can be characterized based on morphological indicators and biochemical markers. A variety of cellular apoptosis assays, including mitochondrial membrane potential assay, cytochrome c release assay, caspase activity assay, annexin V assay, and TUNEL assay (DNA fragmentation) have been designed to measure cell apoptosis. There is a direct link between the selection of a suitable assay and pathways of apoptotic cell which can help to find the appropriate assay and pick the best markers to measure apoptosis.

Mitochondrial membrane potential assay is specific interaction which provides important information regarding cellular energy status,[124] but it cannot isolate of adult and primary viable cells.[125] While, Cytochrome c helps to identify the changes in the early stage of the intrinsic pathway of apoptosis, the protein-protein interaction between native proteins may occur and make a change in fluorescent protein tag.[127] However, the caspases activation is not always defined as an apoptosis,[129] caspase activity can be used to analyze the individual caspases signaling of apoptotic processes.[130] TUNEL can be used to distinguish early apoptosis and necrotic,[132] and is useful assay for assessment of high numbers of apoptotic cells,[127] but it has a risk of false-positives,[127] and the results of this test can be confusing, since necrotic cells and homogenate preparation will produce DNA fragments.[127]

2.2.3.1. Mitochondrial Membrane Potential Assay

Mitochondria play a significant role in generating the cell's primary energy carrier through cellular respiration.[172] Cellular health represents the normal function and structure of mitochondria membrane potential (MMP) and it's an essential parameter in living cells; thus, monitoring changes in MMP reflects the mitochondrial dysfunctions which have been related to intrinsic and extrinsic stress, toxicity through different mechanisms. The toxic compounds affect the mitochondrial functions and reduce the MMP leading to cell death.[173] Therefore, mitochondrial membrane potential assay can be used in evaluation of toxic compounds in cell-based *in vitro* cytotoxicity studies. There are numerous fluorescent dyes including rhodamine-123 (Rh123), JC-1, 3, 3′-dihexyloxacarbocyanine iodide [DiOC6(3)] and tetramethyl rhodamine methyl and ethyl esters (TMRM and TMRE), which have been used for assessment of changes in MMP. These fluorescent dyes are cationic, pH-dependent, and accumulate in the electronegative interior of healthy mitochondria.[174] To perform this assay, at the first step, add 1 mg of JC-1 powder to 200 µl of DMSO, then vortex the solution and allow it to stay at room temperature for 15–20 min to have a homogeneous stock solution (200× JC-1 stock solution). In the next step, use seeded cell suspensions into 96-well plates (5×10^4 cells/100 µl/well) in different test groups which are incubated in a humidified atmosphere with 5% CO_2 at 37 °C. After that, add 2 µl of JC-1 stock solution to each well and incubate the plates in a humidified atmosphere with 5% CO_2 at 37 °C for 15–30 min, and for the positive control use the carbonyl cyanide 3-chlorophenylhydrazone (CCCP) with 50 µl final concentration. In the next step, remove the cell culture medium and add warm PBS to wash the cells. Afterward, 100 µl of warm PBS is added to each well of plates and they keep the plates in a sterile and light-resistant container foil, then measure and analyze the fluorescence of these test plates using an envision plate reader. JC-1 is a green fluorescent cationic dye which generates the J aggregate transport into the mitochondria and produces the green and red fluorescence which can be measured for green fluorescent emission at 520–540 nm and for red fluorescent aggregates 570–590 nm.

2.2.3.2. Cytochrome c Release Assay

Cytochrome c is a small, soluble mitochondrial protein which is located in the intermembrane space (IMS) of mitochondria that plays a significant role in electron transport from cytochrome bc1 (Cbc1)-complex to cytochrome oxidase (CO) on the surface of the mitochondrial inner membrane. It has an undeniable role in cellular metabolism and apoptosis.[175] In the process of programmed cell death, cytochrome c is released into the cytosol by various proapoptotic factors.[176,177] The cytochrome c interaction with Apaf-1 (apoptotic protease activating factor-1), proteolytic processing maturation of death protease caspase-3 and caspase-9 are initiated. Two mechanisms for releasing apoptogenic factors in mitochondrial perme-

