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Development of Probe-Based Ultraweak Chemiluminescence Technique for the Detection of a Panel of Four Oxygen-Derived Free Radicals and Their Applications in the Assessment of Radical-Scavenging Abilities of Extracts and Purified Compounds from Food and Herbal Preparations

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We report here the development of a probe-based ultraweak chemiluminescence (uwCL) method capable of detecting a panel of four oxygen-derived free radicals (ODFRs) including superoxide (O_2^{-}), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), and peroxyl radical (ROO•) using different probes specific for these radicals performed by the same uwCL analyzer. The selected radical-generating systems and their corresponding uwCL-probing emitters were validated. These ODFR-detecting systems were subsequently utilized by us to assess the radical-scavenging ability (RSA) of a variety of extracts and purified constituents derived from foods and herbal preparations. Our approach for assessing RSA for these constituents is based on the suppression of uwCL generated by each ODFR, and the degrees of inhibition have been shown to be dose-dependent. For this reason, the estimation of IC_{50} for each testing compound can be obtained from the curve constructed based on the percent of inhibitions of uwCL versus the concentrations of the compound tested. To illustrate the practical applications of our devised methodology, data for comparative studies of RSA activities of fermented extracts of *Cordeceps sinensis*, purified methylgallate isolated from *Toona sinesis*, resveratrol purified from grape seeds, plus epimedin C from the aerial part of the *Epimedium* plant (yinyanghuo) are to be presented.

KEYWORDS: Probe-based free radical detection; radical-scavenging ability; food extracts; herbal constituents

INTRODUCTION

There is overwhelming evidence to indicate that reactive oxygen species (ROS) have been implicated in the etiology of a host of degenerative diseases including cardiovascular disease, diabetes, Alzheimer's disease, and other neurodegenerative disorders and in aging (*1*−*4*). In addition, they also play a role not only in acute conditions, such as trauma, stroke, and infection, but also in physical exercise and stress. If oxygen-derived free radicals (ODFR) are involved in all of these clinical conditions, then antioxidants should be effective in preventing their occurrence. Indeed, investigations at the cellular, tissue, and whole animal level, as well as epidemiological studies, support the concept that nutritional antioxidant status is inversely related to the occurrence of free radical-mediated diseases (*5*). For this reason, there is a considerable interest in the use of natural food extracts for therapeutic use, aiming to replenish the needed antioxidants to correct the deficit of prooxidant/antioxidant imbalance, also referred to as oxidative stress (*6*, *7*). A detailed assessment of a variety of foodstuffs, herbal remedies, and other agricultural products should provide insight into the antioxidative capacity of these substances to allow eventual identification of the most effective free radical scavengers, so that a natural product antioxidant mixture can be formulated.

Undoubtedly, oxygen radical research has led to a new paradigm of human health, with a shift toward a greater

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Table 1. Free Radical-Generating Systems and Their Respective uwCL-Emitting Probes Used in This Study

ROS or free radical	uwCL-generating system	uwCL-emitting probe	ref
superoxide (O_2^-)	methylglyoxal + arginine	Lucigenin	10 and 11
hydroxyl radical (OH)	Fe^{+2} + H_2O_2 (Fenton's reagent)	indoxyl-β-glucuronide (IBG)	12
peroxyl radical (ROO)	2,2′-azo-bis-[2-amidinopropane (ABAP)]	Luminol	13
hydrogen peroxide (H_2O_2)	3.0% H_2O_2	Luminol	14

emphasis on disease prevention. In addition to the science of free radicals and antioxidants, the public is also bombarded daily with popular information related to the claims of certain health food products that are supposed to possess antioxidant capacity. Surprisingly, all these claims often lack the support of scientific evidence.

A variety of radical-scavenging capacity assays such as the Trolox-equivalent antioxidant capacity (TEAC), total reactive antioxidant potential, or total radical-trapping antioxidant parameter (TRAP), have been devised for screening the potential antioxidant capacity of biological matrices, such as plasma, single compounds, food components, or food extracts (*8*, *9*). However, these methodologies cannot be used to distinguish what type of free radical is being specifically scavenged by the test compound. Along the same vein, although tests for each respective ODFR were available, the principles of these methods were diversified, and instruments varied. For these reasons, they were too cumbersome to setup and to implement. Thus, it is obvious that a simple method that can be used to detect a panel of ODFRs using a single instrument based on an identical chemical principle is definitely desirable.

