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Interference of Antioxidant Flavonoids with MTT Tetrazolium Assay in a Cell Free System

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Introduction: The tetrazolium salt 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) used extensively to measure the quantitative survival and proliferation of mammalian cells. The analysis is based upon the reduction of MTT by metabolically active cells to insoluble formazan crystals. Flavonoids are a large group of natural compounds found in plants with variable phenolic structures. Flavonoids, as they are potential reducing agents, they act as a free radical scavenger. The aim of the study is to assess the reducing effect of some of the flavonoids on tetrazolium salt and their interference with the colorimetric analysis of MTT. The cell viability obtained from the MTT assay was compared with that of SRB assay in the determination of flavonoids cytotoxicity.
Materials and Methods: The present study examined the effect of few bio-flavonoids like Quercetin, EGCG, Rutin and Resveratrol to reduce MTT in the absence of cells under different experimental conditions such as concentration of flavonoids, incubation time and results were compared with SRB assay findings. The study also involves the analysis of flavonoid cytotoxicity on lung cancer cells NCIH-460 and NCIH-522 by MTT and SRB assay to establish the suitable cell viability assay for flavonoids.
Results: All the flavonoids showed the instant formation of the dark blue formazan salt in the absence of the cells with MTT assay. Whereas SRB assay of flavonoids in the absence of cells, results showed the absorbance similar to that of the blank, indicating that SRB did not interfere with flavonoids in a cell-free system.

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Conclusion: From the results, it is evident that MTT is not a suitable method to determine the effect of flavonoids on cell viability and proliferation as flavonoids itself reduces the MTT to formazan crystals. Study also suggests that SRB assay is more suitable method to determine the effect of flavonoids on cell viability.

Keywords: MTT; flavonoids; formazan crystals; antioxidants; phenolic structure.

ABBREVIATIONS

MTT : 3-[4, 5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide;
SRB : Sulforhodamine B;
EGCG : (-)-epigallocatechin 3-gallate;
DMSO : Dimethyl sulphoxide.

1. INTRODUCTION

Determination of cell viability, cell proliferation and cytotoxicity in response to various stimulus is key in monitoring the health of the cultivated cells. Measuring the various activities of the viable cells like metabolic reactions, enzymatic activity, energy production, ion transport, selective cell membrane permeability, reproduction, nucleotide uptake and co-enzyme synthesis reflects the cell damage or cell toxicity. MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide) is one of the simplest, most direct and widely used colorimetric tests using the action of mitochondrial dehydrogenases, an active metabolic enzyme in fast-dividing and healthy cells [1]. The basic concept of MTT assay involves the conversion of a water-soluble, yellow-colored tetrazolium salt by the action of mitochondrial NAD(P)H-dependent cellular oxidoreductase enzymes to water-insoluble purple formazan precipitate. The variations in cell density are further calculated by dissolving the formazan precipitate and spectroscopically quantifying at 540 nm where the optical density linearly represents the activity of the enzyme and thus the number of viable cells [2,3].

Due to certain technical advantages, an alternative assay used for screening is protein-binding dye sulforhodamine B (SRB) assay. SRB assay is a a sensitive and inexpensive method where it measures the cellular protein content by binding electro statically to basic amino acid residues of trichloroacetic acid-fixed cells in a pH-dependent manner.The results obtained are linear with the cell mass and extrapolated for cell proliferation estimation [4,5].

Flavonoids are the main group of phytonutrients which is a polyphenolic class of secondary metabolites in plants and some fungus. They are of considerable health benefits attributed to their wide range of pharmacological activity. Based on the variable phenolic and molecular structures, flavonoids categorized into four groups as flavones, flavanone, catechins and Anthocyanins (Fig. 1) [6].

The well-described working mechanism of almost all flavonoids is their capacity to scavenge oxygen-derived free radicals. Flavonoids are good reducing agents that are attributed to the highly reactive hydroxyl group that donates the electron to free radicals that make radicals stable and inactive afterward. The several health-beneficial properties of flavonoids resulting from antioxidant activities, particularly in cancer, have developed interest in these substances from recent research.Thus the study is initiated to investigate the anticancer activities of some dietary flavonoids like Quercetin, (-)-epigallocatechin 3-gallate (EGCG), Rutin and Resveratrol (structure depicted in Fig. 1) in cancerous cell lines using MTT and SRB assay [7].

