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ARTICLE

A Cobalt-Based Tetrazolium Salts Reduction Test To Assay Polyphenols

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A novel assay was developed to measure the capacity of polyphenols to chelate cobalt(II) by using the reduction of the tetrazolium salts, NBT (nitroblue tetrazolium chloride), MTT (methylthiazolyldiphenyl-tetrazolium bromide), and XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) to formazan products. The reduction of the salts is initiated by a cocktail comprised of cobalt(II), H_2O_2, and selenium(IV), which generates hydroxyl radical, peroxide, and superoxide ions. However, because cobalt(II) could not be replaced either by Fe(II), Mn(II), or Cu(II), the classical Fenton transitional metals, it indicates that cobalt is the key player in the tetrazolium salt reduction. Micromolar concentrations of a large variety of antioxidant polyphenols and minute amounts of fruit beverages rich in polyphenols can readily chelate cobalt, resulting in the inhibition of the reduction of tetrazolium salt to formazan, in a dose-dependent manner. However, this method is unsuitable to measure low molecular weight antioxidants such as ascorbate, uric acid, and vitamin E since these have no chelating properties for cobalt(II). The newly described tetrazolium reduction method is as sensitive as the ferric ion reducing antioxidant power, 2,2-diphenyl-2-picrylhydrazyl hydrate, and the luminol-dependent chemiluminescence antioxidant assays. The practical advantages of using the newly described method to quantify polyphenol levels from various sources are briefly discussed.

KEYWORDS: Tetrazolium reduction; cobalt chelation; selenium; hydrogen peroxide; polyphenols

INTRODUCTION

In 1894, von Pechmann and Runge described the synthesis of certain tetrazolium salts using the oxidation of formazan compounds (*1*). This oxidative process was notable for its reversibility by a variety of reducing substances. Because formazans are conspicuously colored and sparingly soluble in water, their production from tetrazolium compounds can be used to detect reducing substances in tissues (*2*). In 1941, Kuhn and Jerchel sketched their biological application (*3*), and Reid has contributed a documented survey of their significance in chemistry and biology (*4*). In 1976, Altman reviewed the literature on tetrazolium salts and their reduction properties (*5*). The tetrazolium to formazan reactions are now widely exploited as indicators of reducing systems with applications in chemistry and biology (*6*). They are used broadly in cell viability, proliferation, and cytotoxicity assays as a substrate for the electron transfer system of bacterial or mammalian cells and in enzyme assays, histochemical procedures, bacteriological screening, and the assessment of reactive oxygen species (ROS) production by leukocytes and spermatozoa (*7*,*8*). Also, one simple and inexpensive test involves measuring the reduction by superoxide generated by activated neutrophils of the yellow dye nitroblue tetrazolium chloride (NBT) to the insoluble dark blue formazan (*7*, *8*). The dye can be readily extracted by dimethyl sulfoxide (DMSO), and the optical density of formazan is then measured at 600 nm. The simple to perform NBT reduction test is also useful to confirm the diagnosis of chronic granulomatous disease of childhood, a disorder characterized by the complete absence of NADPH oxidase in granules of neutrophils (*8*, *9*). The currently used tetrazolium salt agents include NBT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and the water-soluble tetrazolium XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide], which are all used in common assays to measure cell viability via mitochondrial activities .

Recent findings from our laboratory have confirmed the observations by Liochev et al. (*10*, *11*) that in addition to activated neutrophils and cytochrome C, the enzyme glucose oxidase (GO) from *Aspergillus*, which in the presence of D-glucose generates a flux of hydrogen peroxide, can also reduce NBT to formazan, which was inhibited by superoxide dismutase (SOD). We have recently observed that unlike GO, which reduces NBT directly (*10*), a bolus of reagent H_2O_2 could reduce NBT/MTT and XTT only if the electron donor selenium(IV) [Se(IV)] and exclusively the metal ion cobalt(II) [Co(II)] were added to the reaction mixtures. These novel tetrazolium-reducing "cocktails" probably involve the oxidation of Se(IV) to Se(VI) and also the generation of a Co-catalyzed hydroxyl radical (*12*). Polyphenols

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possess both antioxidant and chelating properties toward transitional metals (*13*, *14*), known to scavenge superoxide and hydroxyl radical (*15*), and in addition, flavanoid−metal ion complexes are also known to chelate superoxide (*16*). Therefore, the chelation of Co by polyphenols can be used as one of the principles for the quantification of polyphenols. The aim of the present study is to show that the tetrazolium salt reduction assay can quantify polyphenols, based on their capacity to inhibit tetrazolium salt reduction.

MATERIALS AND METHODS

Biochemicals. NBT, MTT, the water-soluble tetrazolium XTT, sodium selenite, selenate(VI), seleno-cysteine, seleno-methionine, ebselen, $CoCl_2 \cdot 6H_2O$, $CuCl_2 \cdot H_2O$, $MnCl_2 \cdot 4H_2O$, $FeSO_4 \cdot 7H_2O$, the polyphenols quercetin, gallic acid, tannic acid, catechin, epigallocatechin gallate (EGCG), morin, rutin, curcumin, resveratrol, galangin, hesperetin, apigenin, chrysin, genistein, nor-dihydroguiaretic acid (NDGA), caffeic acid, polyphenon 60 from green tea (a mixture of green tea extract containing 60% catechin), dopamine hydrochloride, L-dopa (3,4-dihydroxy-L-phenylalanine), Folin−Ciocalteu phenol reagent, hydrogen peroxide, hypochlorous acid, AAPH [2,2′-azobis-(2-amidinopropane) dihydrochloride, a donor of peroxyl radical], SOD, horseradish peroxidase (HRP), catalase, glucose oxidase, 3-morpholino-sydnonimine (SIN-1), ascorbic acid, uric acid, trolox, reduced glutathione, and tempol were all obtained from Sigma-Aldrich (St. Louis, MO). Tiron was obtained from Riedel-de Haen (Steinheim, Germany), and desferal (desferoxamine-mesylate) was from Ciba-Geigy (Basel, Switzerland). Red wine (cabernet sauvignon, 2007, Gamla Winery, Israel) and cranberry beverage were purchased from a local supermarket. All of the polyphenols agents used were first dissolved in DMSO at 100 mM and then further diluted by absolute ethanol at 10 and 1 mM. Hank's balanced salt solution (HBSS) was from Biological Industries (Kibbutz Beit Haemek, Israel).

