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Optimization of some parametric values of MTT for the determination of human melanoma (SK-Mel-30) cell viability

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ABSTRACT

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazoliumbromide (MTT) assay is a widely used assessment method for the determination of anticancerogenic effects of active compounds including plant secondary metabolites. Recently, some important plant active ingredients have been widely investigated for anticancerogenic properties on melanoma cancer lines which are the most lethal type of skin cancer. Although some methods including DNA assay, ^{3}H-thymidine incorporation and flow cytometry have been recommended for counting cells in the culture, MTT is one of the most frequent method and therefore, MTT assay needs to be optimized for melanoma cell lines. In this study, the MTT analytical procedure for determination of cell viability of human melanoma cell line (SK-Mel-30) was divided into nine steps and various parameters in each step (reagent amount, incubation time, centrifugation, solvent type, waiting time before spectrophotometric analysis and spectrophotometric parameters) were optimized. Optimum amount of MTT reagent and incubation time after MTT addition were determined as 10 µL and 4 h for 96 well plate, respectively. Various solvents were evaluated for solubility effectiveness of the formed crystals and DMSO was found to be the best solvent to dissolve the crystals. Waiting time before spectrophotometric reading and Uv-vis spectrums were also evaluated. At the end of the study a flowchart, presented the best analytical conditions, was constructed. Obtained findings can be used for the determination of anticancerogenic properties of plant ingredients.

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Introduction

Malignant melanoma is the most widespread aggressive skin cancer type all over the world. Melanoma reports for 3% of all skin cancers but 66% of skin cancer - related deaths. The incidence of melanoma has accelerated by 340 percent since the 1950s; however, the mortality rate from melanoma has increased by only 150 percent. The majority of this is due to melanomas being diagnosed at an earlier phase when they may be more treatable. Melanoma is now the fifth most frequently diagnosed cancer in the United States. Over 70,000 invasive melanomas were diagnosed in the United States last year, with an added 53,000 in situ [1]. One in every 50 people will develop invasive melanoma in their lifetime. If in situ disease is considered, the lifetime risk is one in 34.

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Melanoma took the lives of 8790 Americans last year (approximately one dying every hour) [2]. According to the duration of exposure to sunlight Australia and New Zealand currently have the highest rates in the world (60 people in every 100,000 are diagnosed per year), with the USA (30 per 100,000 per year) and Scandinavia (20 per 100,000 per year) following. Approximately 104,350 new cases of melanoma were determined in the US and 11,650 persons are estimated to die from this cancer type in 2019 [3]. To control the disease and discover the novel drug therapies for the medication of the cancer needs the intensive research studies by using reliable and accurate methods [4]. Melanoma spreads rapidly to distant organs, making treatment tough. The rate of metastasis, its position, and the multitude of metastases all have a significant impact on patient survival, hence why it's critical to find a way to slow this process.[5, 6]

Nowadays, although many methods that can perform the counting cells such as fluorometric DNA assay, ^{3}H-thymidine incorporation and flow cytometry in the culture, one of the most widespread ways for determination of the viable cell number is the rapid colorimetric tetrazolium dye method commonly referred to as the 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay [7]. Besides the cell viability, the test has been frequently used to determine cell proliferation and cytotoxicity because of its low cost, fast and simple test protocol [1, 7]. MTT is reduced by mitochondrial enzyme succinate dehydrogenase in mitochondria of metabolically active cells, and enzymatic reduction of the MTT results into the formation of water-insoluble purple colored formazan precipitate (Figure 1). Formed precipitate can be dissolved in a variety of organic solvents such as dimethyl sulfoxide (DMSO), isopropanol, sodium dodecyl sulfate (SDS) and absorbance of the resulting solution can be measured by a multi-well spectrophotometer [8]. In conclusion, measured absorbance values depend on mitochondrial activity gives an information about the number of metabolically active viable cells [7-9].