ability transition pore (MPTP) are proposed.[178,179] The first one is forming pores in the outer mitochondrial membrane (OMM), which is recruited by the regulation of the Bcl-2 family of proteins,[180] and the second mechanism initiated by rupturing the outer mitochondrial membrane (OMM) and releasing the components from intermembrane space.[178] To perform this assay, prepare the reagents and 250 μl of DMSO is added to the 500X protease inhibitor cocktail and dissolves it well. Then, to prepare the mitochondria extraction buffer, 1 μl of Dithiothreitol (DTT) and 2 μl of protease inhibitor cocktail is added to the mitochondria extraction buffer and mixed with the solution to have a homogeneous mitochondria extraction buffer. In the next step, the 5X cytosol extraction buffer is to be diluted 1X buffer by adding ddH_2O, and to prepare the cytosol extraction buffer for assay, 2 μl of protease inhibitor cocktail is added to 1 μl of DTT and mixed thoroughly. Then add 1 ml of 1X cytosol extraction buffer to the mixture and dissolve thoroughly. To evaluate the apoptotic activities of a compound, seed the cell suspension at the desired concentration and induce apoptosis in cells. In the next step, the cell suspension in different tested groups is centrifuged at 600 × g for 5 min at 4 °C, and cells are collected. Then, cells are washed by adding 10 ml of ice-cold PBS. Afterward, cells are centrifuged at 600 × g for 5 min at 4 °C, and discard supernatant. In the next step 1 ml of 1X cytosol extraction buffer is added to cells and incubated on the crushed ice for 10 min. In the next step, cells are homogenized by using an ice-cold Dounce tissue grinder. Afterward, centrifuge the cell homogenate at 700× g for 10 min at 4 °C, and transfer the supernatant into a 1.5 ml microcentrifuge tube and centrifuge it at 10,000 × g for 30 min at 4 °C and the supernatant as a cytosolic fraction is collected. In the next step, add 100 μl mitochondrial extraction buffer to the pellet and vertex for 10 seconds. Finally, for apoptosis evaluation, 10 μg of mitochondrial and cytosolic fractions are loaded on a 12% SDS-PAGE then probed with mouse monoclonal cytochrome c antibody.

2.2.3.3. Caspase Activity Assay

An essential metabolic mechanism in apoptosis is caspase activation. By preserving the enzymatic cascade in a committed program, the proteolytic activities of the caspases are known to trigger and enhance the apoptotic response. In addition, activated caspases destroy crucial components of cellular structural proteins as well as proteins involved in RNA splicing or DNA repair.[181] The removal of the apoptotic cell and the significant phenotypic alterations are ultimately caused by this group of enzyme activity. The activity profile of the proteins of the caspase family makes it simple to identify them from other cellular proteolytic processes. The aspartic acid residues in the substrates are a need for this class of proteases which use a cysteine residue in its active site.[182] In fact, the term "caspase" originated from these similar characteristics for cysteinyl aspartate-specific proteinase.[183] In this assay, cell-culture manipulation should be performed in a clean room or laminar flow hood using sterile reagents and supplies. An aseptic approach should be used to avoid pollution. Collect cells from the culture. Take out a representative volume for analysis-no more than 1000 μl. Centrifuge at 200 × g for 8–10 min at room temperature. Determine the population viability and cell count by performing a trypan blue exclusion analysis on a diluted cell sample. Add full media with serum and nutrient/cofactor adjuncts if viability is higher than 90% so that there are 200,000 viable cells per milliliter of medium. Plate the cells into a sterile, 96-well plate with an opaque wall in 50 μl volumes (10,000 cells per well). As a cell-free control, add 100 μl of cell culture medium to the wells. If cells require attachment, allow a 2–16 h equilibration time before proceeding. Prepare control agents for caspase induction. For instance, diluted staurosporine to a concentration of 4 μM or rTRAIL to a concentration of 500 ng/ml in cell-culture medium and carried out twofold serial dilutions. Add the solutions to the cell wells in repeated volumes of 50 μl for a final concentration between 2 μM and 15.6 nM. Then, dilute the unknown sample or test compound to 20 μM in the cell-culture medium and add to cell wells in 50 μl volumes to cell wells. After that, fill the wells designated for the negative, non-induction control with 50 μl of cell culture medium. In order to ensure homogeneity and compound dispersion, shake the plate using an orbital shaker. For between four and twenty-four h, incubate the plate at 37 °C in a humidified CO_2 atmosphere. Read absorbance at 405 nm.[184]

