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Full Length Research Paper

Development of bacterial cell-based system for intracellular antioxidant activity screening assay using green fluorescence protein (GFP) reporter

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The novel bacterial cell-based assay was developed for evaluating the intracellular antioxidant activity. The genetically engineered *Escherichia coli* strains harboring the fusions of *sodA*::*gfp* and *fumC*::*gfp* were constructed and applied as reporters in response to cellular superoxide stress. Using this assay, twelve pure compounds and three Thai medicinal plants were investigated for intracellular antioxidant activity in comparison with conventional chemical-based assays; 2,2-diphenyl-1-picrylhydrazyl (DPPH) and superoxide dismutase (SOD) activity assays. Both strains demonstrated that quercetin and α-tocopherol exhibited the most potent and significant antioxidant activity with more than 60% reduction of intracellular superoxide. These compounds also showed high DPPH radical scavenging activity. Interestingly, gallic, caffeic and protocatechuic acids had the most significant DPPH radical scavenging and SOD-like activities but with moderate to weak intracellular antioxidant activity. Our hypothesis was that the lower intracellular antioxidant activity possibly occurs due to poor permeability of compounds into biological membrane based on their structures. Moreover, our results demonstrated that intracellular antioxidant activity of three plant extracts well correlated to results from DPPH assay. Our bacterial-based assay is simple, reproducible, very specific and applicable as an alternative screening tool for assessing the activity of compounds and plant extracts affecting cellular oxidative stress.

Key words: Bacterial cell-based assay, antioxidant activity, oxidative stress, superoxide dismutase, fumarase, green fluorescence protein (GFP) reporter, plant extracts.

INTRODUCTION

Reactive oxygen species (ROS) including superoxide radical ($O^{\cdot}_2^-$), hydroxyl radical ($HO^{\cdot}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) are produced as an unavoidable consequence of the aerobic lifestyle (Imlay, 2002). They can react with and damage many cellular components, such as carbohydrates, lipids, proteins and DNA. ROS can cause oxidative stress that implicates in initiating, accompanying or causing pathogenesis of many diseases and aging (Keller et al., 1998; Halliwell and Gutteridge, 1999; Prasad et al., 1999; Pratico and Delanty, 2000; Lu and Finkel, 2008). In general, living cells possess the protective systems of antioxidants which counteract and prevent the deleterious effects of ROS. These systems include enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) and intracellular antioxidants, such as glutathione (GSH) and some vitamins (Ullmann et al., 2008). Various antioxidants have been derived from foodstuffs, such as fruits and vegetables. Recently,

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interest has increased considerably in finding naturally occurring antioxidants for health promotion and disease prevention with high safety and consumer acceptability. Therefore, several approaches have been established to the evaluation of antioxidant activity *in vitro* and *in vivo* (Shahidi and Ho, 2007; Wolfe and Lui, 2007; Ma et al., 2011). *In vitro* assays based on chemical approaches, such as DPPH radical scavenging assay (Brand-Williams et al., 1995), ferric reducing/antioxidant power (FRAP) assay (Benzie and Strain, 1999) and Trolox equivalent antioxidant capacity (TEAC) assay (van den Berg et al., 1999) are attractive for their simplicity, convenience, reproducibility and cost-effective (Moreno-Sanchez, 2002). They are widely used to determine the capacity of an antioxidant in the reduction of antioxidant, which changes color when reduced. However, they present some disadvantages, such as pre-treatment of colored samples, correction for interfering substances and no biological relevance. Moreover, though they are great methods used for antioxidant evaluation, no approved and standardized method can alone provide an adequate measure, resulting from the complexity of antioxidant action and multiple-method approach for the estimation of antioxidant activity is recommended (Liu et al., 2012). The animal models and human studies are the best approaches to assess the effects of antioxidants *in vivo*. Nevertheless, they are expensive, time-consuming, not affordable and not suitable for initial antioxidant screening of foods and dietary supplements (Liu and Finley, 2005). Mammalian cell models have also been developed and used to examine the antioxidant activity in response to a need for more biologically representative methods than chemical-based assays (Takamatsu et al., 2003; Wolfe and Lui, 2007). These assays are relatively fast and cost-effective as compared to animal models. However, these still require well-trained personnel or specialized technician for practice. Therefore, alternative biological methods are needed. Currently, cellular biosensors based on various recombinant bacteria containing reporters which are specifically induced via selected promoters are widely used in biomedical applications (Alksne et al., 2000; Hansen et al., 2001; Mitchell and Gu, 2004).

Their advantages over the mammalian cell-based assays are that they are inexpensive, very easy to perform and have much shorter generation times as they can be detected within a period of hours. Park et al. (2010) demonstrated a bacterial cell-based methodology as a tool for screening antioxidant activity of natural chemical products. They suggested that this assay is more relevant to the effect of antioxidants at a cellular level than chemical-based assays, however, no results that compared between chemical-based and bacterial-based approaches was stated. The purpose of our study was to develop the bacterial-based antioxidant activity assay used to screen antioxidant substances for potential biological activity. Herein, two *Escherichia coli* biosensor strains that individually carried plasmid that fused *sodA* and *fumC* promoters with *gfp* gene were produced and then used to evaluate antioxidant activity of twelve pure compounds in comparison with two DPPH radical scavenging and SOD activity assays. Furthermore, three Thai traditional medicinal plant extracts were assessed in the biological antioxidant activity using this assay. *Hydnophytum formicarum* Jack. has been used for the treatment of hepatitis, rheumatism and diarrhea (Prachayasittikul et al., 2008). *Spilanthes acmella* Murr. has been used for the treatment of toothache, rheumatism and fever (Prachayasittikul et al., 2009). *Eclipta prostrata* Linn. has been used for the treatment of diverse symptoms, example hyperlipidemia, atherosclerosis and skin diseases (Prachayasittikul et al., 2010). In this study, the green fluorescent protein (GFP) was utilized as a reporter according to its advantages, such as high stability and no need for additional substrates or other cofactors (Cha et al., 1999). Moreover, it is a noninvasive reporter which allowing real-time monitored by continuous quantitative measurement of the GFP emission (Chalfie et al., 1994; Lu et al., 2004). In this way, we were able to evaluate both the chemical and the biological antioxidant activity of compounds and extracts, as well as compare these methods.