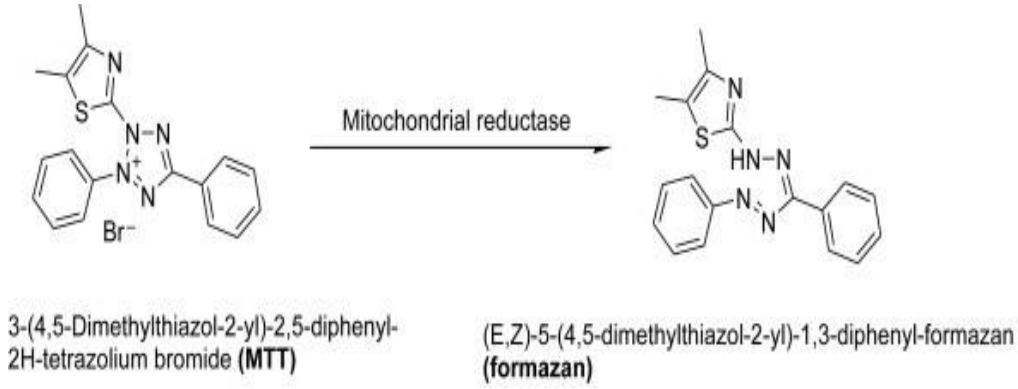

Fig 1 Enzymatic reduction of MTT to formazan [8].

Although, the method has been used in a number of laboratories with minor modifications, some parameters such as concentration and amount of MTT reagent, incubation time for production of formazan crystals, solvent types, centrifugation, cell numbers, waiting time before spectrophotometric analysis and parameters of the spectrophotometers such as wavelengths can affect to reliability of the MTT method [10]. The MTT test was shown to be sensitive to at least 1000 cells/well, whereas fewer cells can cause background high absorbance values [11]. It was also demonstrated that the absorbance signal increases in direct proportion to the incubation time, number of cells and amount of formazan crystals [12, 13]. One of the most important parameters of the MTT test is the solvents used to dissolve formazan crystals. Although the DMSO has been accepted as the most widely used solvent to dissolve formed formazan crystals, there are some other recommended alternative solvents such as isopropyl alcohol and acid isopropyl alcohol due to their lower background absorbance values than DMSO [7, 14]. Various organic solvents such as dioxane, cyclohexane, tetrahydrofuran, dimethylformamide were also used for the assay [15, 16]. In some previous reports, a centrifugation step was performed to precipitate formazan salts before solvent addition and medium removal to dissolve formazan salts [17, 18]. Although the MTT test is one of the effective and reliable method to measure cell viability, this analytical techniques used various kind of studies [19, 20] and it is necessary to optimize its accuracy and minimize the factors that affect MTT activity for specific cell lines. In the study, some critical factors that can affect the reliability and results of the MTT analysis were examined for determination of SK-Mel-30 cell counts in the culture.

Materials and Methods

Chemicals

MTT (Bio Vision Incorporated, California, United States) was dissolved in ultrapure water at the concentration of 10 mg/ml and filtered via 0.22 μm syringe filter (Gelman Sciences, Michigan, United States). The solvents (isopropanol and SDS) for the solubilization of MTT formazan crystals were obtained from Merck (Darmstadt, Germany). DMSO was purchased from ISOLAB (Wertheim, Germany).

Cell line

The human skin melanoma (SK-Mel-30) (ATCC, Virginia, United States) were plated in T75 tissue culture flasks and were cultured in High Glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco, New York, United States) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Cromwell, United States), 1X Antibiotic/Antimycotic (50 units/mL penicillin and 50 mg/mL streptomycin) (Capricorn, Ebsdorfergrund, Germany), 1X MEM Non-Essential amino acids (Gibco, New York, United States).

Instrumentation

The cells were incubated for 24, 48 and 72 h at 37°C, 5% CO_2 in a culture incubator (Thermo Scientific, Ohio, United States). A centrifuge (Hettich, Tuttlingen, Germany) was used to pellet cells before removal of the medium. At the end of the MTT assay, absorbance of the resulting solutions was recorded at 570 and 690 nm using an ELISA plate reader (Thermo Fischer Scientific, Vantaa, Finland). The absorbance spectrum (450–700 nm) was performed using a Cary 60Uv-Vis Spectrophotometer (Agilent Technologies, Malaysia). Results were analyzed with the Soft max pro software (version 2.2.1). The ultrapure water was supplied by an ultrapure (18.2 MΩ cm at 25°C) purification system (Minipure, Ankara, Turkey).

