Limitations of the use of MTT assay for screening in drug discovery

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Abstract: The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay is a routine cell viability assay for cell proliferation and cytotoxicity, which is widely used in many fields, especially in screening for drug discovery. However, this assay exhibits limitations in the presence of particular compounds and under certain assay conditions, which may yield false screening results. For example, polyphenols that are extracted from natural sources can react with MTT in the absence of living cells and thus interfere with the screening results. We measured the absorbance of 15 polyphenols extracted from green tea and showed that the phenolic hydroxyl groups in the polyphenols are responsible for the reduction of MTT to formazan. When three or more phenolic hydroxyl groups were present on a conjugated polyphenol, a significantly increased MTT reduction was observed. Moreover, the type of medium also had an effect on the absorbance value, in the following order: α-MEM + 10% FBS> α-MEM>DMEM/F12>PBS. The absorbance of the MTT assay recorded at 570 nm is more sensitive than that measured at 595 nm. These results will improve the cell-based assay of polyphenols and clarify the limitations of the MTT assay as a method of screening in drug discovery.

Keywords: MTT assay; Polyphenols; Drug discovery; Screening

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1. Introduction

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) can be reduced by mitochondrial dehydrogenase in living cells to produce insoluble purple formazan crystals. The amount of formazan, which can be quantitatively measured using a spectrophotometer, reflects cell viability[1,2]. Based on the correlation between the amount of produced formazan and the number of active cells, the MTT assay is used as a tool to assess cell proliferation and cytotoxicity. This assay is widely used in the field of biology, especially in high-throughput screening for drug discovery[3–5]. However, the MTT assay exhibits limitations in the presence of certain compounds and under certain assay conditions, yielding false screening results[6–8]. Our preliminary data revealed that the use of this assay to screen some compounds extracted from traditional herb medicines led to the identification of false-positive hits. This was further confirmed using an ATP cell viability assay and microscopic examination of cells. To investigate the factors that interfere with the MTT assay, 15 polyphenolic compounds extracted from green tea were used to assess whether the polyphenols themselves affect the results of the MTT assay. Polyphenols extracted from natural sources are attracting increasing attention due to their bioactivities, such as antioxidation and potential treatment for neurodegenerative disease[9,10]. We found that the polyphenols reduced MTT directly in the absence of living cells. Moreover, we found that the type of cell culture medium and the filter set of the spectrophotometer had an effect on the results of the MTT assay.

2. Methods

2.1. Compound library for screening

A "natural compound library" was built for screening using the compounds isolated from traditional herb medicine. The polyphenols *p*-coumaric acid, caffeic acid, chlorogenic acid, gallic acid, catechin,

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epicatechin, scutellarin, quercetin, and baicalein were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and had Chinese SFDA standard medical compound certification. Epicatechin gallate (ECG), epigallocatechin gallate (EGCG), gallocatechin gallate (GCG), epigallocatechin (EGC), kaempferol, and myricetin were purchased from Tianjin Yifang Company (Tianjin, China) and each compound had >95% purity, as assessed by HPLC.

To make the screening library, each compound was dissolved in dimethyl sulfoxide (DMSO) at a 5 mg/mL stock concentration and was dispersed into 96-well storage plates. Stock solutions were stored at –20 °C. For each experiment, a frozen aliquot was thawed and working solutions were prepared in phosphate-buffered saline (PBS). All the reagents used in this study were of analytical grade. The 96-well plates or 384-well plates were purchased from Corning Inc. (New York, USA).

2.2. Cell proliferation assay

DMEM/F12, α-MEM and fetal bovine serum (FBS) were purchased from Gibco Invitrogen (Grand Island, USA). Human mesenchymal stem cells (HMSCs) were kindly provided by Prof. Lin-Song Li at the Stem Cell Research Center of the Peking University Health Science Center. HMSCs were cultured in a volume of 90 μL of α-MEM and 10% FBS culture medium. HMSCs were seeded in 96-well plates at a density of 1×10^3 cells per well. After 24 h incubation, 10 μL of a working solution of the compounds was added to each well, which was done in triplicate. The MTT assay was used to screen for compounds that may induce HMSC proliferation. The hits identified in the primary screening were further assessed for dose-response in MTT assay. To confirm the results, the dose-response was retested using the ATP assay. The ATP assay uses the CellTiter-Glo® Luminescent Cell Viability Assay kit. Examination under a microscope was also used to confirm the proliferation of cells visually.