With the availability of an ultraweak chemiluminescence analyzer, we report herein for the first time that a probe-based uwCL technique that is capable of detecting a panel of four ODFRs, namely, superoxide anion (O_2^-), hydroxyl radical (•OH), peroxyl radical (ROO•), and hydrogen peroxide (H_2O_2) has been developed. To illustrate the application of this proposed system for radical-scavenging ability (RSA) of food extracts, herbal preparations, and purified components or chemicals, we exemplified it by assessing the RSA activities of the proposed panel of ODFRs for fermented extracts of *Cordyeceps sinensis*, purified resveratrol from grape seeds, purified methylgallate from *Toona sinensis*, and Epimedin C form the *Epimedium* plant.

MATERIALS AND METHODS

Materials. Methylglyoxal (pyruvaldehyde; 2-oxopropanol) (6.5 mmol/L), lucigenin, arginine, superoxide dismutase (5100 units/mg), indoxyl-β-glucuronide (IBG), ethylene diaminetetraacetic acid (EDTA), salicylate, luminol, sodium tetraborate, 2,2′-azo-bis (2-amidinopropane) dihydrochloride (ABAP), catalase, and 5,5′-dimethyl-1-pyroline *N*-oxide (DMPO) were purchased from Sigma Chemical Co. (St. Louis, MO). Unless stated otherwise, all chemicals used were of analytical quality, and doubly distilled water was used throughout.

Free Radical-Generating Systems, Their Respective Ultraweak Chemiluminescence(uwCL)-Emitting Probes, and the Operational Procedures. A panel of four types of ODFR-generating systems and their respective uwCL-emitting probes were selected either with the adoption of established methods or developed by us were tabulated in **Table 1**. First, for the superoxide (O_2^-)-generating system, the following reaction mixture in a total volume of 2.1 mL included 1.0 mL of phosphate-buffered saline, pH 7.4; 0.05 mL of 1.0 M arginine; 0.05 mL of 1.4 μM methylglyoxal; and 1.0 mL of 2.0 mM lucigenin. After gently mixing, the quartz round-bottomed cuvette containing the reaction mixture was put in the black-box unit of the uwCL analyzer equipped with a high-sensitivity detector [3.3×10^{-15} W/(cm^2 count)] from Jye Horn Co. (Taipei, Taiwan) (*10*). This analyzer is also available from American Biologics (Chula Vista, CA). Daily calibration of the detector sensitivity was performed with a ^{14}C-light source generating an amount of 10 000 photon counts/s at a voltage ranging between 860 and 867 V. The reproducibility of the ^{14}C-light source was <1.0%.

Next, for the hydroxyl radical (•OH)-generating system, we used Fenton reagents (Fe^{2+} + H_2O_2). The reaction mixture used consisted of the following: 1.0 mL of 3 μM IBG [dissolved in phosphate-buffered saline (PBS), pH 7.4]; 0.1 mL of 1.0 mM $FeSO_4$; 1.6 mL of 3% H_2O_2; and 0.05 mL of 10 mM EDTA. The total volume of the reaction mixture was 2.75 mL. Addition of reagents to the quartz round-bottomed cuvette occurred in a sequential order of EDTA, IBG, H_2O_2, and $FeSO_4$ (*12*). After gently mixing, it was quickly put in the black-box unit of the uwCL analyzer. The uwCL photon measurement was carried out analogus to that of the O_2^--generating system.

For the hydrogen peroxide (H_2O_2)-generating system, the following reaction mixture was used: 1.0 mL of 2 mM luminol (do not freeze, store at 4 °C), containing sodium borate, pH 7.3; 1.0 mL of PBS, pH 7.4; and 1.0 mL of 1.2% H_2O_2 (freshly prepared daily). The total volume of the reaction mixture was 3.00 mL. The quartz round-bottomed cuvette containing the reaction mixture was quickly put in the black-box unit of the analyzer, and uwCL was measured as described previously.

For the peroxyl radical (ROO•)-generating system, a modified 2,2′-azo-bis (2-amidinopropane) (ABAP)-mediated pyrolysis technique was employed (*13*). The reaction mixture consisted of the following: 1.0 mL of 2.0 mM luminol was allowed to mix with 0.5 mL of 0.2 M ABAP, followed by adding 1.0 mL of the 0.2 M $NaHCO_3/Na_2CO_3$ buffer solution, pH 10.2. After a brief mixing, the quartz round-bottomed cuvette was put in the black-box unit of the uwCL analyzer, followed by monitoring uwCL by the data processing unit of the computer.

