REVIEW ARTICLE

Review on the *In Vitro* Cytotoxicity Assessment in Accordance to the International Organization for Standardization (ISO)

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ABSTRACT

Cytotoxicity is a predominant biological evaluation applied to search for a suitable and non-toxic bioactive compound and to determine the biocompatibility of medical devices-related human body. The broad usage of cytotoxicity tests leads to a robust establishment of cytotoxicity assays with high sensitivity and prompt results. *In vitro* assays are always prioritized over *in vivo* due to the reproducible data, reduce numbers of animal used and easily accessible material. Compounds concentration that execute 50% of cell population is determined by calculating the IC50. According to ISO10993, cytotoxicity tests must be performed to determine the biocompatibility of medical devices that has contact with human body. This is crucial to ensure the safety of research and its clinical use. Under the recommendation of ISO10995-Part 5, three categories of tests have been documented; extract elution, direct contact and indirect contact test. Each category plays significant role depending on the nature of experiment and sample used.

Keywords: Cytotoxicity test, biocompatibility, ISO10993, IC_{50}, LD_{50}

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INTRODUCTION

Cytotoxicity is a term relatively referred to a compound or substance that is toxic on cells. This discipline is important to determine the level of toxicity possessed by a compound or chemical substance on cells (1). Cytotoxicity in *in vitro* systems are the chosen method for rapid profiling of the hazardous chemical and environmental samples (2).

The cytotoxicity assessment is a fundamental biological measurement and screening test applied on tissue cells as *in vitro* sample to distinguish the cell proliferation rate, reproduction as well as the morphological effects of substances (3). It has been a crucial preliminary method necessary to develop drugs or biomaterial's compounds and to predict the starting doses that is able to treat diseases without affecting normal cells (4, 5). However, whether a research is measuring the ability of compounds capable to suppress or enhance cell proliferation, and migration or induce cell death solely depends on the objective of a project or an experiment. In certain areas such as in searching for therapeutic anti-cancer drugs, the ability of compounds to inhibit cell growth or reduce cell viability is very important before further molecular analysis can be mapped out. In contrast, in the area of wound healing or stem cells which favour healthy cells turn over, it is indispensable to avoid toxic concentrations that may harm or kill the cells. Moreover, even though cytotoxicity test can be applied on animal models, *in vitro* cell culture has always been prioritized to assess the biological materials or active compounds at the cellular level (6). It is also important to reduce the numbers of animals used as recommended by the ISO 7405:1997 (7, 8). Additionally, cytotoxicity test on cell culture has been considered as the most significant approaches for biological valuation for all medical devices. It is often defined as the quality of a compound to be toxic through cell growth inhibition that destroy the living cells (9, 10).

Cytotoxicity can be determined using several formulas. Cytotoxicity in cells is referred to the concentration required for a test material or compound to kill 50% of cell population. It is expressed using inhibitory concentration or IC_{50} which is calculated as mean percentage increase relative to the untreated control (11). In quantitative cytotoxicity assays, average absorbance of the medium controls which has no test materials is referred as 100% while the percentage of proliferation cells in each treated well is referred as % of the control (9). Data obtained will be plotted to a sigmoidal curve to determine the concentration of compounds causing 50% cell death compared to control (12). However, IC_{50} from a single data set could vary several folds due to the

differences of the software packages that use different methods in calculating the variance of IC_{50} (13).

Hence, to determine the cytotoxic potential of a compound, selectivity index (SI) is used by measuring the ratio of 50% of the inhibitory concentration (IC_{50}) of normal cells to the 50% of cell death population in cancer cells (IC_{50}) (14).

$$SI = \frac{IC_{50} \text{ of normal cells}}{IC_{50} \text{ of cancer cells}}$$

SI can be an indicator whether a compound is non-cytotoxic or not. The greater the SI value, the more selective it is. If the SI value is > 2, the compound is considered to have the selective cytotoxic activity. However, if the SI value is <2, it is considered able to give general cytotoxicity towards the cells (15, 16).