2.3. MTT cell-viability assay

MTT was purchased from Bio Protean Test Tech. (Beijing, China). The primary cell proliferation screening was performed using the MTT assay. Each compound tested in the primary screening was at a final concentration of 10 μg/mL, with three replicates. The hits from the primary screening were assayed again through a secondary screening for dose-response. To obtain a dose-response curve, each hit was brought to a final concentration of 0, 0.12, 0.37, 1.11, 3.33, 10 and 30 μg/mL. After incubation for 48 h in the presence or absence of the screening compounds, 10 μL of MTT (5 mg/mL) was added to the wells, which were then incubated at 37 °C for 4 h to ensure the complete reduction of MTT to formazan by the mitochondrial dehydrogenase in living cells. Subsequently, 100 μL 0.1% NH_4Cl and 10% dodecyl phenyl sodium sulfonate solution was added to the plates, followed by overnight incubation at 37 °C to dissolve the formazan crystals. Absorbance was measured using a Victor^3 V Multilabel reader (PerkinElmer, U.S.A) with the filter set to 595 nm or 570 nm (reference wavelength, 650 nm).

2.4. ATP cell viability assay

The CellTiter-Glo® Luminescent Cell Viability Assay kit for the ATP assay was purchased from Promega (Madison, USA). The ATP assay is a convenient and sensitive luminescence based test, similar to the MTT assay[11,12]. It provides a method to determine the number of viable cells in culture based on the quantification of ATP and allows the direct measurement of samples in the plate using a multiplate reader. Here, we used the ATP assay to confirm the screening results.

HMSCs were plated and incubated, and the addition of compounds followed the protocol described for the MTT assay. After an additional 48 h of incubation, the detection protocol included the addition of 80 μL of the working solution of the ATP assay, which is a mixture of the CellTiter-Glo® buffer and the CellTiter-Glo® substrate, to each well. The luminescence of each well was measured using a Victor^3 V Multilabel reader.

2.5 Data analysis

Data are presented as $\bar{x} \pm$ SD of triplicate samples. Statistical comparisons were performed by one-way ANOVA analysis (Dunnett's post-hoc test) using the SPSS software (version 13.0) to compare mean absorbance values in the presence or absence of compounds. $P<0.05$ was considered statistically significant. For the relativity analysis, we used a linear regression model. Statistical analyses were performed using the SAS software, version 6.12 (SAS Institute, Cary, NC, USA).

3. Results and discussion

3.1. Screening hits identified in the MTT assay were not confirmed by the ATP assay

To identify compounds that had a potential to stimulate stem-cell proliferation *in vitro*, we built a "natural compound library" for screening. All compounds in the library were first screened at a concentration of 10 μg/mL using the MTT assay. The comparison of the absorbance of each compound with the average absorbance of the whole plate allowed us to pick compounds with an absorbance that was more than two-fold higher than the standard deviation of the average absorbance of the whole plate. Three hits (catechin, caffeic acid, and epicatechin) were selected from the primary screening.

These hits were then tested at different concentrations using the MTT assay to obtain a dose-response curve (Fig. 1A). The three hits seemed to promote HMSC proliferation in the following order, caffeic acid> catechin>epicatechin, at 10 μg/mL and 30 μg/mL; however, there was no significant effect at lower concentrations.

To confirm these results, we chose to perform an ATP assay, which yielded very different results (Fig. 1B). The three primary hits did not have similar effect in the ATP assay, as the compounds began to inhibit the growth of HMSCs at 30 μg/mL. This was further confirmed by the visualization of cells under a microscope, which revealed that the three compounds did not induce cell growth.