Therefore the present study was carried to fathom out the reduction effect of flavonoids on MTT's tetrazolium salt in the absence of cells under certain experimental conditions. In addition, they are compared with the SRB assay to show a better option of cytotoxicity assay for flavonoids, which is very significant in providing well grounded results for this agent’s potential anticancer capacity. The comparison between the two assay methods performed in lung cancer-derived NCIH-460 and NCIH-522 cell lines.

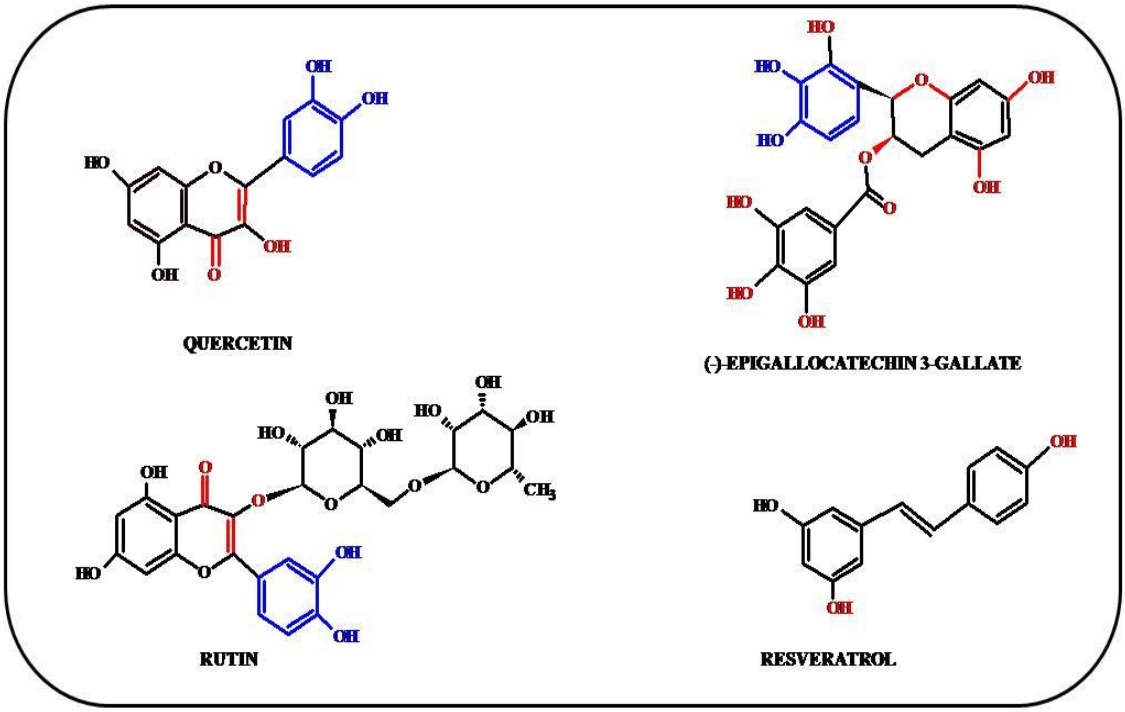

Fig. 1. Chemical structure of the flavonoids used in present study. Structures with functional group and hydroxyl substitution that is responsible for high reducing capability of the compounds are high lightened here (Red:hydroxy group, double bond and oxo functional group; Blue: catechol group in B ring)

2. MATERIAL AND METHODS

2.1 Cell Lines and Reagents

Human non small cell lung carcinoma cell lines NCIH-460 and NCIH-522 obtained from National Centre for Cell Science (NCCS), Pune and cultured in RPMI1640 medium supplemented with 10% fetal bovine serum. The cells are maintained in a humidified atmosphere of 95% of air and 5% CO_2 at 37^{o}C.

Quercetin, EGCG, Rutin and Resveratrol were purchased from Sigma Chemical. Dimethyl sulphoxide (DMSO), 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide and Sulphorhodamine B cell assay kit obtained from HiMedia whereas fetal bovine serum from GIBCO Inc.

2.2 MTT Assay

A total cells of 1×10^{3} per well were seeded in a 96 microplate to carry out the MTT assay and were incubated in a humidified atmosphere for 24hrs. The following test performed by treating the cells with flavonoids at a different concentration ranging from 25 to 200µM for 24hrs. A similar concentration of flavonoids was used for microplate containing no cells. Post incubation period, 10µl of MTT labeling reagent (5mg/ml of final concentration) added to each well in the microplate. After incubation at 37^{o}Cin a humidified atmosphere for 4hr, the supernatant carefully removed from each well and the precipitate obtained was dissolved in DMSO. Plates are tested for the absolute purple formazan crystals solubility and subjected to spectroscopic absorbance measurement at 570nm using a microplate reader.

