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Analysis of the interference of polyphenols and proanthocyanidins on the neutral red assay for cell viability.

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Abstract

The neutral red uptake assay has been wildly used for screening cell viability. However, several natural polyol compounds such as polyphenolics and flavonoids have been observed to interfere with the neutral red assay and give false positive results. This study was designed to determine whether polyphenolic compounds and proanthocyanidins extracted from tamarind seed husk (TSH) affect cell viability. Two kinds of normal cells, retina ganglion cells (RGC-5) and hippocampal cells (HT-22), were used in the study. It was found that by using neutral red assay, the TSH extract increased normal cell viability to the maximum level of 2-folds higher than the control. The artifact was similarly shown in both serum-free and serum-added media which had no cell proliferation. Therefore, neutral red assay may not suitably used for the cell survival test especially in the presence of proanthocyanidins or materials containing polyphenols or proanthocyanidins.

Keywords: Polyphenol, proanthocyanidins, cell viability, neutral red assay.

INTRODUCTION

Many kinds of polyphenolic compounds, especially proanthocyanidins and other flavonoids, have been reported to protect against oxidative stress-induced death in neuronal (Choi *et al.*, 2002; Roychowdhury *et al.*, 2001), retinal (Areias *et al.*, 2001), macrophage (Bagchi *et al.*, 2002), gastric mucosal (Bagchi *et al.*, 2002) and pheochromocytoma cells (Horakova *et al.*, 2002). In some of those studies, the cell viability assays relied on the neutral red assay (Choi *et al.*, 2002; Horakova *et al.*, 2002). These studies have largely reported that flavonoids alone have little or no effect on the cell viability (Choi *et al.*, 2002; Roychowdhury *et al.*, 2001; Horakova *et al.*, 2002). Horakova and his colleagues (2002) reported that the flavonoids from grape seed extract (*Pycnogenol*) at 0-100 ug/ml increased the viability of rat pheochromocytoma cells treated with hydrogen peroxide using the neutral red assay but at the higher doses the extract induced cell death. The cellular uptake of flavonoids depends on both the flavonoid structure and the cell type (Spencer *et al.*, 2004). The interfering effect of some polyphenols on cell viability as determined by neutral red uptake ability is not clearly understood. Sofar, only false positive results of cell viability by neutral red have been reported (Olivier *et al.*, 1995).The simple polyols such as mannitol, sorbitol and xylitol could clearly increase the apparent viability of hepatocytes by the uptake of neutral red up to approximately 60% but

this false positive effect was not found in the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) test (Olivier *et al.*, 1995). This artifact of the measurement may be one explaination of why the assessment of the flavonoid effect on cell uptake and cell function *in vitro* models does not fully reflect the cell uptake and cell function *in vivo*.

From the above studies, it was appeared that the inconsistencies of the colorimetric assay for cell viability depend on cell type, chemical structure of polyphenolic compounds tested and the assay used. To further explore the effects of different types of polyphenol compounds on cell viability *in vitro*, we chose to investigate the effect of proanthocyanidins from tamarind seed husk (TSH). TSH has been found to be a good source of proanthocyanidins (or procyanidins) and has potent antioxidant activity in cell-free systems (Siddhuraju, 2007; Luengthanaphol *et al.*, 2004). The major polyphenol compounds in TSH are proanthocyanidins which include (+)-catechin, procyanidin B_2,(-)-epicatechin, procyanidin trimer, procyanidin tetramer, procyanidin pentamer, procyanidin hexamer along with taxifolin, apigenin, eriodictyol, luteolin and naringenin (Sudjaroen *et al.*, 2005). TSH was confirmed to contain a very large amount of polymeric proanthocyanidins (Sinchaiyakit *et al.*, to be published).

The aims of this study are to investigate the effects of polyphenols and proanthocyanidins from tamarind seed husk on normal cell viability by neutral red assay.

MATERIALS AND METHODS

Chemical reagents

All the chemical reagents used in this study were bought from Sigma Chemical Co.

Biological materials

Oligomeric proanthocyanidin (OPC) isolated from grape seed extract (Akopharma brand) was bought from a local pharmacy. DMEM medium was obtained from Hyclone HyQ cell culture reagents (Cat# SH 30021.01,lot# ANC18374). Retinal ganglion cells (RGC-5), HT-22, an immortalized mouse hippocampal cell line, and SK-N-SH, a human neuroblastoma cell line were kindly obtained from Dr. Neeraj Agarwal, UNTHSC, Texas, USA.

Preparation of tamarind seed husk (TSH) extract

Tamarind seeds were obtained from ripened tamarind fruits and incubated at 140 °C, 45 minutes. After cooling, the seeds were cracked to separate their husk. The husk was ground into fine powder and was extracted with 50% acetone 3 times. The combined acetone extracts were dried by rotary evaporation at 45°C and the dried residue was used throughout the study.