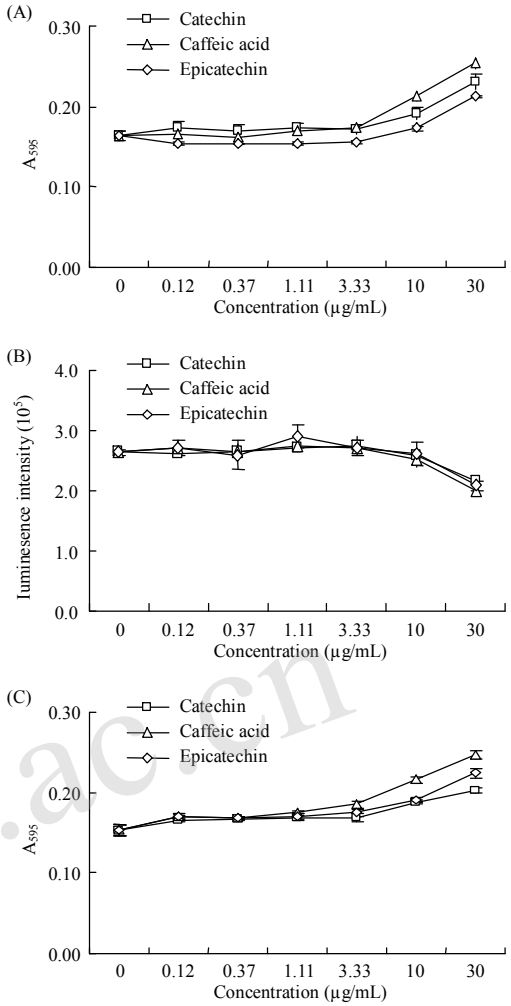

Figure 1. Dose-response curve of the three screening hits: catechin (squares), caffeic acid (triangles), and epicatechin (diamonds). (A) MTT assay in HMSCs; (B) ATP assay in HMSCs; (C) MTT assay in the absence of HMSCs. The final concentration of the hit compounds was 0, 0.12, 0.37, 1.11, 3.33, 10 and 30 μg/mL. Results are presented as $\bar{x} \pm$ SD. The MTT absorbance was measured at 595 nm ($n = 3$).

3.2. Factors that affected the MTT assay: screened compounds, culture medium and filter set-up

To identify the causes of the different results obtained using the same experimental design but different assays, the whole process was evaluated. It seemed that some factors interfere with the MTT assay. The three hits were assayed directly in the absence of cells following the same protocol of the MTT assay. All three hits showed similar dose-response pattern (Fig. 1A and 1C). These results clearly showed that the three hits had the ability to reduce MTT to formazan, which indicates that the use of the MTT assay to perform the cell proliferation screening may yield false-positive hits.

A method was developed previously to assess whether the culture medium is one of the interfering factors[13]. MTT was incubated with the hits (10 μg/mL) in DMEM/F12, α-MEM, or α-MEM and 10% FBS, respectively, which are media commonly used in the culture of stem cells *in vitro*. The incubation of the three compounds with MTT in PBS revealed no significant difference in absorbance (Fig. $2A_1$ and $2A_2$), whereas the absorbance recorded in other culture media showed a significant dose-dependent increase, in the following order: α-MEM + 10% FBS> α-MEM>DMEM/F12>PBS.

The wavelength may also have an effect on absorbance in the MTT assay. The absorbance at both 570 nm and 595 nm was evaluated, as most MTT assays use 570 nm or 595 nm filter. Our data showed that the MTT assay yielded a wider range and higher value at 570 nm compared with 595 nm (Fig. 2), which indicates that the 570 nm absorbance was more sensitive for cell proliferation screening using the MTT assay. For example, the incubation of the three compounds with MTT in α-MEM medium led to a higher absorbance, a wider range (in a dose-dependent manner), and a smaller SD range at 570 nm compared with the values obtained at 595 nm (Fig. $2B_1$ and $2B_2$). Thus, we recommend the 570 nm filter for the MTT assay.