Measurement of Superoxide Radical-Scavenging Ability. Twelve minutes (720 s) after the initiation of the uwCL reaction generated by the methylglyoxal/arginine/lucigenin system and its process until a plateau region was reached (a curve representing the time-dependent increment of uwCL can be visualized on the screen of the data processing unit), 10 μL of the testing compound was then added to the reaction mixture in the quartz cuvette and returned back to the black-box unit for continuous monitoring of uwCL. Varying degrees of sudden drops of uwCL counts could be observed with a stepwise addition of various doses of testing compound. These phenomena represent different degrees of O_2^--scavenging abilities (**Figure 2**, panels **A-1** and **A-2**). However, if one is interested in the estimation of IC_{50} (the concentration of a test compound needed to inhibit 50% of uwCL in the assay system) for a quantitative comparison, a concentration/inhibition curve can be constructed (**Figure 4B**). Thus, a comparison of IC_{50} values will permit one to rank the order of O_2^--scavenging efficiencies of various test compounds.

Measurement of Hydroxyl Radical-Scavenging Ability. Eighteen minutes (1080 s) after the initiation of the uwCL reaction generated by the Fenton reagent (H_2O_2 + Fe^{2+})/IBG system and its process until a plateau region was reached, 10 μL of a test compound was added to the reaction mixture to the black-box unit of the analyzer for continuing monitoring of uwCL. As a result, varying degrees of sudden drops of uwCL counts could be observed with a stepwise addition of various doses of the test compound (**Figure 2**, panels **B-1** and **B-2**). IC_{50} of a test compound could then be estimated as described previously.

Measurement of Hydrogen Peroxide-Scavenging Ability. Ten minutes (600 s) after the initiation of the uwCL reaction generated by H_2O_2/luminol and its process until a plateau region was reached, 10 μL of a test compound was added to the reaction mixture and returned to the black-box unit for continuous monitoring of uwCL. As a result, varying degrees of sudden drops of uwCL counts could be observed with a stepwise addition of various doses of testing compound (**Figure**

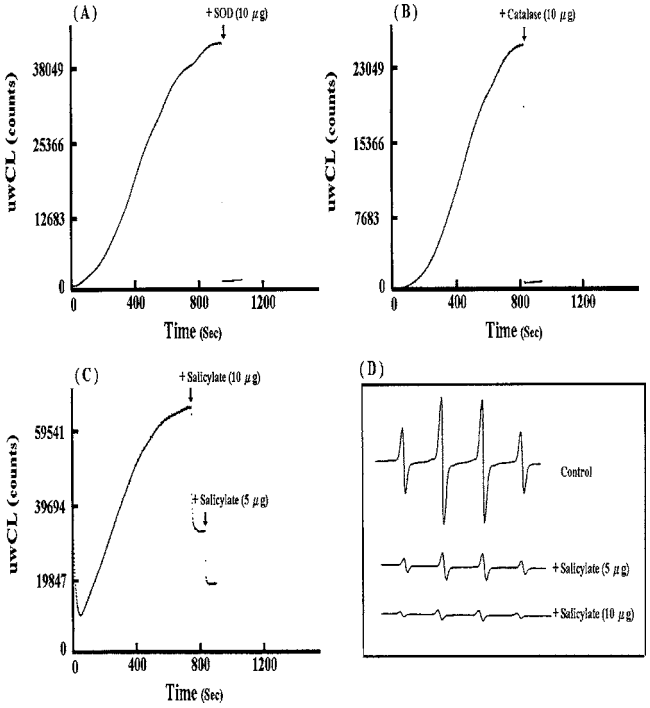

Figure 1. Validation of uwCL-emitting probes specific for each respective ODFR. uwCL generated by the MG/arginine/lucigenin system of O_2^- was completely suppressed by 10 μg of bovine SOD, indicating that lucigenin-derived uwCL was essentially that of O_2^-. (B) uwCL generated by H_2O_2/luminol system was completely suppressed by 10 μg of bovine catalase indicating that luminol-derived uwCL was essentially that of H_2O_2. (C) uwCL generated by the Fenton reagent (H_2O_2 + Fe^{2+})/IBG system was shown to be suppressible by salicylate, an effective •OH radical scavenger. The specificity of the IBG probe was further confirmed by the observation of the inhibitory effect of the ESR signals generated by DMPO-OH adduct by salicylate (D).