In vivo is a more complex system with more than one cell type involved compared to *in vitro* systems that are designed to be as simple as possible. In the *in vivo* model, the concentration that kill 50% of animal populations is determined in accordance to the Organisation for Economic Co-operation and Development (OECD). Lethal concentration or LC_{50} refer to the inhibition concentration of chemicals that causes lethality to 50% of the animal populations while, lethal dose or LD_{50} is referred to drugs or chemicals that kill 50% of animal populations in a single dose of oral administration (17). According to the International Workshop on *In Vitro* Methods for Assessing Acute Systematic Toxicity, a document has been provided to use as guidelines and guidance for using *in vitro* which can reduce the numbers of animals required for *in vitro* lethality assays. Therefore, it is recommended to take advantage of the relationship between *in vitro* IC_{50} and *in vivo* LD_{50} that derived from the Registry of Cytotoxicity (RC). The RC predicted model is a regression analysis of LD_{50} values and *in vitro* cytotoxicity values for 347 chemicals (14).

CYTOTOXICITY AND BIOCOMPATIBILITY

Cytotoxicity test is always favoured as a pilot project test due to the high sensitivity, prompt result and simple assays (10). The constant establishment of cytotoxicity tests leads to several well-defined methods such as determination and measurement of cell damage and cell growth as well as observing the morphological changes which can be analysed through qualitative and quantitative methods. Cytotoxicity has also been used to determine the biocompatibility of medical devices that has contact with the body since they had been widely used in various clinical disciplines. Biocompatibility is the capability of a biomaterial to accomplish its tasks or function without triggering any unwanted response or systemic effect to the recipient or host that receives it (19). In 1922, the International Organization for Standardization has published the international standards for medical devices under the ISO 10993 to ensure the safety of research, manufacture and clinical use. Meanwhile, under the 'Guidance of the Selection of Tests', methods recommended for the biocompatibility evaluation must not restricted to analysing cytotoxicity, sensitization, irritation and acute toxicity (21). Although all cytotoxicity methods employ standard protocol, it must be noted that further analysis is needed to look at the correlating results of these methods with other biological evaluations (10).

As recommended in the ISO 10993-5 (Biological evaluation of medical devices), there are three categories of test listed: extract dilution test, direct contact test and indirect contact test. Extract dilution method is commonly adopted for the *in vitro* cytotoxicity evaluation of materials, while direct contact method enables weak cytotoxic compound or chemical to be detected due to its high sensitivity. The indirect contact test is usually involved agar diffusion which is suitable for medical devices with large toxicity and bulk filtering (10). Selecting the best method for cytotoxicity evaluation is dependent on the characteristic of the sample, the potential site as well as the nature of the use (10, 21, 22). The cellular growth and specific aspect of cellular metabolism can also be evaluated by selecting suitable methods.

EXTRACT DILUTION TEST

The extract dilution test is usually applied for the *in vitro* cytotoxicity evaluation of materials and a broad variety of medical devices. The main objective is to detect toxins leached from exposed surfaces (21) by a wide variety of test material extraction solution for assaying cytotoxic substances (9). The evaluation of extracts cytotoxicity is based on the morphological changes of the cells which include disruption in the normal appearance and functions of the cellular components (9, 23). It can also be used for high-density materials as well as to establish dose-response curves. There are several methods used for the extract dilution test such as tetrazolium salt-based assay; MTT, MTS (10), and WST (24) assay as well as neutral red (NR) assay. Generally, the MTT and NR assays perform similarly in the evaluation of the chemical cytotoxicity and produces good correlation (25). The percentage of cells viability can be calculated using this formulation:

$$\% \text{ of viable cells} = \frac{(\text{Absorbance sample} - \text{Absorbance blank})}{(\text{Absorbance control} - \text{Absorbance blank})} \times 100$$