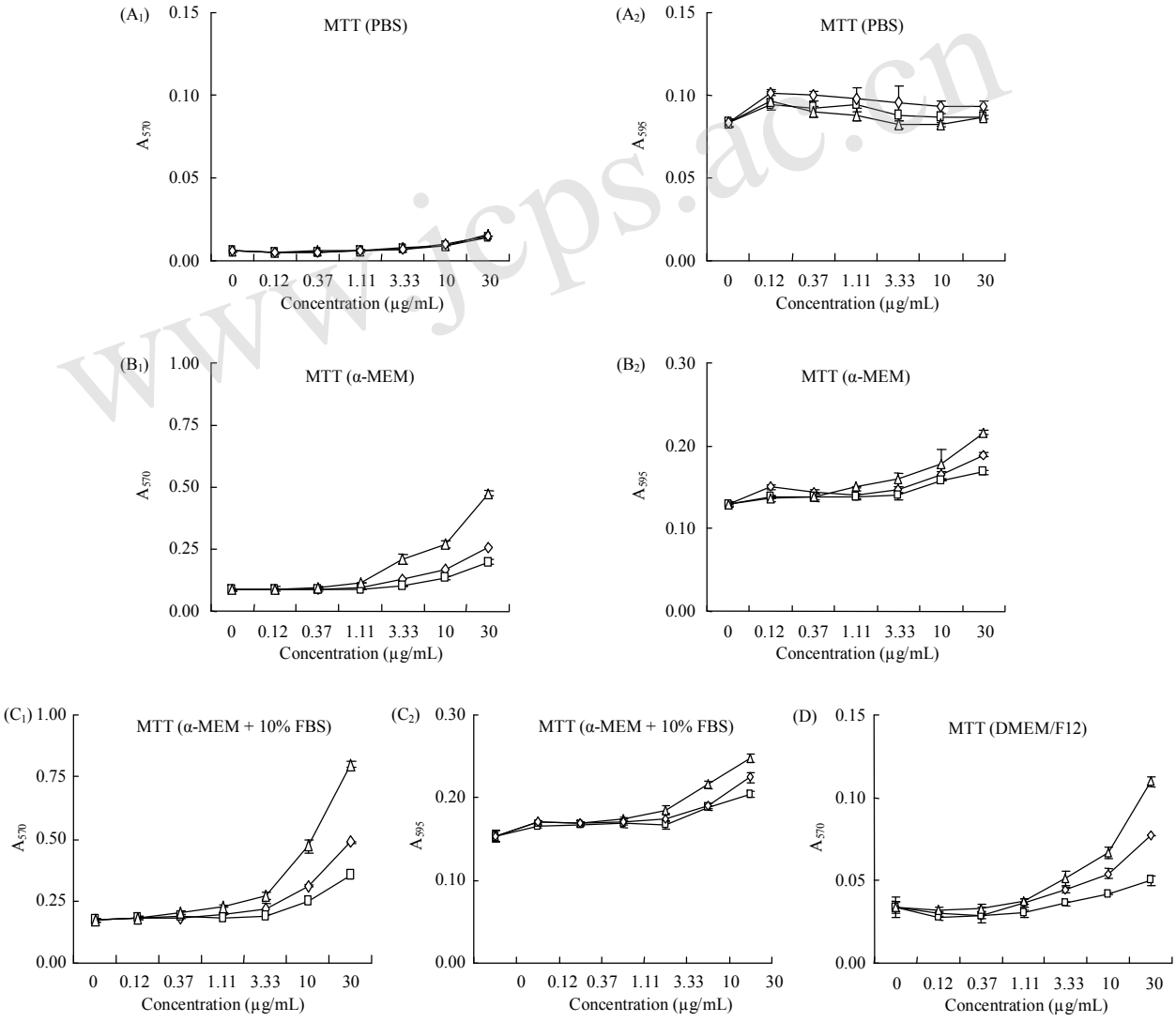

Figure 2. Effect of different media and filter wavelength on the reduction of MTT. MTT (5 mg/mL) was incubated with the three hits: catechin (squares), caffeic acid (triangles), and epicatechin (diamonds) in PBS (A_1 and A_2), in α-MEM medium (B_1 and B_2), in α-MEM + 10% FBS (C_1 and C_2), or in DMEM/F12, in triplicate (without cells). The MTT absorbance was measured at 570 nm (A_1, B_1, C_1 and D) and 595 nm (A_2, B_2 and C_2).