2.3 Sulforhodamine B (SRB) Assay

SRB protein assay used to analyze cell proliferation. NCIH-460 cell suspensions of 100 µl were seeded in a 96 well microtiter plate (1×10^{3} cells per well) and incubated in a 5 % CO2 atmosphere at +37^{o}C. Flavonoids were added to the specified well at different desired concentration (0-200µM) and incubated for 24hrs. Cold fixative solution is layered on the top of the culture medium in each well and kept for 1hr at 2-8^{o}C. After multiple washings, plates are examined under a microscope to confirm cell

fixation and cells were stained with SRB staining solution for at least 30min. The unbound stains removed by washing several times and subsequently, plates were air-dried. Solubilisation solution was added to each well to extract the incorporated dye. The intensity of the colour measured spectroscopically at 450nm. SRB assay conducted according to the manufacturer's instruction (EZ count^{TM}Sulphorhodamine B cell assay kit) and IC50 value calculated from the triplicate reading.

2.4 Statistical Analysis

All the assays were performed in triplicate and subjected to statistical analysis.

Graphs were plotted, taking viability by percent against flavonoid concentrations. The mean absorbance OD (optical density) value obtained for treated samples or of untreated ones, compared with DMSO+Medium using independent Student's *t* test and ANOVA and the level of statistical significance was set at $p<0.05$. Data shown as mean ± standard error of triplicate readings.

3. RESULTS

3.1 Effect of Solvent DMSO on Absorption

To determine whether there is any interference of solvent DMSO with the MTT results, various percentages of DMSO was incubated with 10% of MTT in culture media at 37^{o}C for 4hr. The DMSO at any portion did not show any effect on the absorbance with or without cells. In the culture media, the average maximum absorbance value was just 0.01 to 0.05 higher than the minimal value.

3.2 MTT Assay of Flavonoids on NCIH-460 and NCIH-522

As shown in Figure 2, with the treatment of different flavonoids at different concentrations the percentage viability of both cell lines has been increased. As the concentration rose, the absorbance value for those cells receiving EGCG and Quercetin was substantially increased in comparison to those receiving Resveratrol and Rutin.