2.2.3.4. Annexin V Assay

An established technique for identifying apoptotic cells is the use of annexin V apoptosis assay protocol. An early indicator of apoptosis is the staining of cells with annexin V, which reveals the exposure of phosphatidylserine on the cell surface.[185] This makes it possible to compare cell populations that are

annexin V-positive to those which are annexin V-negative in their membrane. The recommended procedure summarized below for usage with flow cytometry. Stain for mitochondrial potential with $DiOC_6$ (3,3'-dihexyloxacarbocyanine iodide), or TMRM (3,3'-dihexyloxacarbocyanine iodide) or JC-1 in a 1.5-ml Eppendorf tubes. $DiOC_6$, TMRM and JC-1 are dyes that accurately detect the potential of the mitochondrial membrane. Make a stable stock solution of $DiOC_6$ in 100% EtOH at 1 mM or TMRM in DMSO at 1 mM or JC-1 in DMSO at 2.5 mg/ml (−20 °C storage recommended). The stock solution should then be diluted in media to 5 μM for DiOC6 or 50 μM for TMRM or 100 μg/ml for JC-1. In a flow cytometry tube with a minimum volume of 0.3 ml, add cells in medium at a density of 0.2–1.0 million cells/ml. Add 100–500 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP) as a control to the cells and incubate them for 10 min before adding $DiOC_6$, TMRM or JC-1 (Optional). Include $DiOC_6$ at a 50 nM final concentration or TMRM to a final concentration of 2 μM or JC-1 to a final concentration of 10 μg/ml. For 10 min, incubate cells at 37 °C. Aspirate the medium after centrifuging the cells in a microcentrifuge for 7 min at 1200 g. Transfer cells to flow tubes after resuspending them in 0.25 ml of annexin V buffer containing 1 : 1000 (Depending on the cell type) annexin V–Cy5. Cells must be incubated at room temperature for 5 min in the dark. After that, they are put on ice before data collection. Collect cells using a suitable flow cytometer (excitation wavelength: 635 nM; detection: FL4 channel; bandpass filter: 661/16 nM). Utilizing the additional excitation laser and detection channels allows for simultaneous recording of mitochondrial potential as mentioned below.

For $DiOC_6$: [excitation wavelength = 488 nM; detection in the FL1 channel (530/30 nM bandpass filter)].

For TMRM: [excitation wavelength = 488 nM; detection in the FL2 channel (585/42 nM bandpass filter)].

For JC-1: [excitation wavelength = 488 nM; detection in the FL1 channel (530/30 nM bandpass filter) and FL2 channel (585/42 nM bandpass filter)].

There is no need for correction between the mitochondrial potential dyes and the annexin V channel because a second laser is used to detect Cy5. Either FlowJo or Cell Quest software (Becton Dickinson) should be used for data analysis.[186]

2.2.3.5. TUNEL Assay (DNA Fragmentation Assay)

Both basic and applied research fields are very interested in understanding DNA damage. TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining is one of the most popular techniques for detecting DNA damage. TUNEL assay depends on the enzyme terminal deoxynucleotidyl transferase's capacity to integrate labeled dUTP into free 3′-hydroxy termini produced as a result of the fragmentation of genomic DNA into single-stranded DNA.[187] It should be noted that TUNEL assay is not just used to identify apoptotic cells, it has also been found to stain cells undergoing active DNA repair, and it may be used to detect DNA damage linked to non-apoptotic processes such as necrotic cell death brought on by exposure to toxins and other stressors.[188,189] TUNEL assay can therefore be assumed of overall as a method to find DNA damage (DNA fragmentation), and in the right situations, specifically as a technique to identify apoptotic cells. In the following, the method of TUNEL assay on cells and tissue will be explained separately. For cultured cells, cells should be collected by centrifugation, washed in PBS, and then resuspended in PBS at a concentration of 1–2 10^7/ml. Transfer 100 μl of the cell suspension to a 96-well plate with a V-bottom. 100 μl of 2% formaldehyde in PBS, pH 7.4, is added to fix cells. Fifteen minutes of incubation on ice. Cells should be collected using a centrifuge, washed once in 200 μl of PBS, and then put in 200 μl of 70% ice-cold ethanol. Cells can be kept in 70% ethanol at −20 °C for several days. Centrifuge cells, then wash them twice in 200 μl of PBS. Re-suspend $1-5\times10^5$ cells in 50 μl of TdT equilibration buffer (2.5 mM Tris-HCl (pH 6.6), 0.2 M potassium cacodylate, 2.5 mM CoCl2, 0.25 mg/ml bovine serum albumin (BSA)) and incubated for 10 min at 37 °C with frequent gentle mixing. Re-suspend cells in 50 μl of TdT reaction buffer (TdT equilibration buffer containing 0.5 U/μl of TdT enzyme and 40 pmol/μl biotinylated-dUTP (Roche Diagnostics Corp.; Indianapolis, IN)) and incubate for 30 min at 37 °C with frequent gentle mixing. Centrifugally collect the cells, then wash them in 200 μl of PBS. The cells are resuspended in 100 μl of TdT staining buffer (4× saline-sodium citrate (0.6 M NaCl, 60 mM sodium citrate), 2.5 μg/mL fluorescein isothiocyanate-conjugated avidin (Amersham Pharmacia Biotech, Inc.; Piscataway, NJ), 0.1% Triton X-100, and 1% BSA.). Incubate the cell suspension in the dark for 30 min at room temperature. Centrifuge cells, wash twice with 200 μl of PBS, and then re-suspend in PBS at $2-8\times10^6$/ml. Attach coverslips for fluorescence microscopy using Vectashield antifade mounting media. Utilize flow cytometry, confocal microscopy, or fluorescence microscopy to examine cells.