Microorganisms. Clinical isolates of *Escherichia coli* and non-MRSA *S. aureus* were obtained from the clinical microbiology laboratory of Hadassah Hospital (Ein-Kerem, Jerusalem, Israel). The bacterial cells were harvested after overnight growth on surfaces of brain heart infusion agar plates, washed in saline, and adjusted to an OD of $\sim 30^{OD\,540}$. A commercial lyophilized mixture of a probiotic preparation comprised of four *Lactobacillus* species, one *Streptococcus* species, and three *Bifidobacterial* species (VSL#3) was obtained from Seaford Pharmaceuticals Inc. (Mississauga, Ontario, Canada). One hundred milligrams of powder of the VSL#3 preparation was washed twice in a final volume of 20 mL of saline by centrifugation, and the microbial pellets were resuspended in 1 mL of normal saline and adjusted to an absorbance of $\sim 30^{OD\,540}$.

Preparation of Bacteria−Polyphenol Complexes. Complexes between polyphenols and microbial cells were prepared by mixing 100 μL of microbial suspension with 900 μL of saline and 100−500 μM concentrations of various polyphenols (see above). The reaction mixtures were vortexed vigorously, kept at room temperature for 10 min, and washed three times in a total volume of 40 mL of normal saline to remove unbound agents, and the washed cells were resuspended in 800 μL of HBSS.

Tetrazolium Reduction "Cocktails". NBT, MTT, and XTT were dissolved either in ethanol (NBT and MTT) or in distilled hot water (XTT). To 0.8 mL amounts of HBSS, pH 7.4, were added 100 μL of (1) various concentrations of polyphenols, (2) washed bacterial suspensions, and (3) bacterial cells complexed with polyphenols. This was followed by the sequential addition of $CoCl_2$ (10 μM), NBT/MTT or XTT (200 μM), sodium selenite(IV) (2 mM), and reagent H_2O_2 (2 mM). The reaction mixtures were vortexed and incubated for 10 min at room temperature. Under these conditions, both NBT and MTT are reduced to a dark insoluble blue formazan read at 600 nm and XTT to a soluble orange color, which is read at 450 nm. XTT reduction induced by the microorganisms and by the microorganisms complexed with polyphenols (see above) was read at 450 nm after removal of the cells by centrifugation. The degree of inhibition of the tetrazolium salts reduction induced by the various polyphenols agents was expressed as gallic acid equivalents (GAEs).

Luminol-Dependent Chemiluminescence (LDCL) Assay. The antioxidant capacities of polyphenols and of complexes formed between bacteria and polyphenols were also assayed by the LDCL inducing method (*17*, *18*). Briefly, to 1 mL of HBSS (containing 1 mg/mL D-glucose) were added purified polyphenols (0.5−25 μM), 100 μL of microbial cells, or microbial cells coated by polyphenols. This was followed by the sequential addition of luminol (10 μM), GO (2.3 units /mL), Se(IV) (1 mM), and Co (10 μM). This mixture generated strong LDCL signals, which were measured by a LUMAC 2500 M Biocounter (Landgraaf, The Netherlands), attached to a computer. The degree of quenching of light by the various antioxidants indicated their relative antioxidant potencies.

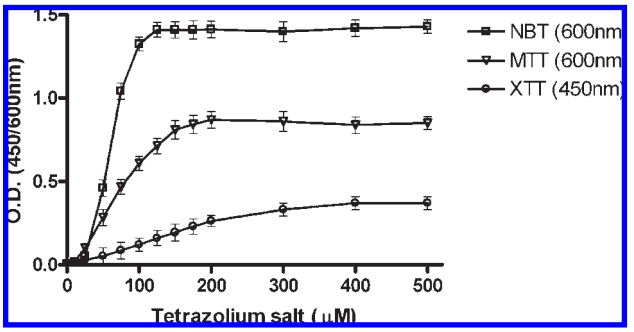

Figure 1. Concentration dependency of tetrazolium salts reduction (0−500 μM). Data are expressed as mean values ± SD; $n = 4$.

Ferric Ion Reducing Antioxidant Power (FRAP) Assay. The FRAP assay was performed according to the modified method of Benzie and Strain (*19*). In this assay, reductants in the sample reduce the Fe (III)/tripyridyltriazine complex, present in stoichiometric excess, to the blue ferrous form, with an increase in the absorbance at 593 nm. Absorbance readings were taken after 5 min using a Biotek PowerWave 340 microplate scanning spectrophotometer (Biotek Instruments, Winooski, VT). Calculations were made on the basis of standard curves obtained for a gallic acid solution and presented as GAE.

2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) Assay. The antioxidant activities of polyphenols were tested by the DPPH assay (*20*), which measures hydrogen atom (or one electron) donating activity and hence provides an evaluation of antioxidant activity, due to the free radical scavenging agent DPPH, a purple-colored stable free radical, which is reduced to the yellow-colored diphenylpicryl hydrazine. A modified spectrophotometric assay was carried out by the following procedure: 50 μL of a sample containing the antioxidant source was added to 800 μL of absolute MeOH solution. DPPH (10 μM) was then added. After 10 min of incubation at room temperature and protected from light, the absorbance at 515 nm was determined with a Cecil CE 1011 spectrophotometer (Cecil, London, United Kingdom). Calculations of the results were made on the basis of a standard curve for a gallic acid solution and presented as GAEs.