MATERIALS AND METHODS

Chemicals and reagents

Paraquat or methyl viologen, resorcinol, 3-hydroxypyridine, 2,2-diphenyl-1-picrylhydrazyl (DPPH), nitroblue tetrazolium (NBT), NADH disodium salt, phenazine methosulfate (PMS) and bovine erythrocyte superoxide dismutase (SOD) were purchased from Sigma-Aldrich (St Louis, MO, USA), hydrogen peroxide was purchased from Merck (Germany). Quercetin, α-tocopherol, caffeic acid, *p*-coumaric acid, 3-hydroxycinnamic acid, gallic acid, orotic acid and dimethyl sulfoxide (DMSO) were obtained from Fluka (USA). Vanillic acid and protocatechuic acid were obtained from Acros organics (Belgium). Salicylic acid was purchased from Unilab (Philippines). Luria-Bertani (LB) medium and agar were purchased from Difco Laboratories (Detroit, MI, USA). Ampicillin was purchased from Bio basic (Canada). All restriction enzymes were obtained from Fermentas (USA). PCR master mix solution and i-Taq™ DNA polymerase were purchased from Intron biotechnology (South Korea). Chemical structures of all tested compounds used in this study are shown in Figure 1. All crude ethyl acetate extracts including that of *S. acmella* (Linn.) Murr., *H. formicarum* Jack. and *E. prostrata* Linn. were prepared as previously described (Prachayasittikul et al., 2008, 2009, 2010).

Construction of *E. coli* reporter strains

pGlow-TOPO (Invitrogen, Carlsbad, CA, USA) was used to construct a recombinant plasmid containing a native oxidative responsive promoter and *gfp* fusion. A total of four oxidative stress responsive promoters, *sodA* (manganese superoxide dismutase), *fumC* (fumarase C) from SoxRS regulon and *katG* (bifunctional catalase), *ahpC* (alkyl hydroperoxide reductase) from OxyR regulon were PCR amplified using *E. coli* TG1 genome as a template. The PCR primers were synthesized referring to the sequence of *E. coli* K-12 MG1655 obtained through Genbank. The amplified PCR products include the native start codon, ribosome binding site (RBS), and –35 and –10 regions plus other regulatory regions.