MTT assay was first developed by Mosman in 1983 and modified by Denizot and Lang in 1986 (1). This assay measures the activity of mitochondrial succinate dehydrogenase enzyme which is also known as the 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (methyl thiozolyl tetrazolium). It is a colometric assay for rapid assessment of cell proliferation and cytotoxicity by evaluating the cell

metabolism. Principally, the tetrazole ring will be cleaved by the mitochondrial dehydrogenase enzyme in the cytochrome b and c of living cells. The reduced form of tetrazolium salt, yellow water-soluble MTT produce a purple crystalline formazan that is resolvable in dimethyl sulfoxide (DMSO) and other organic solvents but unsolvable in water (26). The volume of crystals formed is associated with the number and activity of the cells which is reflected by the colorimetric value of the absorbance (optical density) that measures the number of living cells and their metabolic activity (27, 28). Although the MTT assay has a sensitive response to the proliferation of medical devices and suitable for a quick preliminary screening, it has numerous limitations in terms of application. Its absorbance signal is directly proportional to the cell number and the quantity of formazan crystals produced is not solely dependent on cell number (29). The cells debris and precipitated proteins can also interfere with the optical readings. Additionally, it is not suitable for suspending cells and number of cells seeding must be optimized to obtain reproducible data. The measuring of surviving cells may also be affected by humans and environmental factors leading to errors and inaccurate data (10).

Another assay with tetrazolium-based assay frequently used is MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium). Although the aim of both assays is to measure the viable cell numbers, there may be some changes in the metabolic activity due to the diverse circumstances or chemical treatments which may cause considerable variation in results reported from these assays (30). Meanwhile, water soluble formazan which is also known as WST or (2-(-4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt); is the highly water-soluble formazan which reacts with the cellular dehydrogenase in the presence of intermediate electron acceptor (24). This method demonstrated greater sensitivity and efficiency for measuring bacterial viability and useful for robust NAD(P)H determination (31, 32). It is reported that this assay eliminates the need for washing and solvent solubilization step, and the formazan produced is more soluble than MTT. Thus, it leads to a broader linear range and higher sensitivity (33) and less toxic (30). However, despite the development of this new technique, it has yet to replace the well-established MTT assay (29).

On the other hand, neutral red assay or 3-amino-7-dimethylamino-2-methylphenazine hydrochloride offers a totally different principle. This quantitative assay determines viable cells based on the lysosomal activity. This assay is generally used for the adherent cells and was developed at Rockefeller University (34). Principally, the weak cationic dye of neutral red penetrates the cellular membranes via non-ionic passive diffusion and accumulates in the lysosomes of viable cells. The ability of cells to take up and hold the dye depends on the cells' capacity to sustain pH gradients. Thus, the dye is retained in lysosomes due to the presence of proton gradient that preserve a lower pH than the cytoplasm. The charged dye in the viable cells is then extracted in an ethanol or acetic acid solution and measured using a spectrophotometer at light absorbance of 540 nm. Since the amount of retained dye is proportional to the number of viable cells (35), it is therefore possible to distinguish between viable, damaged or dead cells. In accordance to the ISO guidance, if the NR signal is reduced to <70% of the blank control, the sample is considered potentially cytotoxic (36).

Another assay that uses a different approach is the colorimetric sulforhodamine B (SRB). Principally, this assay measures the cellular protein content with a dye that labels the basic amino acids of the cellular proteins and estimates the total protein mass associated to the cells number. SRB can detect cell densities as low as 1,000-2,000 cells per well while at 7,500- 180,000 cells per well, SRB is still able to exhibit a linear dynamic range (37). The advantages of this method can be summarized as higher sensitivity, independent of cell metabolic activity and better linearity with a stable endpoint (38). In contrast to MTT and MTS assays, SRB is limited to manual or semiautomatic screening, several washing steps and intricate procedure which may result in underestimated OD. It also has higher chances of light contamination since it can be degraded by light exposure. Therefore, it is important to ensure that the staining process is performed within a specific time range (20).

