An Efficient and Economical MTT Assay for Determining the Antioxidant Activity of Plant Natural Product Extracts and Pure Compounds

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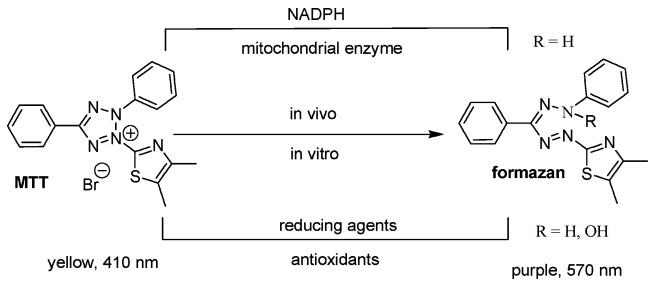
Antioxidants scavenge free radicals, singlet oxygen, and electrons in cellular redox reactions. The yellow MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] is reduced to a purple formazan by mitochondrial enzymes. NADPH is the basis of established in vitro cell viability assays. An antioxidant assay has been developed utilizing the redox reaction between MTT and selected natural product extracts and purified compounds. This simple, fast, and inexpensive MTT antioxidant assay is comparable with the lipid peroxidation inhibitory assay and can be mechanized to achieve high throughput.

Redox reactions are crucial for the success of many biological processes, but they can also be damaging to the human body. Low levels of antioxidants can result in high levels of reactive oxygen species, which cause oxidative stress, result in cell death, and induce many diseases including cancer, heart disease, cataracts, and congestive disorders.[1] Antioxidant compounds play an important role in preventing or delaying the onset of major degenerative diseases. The physiological role of antioxidant compounds is to terminate the oxidation chain reactions by removing free-radical intermediates and inhibit other oxidation reactions by being oxidized themselves. Vitamin E (α-tocopherol) and the FDA-approved food preservatives *tert*-butyl hydroquinone (TBHQ), butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) are excellent examples of antioxidants used for this purpose.[2−4]

Several in vitro methods are in place to determine the antioxidant capacity of natural product extracts and purified compounds. For example, the lipid peroxidation (LPO),[5] the qualitative 2,2-diphenylpicrylhydrazyl (DPPH),[6] the quantitative trolox equivalent antioxidant capacity (TEAC),[7] the 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS),[8] the ferric reducing antioxidant power (FRAP),[9] and the oxygen radical absorption capacity (ORAC)[10] assays have all been used frequently to determine antioxidant capacity of a variety of compounds. However, each of these assays has limitations due to the overall cost, solubility of test compounds in the assay medium, and extrapolation of results to the in vivo outcome.

The [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay is an established colorimetric assay for measuring the activity of mitochondrial enzymes present in healthy cells by monitoring the absorbance of purple formazan (570 nm) formed as the enzymatic reduction product of MTT (410 nm).



This method is used extensively to determine the cellular toxicity of natural and synthetic compounds. In addition, the qualitative use of the MTT method has been reported in the evaluation of the antioxidant potential of plant extracts.[11] We have used this preliminary information and further refined the use of MTT as a tool to measure quantitatively the antioxidant potential of a variety of natural product extracts and pure compounds. Parameters for the assay to yield dependable and reproducible results have been determined, inclusive of reaction conditions such as the solvents, time, concentration of reactants, and reaction chambers.

The present studies have shown that optimum reaction conditions for natural product extracts and pure compounds tested with MTT were rendered in a water−DMSO solvent mixture (final percentage 50:50, v/v), an incubation period of 24 h at ambient condition or 6 h at 37 °C, and reaction in glass vials. Once the reactions between MTT and the test materials were completed in a glass vial or glass microwell plates, the absorbance of the reaction mixture could be read directly at 570 nm using a plate reader or UV−vis spectrophotometer. Tissue culture and cell culture plastic microwell plates interfered with the reaction between MTT and most test compounds.

The antioxidants TBHQ, BHA, BHT, and vitamin C were used as positive controls to validate this proposed MTT antioxidant assay method. MTT was dissolved in water (1 mg/mL) to afford a 1000 μg/mL solution. This MTT solution was then vortexed or shaken with test compounds in DMSO to obtain the final water−DMSO ratio as 50:50. Aqueous-soluble compounds react with MTT, but the formazan crystals formed from the reaction are insoluble in water. Therefore, preparing test compounds in DMSO and achieving a 50% DMSO concentration in the reaction mixture will facilitate direct measurement of absorbance at the end of the reaction. The positive controls were reacted with MTT at the same molar and mass concentrations (150 μM and 25 μg/mL), respectively. At such a molar concentration, the absorbance of the reaction mixture yielded from positive controls was identical at 0.5. At the same mass concentration, the absorbance was slightly different due to the molar differences among the test compounds (Table 1). In general, a solution of MTT in water (90 μL or 90 μg) reacted completely with 5 μg of each test compound in 110 μL of DMSO to afford 200 μL of reaction mixture. The presence of excess MTT in the reaction mixture did not interfere with the absorption of the purple formazan (570 nm) but provided an ample amount of MTT for reaction with potential antioxidant compounds present in 100 μg/mL of natural product extracts.