High Performance Liquid Chromatography (HPLC)

The TSH extract sample (1 g) was hydrolyzed by refluxing in 30 ml methanol and 30 ml HCl (1.5N) for two hours. The hydrolyzed sample was analyzed by HPLC using a reversed phase C18 column and the chromatogram was monitored at 254 nm (Cherdshewasart *et al.*, 2007). The gradient elution was performed by using solvents

of acetonitrile and acetic acid. The HPLC profile of TSH extract hydrolyzate was compared with that of grape seed extract (Akopharma).

Cell culture

Retinal Ganglion Cells(RGC-5) were cultured in DMEM medium(Cat# SH 30021.01,lot# ANC18374, Hyclone HyQ cell culture reagents) with 10%fetal bovine serum and 1% penicillin and streptomycin. Cells were maintained in flasks in incubators 37°C, 5%CO_2 until they were confluent and ready for use.

Normal cell viability test by neutral red assay

RGC-5 cells and HT-22 cells were seeded in 24-well plates (approximately 2,500 or 5,000 cells per well) with 10% fetal bovine serum and 1% penicillin and streptomycin. All cells were incubated at 37°C with TSH or standard flavonoids at different treatment time and doses. After incubation, the cultured media was emptied from wells and the wells were washed twice with HEPES buffer pH 7.2. Then 1 ml of HEPES buffer and 33 μl of neutral red were added to each well. After 2 hours incubation, all wells were washed 3 times with HEPES buffer. After that, 500 μl of solubilization buffer (50% ethanol and 1% glacial acetic acid) was added and incubated for 20 minutes at room temperature. The absorbance at 570 nm was determined.

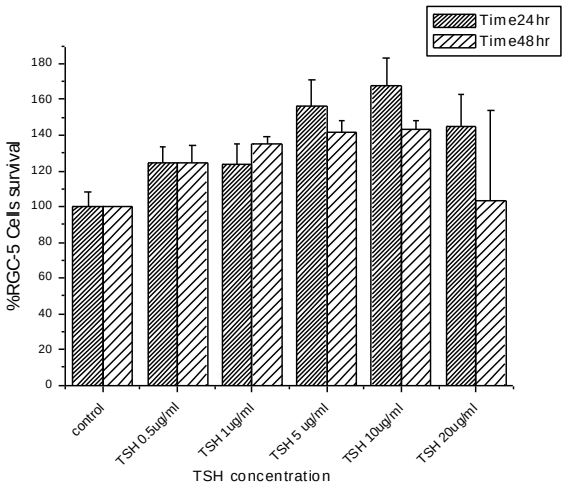
For serum free media experiment, two sets of cells were seeded at 2,500 cells/well in DMEM medium with 10% fetal bovine serum and 1% penicillin and streptomycin. In the first set, the media was changed to serum-free media and then incubated for 24 hours before the treatment while in the second set, the culture was in the same media, cell viability was assessed at 3 hours after adding TSH by neutral red assay.

RESULTS AND DISCUSSION

Extraction and HPLC identification of TSH

The TSH was extracted using 70% acetone which is the best solvent to extract the proantrocyanidin because it inhibits tannin-protein interaction (Hangerman, 1988). The highest peak of HPLC chromatogram emerged at 18 minutes and was identified as catechin by using an internal standard. The HPLC profile of TSH hydrolysate is very similar to that of grape seed extract (data not shown) confirming the presence of proanthocyanidin (Sudjaroen *et al.*, 2005).

(a)



(b)

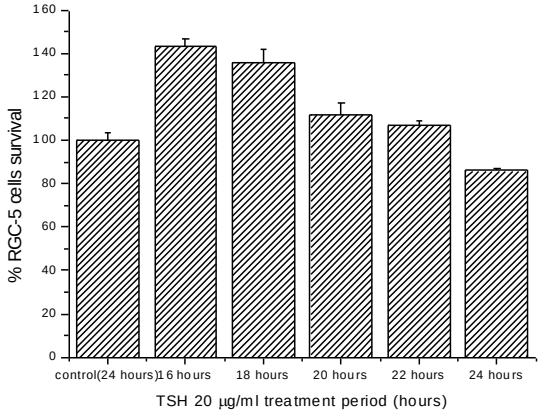


Figure 1 Cell viability response of retinal ganglion cells (RGC-5) exposed to TSHextract at different concentrations after 24 and 48 hours (a); and different treatment period (b). The viability of cells without TSH was designated as 100%. Each shown value represents the mean ± S.E. of four different samples.

Effect of TSH on normal cells using neutral red assay

The principle of the neutral red assay is based on the ability of viable cells to incorporate and bind the dye in the lysosomes. Cell survival is calculated from the increase change in absorbance at 540 nm. Addition of TSH resulted in a higher absorbance of neutral red than controls in the RGC-5 cell survival assay (Figure 1a). However, this absorbance effect decreased with time (Figure 1b). The interactions between the redox-active compounds (polyphenol) and the cells proliferation are influence by the cell culture time.

In a second set of studies addition of TSH still higher absorbance than the control (Figure 2). The cells were growing in DMEM medium with 10% fetal bovine serum and 1% penicillin and streptomycin. Fetal bovine serum contains several kinds of proteins and growth factors which are necessary for cell proliferation. In our experiment, the serum-free medium was changed instead of normal medium after adding the TSH extract. In serum-free medium condition, the cell growth was stopped at G_o- phase with no proliferation. Interestingly, % cell survival of cells in serum-free condition after added TSH extract was still higher than the control. This apparent increased percentage of cell survival was clearly artifactual.