Thus, despite the presence of a broad spectrum of cytotoxicity assays in which they are being claimed as suitable or common, it is important to note that the choice of methods depends on the nature of the sample that is to be evaluated. It is also important to identify samples used in each experiment. For example, the conversion rate of formazan crystal has been shown to have close correlation to the number of mitochondria present in cells (26). Moreover, a study conducted to investigate the proliferative effect of green tea polyphenol showed results obtained from MTT and MTS are different compared to the results obtained from direct measures of ATP and DNA using luminescent cell viability assay kit and cell proliferation assay kit, respectively. The difference is due to the presence of polyphenols that may interfere with the formation of formazan and may change the succinate dehydrogenase activity (30). Further literature review reported that compounds generating superoxide such as nano titanium dioxide, corrosion products of certain metal alloys and many other phytochemicals that demonstrated intrinsic reductive potential including antioxidants able to interact with several tetrazolium-based assays such as MTT and MTS. Increased reduction of MTT dyes also been reported to correlate with the presence of liver fractions as well as defective mitochondria (29). Meanwhile, NR assay

relies on the intracellular accumulation of the dye in cellular lysosomes via active transport. It was reported that NR was the most sensitive assay for time exposure between 3-6 hours. In an experiment conducted to observe the effects of several phenolic compounds on HeLa and BT474 cells, both NR and MTT assays showed similar cytotoxicity profile in all times exposure (18, 24 and 48 hours) (39). Similarly, observation on the effect of cadmium chloride towards hepatoma cell lines also revealed that both NR and MTT assays showed similar sensitivity in detecting the cytotoxicity compound (40). However, studied on effect of cigarette smoke showed NR is the most sensitive assay compared to MTT, XTT, DH, SRB and resazurin binding (39, 41). Therefore, the usage of tetrazolium-based assay and NR assay may give different results.

The comparison of the assay sensitivity between MTT and SRB in an experiment to evaluate the cytotoxic effects of C. nutans on L929 cells showed moderate correlation when using low concentration but excellent similarity at higher concentration (25). A similar study has also been conducted using two human tumor cells lines (MT29 and 11B) which showed that SRB assay had better linearity, higher sensitivity and the staining is not cell dependent (42). Interestingly, SRB assay was also the only assay that the non-linear regression statistics could be fitted closely to the experimental data compared to other assays; MTT, NR and AB (29). This is due to the ability of SRB to measure total cellular protein content and does not rely on the cell functionality. Moreover, SRB assay has been selected as the preferred high-throughput assay of the National Cancer Institute (NCI) in USA and also been used in the NCI's lead compound screening programme (43). Since detection of viable cells is crucial in numerous biological fields, the ability of one data to be reproduced to give enough reliability should be one of the important considerations when selecting a suitable assay. It is also crucial to consider the potential interference, linearity and sensitivity (29)

DIRECT CONTACT TEST

The direct contact methods enable weak cytotoxicity to be detected due to its high sensitivity. This method usually involves low density devices and direct interaction of testing materials to the cells growing in the culture medium. It involves observing the morphological changes as well as detecting the changes in the number of cells which can directly impact the testing of the medical devices (10). Similar to the extract test, there are several methods that fall under these categories such as the Alamar Blue (AB) assay, live or dead cell stain (9) and trypan blue assay (46).

AB assay is an *in vitro* cytotoxicity assay known as resazurin-based (RES) assay that measures cellular metabolic activity. It can be measured by both colorimetry and fluorometry. Principally, this method depends on the conversion of the blue non-fluorescent dye resazurin (7-hydroxy-3H-phenoxazin-3-1-10-oxide) to pink fluorescent resofurin by the mitochondrial enzymes in viable cells that has diaphorase activity such as NADPH dehydrogenase (44). The quantification of resofurin can be used as indicator of metabolic activity (29). Generally, this assay has been used for a broad spectrum of monitoring and drugs screening in cells as well as for bacterial pathogens such as Mycobacterium spp, Staphylococcus spp, Enterococcus spp and Pesudomonas spp (44). The amount of AB dye present in the cells is used to measure the different uptake by the cells. This dye disperses passively through cell membrane and acts as an intermediate electron in the electron transport chain without interference to the normal transfer of electrons (38).