MTT assay

A schematic diagram for MTT Assay was presented in Figure 2. According to the analytical procedure, SK-Mel-30 cells were plated in triplicate wells of a 96-well plate (**1**). The cells were then incubated at 37°C under an atmosphere 5% CO_2 and 95% humidity (**2**). After removal of cell media, each culture well was delicately washed with phosphate buffered saline (PBS) solution and fresh medium was added. In this step, various concentrations of active ingredient (i.e. 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024,

2048 µM) which are extracted from plant materials can be added into the well. The final concentration should be considered (**3**). Various amount of MTT solution (10 and 30µl/well) were added to the cells to find the optimum amount of solution (**4**). The cells were incubated for 2 and 4 hours at 37°C, %5 CO_2 (**5**). After incubation, 96-well plates were centrifuged or non-centrifuged, and medium (0%, 90% and 100%) was removed (**6**). Formed formazan crystals were dissolved in 5 different solvents (DMSO, %20 SDS, isopropanol, two different acid isopropanol concentration (0.01N HCl and 0.04 N HCl) (**7**). The plates were then waited for 3 different time intervals (0.5, 2 and 24h) in a dark place at room temperature (**8**). The absorbance was assessed at 570 and 690 nm by an ELISA microplate reader (**9**). All tests were repeated three times.

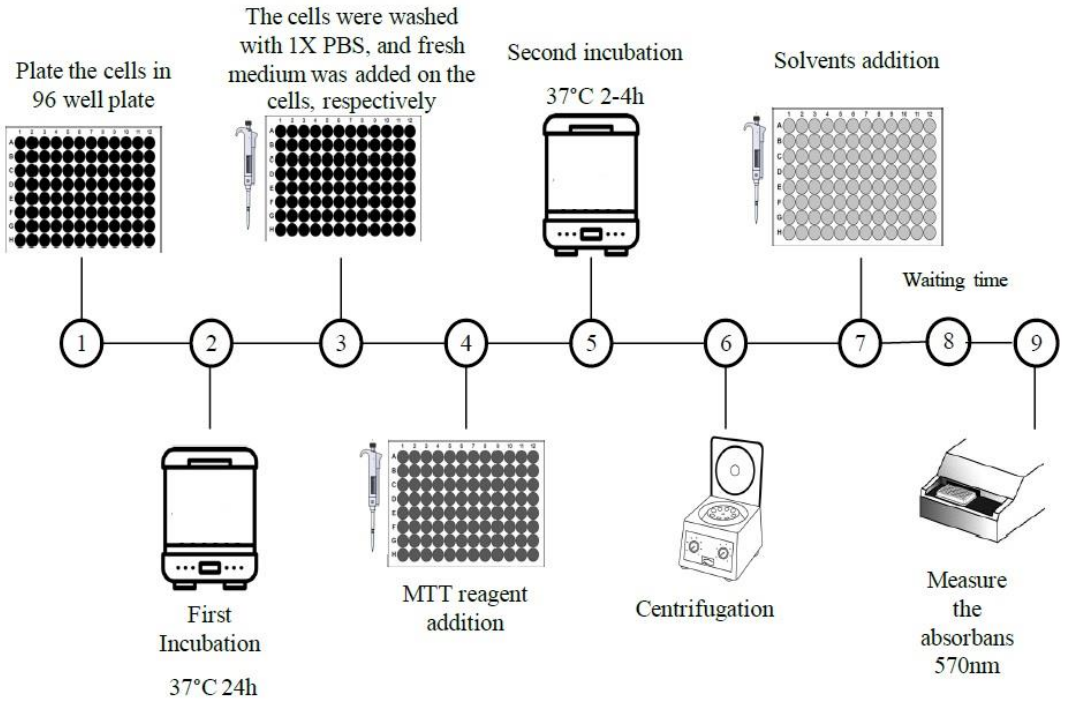

Fig 2 Workflow of MTT assay procedure.

Method validation

Method validation studies (limit of detection (LOD), repeatability and limit of quantification (LOQ)) were conducted according to the Eurachem guide [21]. The LOD was calculated at 3 times the standard deviation (SD), while the LOQ was calculated at 10 times the SD, which was obtained from the 10 independent analyses of samples with known counts of plated SK-Mel-30 cells (approximately 10,000 cells/well). Repeatability

was evaluated by assaying ten replicates of the individual MTT assay and was expressed as the relative standard deviation (RSD; %).