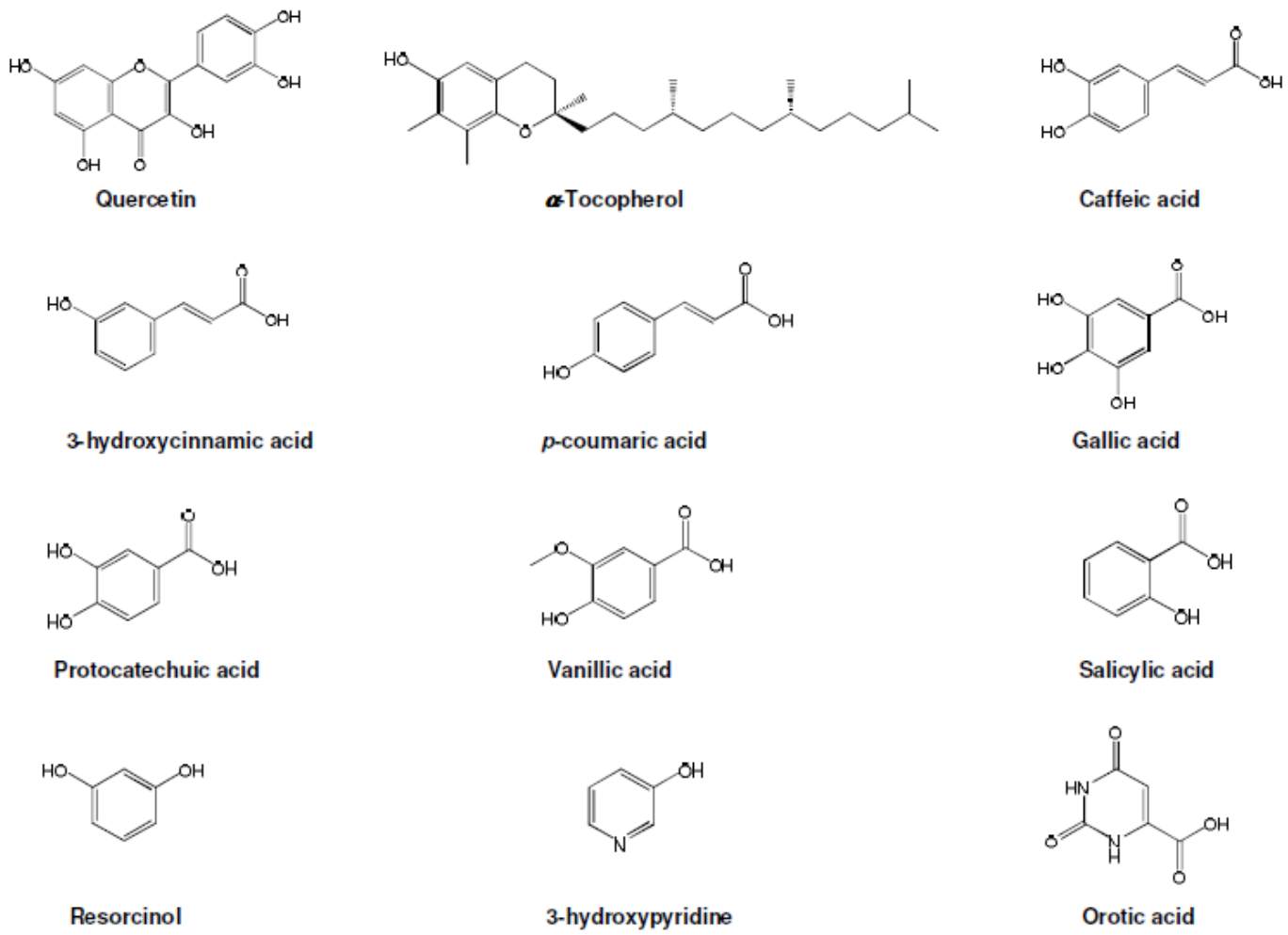

Figure 1. Chemical structures of the tested compounds used in this study.

Sequences of PCR primers and the sizes of the amplified promoters are shown in Table 1. Expected products were confirmed by gel electrophoresis. Following amplification without purification, PCR products were mixed with pGlow-TOPO vector at room temperature for 5 min. The resulting recombinant plasmids were transformed into *E. coli* strain Top10. The positive colonies were screened by plating on LB agar plates containing 100 µg/ml ampicillin. The positive clones were extracted the plasmids and the insertions were proved by restriction enzyme digestion. The orientation of the promoter insert was confirmed by PCR using the forward primer of each promoter and downstream GFP primer (5′ GGG TAA GCT TTC CGT ATG TAG C 3′). The sequences of the fusions were verified by DNA sequencing.

Determination of optimal culture conditions and intracellular antioxidant activity testing

E. coli reporter strains containing the fusions of promoters and *gfp* were used to monitor the intracellular oxidative stress generation. The overnight cultures were grown aerobically in 250 ml flasks containing 50 ml of LB medium and 100 µg/ml ampicillin at 37°C with vigorous shaking (150 rpm) until the optical density at 600 nm (OD_{600}) reached approximately 0.4 as measured by a UV-Visible spectrophotometer (UV-1601, Shimadzu, Japan). Cultures were divided into 5 ml aliquots in 50 ml tubes (Fisher Scientific, Pittsburgh, PA, USA); then paraquat or hydrogen peroxide was added at various concentrations. For antioxidant activity testing, the tested compounds or plant extracts were added immediately at the desired concentrations after addition of inducers. Cultures were further incubated at 37°C with shaking (150 rpm). The raw fluorescence intensity and OD measurements were taken every hour using FLx800 microplate fluorescence reader (Bio-Tek instruments, VT, USA) at an excitation wavelength of 395 nm and emission of 509 nm and UV-Visible spectrophotometer at a wavelength of 600 nm until 8 h. For data analysis, raw fluorescence intensity (FL) and OD measurements of triplicate experiments were averaged and the standard errors of means were calculated. Specific fluorescence intensity (SFI) was calculated from the following equation; raw fluorescence intensity/OD_{600}. Fold induction (FI) was calculated by the equation; $SFI_{stress}/SFI_{control}$ or $SFI_{test}/SFI_{control}$ where SFI_{stress} represents the specific fluorescence intensity of the tubes with oxidants, SFI_{test} represents the specific fluorescence intensity of the tubes with tested compounds plus oxidants and $SFI_{control}$ represents the specific fluorescence intensity of the tubes without any oxidants. The percent relative fold induction (RFI) was calculated by the equation; $(FI_{test}/FI_{stress}) \times 100$ where FI_{test} represents the fold induction of tubes with tested

Table 1. PCR primers used in this study.

Gene	Primer sequences (5′→3′)	PCR product size (bp)
katG	Forward; GTG TGG CTT TTG TGA AAA TCA Reverse; TCA TCA ATG TGC TCC CCT CT	329
ahpC	Forward; GAG CTT AGA TCA GGT GAT TG Reverse; ACA TCT ATA CTT CCT CCG TG	309
sodA	Forward; GTA ATC GCG TTA CTC ATC TT Reverse; TCA TAT TCA TCT CCA GTA TT	305
fumC	Forward; CAC AAT GCA CCC GCT GTG TG Reverse; TCA TGA CCT GCT CCT CAC CTG	172

compounds plus oxidants and FI_{stress} represents the fold induction of tubes with oxidants.

DPPH radical scavenging assay

DPPH radical scavenging capacity was determined by the method as previously described (Prachayasittikul et al., 2009). The 2,2-diphenyl-1-picrylhydrazyl (DPPH) was dissolved in 100% methanol to prepare 0.1 mM DPPH solution. The 1 ml of this solution was added to 0.5 ml, 3 mM of sample solution which dissolved in DMSO (final concentration of sample equals to 1 mM). After 30 min of incubation at room temperature, the absorbance of the reaction mixture was measured using UV-Visible spectrophotometer at 517 nm. The percentage of radical scavenging activity was calculated according to the equation:

Radical scavenging activity (%) = [(Abs.control - Abs.sample) / Abs.control] × 100

Where, Abs.control is the absorbance of the control reaction and Abs.sample is the absorbance of the tested compound.

SOD activity assay

Superoxide dismutase (SOD) activity was measured using the method as described previously (Grey et al., 2009) with some modifications. In principle, this assay is based on the ability of SOD to inhibit NBT reduction by an aerobic mixture of NADH and PMS, which produces superoxide at non-acidic pH. The complete reaction system (1 ml total volume) consisted of 50 mM phosphate buffer, pH 7.4, 0.1 mM EDTA, 50 µM NBT, 78 µM NADH and 3.3 µM PMS (final concentrations). For the assay, 100 µL of sample or standard at various concentrations were added into cuvettes containing 900 µL of reaction mixture. The absorbance at 560 nm was monitored during 5 min as an index of NBT reduction using a UV-Visible spectrophotometer and SOD activity was calculated from the following equation:

Enzyme inhibition (%) = [(Abs.control - Abs.sample) / Abs.control] × 100

Where, Abs.control is the absorbance of the control reaction and Abs.sample is the absorbance of the tested compound. Enzyme inhibition (%) was used for graph plotting and calculated for IC_{50} values.