Quantification of Polyphenols by the Folin−Ciocalteu Phenol Reagent Method. The total amounts of polyphenols were determined according to the modified Folin−Ciocalteu phenol reagent method and expressed as GAEs (*21*). Briefly, to 1 mL of HBSS were added polyphenols followed by the addition of 50 μL of Folin reagent and immediately by 150 μL of saturated sodium carbonate solution (25% w/v). After 10 min at room temperature, the O.D. was determined at 760 nm.

Statistical Analysis. Statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA). Results are presented as the means ± SD (except for luminescence experiments presented in **Figures 1** and **5**). An unpaired Student's t test was performed as specified in figures (differences were considered significant when $p < 0.05$).

RESULTS

Reduction of NBT/XTT and MTT by Combinations among Co(II), Se(IV), and H_2O_2. Table 1 shows that a maximal reduction of NBT, XTT, and MTT to formazan salts takes place only if a "cocktail" comprised of combinations among Co(II), tetrazolium salts, Se(IV), and H_2O_2 was employed. On the other hand, neither selenate(VI), seleno-cysteine, seleno-methionine, nor the organic Se compound, ebselen, could replace Se(IV). Also, neither

Table 1. Reduction of Tetrazolium Salts (200 μM) by Combinations among Hydrogen Peroxide (2 mM), Se (2 mM), and Co (10 μM)[a]

	H_2O_2	Se	Co	O.D. 450/600 nm
NBT (200 μM)	+			0.004 ± 0.002
	+	+		0.028 ± 0.013
	+		+	0.538 ± 0.043
	+	+	+	1.410 ± 0.083
MTT (200 μM)	+			0.003 ± 0.002
	+	+		0.004 ± 0.002
	+		+	0.448 ± 0.062
	+	+	+	0.870 ± 0.102
XTT (200 μM)	+			0.008 ± 0.002
	+	+		0.011 ± 0.004
	+		+	0.105 ± 0.046
	+	+	+	0.722 ± 0.094

[a] Data are expressed as mean values ± SD; n = 6.

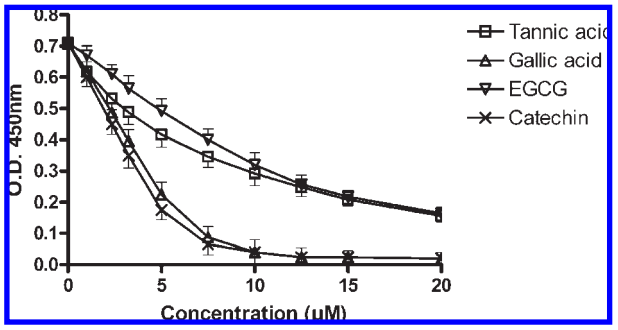

Figure 2. Inhibitory effect on XTT reduction by increasing concentrations of tannic acid, gallic acid, EGCG, and catechin. Data are expressed as mean values ± SD; n = 6.

SIN-1 (a generator of superoxide, nitric oxide, and peroxynitrite) and AAPH nor hypochlorous acid could replace H_2O_2. When tested at 10−100 μM, it is enigmatic why neither the divalent transitional metals copper, manganese, nor iron, the classical metals involved in the Fenton reaction to generate hydroxyl radical (*22*), could not replace Co(II). The results suggest that Co-catalyzed hydroxyl radical (*12*) is the key player in the tetrazolium reduction assay, which acts in concert with H_2O_2/oxidized Se(IV) to reduce tetrazolium salts. **Figure 1** shows the dose dependency of tetrazolium salts reductions when the novel "cocktail" was employed. While at 200 μM both NBT and MTT reduction reached a plateau level, the reduction of XTT continued to rise slowly, reaching a maximal plateau at 500 μM.

Effect of Inhibitors on Tetrazolium Salts Reduction Induced by the "Cocktail". The reduction of the tetrazolium salts by the "cocktail" was totally inhibited by 100 units of SOD, 5 units of HRP, 250 units of catalase, a 100 μM concentration of tiron (a superoxide scavenger), and a 10 μM concentration of deferoxamine (a metal chelator) (*23*). The results indicate that the cocktail generated peroxide, superoxide, and Co-catalyzed hydroxyl radical (*17*), which plays a key agent in the reduction process (see **Figure 6**). However, tested at greater than 100 μM, neither the classical low molecular weight antioxidants (LMWAs) ascorbic acid, uric acid, trolox (a vitamin E analogue), reduced glutathione, nor tempol (a nitroxide antioxidant) had any inhibitory activities on NBT reduction. **Figure 2** shows the inhibitory effect on XTT reduction of increasing concentrations of tannic acid, gallic acid, EGCG, and catechin. As can be seen, all four agents inhibited XTT reduction in a dose-dependent manner. However, gallic acid and catechin had stronger reductive capacities than EGCG and tannic acid. The reason why the XTT reduction assay was preferred to the use of MTT and NBT is because the XTT formazan product generated is water-soluble and technically, when used with microorganisms (**Figure 4**, see below), did not necessitate dye extraction.

Measurements of Polyphenols by the Tetrazolium Salt Reduction Cocktail, DPPH, FRAP, Luminescence, and Folin−Ciocalteu Methods. The sensitivity of the tetarazolium salts reduction test described here was compared to other classical methods, which measure antioxidant capacities such as the DPPH (a single free radical), FRAP, and LDCL methods (see above). **Table 2** shows that normalized to GAE, the antioxidant assays DPPH and FRAP and the novel XTT/MTT reduction tests can all detect micromolar amounts of several single polyphenols and agents present in red wine and cranberries beverages. As can be seen, on a molar basis, while the most potent inhibitors of the reduction of XTT were catechin and gallic acid, when used with the MTT test, the most potent inhibitors were catechin, followed in a descending order by gallic acid, polyphenon-60, and curcumin. See the Discussion for explanations of the possible differences between the two tetrazolium salts used. However, we did not use the NBT test to assay the effect of polyphenols. Also, when tested by the XTT, MTT, DPPH, FRAP, and Folin−Ciocalteu methods, red wine and cranberry beverages (containing 20% fruit), both rich in polyphenols, showed the highest total oxidant scavenging capacity (TOSC) patterns, as compared to the other agents tested. **Figure 3** shows, by comparison, that the LDCL method yielded results similar to those obtained by the other methods (see the Materials and Methods) (*17*, *18*). As can be seen, gallic acid, quercetin, NDGA, tannic acid, caffeic acid, catechin, and dopamine were the strongest inhibitors of luminescence. Under similar conditions, red wine and cranberry beverages at 10 μL, containing 19 and 12 μM GAEs, respectively, totally quenched LDCL. However, there was a clear but not a complete correlation between the amount of polyphenols as measured by Folin−Ciocalteu reagent (see the Discussion) and the inhibitory effects of polyphenols when tested either by the tetrazolium salt reduction method or also by the three other antioxidant assays used (**Table 2** and **Figure 1**).