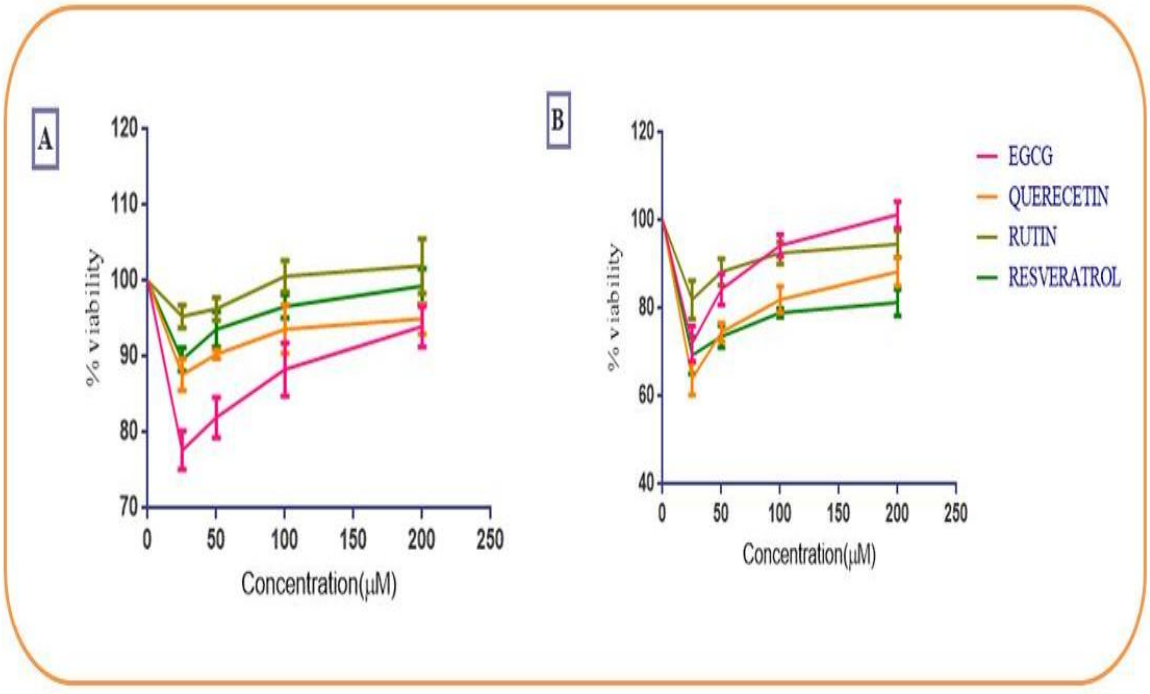

Fig. 2. MTT results showing the percent viability at different concentrations of cancer cells treated with various flavonoids, i.e., EGCG, Quercetin, Resveratrol and Rutin. NCIH-460(A) and NCIH-522(B) treated flavonoids displays reversed profiles of cell viability that start at a concentration of 25 µM

Values are shown as mean percentage of ± S.E.M (n = 3)

3.3 SRB Assay of Flavonoids on NCIH-460 and NCIH-522

The graph plotted in Fig. 5 shows the findings when SRB assay was used to evaluate cancer cell viability. The test confirms the cytotoxic and ant proliferative effect of flavonoids in both cell lines in a dose-dependent manner, as shown in the figure.

3.4 Effect of Flavonoid Concentration on Absorbance without Cells

The above findings clearly show that the results of MTT assay does not correspond to the SRB assay findings and also a microscopic examination of cells after treating with the different flavonoid. These findings suggest the inherent effect of flavonoids directly on MTT without cells. To validate this hypothesis, experiments are carried out by incubating various concentrations of the MTT reagents with specific flavonoids. The graph plotted for the corresponding formazan salt formed, taking the absorbance value against the varying flavonoid concentration shown in Figu. 3.In the absence of cells, it was observed that all the flavonoids reduced tetrazolium into insoluble purple precipitate of formazan salt in a dose-dependent manner. The potential for reduction of MTT by various flavonoids follows the orderEGCG>Querscetin> Rutin > Resvertrol.

The absorbance obtained is identical to that of the blank (data not given) when SRB assay of these flavonoids conducted in a cell-free system and thus shows no interference.

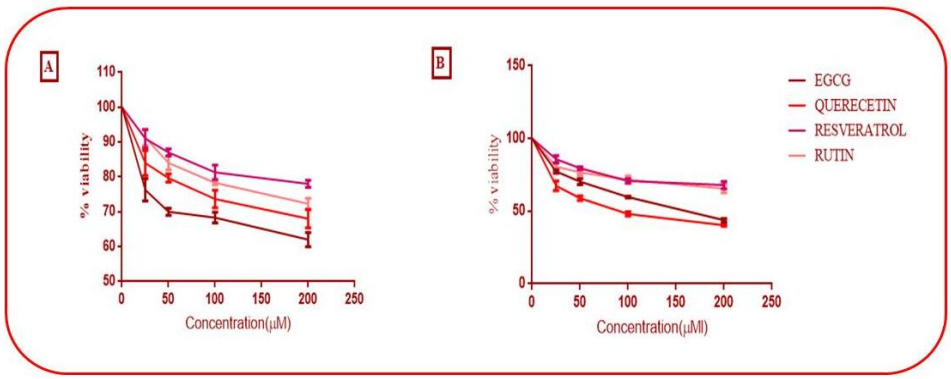

Fig. 3. Details of the SRB assay showing the percentage viability of the cancer cells NCIH-460(A) and NCIH-522(B) treated with EGCG, Quercetin, Resveratrol and Rutin. The treatment prevents growth of cancer cells in a way dependent on its concentration

Values are shown as mean absorbance by ±S.E.M (n=3)

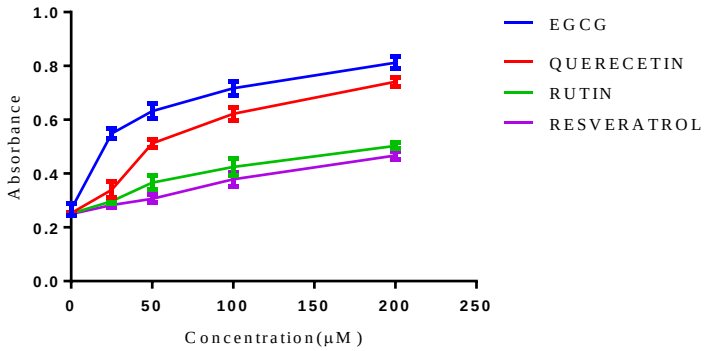

Fig. 4. Effects of flavonoid concentrations on absorption when MTT (10 per cent) was incubated with different concentrations of EGCG, Quercetin, Resveratrol and Rutin (25, 50, 100 and 200ug / ml) for 4 h, at 37°C