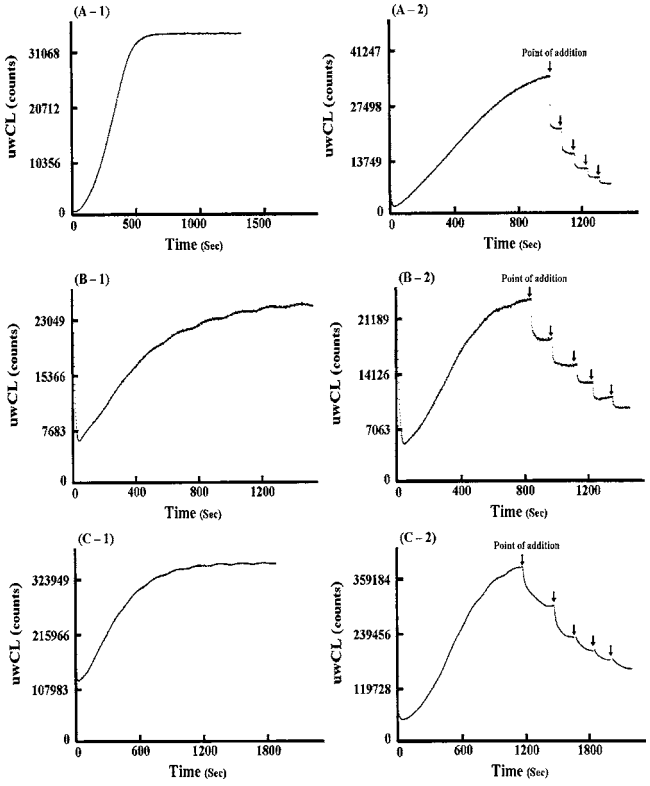

Figure 2. Representative time-dependent probe-based uwCL profiles for various ODFR and their sequential suppression of uwCL by the addition of various concentrations of test compounds. O_2^- (A-1 and A-2); •OH (B-1 and B-2); and H_2O_2 (C-1 and C-2).

2, panels **C-1** and **C-2**). The obtainment of IC_{50} of a test compound can be carried out as described previously.

Measurement of Peroxyl Radical-Scavenging Ability. After the initiation of the uwCL reaction was generated by adding ABAP and alkaline-buffered luminol in the round-bottomed quartz cuvette held at constant temperature of 37 °C as soon as uwCL reached around 50 000 counts, 10 μL of a test compound was allowed to add to the reaction mixture in the round-bottomed quartz cuvette and returned to the black-box unit of the analyzer for continuous monitoring of uwCL. As a result, a rapid and sudden drop of uwCL could be observed. The depressed uwCL could steadily be maintained for a period of time (referred to as the time of depressed uwCL; T_d). The uwCL would then return to normal pattern once the contents of the scavenging constituent was completely exhausted. Thus, the length of the time of depressed uwCL can be used as an index for peroxyl radical scavenging ability of a test compound (**Figure 3A**).

To standardize the system, we used Trolox (H_2O-soluble vitamin E analog) as the standard (**Figure 3B**). Thus, the IC_{50} value of a test compound can be converted to a Trolox-equivalent unit (T.E.U.) using the following formula:

$$\text{T.E.U. of a test compound} = \text{1 unit } (\mu\text{mol})\ T_{d(\text{test})}/T_{d(\text{Trolox})}$$

Electron Spin Resonance Spectroscopy. The spin-trapping technique, a general method to convert transient free radicals to stable free radicals, was used to measure •OH formation. The spin trap used in this study was 5,5′-dimethyl-pyrolline-1-oxide (DMPO), which was purified by treatment with activated charcoal under N_2 atmosphere and protected from light. A mixture containing 1% H_2O_2, 100 μM $FeSO_4$, 0.1 mM EDTA, 0.1 M DMPO, and various concentrations a testing compound was transferred to a quartz capillary and put in the cavity of an ESR spectrometer (Bruker E-200 ESR spectrometer). ESR measurement conditions were power 10 mW; *x*-band modulation frequency 100 kHz; modulation amplitude 2 G (0.2 mT); central magnetic field 3380 G; scan width 20 mT; time constant 20 ms; and temperature 25 °C (*15*).