Determination of the Tetrazolium Salts Reductive Capacities of Polyphenol−Bacteria Complexes. We have observed that many microbial species have the capacity to irreversibly absorb on their surfaces a variety of antioxidant polyphenols (*24*). Therefore, it was of interest to establish whether bacteria−polyphenol complexes might acquire enhanced antioxidant capacities. Various bacterial strains (see the Materials and Methods) were coated either by catechin (500 μM) or by red wine (100 μL) and tested by the XTT reduction assay. **Figure 4** shows that the polyphenol catechin, complexed with *E. coli* and with *S. aureus*, significantly increased XTT reduction. However, there was an insignificant increase of XTT reduction by the VSL#3 preparations coated with catechin. Complexes formed between red wine and bacteria also had significantly higher GAE values. **Figure 5** shows that employing the LDCL assay, while untreated *S. aureus* (**Figure 5a**) and *E. coli* (**Figure 5b**) had practically no inhibitory effects on light emission, and coating the cells either with catechin or with red wine very significantly increased their antioxidant capacities. However, while VLS#3 alone had only a moderate antioxidant capacity, which did not change by coating cells with catechin, a marked inhibition of LDCL took place if the cells had been precoated with red wine (**Figure 5c**). Taken together, it is shown that there is a definite parallelism between the chemiluminescence and the XTT assays, which both measure reductive capacities.

DISCUSSION

The tetrazolium salts reduction phenomenon is widely used as an indicator of reducing systems having a wide applications in

Table 2. Measurements of TOSC by Single Polyphenols at 10 μM and by Beverages (10 μL) Using the Tetrazolium Salt Reduction "Cocktails", DPPH, and FRAP Methods[a]

			μM GAE				
		polyphenol/beverage	DPPH	FRAP	XTT	MTT	Folin−Ciocalteu
nonflavonoids	phenolic acids	gallic acid	>7.5	>7.5	9.91 ± 0.29	9.96 + 0.04	9.78 ± 0.76
		tannic acid	6.54 ± 0.78	2.78 ± 0.13	5.83 ± 0.37	5.93 ± 0.10	8.82 ± 0.28
		caffeic acid	3.09 ± 0.12	1.72 ± 0.06	3.01 ± 0.01	5.80 ± 0.24	4.95 ± 0.64
		NDGA	2.60 ± 0.05	2.71 ± 0.11	5.28 ± 0.27	5.37 ± 0.38	5.47 ± 0.12
	stilbenes	resveratrol	1.46 ± 0.23	2.30 ± 0.17	4.21 ± 0.32	3.08 ± 0.12	5.56 ± 0.21
	others	curcumin	0.75 ± 0.07	0.49 ± 0.02	3.14 ± 0.08	9.39 ± 0.42	5.81 ± 0.05
		L-Dopa	4.60 ± 0.32	3.02 ± 0.07	0.44 ± 0.01	3.40 ± 0.23	7.10 ± 0.57
		dopamine	4.08 ± 0.12	3.45 ± 0.01	1.36 ± 0.01	0.40 ± 0.04	7.01 ± 0.26
flavonoids	flavanols	catechin	1.25 ± 0.05	1.00 ± 0.05	10.28 ± 0.81	10.14 ± 0.38	4.99 ± 0.68
		EGCG	3.99 ± 0.66	1.24 ± 0.09	1.36 ± 0.01	1.85 ± 0.32	5.67 ± 0.10
		polyphenon-60	1.20 ± 0.02	0.73 ± 0.01	2.67 ± 0.04	9.76 ± 0.09	4.20 ± 0.07
	flavonols	quercetin	5.32 ± 0.13	2.52 ± 0.12	8.48 ± 0.94	3.02 ± 0.35	12.00 ± 0.19
		rutin	1.06 ± 0.03	0.93 ± 0.03	4.03 ± 0.05	9.20 ± 0.05	4.91 ± 0.12
		morin	0.98 ± 0.02	0.91 ± 0.03	0.89 ± 0.02	0.90 ± 0.06	5.50 ± 0.17
		galangin	1.22 ± 0.08	1.84 ± 0.15	3.22 ± 0.43	1.47 ± 0.09	4.06 ± 0.12
	flavanone	hesperetin	0.24 ± 0.04	0.88 ± 0.10	9.30 ± 0.22	6.02 ± 0.17	5.45 ± 0.34
	flavones	apigenin	0.12 ± 0.03	[b]	12.01 ± 0.42	3.33 ± 0.34	4.44 ± 0.36
		chrysin	[b]	[b]	13.01 ± 0.36	3.32 ± 0.40	2.94 ± 0.25
	isoflavons	genistein	[b]	[b]	4.69 ± 0.18	4.00 ± 0.21	3.95 ± 0.17
proanthocyanidins	beverages	wine	5.33 ± 0.42	3.88 ± 0.11	9.47 ± 0.66	9.29 ± 0.45	19.23 ± 0.37
		crannberries	2.51 ± 0.15	2.25 ± 0.02	9.94 ± 0.50	9.80 ± 0.90	12.21 ± 0.32

[a] The polyphenol content measured by Folin−Ciocalteu's phenol reagent method is also presented. Data are expressed as GAE as mean values ± SD; $n = 6$. [b] Below detection.