RESULTS

Effects of oxidative stress to promoters in *E. coli* reporter strains

In this study, we successfully constructed the recombinant *E. coli* TOP10 reporter strains. A total of four oxidative stress promoters, *sodA*, *fumC*, *katG* and *ahpC* were fused individually to *gfp* located on pGlow-TOPO. To investigate the appropriate concentration and time that these promoters can be maximally induced, each reporter strain was grown to reach mid log phase, then exposed to various concentrations of either paraquat (0 to 1.0 mM), or hydrogen peroxide (0 to 2.0 mM), then the raw fluorescence intensity and OD_{600} were measured over a period of time. Since the cell density was different according to oxidant concentration, the specific fluorescence intensity was used instead of raw fluorescence intensity to normalize the data. The fold induction over the control was also calculated to eliminate the basal level of the promoter expression in the cells. The fold induction was plotted to demonstrate the net induction directly occurred from the applied stresses (Figure 2). The induction of these promoters appeared to be dose-dependent particularly after longer exposure times. However, the highest fold induction of *sodA* promoter was observed when cells were treated with 0.2 and 0.3 mM of paraquat at approximately 240 min after induction (Figure 2A), while *fumC* promoter had the highest fold induction when cells were exposed to 0.7 and 0.8 mM of paraquat at about 360 min after induction (Figure 2B). According to these conditions, the *fumC* promoter was higher responsive than *sodA* promoter in which they were induced to 13-fold and 7-fold respectively. In addition, these promoters showed the similar patterns that their induction by hydrogen peroxide (< 2-fold) was much smaller than those of the paraquat effect (data not shown). The cultures harboring fusions of *katG* and *ahpC* promoters were also tested for their activity in response to various concentrations of hydrogen peroxide and paraquat. Interestingly, the induction caused by these stresses was

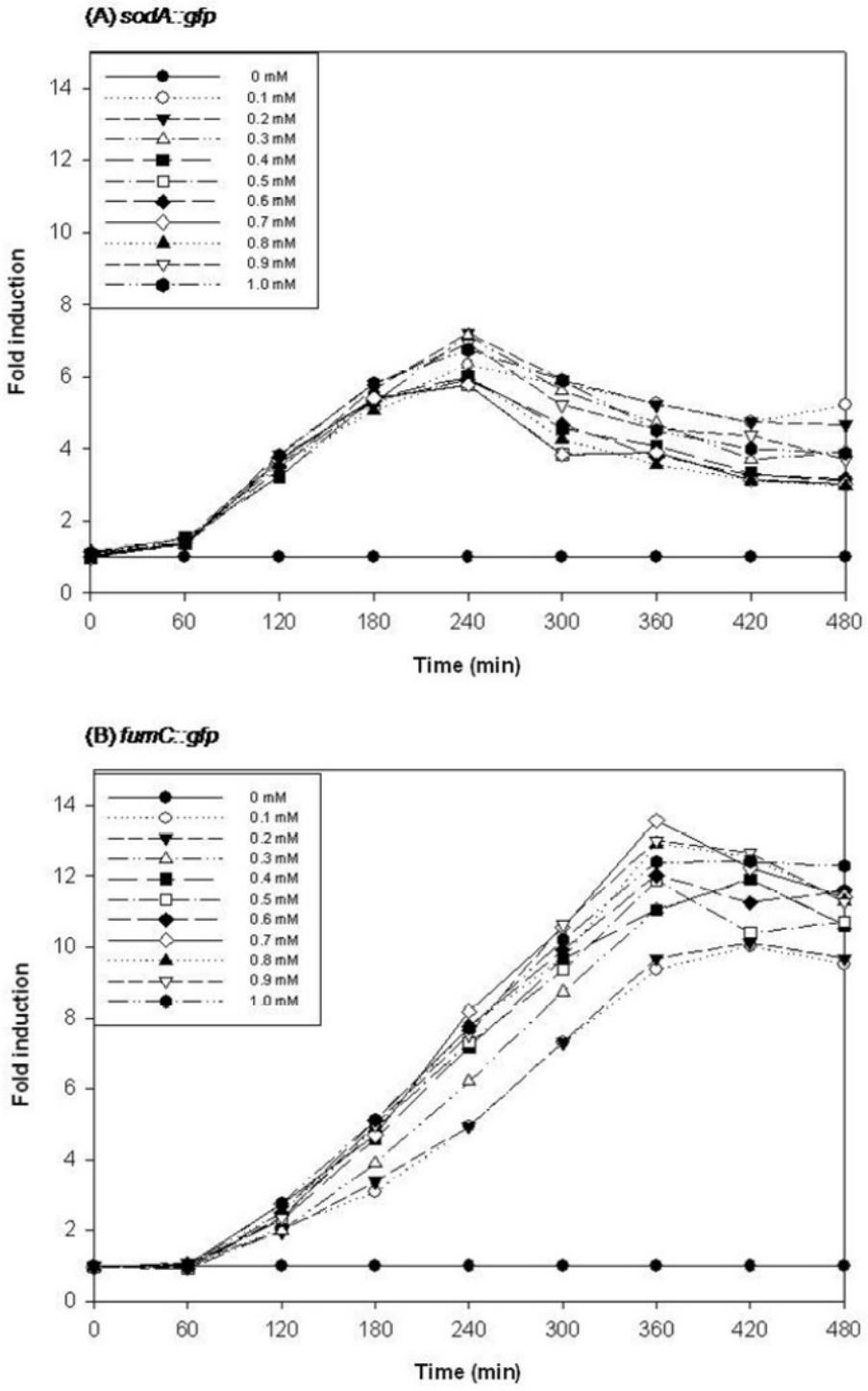

Figure 2. Fold induction profiles of *sodA*::*gfp* (A) and *fumC*::*gfp* (B) towards paraquat. Cultures were grown until mid log phase, then, induced by paraquat (0 to 1.0 mM). The raw fluorescence intensity and OD_{600} were measured over a period of time. The experiments were performed in triplicates. Means ± SD were calculated but graphs were plotted using only means.