Statistical study

The data were expressed as means ± SD of at least 3 independent analyses. Significant differences were determined by one-way and/or two-way ANOVA followed by Tukey's multiple comparisons test employed by Minitab 17 statistical analysis program (Software, La Jolla, California). P value < 0.05 was considered statistically significant.

Results and Discussion

Preparation of the cells for MTT assay (Step 1-3)

The cells were plated at the concentration of $1x10^4$ number/well in 96-well plates (Step 1) and the cells were incubated at 37°C for 24h (Step 2). The number of the cell counts were in agreement with those reported by [22]. The medium in the wells were replaced with 1X PBS 24h later (Step 3). After the cells were grown in the cells, various concentration of plant active ingredients extracted and/or purified from the plants such as phenolic acids can be added to the cells. In this case, the final concentrations of active ingredients and living cell counts should be taken into consideration.

Throughout the first three steps of experimental workflow, schematized in Figure 2, the tested parameters were not changed. However, some parameters for the next steps were varied to find the most suitable parametric values.

Optimization of the other parameters (Step 4-9)

Effects of the other parameters (Step 4-9, illustrated in Fig 2) on the absorbance values for the MTT assay were evaluated. After the cells plating, the first incubating and washing procedure, various amounts of MTT solution was added to the cells in step 4. Two different volumes of MTT reagent (10 and 30 µL of 10 mg/mL) were selected in this experiment according to the various manufacturer protocols. The results showed that a significant increment in absorbance values were found related to the addition of MTT reagents. In the experiment, 10 µl of MTT solution was found to be a better concentration than 30 µl for production of formazan crystals as shown in Figure 3. The result were consistent with those reported previously [11, 23].