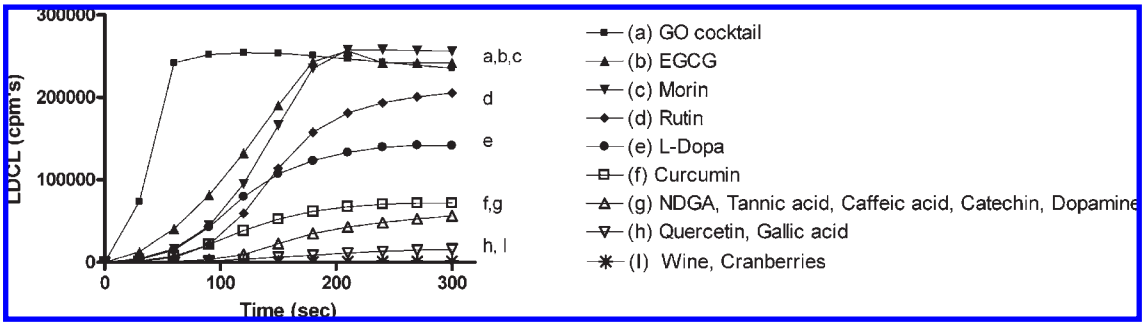

Figure 3. Effect of single polyphenols at 10 μM and of two beverages (10 μL) on LDCL, induced by the glucose oxidase cocktail. The data presented represent a typical luminescence curve obtained from the samples shown in table **2.**

chemistry and biology (*5*). These salts have been primarily used as substrates for the electron transfer system in bacterial and in mammalian cells to measure cell viability and cell proliferation and to determine oxidative functions of activated leukocytes (*5*). The present communication describes a novel additional use for tetrazolium salt reduction tests, which can quantify polyphenols and polyphenol−bacteria complexes. The method utilizes a "cocktail" comprised of Se(IV), reagent H_2O_2, and Co(II) (**Table 1**), which when mixed together, rapidly reduces NBT and MTT to a dark blue insoluble formazan and XTT to a soluble orange formazan product. These reduction tests are based on the ability of polyphenols to chelate Co, the "key player" in the tetrazolium salt reduction test described (see below), to scavenge ROS (*25*), and thus is suitable for quantifying polyphenol antioxidants from a variety of sources. Therefore, this is why the tetrazolium reduction tests described are not suitable to measure antioxidants lacking chelating capacities for Co such as classical LMWA, for example, ascorbate, glutathione, vitamin E, and also body fluids. However, H_2O_2 could not be replaced either by the peroxyl radical initiator AAPH, hypochlorous acid, nor by SIN-1 (a generator of nitric oxide, superoxide, and peroxinitrite). Also, Se(IV) could not be replaced either by selenate(VI), seleno cysteine, seleno methionine, or the organo seleno compound, ebselen, indicating that Se(IV) needs to be in its free form. It should also be stressed that Co(II), which plays a "key role" in the salt reduction, could not be replaced by an equimolar concentration of either Cu(II), Fe(II), Mn(II) (transition metals), molybdate(II), or vanadium(II). To study the nature of the ROS generated by the cocktail involved in the reduction of the tetrazolium salts, we tested a series of putative antioxidant agents. A total inhibition of the tetrazolium salt reduction was induced by SOD, which catalyzes the dismutation of superoxide to oxygen and hydrogen peroxide (*10*); by HRP, which is a hydrogen peroxide oxidoreductase; by catalase, which decomposes hydrogen peroxide to water and oxygen; and by tiron, a superoxide scavenger. These results suggest that the reduction of tetrazolium salts to formazan products is mediated by combinations among several oxidants generated by the cocktail.

The dependency on the concentrations the three tetrazolium salts (0−500 μM) is shown in **Figure 1** showing major differences among the salts tested. This can be explained on the ground that the various salts have different affinities for electrons, dependency on their molecular structure, where one agent is more easily reduced than the other (*5*). Tetrazolium

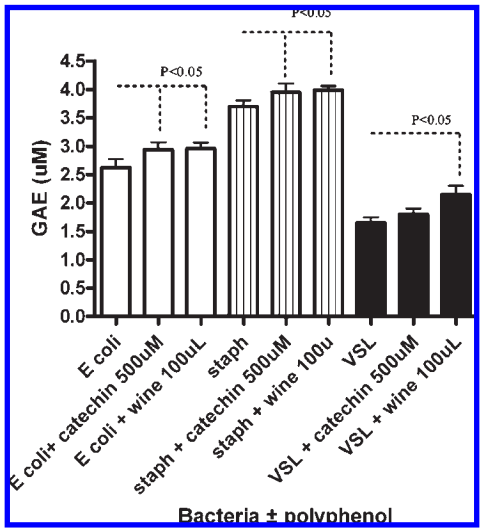

Figure 4. Total GAE values as measured by the XTT reduction method of *E. coli*, *S. aureus*, and a lactobacilli mixture (VSL#3) precoated by catechin (500 μM) and red wine (100 μL). Data are expressed as mean values ± SD; $n = 6$.