smaller than 2-fold in all tested concentrations over a time period (data not shown). From the above mentioned observations, the strains harboring the fusions of *sodA* and *fumC* promoters were chosen for antioxidant activity

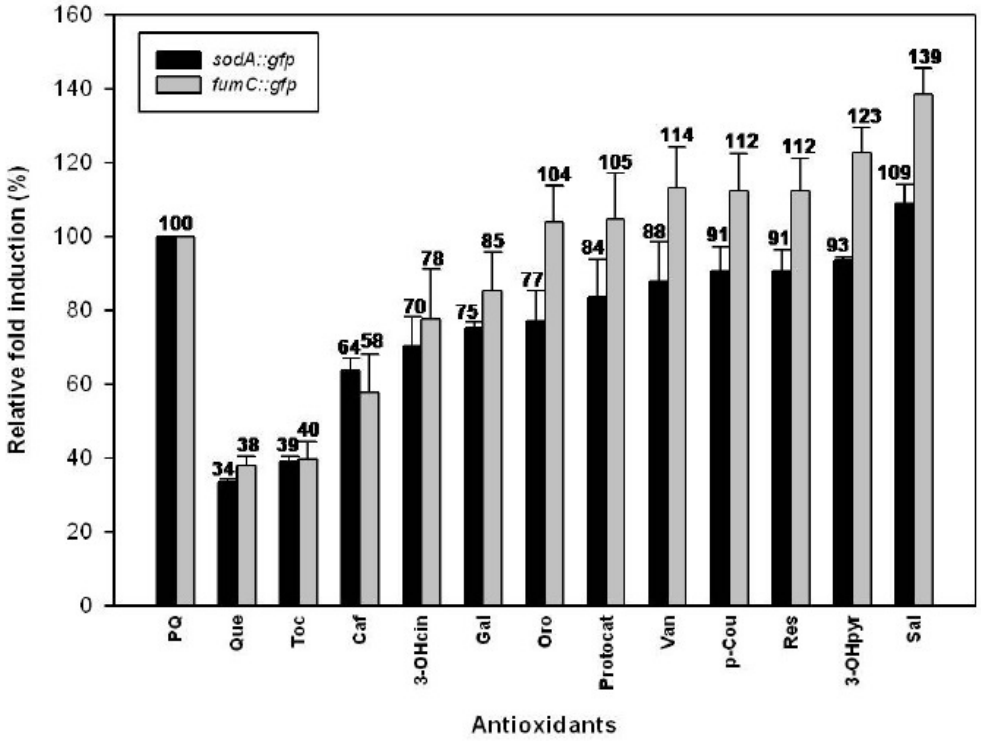

Figure 3. Determination of intracellular antioxidant activity using bacterial cell-based assay. Two *E. coli* reporter strains harbored the fusion of *sodA*::*gfp* (black bar) or *fumC*::*gfp* (light grey bar). Cells were treated with 0.2 mM paraquat or 0.7 mM paraquat, respectively. 1 mM of each tested compound was added immediately to the culture; quercetin (Que), α-tocopherol (Toc), caffeic acid (Caf), 3-hydroxycinnamic acid (3-OHcin), gallic acid (Gal), orotic acid (Oro), protocatechuic acid (Protocat), vanillic acid (Van), *p*-coumaric acid (p-Cou), resorcinol (Res), 3-hydroxypyridine (3-OHpyr), salicylic acid (Sal) and paraquat (PQ) as a control. The raw fluorescence intensity and OD_{600} were measured at 240 and 360 min, respectively. Graph represented % relative fold induction of each compound. The experiments were performed in triplicates. Means ± SD were calculated and used for plotting graph with error bars.

testing using the optimal conditions whereby these two promoters had the highest induction of these two promoters was achieved.

Determination of intracellular antioxidant activity of compounds using bacterial cell-based assay

To investigate whether these genetically engineered bacterial strains can be applied to determine the antioxidant activity of putative compounds at cellular level, therefore, twelve flavonoids and phenolic compounds (Figure 1) previously reported as antioxidants were tested. Each compound was added to the cultures carrying two promoter fusions immediately after paraquat treatment to make the final concentration of compound of 1 mM, then, the raw fluorescence intensity and OD_{600} were measured to investigate a role of compound to reduce intracellular oxidative stress. Expectedly, both promoters showed similar patterns in response to all tested compounds although there were the different relative fold induction values between these promoters (Figure 3). The results show that quercetin and α-tocopherol exerted the most significant intracellular antioxidant activity as they could reduce the paraquat generated superoxide stress > 60%. Moreover, the other compounds; caffeic acid, 3-hydroxycinnamic acid and gallic acid displayed significantly moderate intracellular antioxidant activity by reducing superoxide stress approximately 20 to 40%, while the rest of the compounds; orotic acid, protocatechuic acid, vanillic acid, *p*-coumaric acid, resorcinol, 3-hydroxypyridine and salicylic acid possessed weak intracellular antioxidant activity with < 20% reducing superoxide stress when they were investigated by *sodA* promoter or no obvious intracellular antioxidant activity when they were determined by *fumC* promoter (Figure 3). By comparing the percent relative fold induction of two promoters, we could observe that after adding various compounds, the decreased induction of *sodA* promoter by paraquat was detected as compared to the induction of *fumC* promoter. As shown in Figure 3, some compounds increased the relative fold induction when evaluating by *fumC* promoter.