Therefore, the absorbance would record the unreacted and highly dichromatic resazurin whilst the fluorescence records the reduced form, resorufin. Absorbance is taken at two wavelengths; 570 and 600 nm or 540 and 630 nm depending on the type of microtiter plate (flat-bottomed or rounded). Meanwhile, fluorescence can be monitored at 530-560 nm wavelength and an emission wavelength at 590nm. Although this assay is non-toxic at low concentration, simple and sensitive, the reduction of AB dye is non-linear due to the non-linear correlation between dye reduction and cell number. It is therefore not suitable to measure proliferation of cells (44). Moreover, since resofurin is continuously developed in cells, the reaction needs to be read at a precisely defined time and temperature. This assay has also been used to monitor the proliferation and function of immune cell (45) including lymphocytes, monocytes since no cell lysis is required. Thus, it is possible to do a continuous monitoring through time-course experiment (44).

Staining of live or dead cell is another direct contact assay that allows direct count of viable and dead cells. Several staining such as trypan blue, SYTO-13 and ethidiumhomodimer-2 are used to distinguish viable and dead cells depending on the ability of cells to uptake the dye. Trypan blue or 3,3′-[(3,3′-dimethyl(1,1′-biphenyl)-4,4′-diyl)bis(azo)]bis(5-amino-4-hydroxy-2,7-naphthalenedisulfonic acid) is a diazo dye commonly used to stain and quantify live cells via labelling the dead cells. This technique is usually performed on a relatively small number of samples. In live cells, the cell membrane is intact thus disabling trypan blue dye from penetrating cell membrane and entering the cytoplasm (46). Nonetheless, in dead cell, the cell membrane becomes permeable enabling the negatively charged trypan blue to pass through and enter the cytoplasm. Hence, observation under light microscope reveals only dead cells as they are stained with blue colour. Although this method has been performed by many researchers, it is relatively time-consuming (47).

Meanwhile, SYTO-13 is a fluorescent cyanine dye that

provide quantitative and sensitive nucleic acid staining. It is a member of the SYTO dyes that is used to stain RNA and DNA in live and dead cells. In contrast to trypan blue, this dye able to penetrate almost all living cell types and yields bright fluorescence upon binding to DNA or RNA. Another fluorescent cell permeant dye is ethidumhomodimer-2 that has green and red fluorescent dyes. Although this dye able to only enter dead cells, it has higher binding affinity to DNA compared to SYTO-13 (48). Thus, despite the commonly used cell exclusion-based assays for cytotoxicity measurement, they also have advantages and disadvantages depending on the samples used and the nature of the experiments. Similar to these staining methods, the advantages are able to directly count the viable and dead cells via microscope or plate fluorometer. Although these staining are claimed to be highly sensitive, dyes that label the living cells for example may have a drawback since some dying cells may retain their membrane integrity for a substantial period. Moreover, dyes used to label nucleic acid are toxic and therefore it is important to avoid staining cells for longer period (49).

Therefore, it is necessary to determine the adverse outcome (AO) of each experiment based on the objectives of the research. AB and trypan blues assays are not suitable when assessing the cytotoxicity of compounds (45). Similarly, SYTO13 has its drawback since the dye can be retained in dying cells by holding the integrity of membrane. However, there are several other methods that can be used in evaluating the compounds cytotoxicity such as MTT, MTS, NR and SRB assays. If the test materials are polyphenols, phytochemicals or able to generate superoxide, methods involving the tetrazolium-based are best to be avoided since they can interact with tetrazolium salt. Moreover, single biochemical readouts are not suitable to be used as unequivocal indicators of a precise cytotoxicity.