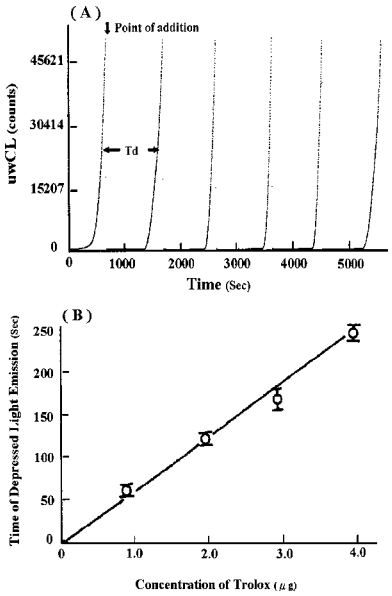

Figure 3. Representative time-dependent probe-based uwCL profile for ROO• using the ABAP/luminol system. (A). T_d denotes the time of depressed uwCL. (B) A standard curve of T_d vs concentrations of Trolox was presented. In an actual measurement, T_d obtained for a typical test compound was converted to a Trolox-equivalent unit (T.E.U.).

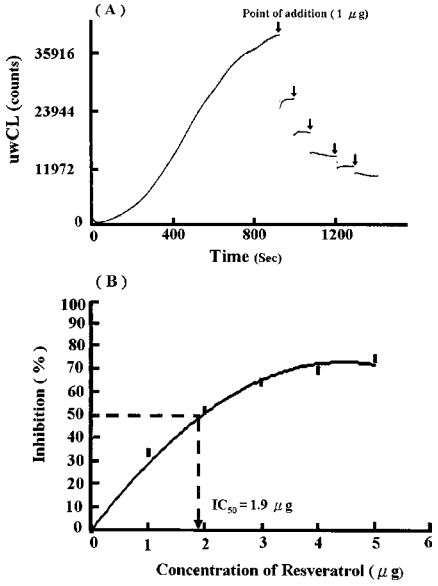

Figure 4. Calculation of IC_{50} values for each test compound can be carried out as exemplified by a case of resveratrol by transforming the data points obtained from a time-dependent uwCL profile (A) into a concentration–inhibition curve (B). As revealed by panel B, the IC_{50} value of resveratrol was 1.9 μg.

RESULTS AND DISCUSSION

We report here for the first time that a panel of four oxygen-derived free radicals (O_2^-, •OH, ROO•, and H_2O_2) can be simultaneously detected by a single instrument using the same chemical principle of probe-based ultraweak chemiluminescence (uwCL). The final selection of a suitable respective radical-generating system and uwCL-emitter probe deserves some comments here. First, among a variety of O_2^--generating systems documented in the literature, the xanthine/xanthine oxidase method was most commonly used. However, we found that the lucigenin-based uwCL generated by this system reached a plateau rapidly and thereafter decayed very quickly. Moreover, xanthine oxidase-catalyzed oxidation of the substrate produced a mixture of O_2^- and H_2O_2 (*16*). Therefore, as far as the standpoint of generating pure O_2^- is concerned, the utilization of xanthine/xanthine oxidase system is not considered to be specific.

Conversely, we found that O_2^- generated by the interaction between arginine and the three-carbon α-dicarbonyl compound of methylglyoxal (pyruvaldehyde; 2-oxopropanol; MG) was quite specific because a complete suppression of lucigenin-based uwCL could be achieved with the use of a proper concentration of SOD (**Figure 1A**). Another advantage of the MG/arginine/lucigenin system for generating O_2^--based uwCL was that the intensity of the latter could remain steadily stable without fluctuation at the plateau for at least 15 min (**Figure 1A**). This period of time would allow the addition of a test compound to assess its radical-scavenging activity (RSA) at any time-point without compromising the precision of the result.

Lucigenin (bis-*N*-methylacridinium nitrate) has been used as a chemiluminigenic probe for detecting O_2^- (*29*−*31*). The utilization of lucigenin for probing O_2^- was considered to be specific because the interaction of this probe with O_2^- results in the release of photons that was ascribed to the formation of endoperoxide (*10*). Upon the breakdown of the latter, an excited *N*-methylacridone molecule was formed, which could release photons upon its relaxation (*17*).