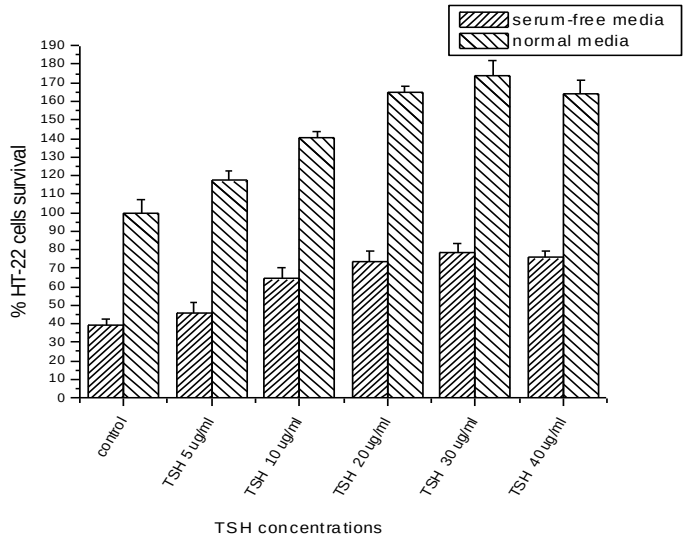


Figure 2 Cell viability response of hippocampal ganglion cells (HT22) exposed to TSH extract at different concentrations. The viability of cells without TSH was designated as a control of 100% cell viability. Each value represents the mean ± S.E. of four different samples.

To further explore the basic for the effect of flavonoids on the cell viability test, catechin and quercetin were used in the test. It was found that catechin, a proanthocyanidins monomer, increased the HT-22 apparent cell survival 18%, while quercetin increased the effect up to 70% (Figure 3). The false positive artifact of TSH may be associated with the presence of catechin in TSH.

It has been reported that neutral red uptake may be induced by osmotic swelling agents such as polyols (Olivier *et al.*, 1995; Chiba *et al.*, 1998). Because TSH extract is composed of monomers, oligomers and polymers of proanthocyanidins with many hydroxyl groups (polyol), the false positive may be due to excessive osmotic swelling and abnormal neutral red uptake (false positive increase in cell viability).

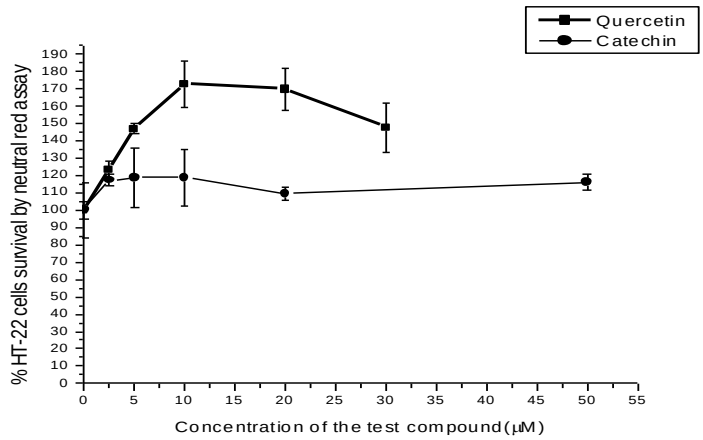


Figure 3 Cell viability response of hippocampal ganglion cells (HT22) exposed to quercetin and catechin (as standard flavonoids). Cells were seeded 5,000 cells/well in DMEM medium with 10% fetal bovine serum and 1% penicillin and streptomycin. Cell viability response was assessed at 3 hours after adding quercetin or catechin using neutral red assay. The viability of cells without the tested samples was designated as 100% as control. Each value represents the mean ± S.E. of four different samples.

CONCLUSION

This study demonstrated that proanthocyanidins isolated from TSH extract interfere the apparent viability of RGC-5 and HT-22 cells when using the neutral red assay. It is proposed that this artifact may be due to abnormal osmotic uptake of the dye and the high level of polyphenols in the TSH. Thus it is suggested that the neutral red assay is not suitable to determine cell viability in the presence of materials containing polyphenols or proanthocyanidins. Although Olivier *et al.* 1995 reported that the 3-[4,5-dimethylthiazol-2 yl]-2,5-diphenyl tetrazolium bromide (MTT) assay was unaffected by concentrations of polyols. However, the artifacts in cells-base studies resulting from the reactivity of polyphenols by MTT assay was investigated by Wisman *et al.* (2008). Many of polyphenolic compounds including

proanthocyanidin, catechin, quercetin and tannin can reacted with MTT, leading to the conclusion that polyphenolics often give false positive responses in the MTT cell viability assay (Wisman *et al.*, 2008). Wisman *et al.* (2008) suggested that the better method for determining cell viability is the luciferin/luciferase ATP assay.

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