The plant extracts tested at 100 ppm in this assay gave absorbances between 0.5 and 1.0 (Table 1). The extracts from mushrooms (*Agrocybe aegerita*, *Pleurotus ostreatus*, and *Lentimula edodes*) also showed similar trends, and the results were in agreement with an earlier report.[12] A number of known antioxidant

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Table 1. Absorbances of Reaction Mixtures at 570 nm Obtained after Reaction between Natural Product Extracts (100 ppm) and Compounds (25 ppm) with MTT at 37 °C

extract/compound	absorbance	extract/compound	absorbance
Positive controls		quercetin	0.75
L-ascorbic acid	0.50	quercetin dihydrate	0.30
BHA[a]	0.51	quercetin-3-rhamnoside	0.30
BHT[a]	0.51	quercetin tetramethyl ether	0.23
TBHQ[a]	0.51	7,5,4′-trihydroxyflavonone	0.14
Natural product extracts		**Phenolics: others**	
MAA[b]	0.80	3-methylcatechol	0.86
MCL[b]	0.59	7-methoxycoumarin	0.18
MCM[b]	0.73	pyrogallol	0.86
MPO[b]	0.61	**Vitamins**	
WAA[b]	0.95	α-tocopherol	0.56
WCL[b]	0.57	vitamin K	0.23
WCM[b]	0.72	**Natural colorants**	
WPO[b]	1.10	betacyanin	0.60
Phenolics: acids		bixin	0.61
caffeic acid	0.69	*β*-carotene	0.32
chlorogenic acid	0.72	chlorophyll	0.57
p-coumaric acid	0.15	cyanidin-3-*O*-glucosyl malonate	0.20
ferulic acid	0.29	cyanidin-3-*O*-glucosyl rhamnoside	0.17
4-hydroxybenzylacetic acid	0.17	cyanidin-3-*O*-glucosyl rutinoside	0.28
p-hydroxybenzoic acid	0.13	lycopene	0.59
4-hydroxyl-3-methoxybenzoic acid	0.19	**Fatty acids**	
3,4-methylenedioxy cinnamic acid	0.19	decanoic acid	0.51
Phenolics: curcuminoids		docosanoic acid	0.25
curcumin I	0.85	*cis*-5-dodecanoic acid	0.17
curcumin II	0.61	elcosanoic acid	0.25
curcumin III	0.37	erucic acid	0.68
Phenolics: flavonoids		heneicosanoic acid	0.22
biochanin A	0.37	heptadecanoic acid	0.91
(−)-catechin	0.53	lauric acid	0.19
(+)-catechin	0.88	lignoceric acid	0.16
DL-catechin trihydrate	1.18	linoleic acid	0.68
chrysin	0.30	methyl linolenate	0.25
7,8-dihydroxyflavone	0.49	myristic acid	0.58
5,7-dihydroxy-4′-methoxyisoflavone	0.38	nervonic acid	0.89
7,4′-dihydroxyisoflavone	0.48	nonadecanoic acid	0.53
5,2′-dimethoxyflavone	0.27	nonanoic acid	0.16
7,4′-dimethoxyflavone	0.17	octanoic acid	0.17
(−)-epicatechin	0.17	*cis*-10-pentadecenoic acid	0.19
(−)-epicatechin gallate	1.18	stearic acid	0.47
formononentin	0.20	tricosanoic acid	0.27
(−)-gallocatechin	0.99	tridecanoic acid	0.47
(−)-gallocatechin gallate	0.92	undecanoic acid	0.19
genistein	0.29	**Isoprenoids**	
genistin	0.33	camphor	0.24
5,7-hydroxy-4′-methoxyflavone	0.26	cholesterol	0.22
7-hydroxy-4′-methoxyflavone	0.23	cholesterol glycoside	0.30
luteolin	0.66	ursolic acid	0.43
4′-methoxyflavone	0.24		

[a] TBHQ: *tert*-butyl hydroquinone; BHA: butylated hydroxyanisole (BHA); BHT: butylated hydroxytoluene (BHT). [b] Aqueous and methanolic extracts of *Curcuma longa* (WCL and MCL), *Curcuma mangga* (WCM and MCM), *Agrocybe aegerita* (WAA and MAA), *Pluratus ostreatus* (WPO and MPO), and *Lentinula edodes* (WLE and MLE).