INDIRECT CONTACT TEST

The indirect contact test measures the cytotoxicity that diffuse through a barrier to avoid direct interaction of any tested by-products with the cells such as agar diffusion. It is usually suitable for high toxicity and density materials since these types of materials may cause physical impairment to cells (21, 23). Common example that describe the indirect contact test is agar overlay assay. This assay has been introduced after the 22nd US Pharmacopeia (USP) review to evaluate the safety of plastic for medical purposes (27, 28, 50). This method is appropriate for medical devices that contain large amount of toxicity. Meanwhile, for the biocompatibility evaluation of toxic components of small molecular weight medical devices, it is suggested to use the molecular filtration method (10).

Agar overlay assay is a qualitative assessment of cytotoxicity. This method involves the use of agar where monolayer cells will be cultured or overlaid by the agar (19, 27). Following this, cells will be exposed indirectly to the test material by adding it on top of the thin layer of agar. The added compound will be released via radial diffusion that creates concentration gradient in the agar. The cushioning effect of the agar layer protects the cells from the mechanical damage caused by test material. The point where the compound is added will have the highest concentration and cells plated below it will receive the strongest effect. The evaluation of the degree of cells destruction is estimated based on the radius of dissolution or via electron microscope and size of the dead cells zone. However, it has been reported that some potential cytotoxic leachates unable to diffuse across the plate due to the ability to bind to the agar. For example, in the *in vitro* experiments for dental filling materials and erythrocytes lysis test, both tests did not correlate well with the *in vivo* results. This is suggested due to the failure of tested materials to diffuse through the agar overlay. Therefore, although this method is simple and rapid, the agar cannot effectively mimic the barrier *in vivo* (9, 10).

ASSESSMENT OF CYTOTOXICITY DEGREE

Cytotoxicity test provides a degree of toxicity when a material or substance or medical device reacts with cells or human body. Both agar diffusion and direct contact method have similar evaluation of cytotoxicity degree since both approaches offer qualitative evaluation of *in vitro* cytotoxicity (27). Although qualitative research is more subjective compared to quantitative research, it is essential to select applicable methods according to the nature of the experiment that is being conducted. Therefore, both qualitative and quantitative research is often regarded as equivalent (51).

The degree of reactivity around or under the sample is determined by the reactivity zone (cellular degeneration or malformation) as in agar diffusion and direct contact test (Table I). However, elution test does not depend on the reactivity zone (Table II). This is because the whole monolayer is exposed to the extract. Therefore, the reactivity degree depends on the cells viability which are indicated by the changes in the cell morphology such as loss of membrane permeability and size of cells. Grading has been assigned and the scoring criteria is

Table I: Reactivity Grades for Agar Diffusion and Direct Contact Test (10)

Grade	Reactivity	Conditions of all cultures
0	Absence	No cell lyses observed around or under sample
1	Slight	Some deformed or deteriorated cells observed under sample
2	Mild	Vicinity that restricted to area under sample
3	Moderate	Vicinity that ranges from 0.5 to 1.0 beyond sample
4	Severe	Vicinity that covers more than 1.0 cm beyond sample but does not involve the whole plate

Table II: Reactivity grades for elution test (10)

Grade	Reactivity	Conditions of all cultures
0	Absence	Discrete cytoplasmic granules; no cell lysis
1	Slight	Approximately 20% of the cells are round, loosely attached with no cytoplasmic granules observed; Lysed cells are periodically observed.
2	Mild	Approximately 50% of the cells are round and lacking cytoplasmic granules with substantial cell lysis and empty areas between cells.
3	Moderate	Approximately 70% of the cell layers contain rounded cell and/or are lysed
4	Severe	The destruction of the cell layers is almost thorough.

described under the reactivity category as absence, slight or mild (21, 27). Table III is the comparison of cytotoxicity testing methods used as accordance to ISO guidance.