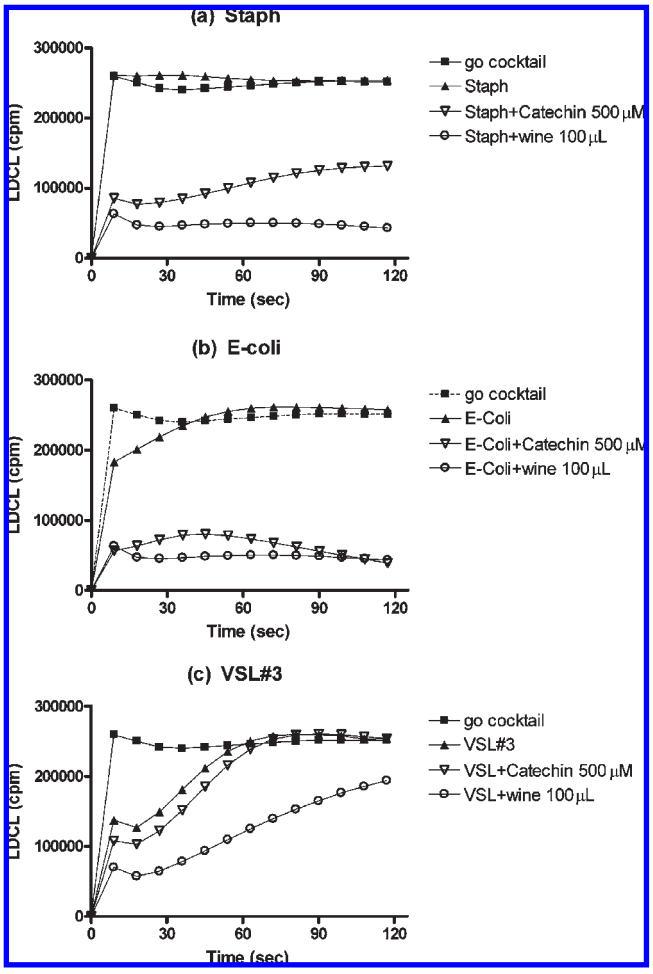

Figure 5. Effect of complexes formed between catechin, red wine, *S. aureus*, *E. coli*, and a lactobacilli mixture (VSL#3) on LDCL, induced by the glucose oxidase cocktail. The data presented represent a typical luminescence curve obtained from the samples shown in **Figure 4**.

salts can be compared for their reductive activities by their redox potential values, which can also be measured by the cyclic voltammetry method that measures electrochemical properties (*26*).

As shown in **Table 2** and **Figure 3**, the inhibition by antioxidant polyphenols of the reduction of the tetrazolium salts partially correlates with the antioxidant reductive capacities measured by the FRAP, DPPH, and LDCL methods (see the Materials and Methods). While the FRAP method determines ferric reducing capacities, the DPPH method uses this agent as a single radical, which can be scavenged by antioxidants, and the Folin−Ciocalteu phenol reagent assay can detect and quantify only the number of hydroxyl groups that has no absolute correlation with their antioxidant properties. However, the luminescence assay used, which simultaneously generates H_2O_2, superoxide, and Co-catalyzed $OH^{\bullet}$, measures in a dose-dependent manner a large variety of reducing agents and agents possessing chelating properties for Co. It is also paradoxical that both the tetrtazolium reduction cocktail and the luminescence-inducing cocktail utilize the same reagents, that is, hydrogen peroxide, Se(IV), and Co(II). However, while the tetrazolium reduction cocktail is able to detect only agents such as antioxidant polyphenols present in extracts from various plants, which possess chelating properties, the luminescence test can measure both chelating agents and also classical LMWA, which do not possess chelating properties. While the luminescence test can detect small amounts of LMWA such as ascorbic acid, uric acid, vitamin E, and reduced glutathione, even large amounts (100−500 μM) of these classical LMWA failed to inhibit the tetrazolium salt reduction presumably due to their inability to chelate Co. Therefore, it stands to reason that analysis by different methods of phenolic content or TOSCs of various agents cannot always be compared since they are based on different mechanisms of action and also diverse modes of detection. In this respect, it is also important to stress that the partial correlation displayed among the various methods tested might be explained on the ground that "Individual differences within each polyphenol group result from the variation in number and arrangement of the hydroxyl groups as well as from the nature and extent of alkylation and/or glycosylation of these groups" (*27*). Chen et al. (*28*) also suggested that polyphenol scavenging activities may be closely related to the position rather than the numbers of phenolic hydroxyl groups. When tested by the XTT assay (see **Table 2**), one example for the apparent lack of structural−activity correlation among the various agents is the finding that while EGCG (1.36 GAEμM) contains eight hydroxyl groups, catechin (10.28 GAE μM) possesses only five hydroxyl groups. Furthermore, catechin and quercetin, which both possess five hydroxyl groups, have 10.28 GAE and 8.48 GAE, respectively. It seems, therefore, that the numbers of OH groups on the phenol rings do not necessarily predict their TOSC or Co chelating properties. Furthermore, it has been proposed (*28*) that the location and number of hydroxyl groups present on the A and B rings of the polyphenol structure are important OH-scavenging active groups, as the ones generated by our cocktail. Judging from the results using the XTT reduction assay (**Table 2**), it appears that the position of the OH groups on ring A in all four agents (catechin, quercetin, rutin, and EGCG) is the same. On the other hand, while EGCG, which has the lowest GAE, has three OH groups on its B ring, the other three polyphenols contain two OH groups at the same location. It is proposed therefore that at least for the XTT reduction assay there is no direct correlation between the number and the location of the OH groups on the phenol rings and their TOSC. Therefore, the tetrazolium reduction test developed is probably able to quantify polyphenols both as hydroxyl radical scavengers and also due to their ability to chelate Co, the catalyst leading to $OH^{\bullet}$ formation (*12*).

As shown in **Figure 4**, the antioxidant capacities of bacterial cells and of bacteria−polyphenol complexes were measurable by the XTT reduction assay. **Figure 5** shows that the LDCL method correlates well with the XTT method except for catechin, which was detected only with the luminescence technique. We hereby present an additional function for TOSC assays to measure polyphenols attached to microbial surfaces.

It might be postulated that during the consumption of nutrients rich in antioxidants polyphenols, both the oral microbiota and the flora of the large intestine can be coated by polyphenols, which endow upon them enhanced TOSC (*24*). Therefore, bacteria−polyphenol complexes might act as "depots" for polyphenols capable of protecting epithelial cells against oxidative stress in inflammatory sites in the oro/intestinal areas. It is also of importance that measurements by the XTT reduction test of the TOSC of microbial cells and of microbial cells coated by polyphenols have advantages over the use of the MTT or NBT tests since reduced XTT is sparingly soluble in water and spectrophotometric readings of the color intensities generated can be performed in supernatant fluids obtained after a short centrifugation.