The paucity of papers dealing with the detection of •OH by the chemiluminescence approach could understandably be due to the lack of a suitable probe. During an extensive literature search, we found only one published paper dealing with the detection of •OH by using luminol as a CL probe (*18*). However, luminol has been used extensively as a CL probe for H_2O_2; thus, its utilization in the detection of •OH should be considered nonspecific. For this reason, a specific CL emitter for •OH has to be sought by us. Agatsuma et al. (*19*) reported that plasma from hemodialysis patients evoked weak photon emission in a characteristic emission spectrum with a peak at 430 nm, attributed to an attack by •OH generated from the iron-catalyzed breakdown of H_2O_2. Subsequently, the compound responsible for this unique phenomenon had been purified and identified to be indoxyl-β-glucuronide (IBG) (*20*). We thus proceeded to assess if this compound was capable of serving as a uwCL emitter in the Fenton reaction ($Fe^{2+} + H_2O_2$). We demonstrated that indeed, IBG could elicit a very strong intensity of uwCL (**Figure 1C**). Conversely, IBG was shown to be insensitive to either O_2^- or H_2O_2 with their uwCL intensities nearly close to background values when both radicals were allowed to interact with IBG (data not shown). Furthermore, we also demonstrated that IBG elicited uwCL could be effectively quenched by salicylate, an •OH scavenger, which was in parallel with the ability of the same compound to suppress the signal of the DMPO-OH adduct detected by ESR (**Figure 1D**). These data clearly indicate that IBG is indeed a specific uwCL probe suitable for monitoring the production of •OH.

Direct addition of 3% H_2O_2 to serve as the H_2O_2-generating system to a luminol probe elicited a time-dependent increment of uwCL and eventually reached a plateau at approximately 10 min. The specificity of the luminol probe for H_2O_2 was demonstrated by adding catalase at the plateau region and observed a near complete suppression uwCL (**Figure 1B**).

Quantitative measurement of the total peroxyl radical (ROO•)-trapping capability of human blood plasma by controlled peroxidation was reported originally by Wayner et al. (*21*). This method was subsequently modified to become a chemiluminescence-based TRAP assay (*22*) using AAPH instead of ABAP to oxidize luminol, leading to the formation of luminol radicals that emit photons. In this study, we adoptedABAP as the initiator of luminol radicals that ultimately reacted with molecular oxygen to generate ROO• radicals. This possible application was proved to be successful via the following mechanism:

$$\mathrm{ABAP} \xrightarrow[\Delta]{\text{pyrolysis}} 2\mathrm{R}\bullet$$

$$\mathrm{R}\bullet + \mathrm{O2} \rightarrow \mathrm{ROO}\bullet$$

$$\mathrm{ROO}\bullet + \text{luminol} \rightarrow (\mathrm{LH_2})\mathrm{ROOH} + \mathrm{LH}\bullet$$

$$\mathrm{LH}\bullet \rightarrow \mathrm{L}\bullet + \text{uwCL}$$

The ability of a test compound to quench the luminol-derived uwCL could then be related to its capacity to trap ABAP-derived radicals as reflected by the magnitude of time elapsed in the depression of uwCL. For comparative purposes, Trolox was used as standard, and anti-peroxyl radical activities of various compounds were ultimately expressed as Trolox-equivalent units (T.E.U.) (**Figure 3**).

To illustrate the application of the proposed method for the RSAs of O_2^-, •OH, H_2O_2, and ROO•, three purified constituents isolated from various agricultural plants and one herbal extract were measured for comparative purposes. The reasoning behind the selection of these compounds needs further qualification.

Table 2. Comparison of RSA Activities of Compounds or Extracts Derived from Various Sources

	RSA [IC_{50} value (μg) or Trolox-equivalent unit (T.E.U.)]			
compound or extract	superoxide (O_2^-)	hydroxyl radical (OH)	hydrogen peroxide (H_2O_2)	peroxyl radical (ROO)[a]
methylgallate[b]	0.02	0.20	38.00	0.40 (T.E.U.)
fermented extracts of *C. sinensis*	26.00	18.10	4.10	1.70 (T.E.U.)
Resveratrol	1.90	1.40	38.00	0.12 (T.E.U.)
Epimedin C[c]	14.20	trace	6.50	3.40 (T.E.U.)

[a] Expressed as T.E.U. by comparing to RSA generated by 1.0 μM Trolox. [b] Isolated from *T. sinensis* (Meliaceae). [c] Isolated from aerial part of Epimedium plant (yinyanghno).