For tissue, two options are available thus; 1) Colorimetric staining for light microscopic examination and 2) Fluorescent staining for colorimetric staining, 4% formaldehyde in PBS is used to fix tissue samples for 24 h and embedded in paraffin. Prepare 4–6 μm paraffin sections on glass slides pretreated with 0.01% poly-L-lysine (w/v) in distilled H_2O. Slides are heated for 30 min at 60 °C (or 10 min at 70 °C) to deparaffinize sections, then two 5-min xylene baths are incubated at room temperature in Coplin jars. Transferring the slides through a graded ethanol series: 2×3 min at 96% ethanol, 1×3 min at 90% ethanol, 1–3 min at 80% ethanol, 1×3 min at 70% ethanol, and 1×3 min at double distilled water (DDW) will rehydrate the tissue samples. Remove any extra water carefully before covering portions with a 20 μg/ml proteinase K solution. Incubate for 15 min at room temperature. After proteinase K treatment, rinse slides with DDW for three to five min. Covering sections with 2% hydrogen peroxide for 5 min at room temperature will inactivate endogenous peroxidases. Wash slides 3×5 min with DDW. Remove any remaining water carefully, then cover the sections with TdT equilibration buffer and incubate for 10 min at room temperature. Sections are covered with TdT reaction buffer after the TdT equilibration buffer has been removed. Slides should be incubated for 30 min at 37 °C in a humidified atmosphere. A small amount of TdT buffer can be carefully covered with a glass coverslip during incubation to save reagents. Be careful not to trap air bubbles because they could cause staining artifacts. Slides should be in 2×SSC (300 mM NaCl, 30 mM sodium citrate) for 2 ×10 min to stop the reaction. After washing slides in PBS, cover tissue slices with a 2% BSA solution for 30 to 60 min to prevent nonspecific binding. Slides must be washed for 2×5 min in PBS before being incubated with Vectastain ABC-peroxidase solution (Vector Laboratories, Burlingame, CA) for 1 h at 37 °C. Slides should be washed in PBS for 2×5 min, then stained with a DAB (3,3′-Diaminobenzidine) staining solution at room temperature (10 to 60 min). The reaction can be stopped by washing slides in DDW. Hematoxylin stain should be used as a light counter-stain on tissue sections. Apply coverslips to tissue slices using Aqua-Poly/Mount mounting medium. Use a light microscope to check slices.

For fluorescent staining, perform a colorimetric staining procedure till cover tissue slices with a 2% BSA solution for 30 to 60 min to prevent nonspecific binding, except inactivating hydrogen peroxide step. To cover tissue sections with TdT staining buffer, first wash slides in PBS for 2 ×5 min. Slides should be left at room temperature and in the dark for 30 min. Rinse slides on PBS for 2×5 min. Use hematoxylin, Hoechst 33342, or another suitable counter-stain to lightly stain sections. Wash the slides with PBS, let them air dry, and use Vectashield antifade mounting material to attach coverslips. Use confocal or fluorescence microscopy to examine tissue slices.[190]