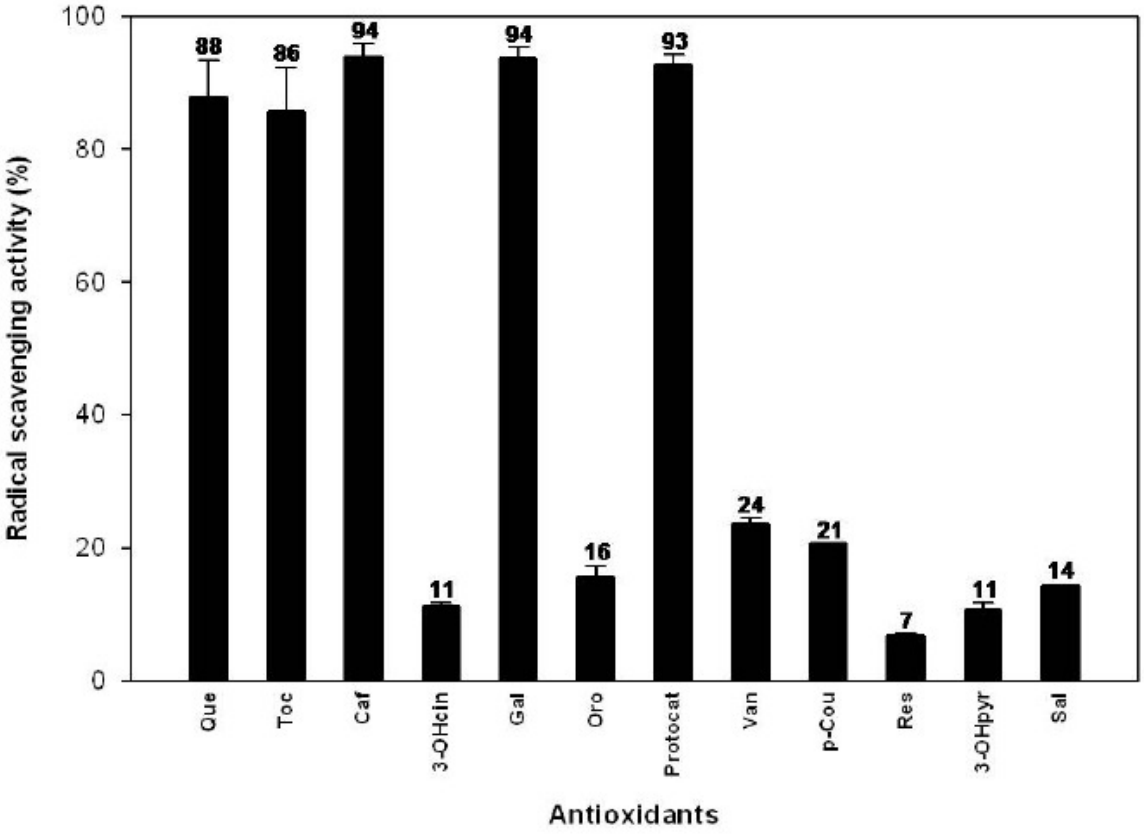

Figure 4. Evaluation of percent radical scavenging activity using DPPH assay. 1 mM of each tested compound was added to the 0.1 mM DPPH-methanol solution to evaluate the DPPH free radical-scavenging capacity; quercetin (Que), α-tocopherol (Toc), caffeic acid (Caf), 3-hydroxycinnamic acid (3-OHcin), gallic acid (Gal), orotic acid (Oro), protocatechuic acid (Protocat), vanillic acid (Van), *p*-coumaric acid (p-Cou), resorcinol (Res), 3-hydroxypyridine (3-OHpyr) and salicylic acid (Sal). The assays were measured in triplicates. Means ± SD were calculated and used for plotting graph with error bars.

Comparison of antioxidant activity determined by bacterial cell-based, DPPH and SOD activity assays

All compounds were also tested for their antioxidant activity using DPPH radical scavenging activity and SOD activity assays to determine whether there are correlations between our cell-based and such conventional chemical-based antioxidant assays. The DPPH radical scavenging activity (%) and IC_{50} values for SOD-like activity of all tested compounds are shown in Figure 4 and Table 2, respectively. A roughly similar pattern among three methods was observed with some exception. In agreement with the bacterial cell-based assay, quercetin exhibited very strong DPPH radical scavenging activity (> 85%) and high SOD like activity (IC_{50} < 5 µg/ml), whereas vanillic acid, resorcinol, *p*-coumaric acid, orotic acid, salicylic acid, 3-hydroxycinnamic acid and 3-hydroxypyridine showed weak DPPH radical scavenging activity (< 25%) and low SOD -like activity. Remarkably, there were some significant differences among these methods: α-tocopherol had no SOD –like activity, However, it showed very strong DPPH radical scavenging activity (> 85%) and high intracellular antioxidant activity (> 60%). Although, gallic acid and caffeic acid showed the strongest radical scavenging activity (~ 94%) as well as highest SOD-like activity (IC_{50} = 0.52 and 1.92 µg/ml), they revealed moderate intracellular antioxidant activity (20 to 40%). Moreover, protocatechuic acid demonstrated very strong DPPH radical scavenging activity (> 85%) and high SOD-like activity (IC_{50} < 5 µg/ml), but it showed weak intracellular antioxidant activity (< 20%). Although, the results of two chemical-based methods showed good correlations, no absolute correlation was observed among these results derived by three different methods.