Results shall be shown as mean absorbance±S.E.M (n=3)

3.5 Effect of Incubation Time on MTT Reduction

Flavonoids are made to react with MTT for different incubation times (1, 2, 4 and 8hrs) at a concentration of 50(µM). The results in Figure 4 show that with increasing incubation period, EGCG, Quercetin, Rutin reduced MTT according to time while Resveratrol showed little change in absorbance.

3.6 MTT and SRB Assay of Doxorubicin as a Validation Control

Validation control assay performed using standard drug doxorubicin. As shown in the Fig. 6 the absorbance pattern of both MTT and SRB assay of doxorubicin was found to be similar in both the cell lines implies that there is no reduction of MTT by doxorubicin.

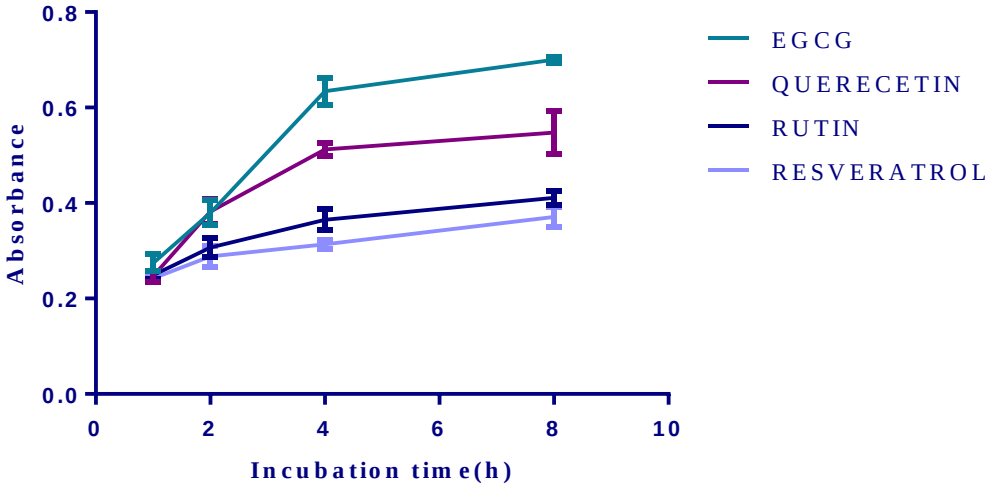

Fig. 5. Incubation time effects on absorbance when MTT (10 percent) has been incubated with 50µM of EGCG, Quercetin, Resveratrol and Rutin at 37°C for different times (1, 2, 4 and 8 h)

Results are given as mean absorbance±S.E.M (n = 3)