2.2.4. Fluorometric Assays

However, due to the wide variability in colony diameter, counting colonies may not necessarily indicate cell proliferation. A semi-automated technique that integrates the surface occupied by the colonies has already been published as a way to get around this issue.[191] Although dye exclusion techniques are frequently employed for cells growing in suspension, the method is not appropriate for low cell populations. For typical large-scale cytotoxicity studies, assays utilizing uptake of radioactive precursors are not always practical and can be dangerous. We discuss here how Alamar Blue and CFDA-AM assays are used to evaluate cytotoxicity *in vitro*. Alamar blue is a non-radioactive and simple assay for rapid assessment,[133] but it does not give information about cell counting like hemacytometer. While, CFDA-AM appropriates for parallel assay on the same cell lines, its fluorescent compounds may interfere in assay.[134]

2.2.4.1. Alamar Blue Assay

Alamar blue reagent, a resazurin-based solution, determines viability using the reducing power of living cells. Alamar blue reagent's active component, resazurin, is a non-toxic, cell-permeable substance that is blue in color and essentially non-fluorescent. Resazurin is transformed into the red, highly fluorescent chemical resorufin when it enters live cells. Either an absorbance-based plate reader or a fluorescence-based plate reader can be used to quickly identify changes in viability. A wide range of human and animal cell lines, bacteria, plants, and fungi can all be employed with the alamar assay.[192] Put 50–50,000 cells in each well of a 96-well plate. The assay should be done in three replicates for each set of tests. Include an untreated control cell and a control well without cells. Wells with the transfection components but no cells, as well as a cytotoxicity positive control can also be added if desired. Calculate the well's culture medium volume. Alamar blue should be added to the well equal to 0.1 volume of culture medium. Incubate in a tissue culture incubator for 1 to 4 h at 37 °C. Depending on the cell

line's capability for metabolism and the amount of time spent incubating with the reagent, different cell types have varying degrees of ability to decrease resazurin to resorufin. 100 µl of the cell culture supernatant should be transferred to a 96-well plate with a flat bottom. If the reading is not done quickly, stop the reaction and stabilize it by adding 50 µl of 3% SDS for every 100 µl of the initial culture volume. Before continuing, keep the plate at room temperature for up to 24 h, as long as the contents are covered to avoid evaporation and shielded from light. Read the plate at 560 nm for the excitation and 590 nm for the emission wavelengths. With a reading at 600 nm as a reference, alamar blue data can also be read as absorbance on a spectrophotometer at 570 nm. Measurements of fluorescence, however, are significantly more sensitive than those of absorbance. Plot the results after subtracting the blank value (wells with no cells) from each reading. The values are positively associated with metabolic activity, which in turn affects cell viability.[193]

2.2.4.2. CFDA-AM Assay

Non-polar substrate molecules that can passively load into cells are suggested for staining live cells. Examples of these molecules include the most widely used esterified fluorogenic substrates, fluorescein diacetate (FDA) and carboxyfluorescein diacetate (CFDA), as well as their derivatives. CFDA has more negative charges than FDA and is consequently better maintained in cells. The electrically neutral carboxyfluorescein diacetate acetoxymethyl ester of CFDA (5-CFDA-AM), which can be loaded into cells at lower concentrations than CFDA, is one of the frequently used derivatives. This is a lipophilic substrate that is only mildly permeant to most cell membranes, like other CFDA derivatives.[194] It is best to put cells onto culture plates that can be read by plate readers. To detect the background fluorescence, blank wells (cell-free wells) should be included. Using the serum- and amino acid-free culture medium, a stock solution of 5-CFDA-AM (4 mM in DMSO) is diluted (1:1,000). Adherent cells' culture medium is removed from their wells, and the diluted 5-CFDA-AM solution, and 4 µM working solution are then added to the cells. For suspension cell cultures, a working solution of 8 µM 5-CFDA-AM can be prepared by diluting a 4 mM stock solution by a factor of 1:500 in an amino acid and serum free culture medium. Cells are divided into the appropriate number of wells, and an equal volume of 8 µM 5-CFDA-AM working solution is added to each well. At 18 to 22 °C and in the dark, cells are treated with 5-CFDA, AM for 30 min. Using a fluorescence plate reader, fluorescence is measured at 493 and 541 nm for the excitation and emission wavelengths, respectively. The equations provided below can be used to determine the proportion of cell growth and growth inhibition. If an exact cell number computation is required, a cell number standard curve for each type of cell should be created.[195]