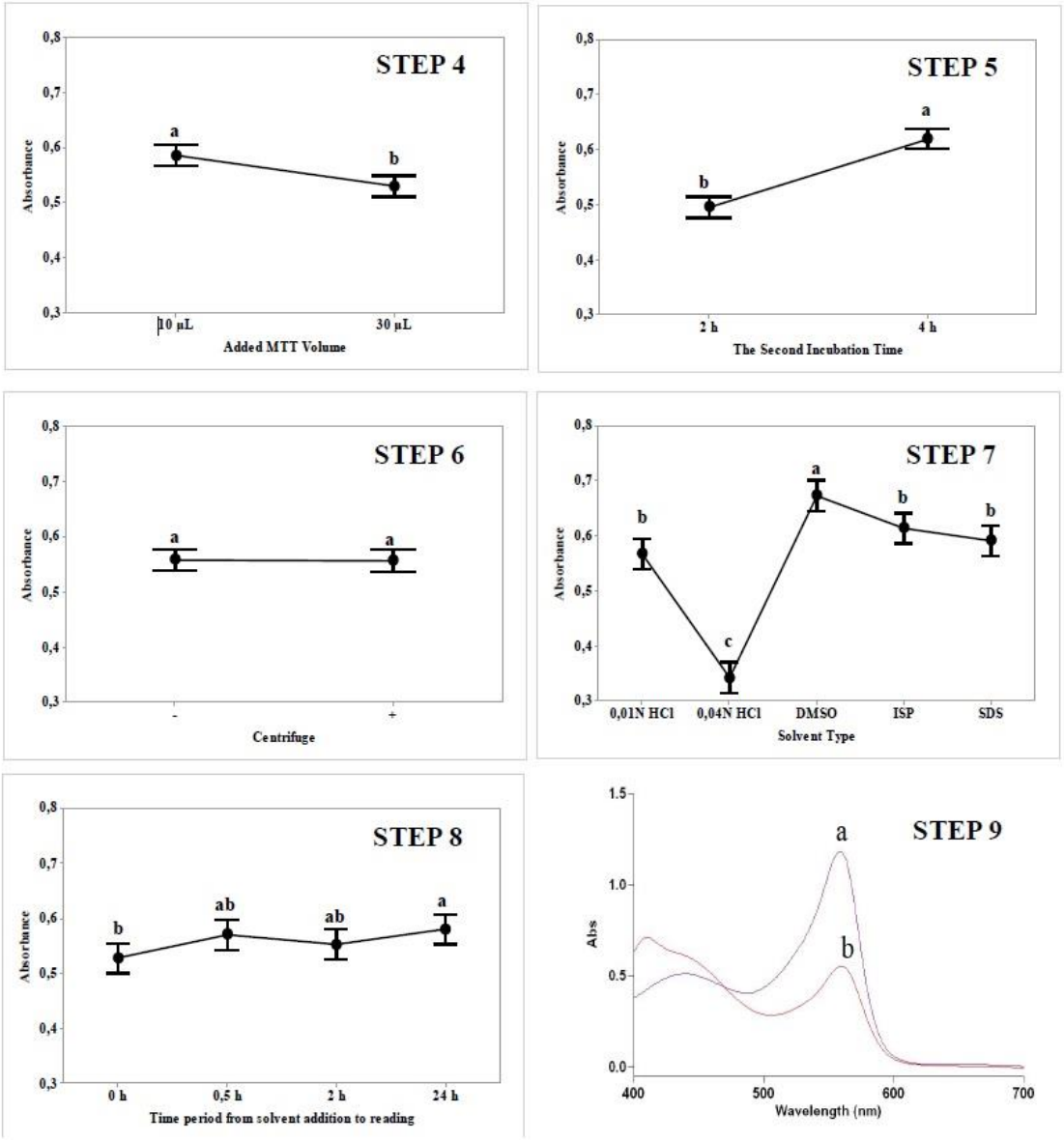

Fig 3 Affecting parameters of MTT assay for the determination of cell viability

After adding the MTT solution, the cells were incubated for two different time intervals as 2 and 4 hours for optimization of the second incubation time (Step 5). According to the results, the cells incubated for 4 hours showed a greater absorbance value than that of 2 hours (Figure 3).

A centrifugation procedure (500 x g for 5 min) was applied for the fast sedimentation of formed formazan crystals after the second incubation of the cells in the well plate (Step 6). To compare the sedimentation effects on the method performance, two different applications (centrifugation and without centrifugation) were performed. According to the results, there was no statistical difference among the absorbance values between with

and without centrifugation application (Figure 3, Step 6). Although centrifugation was recommended in many studies [17, 18], we could not achieved statistically significant results for centrifugation.

Small amount of formazan crystals, related to the viable cell counts, were observed at the bottom of the wells. The solubility of the crystals can be affected by medium fraction found in the upper phase of the wells when adding the solvent at the next step. Therefore, different amounts of medium fraction (0%, 90% and 100%) after the centrifugation procedure were removed from the wells and absorbance values of the crystals was evaluated. According to the results, the highest absorbance values were obtained in the experiment of without removing of the medium (0%), whereas the lowest value was found in 100% removing medium. It can be attributed to the additional absorbance values of the medium may increase the total absorbance value. To find the exact value of absorbance that can be result from the formazan crystals the medium content in the upper phase must be removed from the well. This procedure can increase the dissolving of formazan crystals when adding the solvent at the next step.

Various solvent types (DMSO, %20 SDS, isopropanol, two different concentration of acid isopropanol (0.01N HCl and 0.04 N HCl)) were applied to dissolve the formed formazan crystals (Step 7). The results showed that the lowest absorbance value was obtained for isopropanol with 0.04N HCl application. As shown in Figure 3 Step 7, there was no statistically significant difference among the SDS, ISP and ISP with 0.01N HCl ($P<0.05$). The highest absorbance value was achieved for the application of DMSO. It was reported that the DMSO [24, 25] and acid-isopropanol (0.04 N HCl in isopropanol) [7, 26] were widely used for the dissolving solvents of formed formazan crystals.

DMSO is one of the important organosulfur compounds with the formula of $(CH_3)_2SO$. This compound has a polar character that dissolves both nonpolar and polar compounds and is miscible in a wide variety of organic solvents as well as water. DMSO has a relatively high boiling point. It is also a reliable compound for toxicological aspects and it has a median lethal dose higher than ethanol (DMSO: LD_{50}, oral, rat, 14,500 mg/kg; [27], ethanol: LD_{50}, oral, rat, 7,060 mg/kg [28]).

Absorbance values in wells were measured at various time intervals after the addition of DMSO to find the effects of waiting periods (Step 8). The lowest absorbance value was observed following addition of medium within 0.5 h. There was no statistically difference

between the 0.5 and 2 h for the absorbance values, but that the values increased for 24 h (Figure 3, Step 8). After the MTT formazan dissolved in DMSO, the optical density remained the same for several hours, but the resolution increased after 24 hours. In conclusion, prepared solutions should be read within 2 hours. Obtained result agreed with the study of [29].