Determination of intracellular antioxidant activity of plant extracts using bacterial cell-based assay

Recently, several biologically relevant assays using some enzymes or molecules or cells have been developed for antioxidant activity measurement to screen the potent

Table 2. SOD-like activity of twelve tested compounds by NBT reduction assay.

Antioxidant	IC_{50}* (µg/ml)
Gallic acid	0.52
Quercetin	1.55
Caffeic acid	1.91
Protocatechuic acid	4.91
Vanillic acid	35.92
3-hydroxycinnamic acid	420
p-coumaric acid	500
Salicylic acid	> 500**
Orotic acid	> 500**
3-hydroxypyridine	> 500**
Resorcinol	> 500**
α-tocopherol	no activity***
SOD**** (Bovine erythrocytes)	0.24

*IC_{50} values mean the minimal concentration of tested compounds that inhibits NBT reduction of 50%. **The values in parentheses represent the measured % inhibition of NBT reduction when the final concentration of tested compounds in reactions was 500 µg/ml. *** No activity means % inhibition of NBT reduction when the final concentration of tested compounds in reactions was 500 µg/ml. ****Superoxide dismutase (SOD, 3,400 U/mg) from bovine erythrocytes was used as a standard.

antioxidants from natural products and dietary supplements (Mello et al., 2003; Cortina-Puig et al., 2009; Oktyabrsky et al., 2009; Song et al., 2010). To investigate whether our bacterial cell-based assay can be applied for antioxidant screening from natural products, we also tested our system using the extracts from different plants. Hence, the ethyl acetate extracts from *H. formicarum* Jack., *S. acmella* Murr. and *E. prostrata* Linn. were examined for their intracellular antioxidant activity since they had the highest radical scavenging activity by determined DPPH assay (Prachayasittikul et al., 2008, 2009, 2010; Prachayasittikul, unpublished data). These extracts were dissolved in dimethyl sulfoxide (DMSO) and added immediately to the cultures after paraquat treatment, the final concentration of extracts in cultures were 100, 300 and 500 µg/ml. The reporter strain harboring *fumC*::*gfp* fusion was used for testing since it exhibited higher fold induction in response to paraquat comparing with strain harboring *sodA*::*gfp* fusion. The relative fold induction of these plant extracts are shown in Figure 5. The intracellular antioxidative effect of extracts was inversely proportional to relative fold induction. The extract of *H. formicarum* Jack. had the highest intracellular antioxidant activity followed by *S. acmella* Murr. and *E. prostrata* Linn. Nevertheless, the results show no significant differences among the tested extracts of the two latter. Notably, these extracts could alleviate intracellular superoxide stress in a dose dependent fashion (Figure 5).

DISCUSSION

In *E. coli*, there are two main transcriptional regulatory proteins for oxidative stress sensing; SoxR and OxyR which respond to various ROS (Pomposiello and Demple, 2001). In response to superoxide, SoxR can only trigger the expression of SoxS, in turn SoxS can activate the transcription of many genes, such as *sodA* (manganese superoxide dismutase), *fumC* (fumarase C), *zwf* (glucose-6-phosphate dehydrogenase) and *nfo* (exonuclease IV). While in response to peroxide, OxyR can stimulate the transcription of many genes, such as *katG* (hydroperoxidase I), *ahpCF* (hydroperoxide reductase), *grxA* (glutaredoxin I) and *gorA* (glutathione reductase). Using these oxidative stress sensing systems, we developed the bacterial assay and used for evaluation of intracellular antioxidant efficiency of pure compounds and plant extracts. In our system, we used the GFP as a reporter since it is a convenient system throughout the monitoring process which does not require cell lysate preparation and any cofactors or substrates (Kain and Kitts, 1997). Initial experiment was conducted to determine the optimal conditions for the promoter inductions in the presence of paraquat and H_2O_2. Interestingly, the induction of *sodA* and *fumC* promoters was appeared to be in dose-dependent manner particularly after longer exposure times. Plausible explanation could be drawn as the visible induction was delayed due to the rate-limiting step of chromophore formation which