ACRONYMS AND ABBREVIATIONS

Alamar Blue test (AB), Deoxyribonucleic acid (DNA), dimethyl sulfoxide (DMSO), cytotoxic concentration (CC), hydrochloric acid (HCL), inhibitory concentration (IC), International Organization for Standardization (ISO), lethal concentration (LC), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxylphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), 3-(4, 5-dimethyl-2-thizolyl)-2,5-diphenyl-2H-tetrazolium (MTT), nanometers (nm), Organisation for Economic Co-operation and Development (OECD), Ribonucleic acid (RNA), sulforhodamine B (SRB), 2-(-4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST), 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium (XTT).

CONCLUSION

Cytotoxicity test is a fundamental yet the most crucial indicators that is simple, rapid and sensitive. Methods selection must be done according to the designated experiment. It is also important to take note on the advantages and disadvantages of selected methods to maintain the reproducibility of the data and reduce data interference. Despite the usage of animal models, *in vitro* assays are often prioritized in performing the cytotoxicity test due to its sensitivity, reproducibility and easy access to the sample.

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Table III: Comparison of cytotoxicity testing methods

	Advantages	Disadvantages	Outcome of assay	Setting used
Tetrazolium-based assay (MTT, MTS, XTT and WST)	1. Sensitive response to the cell proliferation (33) 2. Quick preliminary screening (33) 3. WST eliminates the needs for washing and less toxic (24)	1. Not suitable for suspending cells (26,29) 2. Conversion of tetrazolium salts has several interferences depend on the compounds used such reducing agent (30). 3. This assay depends on the mitochondria and metabolic rate (29)	The reduced form of tetrazolium salts produced formazan crystal. The amount of this crystal is the used to plot the sigmoidal curves graph to obtain the IC50 (Tonder et al. 2015).	Colorimetric assay (26,27,28,29,30)
Neutral red uptake assay	1. Enumeration of cell is not dependent on enzymatic conversion dye (35) 2. Sensitive assay for short time exposure of 3-6 hours (39)	1. May have some interference of test compound (39)	The NR dye that retained in the viable cells is propotional to the number of viable cells (Ates et al. 2017). If the signal is reduced to <70% of the blank control, the sample is considered potentially cytotoxic (Bruinink & Luginbuehl 2011).	Colorimetric assay (34)
Sulforhodamine B	1. Measure total protein content and does rely on cell functionality (37) 2. Highly sensitive and reproducible (38)	1. Limited to manual or semiautomatic screening (20) 2. Consist of several washing step (20)	Cell viability was expressed as a percentage of control values using the intra-class correlation coefficient (ICC) and limits of agreement statistics to compare the scores (Vajrabhaya & Korsuwannawong 2018).	Colorimetric assay (37)
Alamar blue assay or Resazurin assay	1. Able to monitor the cell proliferation and function of immune cells (Zhang et al. 2011).	1. Not suitable for assessing cytotoxic compound (Rampersad 2012, Tonder et al. 2015)	The mitochondrial enzyme converts the resazurin to resofurin. The quantification of resofurin can be used as indicator to the metabolic activity if the cells (Rampersad 2012, Tonder et al. 2015).	Colorimetric and fluorometry assay (44)
Indirect contact test (Agar overlay)	1. Suitable for investigating the high toxicity and density materials (USP 2017). 2. Cells will be protected with present of agar that overlaid on top of the cells (USP2017) 3. Simple and rapid for the testing of high-density materials (Li et al. 2015).	1. Unable to mimic the barrier in vivo. 2. Some potential cytotoxic leachates able to bind to the agar and unable to diffuse across the plate (Liu et al. 2018)	The potential high toxicity test material will diffuse across the agar thus causing cytotoxicity towards the cells. However, the results were directly related to the agar thickness (which represents the barrier in vivo) and serum concentration (Liu et al.2018).	Agar overlay/ diffusion (19)

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