$$\text{Cell growth } (\%) = \frac{\text{Mean OD sample}}{\text{Mean OD blank}} \times 100 \quad (14)$$

$$\text{Growth inhibition } (\%) = 1 - \text{Cell growth } (\%) \quad (15)$$

2.2.5. Luminometric Assays

The basis for bioluminescence tests is the relationship between a bioluminescent reaction and a compound's action. An increase in cell growth or cell death could result from this action.[196] Since the 1970s, luminometers have been used to measure bioluminescent phenomena. Modern luminometers have a photon counter, and the signal they produce is proportional to the photons they release but not exactly equal to them.[197]

2.2.5.1. Adenosine Tri-Phosphate (ATP) Assay

Using the luciferin-luciferase reaction, ATP bioluminescence was first created to test whether there was a linear relationship between the number of cultivated cells and luminescence measurements.[198] Cells must first become ATP permeable in order for the luciferase enzyme to interact with intracellular ATP in luminometric ATP cell viability studies. Finally, intracellular ATP levels are determined by measuring the light using luminometers after intracellular ATPases have been deactivated. The majority of assays are so sensitive that the luminescent signal can be detected even from 50 cells. The luminescent signal is relatively stable and can be evaluated in a few hours. In 100 µl of culture medium, cells are divided into 96-well plates. No-cell wells should be included for blank subtraction and no-treatment cells should be included as controls. Plates are equilibrated to room temperature for 30 min after the desired incubation or treatment period. Most kits only comprise one working solution that contains all of the assay's components, including detergents, ATPase inhibitors, luciferin, and luciferase. Equal amounts of assay buffer (100 µl) are

added to each well and incubated at room temperature for 10–20 min on an orbital shaker. When using a kit with different solutions, 50 µl of detergent-ATPase inhibitor buffer must be added first, followed by 50 µl of luciferin-luciferase buffer, then it must be incubated. The standard ATP stock solution is used to prepare the ATP standard curve for calculation. It is estimated that a standard curve with a range of 10 pM to 10 M will be acceptable for comparison. The standard curve plate contains a working solution as well and is incubated at room temperature for the same time as the experimental groups. After incubation, luminescence at a wavelength of 560 nm is measured by a luminometer.[195]

2.2.5.2. Real-Time Viability Assay

The only cell viability method that enables real-time monitoring of cell viability is the real-time viability assay, a novel luciferase method approach. A pro-substrate of luciferase and an engineered luciferase obtained from marine shrimp are used in this novel method. This approach also incorporates luciferase and a cell-permeable pro-substrate into the culture medium, but the cells are not lysed to release intracellular ATP. The pro-substrate is instead absorbed by live cells and transformed into a "substrate", which diffuses into the culture medium. The luciferase enzyme then produces a luminescent signal using the diffused substrate. Cells are divided into wells and placed in media containing the selected treatment chemical and a buffer for the real-time viability experiment. Then, depending on the experimental design, cells are incubated at 37 °C while continuous luminescence measurement is done every 30 min or hour. Real-time cell viability assay buffers are stable at 37 °C for up to 72 h. However, for each cell type, an optimization step is needed to identify the required time until the limiting factor pro-substrate is completely used and finally, luminescent signal is measured. Standard ATP measurements or percentage cell growth can be used to calculate cell viability.[195]

3. Conclusion

A wide range of assays has been widely used to evaluate the adverse effects and unwanted consequences of novel drugs and new agents. The commonly used cytotoxicity/cell viability testing techniques include the dye exclusion test, colorimetric assays, fluorometric assays and luminometric assays which play crucial roles in drug efficacy and development. Fit-for-purpose cytotoxicity assay should be considered based on compound mechanism of action, drug solvent, concentration of compounds, drug exposure duration, and cell culture. This would undoubtedly enhance a good and well-conducted experimental design. Generally, a robust cell health assay should be safe, time- and cost-effective, reliable, efficient and rapid. In this review, cytotoxicity assays have been described in more details to help decide which assay method is best suited for a research study.

Acknowledgments

We thankfully acknowledge the University of Würzburg for supporting Eman M. Othman with the Scientia grant. Open Access funding enabled and organized by Projekt DEAL.

Conflict of Interest

The authors declare no conflict of interest.

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Received November 25, 2022
Accepted January 3, 2023