3.3. Polyphenols reduced MTT

The hits identified in our screen have a common structural feature: the phenolic hydroxyl group. To test whether polyphenols have an effect on the outcome of the MTT assay, we assayed 15 polyphenol compounds extracted from green tea. These polyphenols were divided into 4 groups: phenolic acids, flavanols, flavonols, and flavones. The results of this experiment and the test conditions are provided in Figure 3.

Our results clearly showed that the reducing ability of polyphenols obeys the following order: gallic acid>caffeic acid>chlorogenic acid>*p*-coumaric acid (for phenolic acids); EGC>GCG>EGCG>ECG> catechin>epicatechin (for flavanols); myricetin> quercetin>kaempferol (for flavonols); and baicalein> scutellarin (for flavones), which suggests that the absorbance increases with the increase in the number of phenolic hydroxyl groups within the same group of polyphenols (Fig. 3A). The absorbance remained low when one or two phenolic hydroxyl groups were present on one conjugated system (such as caffeic acid, chlorogenic acid, *p*-coumaric acid, catechin, epicatechin, kaempferol, and scutellarin); in contrast, it increased significantly when three phenolic hydroxyl groups were present on one benzene ring or a gallic ring (such as gallic acid, ECG, EGCG, GCG, EGC, and baicalein) as well as when three phenolic hydroxyl groups were present on a conjugated flavonol ring (such as quercetin and myricetin). Evidently, the highest absorbance was from the compound that contained both a gallic ring and a flavonol ring, such as myricetin (Fig. 3B).

In conclusion, although the MTT assay is a useful cell viability assay for cell proliferation and cytotoxicity study and it is widely used in many fields, especially in screening for drug discovery, many factors can interfere with the results, such as compounds that contain polyphenolic hydroxyl groups, types of culture media, and filter sets. For this reason, screening results should be carefully confirmed. Our results will improve the cell-based study of polyphenols and clarify the limitations of the MTT assay for screening.