In addition to their antioxidative properties, polyphenols are known to act as strong chelators for transitional divalent metals (*13*, *14*) and also as hydrogen peroxide, superoxide, and hydroxyl radical scavengers (*15*, *16*). However, it is unreasonable to expect that polyphenols might inhibit the Co-catalyzed reduction of tetrazolium salts by decomposing hydrogen peroxide since absolutely no reduction of tetrazolium occurs if Co is removed from the reaction mixture. This assumption is further strengthened by the finding that deferoxamine [a potent metal chelating agent (*23*)], which is not expected to possess any hydrogen peroxide decomposing capacities, also inhibited tetrazolium salt reduction in a dose-dependent manner (data not shown).

The mechanisms by which combinations among Co(II), Se(IV), and hydrogen peroxide reduce tetrazolium salts are still not fully understood. It is still enigmatic why oxidizing agents such as H_2O_2 and Co-catalyzed hydroxyl radical have the ability to induce the reduction of tetrazolium salts to formazan and also why free selenite(IV) is exclusively necessary for this phenomenon to occur. **Figure 6** proposes the main events, which lead to the reduction of tetrazolium salts. It is postulated that it involves mainly interactions among Co-catalyzed hydroxyl radical formation (*12*, *29*), which is accompanied by oxidation by H_2O_2 of Se(IV) to Se(VI). Electrons transferred from Se oxidation possibly contribute to the reduction of tetrazolium salts to formazans by acting as acceptors of electrons from the oxidized Se(IV) (*5*). The exclusive involvement of Co, as a catalytic transitional metal at 5−10 μM, in this reaction is also not fully understood since catalytic equimolar amounts of other classical transitional metals, for example, Fe(II), Cu(II), and Mn(II), which also catalyze the Fenton reaction to form hydroxyl radicals (*22*), failed to act in concert with Se and with hydrogen peroxide to reduce tetrazolium salts.

Taken together, our findings suggest that the Co-catalyzed tetrazolium salt reduction is unique and that this assay is a simple to perform test to assay the chelating properties of polyphenols, one of the important properties of these group of compounds. In addition, it might also be postulated that the capacity of polyphenols to inhibit the tetrazolium salt reduction might be due perhaps to both their ability to chelate Co and also to scavenge ROS generated by the cocktail. However, because the cocktail used to reduce tetrazolium salts involves combinations of millimolar amounts of H_2O_2 but only micromolar catalytic amounts of Co, it excludes the possibility that H_2O_2 decomposition plays any major role is the reaction whereby polyphenols inhibit tetrazolium salts reduction.

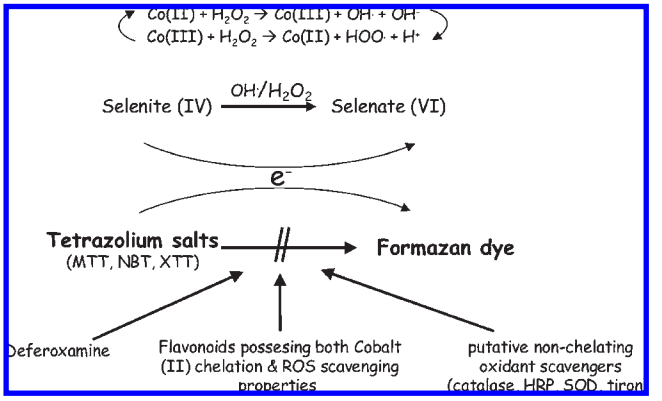

Figure 6. Proposed mechanism for the reduction of tetrazolium salts to formazans, induced by a "cocktail" comprised of sodium selenite(IV), Co(II), and hydrogen peroxide.

The evaluation of antioxidant capacities plays a pivotal role in the understanding of many biological and biochemical systems, especially in clinical disorders characterized by oxidative stresses (*22*). The present communication offers a use for a novel, easy to perform, "Co-based cocktail", which generates an assortment of oxygen reactive species capable of reducing tetrazolium salts. The inhibition of tetrazolium salt reduction by polyphenols depends on their ability to chelate Co and thus to inhibit in a dose-dependent manner the reduction of the tetrazolium salts. It is therefore not surprising that this newly described method is not suitable for measurements of antioxidants such as ascorbic acid, reduced glutathione, vitamin E, and uric acid or antioxidant capacities in body fluids or in mammalian cells in culture supplemented with LMWAs (*30*), since these do not possess significant chelating properties for Co.

ABBREVIATIONS USED

ROS, reactive oxygen species; LMWA, low molecular weight antioxidant; TOSC, total oxidant scavenging capacity; LDCL, luminol-dependent chemiluminescence; NBT, nitroblue tetrazolium chloride; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; FRAP, ferric ion reducing antioxidant power; DPPH, 2,2-diphenyl-2-picrylhydrazyl hydrate; DMSO, dimethyl sulfoxide; SOD, superoxide dismutase; GO, glucose oxidase; EGCG, epigallocatechin gallate; NDGA, nor-dihydroguiaretic acid; AAPH, 2,2′-azobis-(2-amidinopropane) dihydrochloride; HBSS, Hank's balanced salt solution; GAE, gallic acid equivalent; HRP, horseradish peroxidase; SIN-1, 3-morpholino-sydnonimine.

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LITERATURE CITED

(1) Pechmann, H. V.; Runge, P. Oxidation der Formazylverbindungen II. *Ber. Deutsch. Chem. Ges.* **1894**, *27*, 2920–2930.

(2) Findlay, G. H. The value of some tetrazolium salts as histochemical reagents for sulphydryl groups. *J. Histochem. Cytochem.* **1955**, *3* (5), 331–338.

(3) Kuhn, R.; Jerchel, D. Über Invertseifen, VIII. Reduktion von Tetrazoliumsalzen durch Bakterier gärende Hefe und keimende Samen. *Ber. Deutsch. Chem. Ges.* **1941**, *74*, 949–952.

(4) Reid, W. Formazane und Tetrazoliumsalze, ihre Synthesen und ihre Bedeutung als Reduktionsindikatoren und Vitalfarbstoffe. *Angew. Chem.* **1951**, *64* (14), 391–396.