First, methylgallate (A) was one of the 15 compounds isolated from crude extracts of *Toona sinensis* (Meliaceae) that was distributed in southerm Taiwan and was widely cultivated in China. This plant is very popular for vegetarians in Taiwan, and its crude extracts have been demonstrated to possess anticancer and hypoglycemic effects (*23*). Furthermore, methylgallate has been demonstrated to possess antioxidative capacity and lipid peroxidation inhibitory activities (*24*, *25*). Recently, methylgallate has been shown to be able to protect H_2O_2-induced oxidative stress and DNA damage in MDCK cells (*26*). Therefore, it will be of interest to delineate specifically what type of free radical methylgallate can scavenge most effectively. Second, resveratrol (B) was a purified compound isolated from seeds of grapes and was demonstrated to possess anti-carcinogenic activity because of its capability to serve as an antioxidant, anti-inflammatory agent, as well as a phase II enzyme inducer (*27*). Therefore, it is of interest to relate its anti-carcinogenic activity to the RSA of this compound. Third, Epimedin C (C) was a compound isolated from the aerial part of the *Epimedium* plant and was used in China for the treatment of impotence, atrophy, neurasthenia, amnesia, and climacteric hypertension (*28*). Studies have demonstrated that this constituent possesses anti-tumor as well as immunomodulatory activities (*29*, *30*). Recently, we have shown that Epimedin C induces cell cycle blockage of hepatoma SK-Hep-1 cells (*31*). It will be of interest to examine if the anti-tumor activity of this compound is also related to its RSA. Last, fermented extract of *C. sinenesis* (D) has previously been demonstrated by us to exert excellent capability of scavenging the •OH radical (*12*). On the basis of the foregoing discussion, all four compounds we selected demonstrate varying degrees of antioxidative capacities. Therefore, we performed their relative RSA against a panel of four free radicals using our proposed method. The results of these measurements are categorically outlined in **Table 2**. First, the efficacy of RSA for O_2^- of these compounds yields a descending order of A > B > C > D, respectively.Second, the efficacy of RSA for the •OH radical of these compounds also yields a descension of A > B > D > C, respectively. Third, the efficacy of RSA for H_2O_2 of these compounds yields the order of ranking D > C > A = B. Last, the efficacy of RSA for the ROO• radical of these compounds yields a descending order C > D > A > B. Overall, methylgallate was demonstrated to be a super antioxidant for O_2^- and •OH radicals. Resveratrol was proved to be an excellent antioxidant against O_2^- and •OH but relatively poor in scavenging the H_2O_2 and ROO• radical. Conversely, Epimedin C and fermented extracts of *C. sinenesis* were proven to be excellent scavengers for H_2O_2 and ROO• radicals albeit their poor abilities to scavenge O_2^- and •OH radicals. These results suggest that the combination of these four compounds could yield an excellent antioxidant cocktail. Further experiments are warranted to attest as to whether this designed antioxidant cocktail is beneficial in replenishing the antioxidant capacity for the individual who may suffer from antioxidant deficits.

In conclusion, we have developed an ultraweak, probe-based chemiluminescence method for the rapid and specific detection of a panel of four ODFRs using a single instrument. The detection system for each respective ODFR has been successfully adopted to assess the relative RSA of constituents or crude extracts from foods and herbal preparations. By so doing, it can provide insight into the antioxidative capacity of these substances to allow actual comparison of their RSA activities. Finally, the system can be extended to screen and to identify the most effective free radical scavengers from various sources so that a natural product of an antioxidant mixture suitable for clinical use can be formulated. Our proposed method can be a rapid and simple tool for facilitating this practice.

ABBREVIATIONS USED

ODFR, oxygen-derived free radical; RSA, radical scavenging activity; ROS, reactive oxygen species; TEAC, Trolox-equivalent antioxidant capacity; TRAP, total radical trapping antioxidant parameter; uwCL, ultraweak chemiluminescence; IBG, indoxyl-β-glucuronide; ABAP, 2,2-azo-bis-(2-amidinopropane) dihydrochloride; DMPO, 5,5′-dimethyl-1-pyroline *N*-oxide; EDTA, ethylene diaminetetracetic acid; MG, methylglyoxal; T.E.U., Trolox-equivalent unit; Trolox, 6-hydroxyl-2,5,7,8- tetramethylchroman-2-carboxylic acid; AAPH, 2,2′-azio-bis (2-amidinopropane); O_2^-, superoxide anion; •OH, hydroxyl radical; ROO•, peroxyl radical.

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