compounds were evaluated belonging to various classes (Table 1). The phenolic compounds studied showed absorbances ranging from 0.13 to 0.86 and suggested that the number of hydroxy groups present correlated with enhanced antioxidant capacity.[13] Curcuminoids I−III are well-known antioxidants, and the present results showed that the activity profile was in agreement with the published lipid peroxidation inhibitory (LPO) activity profile for these compounds.[14] Our group has reported the antioxidant activity of 29 fatty acids using the LPO assay.[15] In the MTT antioxidant assay, these fatty acids showed formazan absorbtions between 0.1 and 0.9 (Table 1). The activity profile correlated well with the unsaturation and chain length of the fatty acid, as observed in the case of the LPO assay.[15]

Natural colorants or pigments possess strong antioxidant activity and are implicated as protecting cells against oxidative damage caused by free radicals. The pigments studied in this assay gave absorbances between 0.17 and 0.63 (Table 1). Both bixin and cyanidin-3-*O*-glucoside gave absorbances at about 0.6 in this assay and indicated that the antioxidant data and trend observed in the MTT assay were in agreement with the published LPO assay results.[16] With the exception of quercetin (absorbance 0.75), the other flavonoids gave moderate absorbances between 0.1 and 0.5 (Table 1). Among the phenolics studied, the catechin derivatives showed the strongest absorptions and again were in agreement with the antioxidant activity reported for such compounds using lipid peroxidation.[17] Isoprenoids studied in this assay showed weak absorbances, confirming their poor antioxidant activity.

In conclusion, the MTT antioxidant assay proposed herein is rapid and inexpensive because it requires only one reagent and universal solvents such as water and DMSO. Since the reaction time is short and the absorbance is read by using a plate reader or by a UV−vis spectrophotometer, antioxidant potential of large batches of samples can be generated in a short period of time. The method is versatile and overcomes the solubility issues associated with compounds and extracts. Finally, this method could be

developed as a high-throughput assay for determining the antioxidant potential of large volumes of samples.

Experimental Section

General Experimental Procedures. MTT, the antioxidant positive controls, and the pure natural product compounds used in the assay were purchased from Sigma-Aldrich Chemical Co. DMSO used for the assay was of ACS reagent grade (EMD Chemicals Inc., Gibbstown, NJ). The water used was reverse osmosis water purified using a Millipore cartridge filter. Absorbances were measured using a plate reader (Microplate Autoreader EL311; Biotek Instruments). The glass vials (2 mL) utilized were Fisherbrand with Titeseal closure (Fisher Scientific).

Plant and Fungal Material. The rhizomes of *Curcuma mangga* Valeton & Zijp. (Zingiberaceae) and *Curcuma longa* L. (Zingiberaceae) were harvested from plants grown in the greenhouses of the Department of Horticulture at Michigan State University, East Lansing, MI. Voucher plant specimens, MSC 396404 and MSC 396405 for *C. longa* and *C. mangga*, respectively, were filed with the Beal-Darlington Herbarium, Department of Botany and Plant Pathology, Michigan State University. Plants were grown in 1-gallon pots containing sterile potting medium and were watered with fertilizer (20:20:20, NPK) every two weeks and kept at a 12 h photoperiod at 80 °C. The rhizomes harvested in May 2009 were from plants grown for about 10 months and used for extraction immediately.

The dried powders of the mushrooms *Agrocybe aegerita* (strain # AA-1), *Plurotus ostreatus* (strain # PO-101), and *Lentinula edodes* (strain # LE-106) were supplied by Diversified Natural Product Company, Scottville, MI.

Extracts of *C. mangga, C. longa*, and mushroom powders were separately blended with water and centrifuged (10 min). Each supernatant was lyophilized to yield the dried water extracts of *C. longa* (WCL), *C. mangga* (WCM), *A. aegerita* (WAA), *P. ostreatus* (WPO), and *L. edodes* (WLE). Similarly, the residues from centrifugation were separately soaked in methanol (3 h) and centrifuged (10 min), and the supernatant was evaporated under vacuum to obtain the methanolic extracts of C. *longa* (MCL), *C. mangga* (MCM), *A. aegerita* (MAA), *P. ostreatus* (MPO), and *L. edodes* (MLE). The extracts were prepared earlier and stored at −20 °C.

MTT Antioxidant Assay. Stock solutions of test compounds and extracts were prepared in DMSO (1 and 4 mg/mL, respectively). The MTT (1 mg/mL) was dissolved in water. An aliquot of 190 μL of MTT solution in water and 10 μL of test compounds or extracts in DMSO were vortexed in a capped glass vial (2 mL) for 1 min. To this was added DMSO (200 μL), and the solution was vortexed again. The reaction mixture was then incubated at 37 °C for 6 h, 200 μL of the reaction mixture was pipetted to a 96-well cell culture plate, and the absorbance was measured at 570 nm. Each sample was assayed in triplicate, and the experiment repeated 10 times. The average absorbance was tabulated in Table 1 for all compounds, extracts, and antioxidant controls.

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