After the waiting period obtained absorbance was read by a spectrophotometer at the wavelength of 570 nm. Although the formed formazan crystals in DMSO gives some absorbance values in spectrophotometers, used medium and DMEM result in additional absorbance values as shown in Figure 4. According to the figure, DMSO has no absorbance value in the tested wavelength range (a) and formazan crystals solved in DMSO has an absorbance value depending on the viable cell number. Therefore, the medium fractions which are found in upper phase after the centrifugation step before the addition of solvents should be carefully removed to increase the absorbance value for the formazan crystals. In addition, absorbance values of DMEM (b), MTT (c) and FBS + DMEM (d) were found to be higher than the DMSO absorbance. The results show that additional absorbance values can be observed when these compounds are used in experiments. Therefore, these values should be considered as control samples.

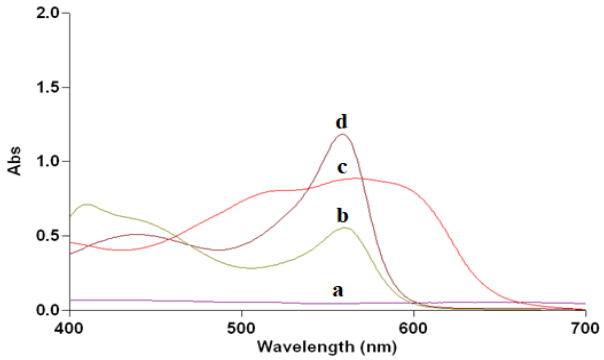

Fig 4 Absorbance values of DMSO (a), DMEM (b), MTT (c) and FBS + DMEM (d)

Method validation

Repeatability of the method was found in the range between 4.939%. LOD and LOQ values were found to be 495 and 1651 cells/well. As a result, the optimized method can give reliable results if the cell number is higher than 1651 cells/well.

Conclusion

The metabolic activities among different cell lines have been reported a great variability and therefore MTT assay needs to be characterized and optimized for melanoma cell lines. In the assay, metabolically active cells can convert the MTT dye (yellow water-soluble tetrazolium salt) in water to insoluble dark formazan crystals by reductive cleavage and the conversion can be affected by many factors during the analytical procedure. MTT assay has great potential as a fast method for determining cell viability of cell lines. In this study, a tetrazolium salt (MTT) based colorimetric assay was optimized and validated. Constructed flowchart based on the analytical results was presented in Table 1. Optimized MTT assay can be reliably applied to determine cell viability of SK-Mel-30.

Table 1 Constructed flowchart based on the analytical results

Step	Actions	Optimum parameter
1	Seeding the cells	10000 cells/well
2	First incubation	37°C 24h
3	Washing the wells	The medium is replaced with 1X PBS after 24h
4	MTT reagent addition	10 µl from 10 mg/ml
5	Second incubation	37°C 4h
6	Centrifugation	No difference
7	Solvent addition	DMSO
8	Waiting time for reading	0.5-2 hours
9	Wavelength	570 nm