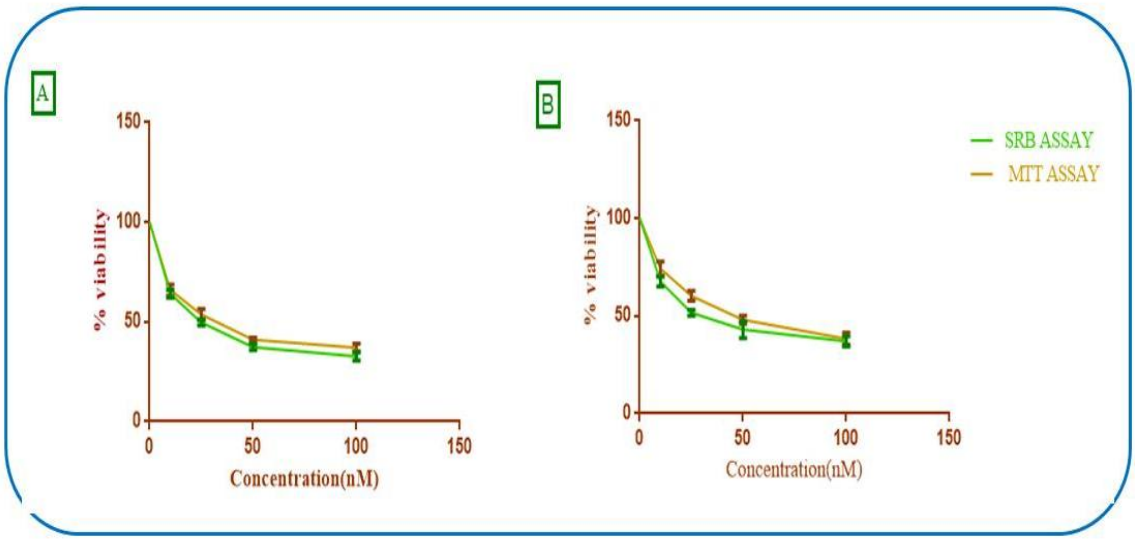

Fig. 6. The results of the MTT and SRB test show the viability percentage of doxorubicin-treated cancer cells as a validation control and indicate that doxorubicin inhibits cancerous cell growth in both assays and inhibition is dose dependent

Values are shown as mean percentage of ± S.E.M (n = 3)

4. DISCUSSION AND CONCLUSION

MTT is a direct, widely used assay to quantities the viability of cells and therefore the cytotoxic potential of compounds under test. Since its action mechanism is reduction reaction, the results of this assay does not reflect the actual cell viability when evaluating substances with intrinsic reductive potential.

Flavonoids belong to the family with excellent antioxidant potential, reported inhibiting the growth and proliferation of cancer cells. But when tested using MTT assay, the selected flavonoids such as EGCG, Quercetin, Resveratrol and Rutin, previously known for inhibiting cancer cell growth, were no longer inhibitive. . These compounds, irrespective of any cellular mechanism, may reduce MTT to form purple formazan leading to a fallacious interpretation of the results. There are also other indications that some herbal extracts, antioxidants such as vitamin E and their isomers have also been found to reduce MTT into formazan salt in a cell-free system [8,9]. Therefore, the assay includes vigilance when research involves screening natural products, like antioxidant phytochemicals extracted from plants.

The antioxidants interfere and possess the ability to reduce MTT in the cell system as reported by natarajan *et al.* [10]. The existence of the number of phenolic groups makes dietary polyphenols act as a reduction agent. The phenolic group can accept an electron form fairly stable phenolic radicals. They contain highly reactive hydroxyl group especially on B ring with a favorable electron donating properties. The hydroxyl group has the ability to transfer the electrons to 3-[4, 5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and reduces in to MTT-formazan [11]. Flavonoids, has a wide number of polyphenols that occur naturally, vary considerably in their structure like variation in the type of functional group and types of heterocycle involved. These variations bound to have an impact on their reducing power and thus show differences in reducing the MTT into formazan salt. On a general note, as the number of hydroxyl groups increases, the antioxidant potential of flavonoids also increases. Similarly, the catechol moiety with a hydroxyl group on carbon 3(3-OH) and the presence of a 2, 3 double bond also contributes to the activity [12]. Thus quercetin and EGCG show greater reducing activity, while absence of 3-OH in the C ring of rutin explains its lower reducing activity than that of quercetin [13]. Resveratrol found least or no reducing due to lack of these structural requirements.

Sulfarhodamine B assay appeared to be more sensitive, reproducible and rapid assay when compared with the MTT Assay. It provides better linearity with cell number at higher cell density with little intracellular and inter-assay variation [14]. The flavonoids under study showed substantial cytotoxic impact on cancer cells when the SRB assay is used to determine cell viability and thus supported microscopic findings. In the absence of cell tests, when we conducted SRB assay of flavonoids showed the absorbance close to that of the blank suggesting that SRB did not interact with flavonoids in the cell free environment. Nonetheless, SRB assay and MTT assay of doxorubicin yielded similar findings in validation control analysis where it was found that the MTT assay was not affected. Therefore SRB assay is more suitable for determining the growth kinetics particularly in the case of plant-derived antioxidants than MTT assay.

In conclusion, therefore, the results obtained by MTT assay gives false results in cases of natural products with intrinsic reduction potential such as antioxidant flavonoids. Thus applications of this assay demands precautions like inclusion of control without cells or washing of cells several times before adding MTT is recommended to avoid the false research conclusions whereas flavonoid does not interfere with the SRB assay method in absence of the cells. Thus it is recommended that SRB assay is preferred over MTT assay in case of testing compound like antioxidant flavonoid or other compound with reducing potential.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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