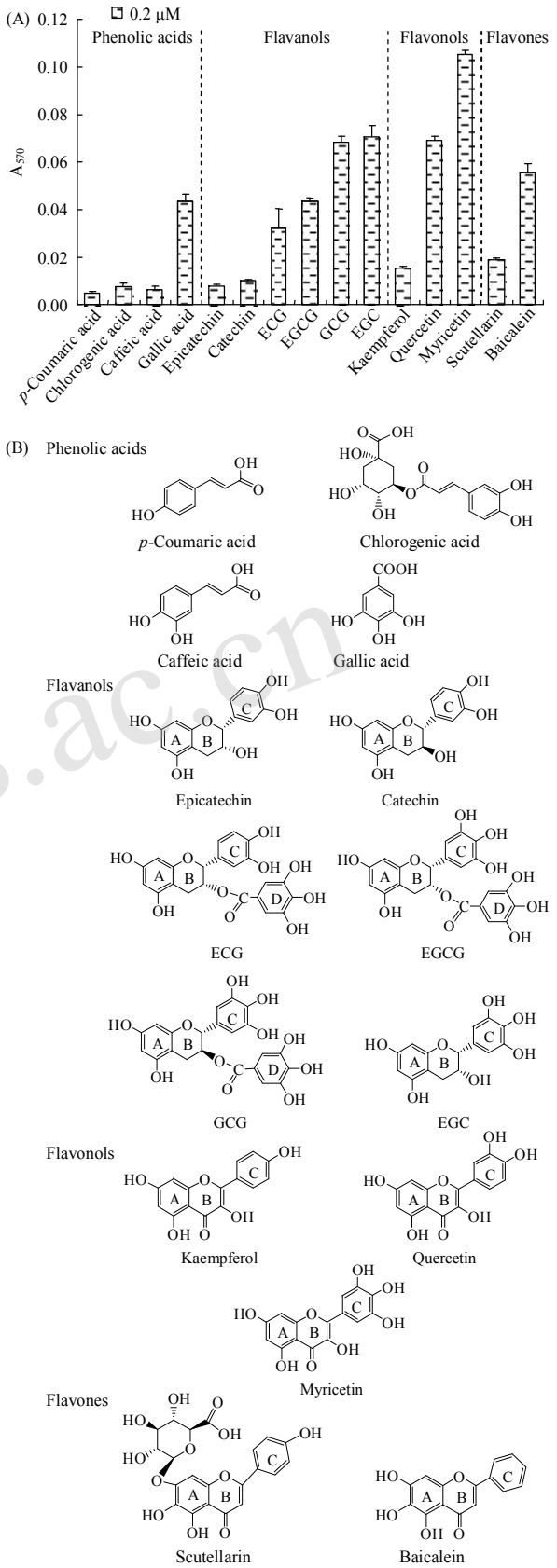

Figure 3. Correlation between the absorbance and the structure of the polyphenolic compounds in MTT assay. The association between the absorbance and the phenolic compounds examined (A). The structures of the phenolic compounds are shown in (B). Compounds are listed from left to right and top to bottom.

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References

[1] Mosmann, T. *J. Immunol. Methods.* **1983**, *65*, 55–63.

[2] Campling, B.G.; Pym, J.; Galbraith, P.R.; Cole, S.P. *Leuk. Res*. **1988**, *12*, 823–831.

[3] Berridge, M.V.; Horst, P.M.; Tan, A.S. *Biotechnol. Annu. Rev.* **2005**, *11*, 127–152.

[4] Young, F.M.; Phungtamdet, W.; Sanderson, B.J.S. *Toxicol. In Vitro.* **2005**, *19*, 1051–1059.

[5] Niles, A.L.; Moravec, R.A.; Riss, T.L. *Exp. Opin. Drug Discov.* **2008**, *3*, 655–669.

[6] Shoemaker, M.; Cohen, I.; Campbell, M. *J. Ethnopharmacol.* **2004**, *93*, 381–384.

[7] Talorete, T.P.N.; Bouaziz, M.; Sayadi, S.; Isoda, H. *Cytotechnology*. **2006**, *52*, 189–198.

[8] Peng, L.; Wang, B.C.; Ren, P. *Colloids Surf. B. Biointerfaces.* **2005**, *45*, 108–111.

[9] Lin, C.L.; Chen, T.F.; Chiu, M.J. *Neurobiol. Aging*. **2009**, *30*, 81–92.

[10] Yang, C.S.; Lambert, J.D.; Sang, S.M. *Arch. Toxicol.* **2009**, *83*, 11–21.

[11] Ulukaya, E.; Ozdikicioglu, F.; Oral, A.Y.; Demirci, M. *Cancer Chemother. Pharmacol.* **2008**, *22*, 246–250.

[12] Weyermanna, J.; Lochmanna, D.; Zimmerb, A. *Int. J. Pharm.* **2005**, *288*, 369–376.

[13] Funk, D.; Schrenk, H.H.; Frei, E. *Biotechniques*. **2007**, *43*, 178–182.

MTT方法在药物筛选研究中的应用限制

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摘要: 3-(4,5-二甲基噻唑-2)-2,5-二苯基四氮唑溴盐(MTT)法是一种常规的检测细胞活性和药物毒性的方法, 应用范围十分广泛, 是在细胞水平的药物筛选研究中重要的检测手段之一。然而, 这种检测方法由于化合物或测试条件的原因, 可能给出不真实的筛选结果。例如, 从天然植物中所提取多酚类化合物可以直接与MTT反应, 即使在没有细胞的情况下, 随着其浓度的增加, MTT的吸光度值也随之增加, 因此不能真实地反映筛选结果。我们从绿茶中提取的15个多酚类化合物进行测定, 表明MTT与多酚类化合物反应能力的大小与多酚类酚羟基的还原性有关。当三个或更多的酚羟基形成共轭的多酚时, 其还原能力显著增加。同时常用的细胞培养基也能影响MTT的吸光度值, 其影响顺序的大小为: α-MEM培养基 + 10% 胎牛血清(FBS)>α-MEM培养基>DMEM/F12>PBS。而且我们还发现MTT法在570 nm处的吸收测定比在595 nm处更敏感。这些发现不仅有助于多酚类化合物的细胞水平活性研究,还将有助于MTT方法在药物筛选研究中的正确应用。

关键词: MTT方法; 多酚类化合物; 药物发现; 筛选