(5) Altman, F. P. Tetrazolium salts and formazans. *Prog. Histochem. Cytochem.* **1976**, *9* (3), 1–56.
(6) Seidler, E. The tetrazolium-formazan system: design and histochemistry. *Prog. Histochem. Cytochem.* **1991**, *24* (1), 1–86.
(7) Park, B. H.; Fikrig, S. M.; Smithwick, E. M. Infection and nitroblue-tetrazolium reduction by neutrophils. A diagnostic acid. *Lancet* **1968**, *2* (7567), 532–534.
(8) Baehner, R. L.; Boxer, L. A.; Davis, J. The biochemical basis of nitroblue tetrazolium reduction in normal human and chronic granulomatous disease polymorphonuclear leukocytes. *Blood* **1976**, *48* (2), 309–313.
(9) Nathan, D. G.; Baehner, R. L.; Weaver, D. K. Failure of nitro blue tetrazolium reduction in the phagocytic vacuoles of leukocytes in chronic granulomatous disease. *J. Clin. Invest.* **1969**, *48* (10), 1895–1904.
(10) Liochev, S. I.; Fridovich, I. Superoxide from glucose oxidase or from nitroblue tetrazolium? *Arch. Biochem. Biophys.* **1995**, *318* (2), 408–410.
(11) Picker, S. D.; Fridovich, I. On the mechanism of production of superoxide radical by reaction mixtures containing NADH, phenazine methosulfate, and nitroblue tetrazolium. *Arch. Biochem. Biophys.* **1984**, *228* (1), 155–158.
(12) Moorhouse, C. P.; Halliwell, B.; Grootveld, M.; Gutteridge, J. M. Cobalt(II) ion as a promoter of hydroxyl radical and possible 'crypto-hydroxyl' radical formation under physiological conditions. Differential effects of hydroxyl radical scavengers. *Biochim. Biophys. Acta* **1985**, *843* (3), 261–268.
(13) Hider, R. C.; Liu, Z. D.; Khodr, H. H. Metal chelation of polyphenols. *Methods Enzymol.* **2001**, *335*, 190–203.
(14) Ozyurek, M.; Bektasoglu, B.; Guclu, K.; Apak, R. Hydroxyl radical scavenging assay of phenolics and flavonoids with a modified cupric reducing antioxidant capacity (CUPRAC) method using catalase for hydrogen peroxide degradation. *Anal. Chim. Acta* **2008**, *616* (2), 196–206.
(15) Naderi, G. A.; Asgary, S.; Sarraf-Zadegan, N.; Shirvany, H. Antioxidant effect of flavonoids on the susceptibility of LDL oxidation. *Mol. Cell. Biochem.* **2003**, *246* (1−2), 193–196.
(16) Moridani, M. Y.; Pourahmad, J.; Bui, H.; Siraki, A.; O'Brien, P. J. Dietary flavonoid iron complexes as cytoprotective superoxide radical scavengers. *Free Radical Biol. Med.* **2003**, *34* (2), 243–253.
(17) Ginsburg, I.; Sadovnic, M.; Oron, M.; Kohen, R. Novel chemiluminescence-inducing cocktails, part II: measurement of the antioxidant capacity of vitamins, thiols, body fluids, alcoholic beverages and edible oils. *Inflammopharmacology* **2004**, *12* (4), 305–320.
(18) Ginsburg, I.; Sadovnic, M.; Oron, M.; Kohen, R. Novel chemiluminescence-inducing cocktails, part I: The role in light emission of combinations of luminal with SIN-1, selenite, albumin, glucose oxidase and Co2+. *Inflammopharmacology* **2004**, *12* (4), 289–303.
(19) Benzie, I. F.; Strain, J. J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Anal. Biochem.* **1996**, *239* (1), 70–76.
(20) Blois, M. S. Anti-oxidant activity was measured according to the elimination method of DPPH free radicals. *Nature* **1959**, *181* (1), 1199.
(21) Singleton, V.; Orthofer, R.; Lamuela-Raventos, R. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Oxidants Antioxid., Part A* **1999**, *299*, 152–178.
(22) Halliwell, B.; Gutteridge, J. *Free Radicals in Biology and Medicine*, 4th ed.; Oxford University Press: Oxford, 2007.
(23) Smith, R. S. Chelating agents in the diagnosis and treatment of iron overload in Thalassemia. *Ann. N.Y. Acad. Sci.* **1964**, *119*, 776–88.
(24) Koren, E.; Kohen, R.; Ovadia, H.; Ginsburg, I. Bacteria coated by polyphenols acquire potent oxidant-scavenging capacities. *Exp. Biol. Med. (Maywood)* **2009**, *234*, 940–951.
(25) Pietta, P. G. Flavonoids as antioxidants. *J. Nat. Prod.* **2000**, *63* (7), 1035–1042.
(26) Kohen, R.; Vellaichamy, E.; Hrbac, J.; Gati, I.; Tirosh, O. Quantification of the overall reactive oxygen species scavenging capacity of biological fluids and tissues. *Free Radical Biol. Med.* **2000**, *28* (6), 871–879.
(27) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20* (7), 933–956.
(28) Chen, J. W.; Zhu, Z. Q.; Hu, T. X.; Zhu, D. Y. Structure-activity relationship of natural flavonoids in hydroxyl radical-scavenging effects. *Acta Pharmacol. Sin.* **2002**, *23* (7), 667–672.
(29) Kadiiska, M. B.; Maples, K. R.; Mason, R. P. A comparison of cobalt(II) and iron(II) hydroxyl and superoxide free radical formation. *Arch. Biochem. Biophys.* **1989**, *275* (1), 98–111.
(30) Koren, E.; Zverev, I.; Ginsburg, I.; Kohen, R. Supplementation with antioxidants fails to increase the total antioxidant capacity of several cell lines in culture. *Biomed. Pharmacother.* **2008**, *62* (3), 179–188.

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