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References

1. Chacon, E., D. Acosta, and J.J. Lemasters, 9 - Primary Cultures of Cardiac Myocytes as In Vitro Models for Pharmacological and Toxicological Assessments, in In Vitro Methods in Pharmaceutical Research, J.V. Castell and M.J. Gómez-Lechón, Editors. 1997, Academic Press: San Diego. p. 209-223.
2. Dzwierzynski, W.W., Managing Malignant Melanoma. Plastic and Reconstructive Surgery, 2013. 132(3): p. 446e-460e.
3. Melanoma. Nature Reviews Disease Primers, 2015. 1(1): p. 15055.
4. Senapati, S., et al., Controlled drug delivery vehicles for cancer treatment and their performance. Signal transduction and targeted therapy, 2018. 3: p. 7-7.
5. Sandru, A., et al., Survival rates of patients with metastatic malignant melanoma. J Med Life, 2014. 7(4): p. 572-6.
6. Schadendorf, D., et al., Melanoma. The Lancet, 2018. 392(10151): p. 971-984.
7. Mosmann, T., Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods, 1983. 65(1): p. 55-63.
8. Kuete, V., O. Karaosmanoğlu, and H. Sivas, Chapter 10 - Anticancer Activities of African Medicinal Spices and Vegetables, in Medicinal Spices and Vegetables from Africa, V. Kuete, Editor. 2017, Academic Press. p. 271-297.
9. Pascua-Maestro, R., et al., The MTT-formazan assay: Complementary technical approaches and in vivo validation in Drosophila larvae. Acta Histochemica, 2018. 120(3): p. 179-186.
10. Sylvester, P.W., Optimization of the Tetrazolium Dye (MTT) Colorimetric Assay for Cellular Growth and Viability, in Drug Design and Discovery: Methods and Protocols, S.D. Satyanarayanajois, Editor. 2011, Humana Press: Totowa, NJ. p. 157-168.
11. van Tonder, A., A.M. Joubert, and A.D. Cromarty, Limitations of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay when compared to three commonly used cell enumeration assays. BMC Research Notes, 2015. 8(1): p. 47.
12. Jabbar, S.A., P.R. Twentyman, and J.V. Watson, The MTT assay underestimates the growth inhibitory effects of interferons. British journal of cancer, 1989. 60(4): p. 523-528.
13. Hayon, T., et al., Appraisal of the MTT-based assay as a useful tool for predicting drug chemosensitivity in leukemia. Leukemia & lymphoma, 2003. 44(11): p. 1957-1962.
14. Carmichael, J., et al., Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer Res, 1987. 47(4): p. 936-42.
15. Denizot, F. and R. Lang, Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. Journal of immunological methods, 1986. 89(2): p. 271-277.
16. Abe, K. and N. Matsuki, Measurement of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction activity and lactate dehydrogenase release using MTT. Neuroscience research, 2000. 38(4): p. 325-329.
17. Ali, B., et al., Cytotoxic effects of a novel maleimide derivative on epithelial and tumor cells. Bioorganic Chemistry, 2017. 72: p. 199-207.
18. Marks, D.C., et al., The MTT cell viability assay for cytotoxicity testing in multidrug-resistant human leukemic cells. Leukemia Research, 1992. 16(12): p. 1165-1173.
19. Sönmez, M., et al., Investigation of antioxidant, enzyme inhibition and antiproliferative activities of blackthorn (Prunus spinosa L.) extracts. International Journal of Life Sciences and Biotechnology, 2021.
20. Genç, S., et al., Investigation of the Exosome-Based Drug Delivery System Potential in theTreatment of Glioblastoma in vitro Experimental Models. International Journal of Life Sciences and Biotechnology, 2021.

21. EUORACHEM, The fitness for purpose of analytical methods. A laboratory guide to method validation and related topics. EURACHEM Guide, ed. B. Magnusson and U. Örnemark. 2014.
22. Plumb, J.A., Cell sensitivity assays : the MTT assay. Methods Mol Med, 1999. 28: p. 25-30.
23. Doustvandi, M.A., et al., Photodynamic therapy using zinc phthalocyanine with low dose of diode laser combined with doxorubicin is a synergistic combination therapy for human SK-MEL-3 melanoma cells. Photodiagnosis and Photodynamic Therapy, 2019. 28: p. 88-97.
24. Jo, A., et al., Cell viability and measurement of reactive species in gas- and liquid-phase exposed by a microwave-excited atmospheric pressure argon plasma jet. Current Applied Physics, 2020. 20(4): p. 562-571.
25. Suntravat, M., et al., Expression, purification, and analysis of three recombinant ECD disintegrins (r-colombistatins) from P-III class snake venom metalloproteinases affecting platelet aggregation and SK-MEL-28 cell adhesion. Toxicon, 2016. 122: p. 43-49.
26. Tubaro, A., et al., Suitability of the MTT-based cytotoxicity assay to detect okadaic acid contamination of mussels. Toxicon, 1996. 34(9): p. 965-974.
27. MSDS, Material Safety Data Sheet: Dimethyl Sulfoxide. https://fscimage.fishersci.com/msds/07770.htm (Accessed date:01.03.2021), 2007.
28. MSDS, Material Safety Data Sheet: Ethanol. https://fscimage.fishersci.com/msds/89308.htm (Accessed date: 01.03.2021), 2003.
29. Twentyman, P.R. and M. Luscombe, A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. British journal of cancer, 1987. 56(3): p. 279-285.