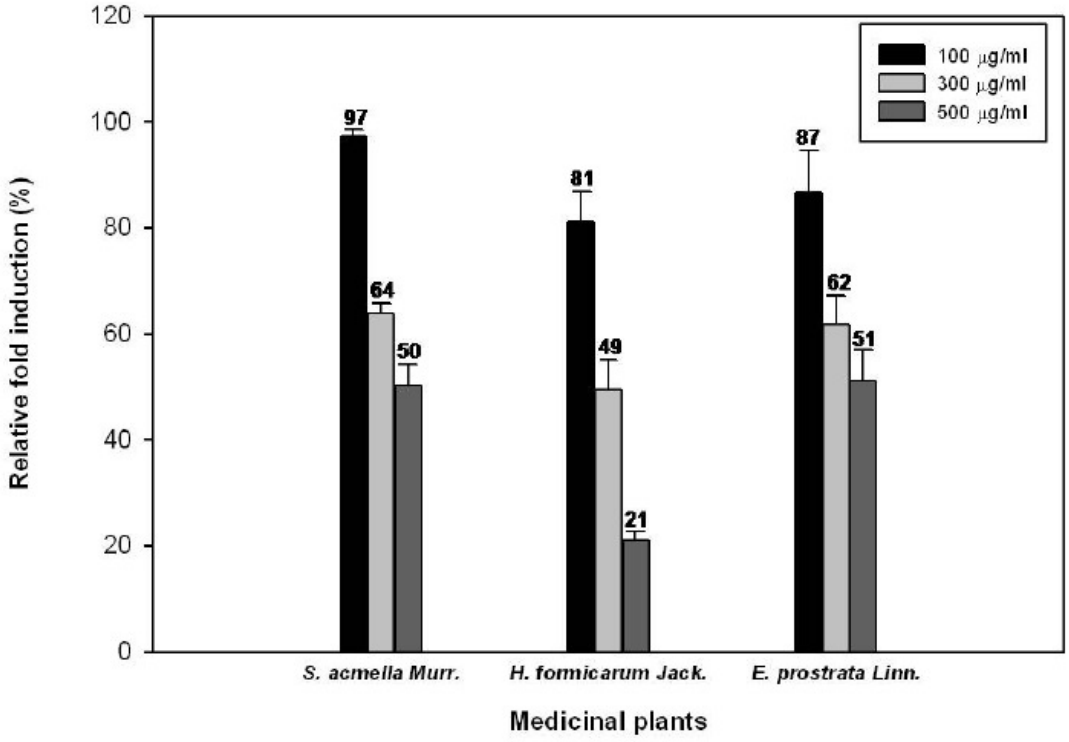

Figure 5. Determination of intracellular antioxidant activity of plant extracts using *fumC*::*gfp* reporter strain. All three Thai medicinal plant ethyl acetate extracts were tested for their anti-superoxide activity. Cells were treated with 0.7 mM paraquat, then, 100 (black bar), 300 (light grey bar) and 500 (dark grey bar) µg/ml of each plant extract were added immediately to the culture. The raw fluorescence intensity and OD_{600} were measured at 360 min. Graph represented % relative fold induction of each plant extract at various concentrations. The experiments were performed in triplicates. Means ± SD were calculated and used for plotting graph with error bars.

requires at least 95 min (Cha et al., 1999; Lu et al., 2005). Notably, the maximal peak of *fumC* was delayed comparing with the peak of *sodA* in which their highest expressions were reached at 6 and 4 h, respectively after stress. The *sodA* encodes manganese containing superoxide dismutase which converts $O^{\cdot}_2^{-}$ to H_2O_2, thus it is a first line of defense in detoxifying this ROS, suggesting a possible reason that *sodA* had high sensitivity in response to low concentration of paraquat and its expression reached to maximum faster than *fumC*. Remarkably, the genes in OxyR regulon, *katG* and *ahpC* were not highly responsive to H_2O_2 as observed by less than 2-fold induction throughout the experiment comparing with the genes in SoxRS regulon. These results were consistent to the previous work reported by Lu et al. (2005) that they characterized the gene regulation pattern of many oxidative stress responsive genes in response to stressors using similar plasmid system. Their results show that fold inductions of *katG* and *ahpC* promoters induced by 0.01 to 10 mM H_2O_2 varied from 1.0 to 1.6 during 120 to 360 min comparing to uninduced condition. They discussed that both *katG* and *ahpC* had high basal levels even in the absence of H_2O_2, therefore their induction folds were small. They also elucidated that high concentration of H_2O_2 had no deleterious effects on GFP reporter property as they excluded the low induction of these genes caused by H_2O_2 inhibitory effects. Our results were also supported by Gonzalez-Flecha and Demple (1997) in which the induction of *katG::lacZ* fusion was less than 2-fold in response to 0.002 to 1 mM H_2O_2. However, Belkin et al. (1996) revealed that significant induction of *katG::luxCDABE* could be observed at H_2O_2 concentrations as low as 2.9 µM since the great sensitivity of *lux* reporter. Based on these published studies, these discrepancies were due to different reporter systems. In general, the enzymatic-based systems, such as *lux* gene have higher sensitivity owing to the signal amplification effect. Despite the high sensitivity of *lux* gene, the damage by high concentration of H_2O_2 is a drawback of this system (Belkin et al., 1996). Considerably, inconsistent results could be occurred by different experimental conditions, such as growth phase of cells, time after induction, etc.).

Because of their high responses to paraquat, *sodA*::*gfp* and *fumC*::*gfp* fusions were used to assess the anti-oxidant potential of various phenolic compounds and flavonoids for specific intracellular superoxide anion alleviation.

The results reveal that quercetin and α-tocopherol had the highest intracellular antioxidant activity for superoxide alleviation (Figure 3). Quercetin is a flavonoid compound with better antioxidant activity than others in many different assays (Rice-Evans et al., 1996; Fernandez-Panchon et al., 2008; Holst and Williamson, 2008). The α-tocopherol is well known as a lipid soluble antioxidant which can protect cell membranes from oxidation and it is commonly used as a standard compound for many antioxidant activity assays. This study also showed that caffeic acid, 3-hydroxycinnamic acid and gallic acid possessed significant moderate intracellular antioxidant activity. There was an earlier work performed by utilizing mammalian cell-based assay, L-929 murine fibrosarcoma cell line containing fluorescence probe 2′,7′-dichlorofluorescin-diacetate (DCFH-DA), as an indicator of intracellular ROS to evaluate the antioxidant properties of compounds and mixtures (Girard-Lalancette et al., 2009). Their results were in good agreement with our results which showed that the order of antioxidant potentials of the tested compounds were as following: quercetin > caffeic acid > gallic acid > α-tocopherol. However, it was different in the order of α-tocopherol which is possible that DCFH-DA probe can be directly oxidized by several intracellular ROS intermediates (LeBel et al., 1992; Wang and Joseph, 1999). In their experiment, they challenged cells with *tert*-butylhydroperoxide (*t*-BOOH) that can produce the peroxide stress, another type of ROS, while our bacterial system mainly assessed the intracellular superoxide anions. Surprisingly, our results showed significant differences in relative fold induction and sensitivity between the *sodA* and *fumC* in response to some tested compounds (Figure 3). Apparently, *sodA* was less inducible by paraquat than *fumC*. However, *sodA* showed higher sensitivity as compared to *fumC* when testing the intracellular anti-oxidative effect of compounds. The *sodA* gene encodes manganese superoxide dismutase which converts $O^{\cdot}_2^-$ to H_2O_2, whereas, *fumC* encodes stable fumarase C that can replace the oxidatively unstable fumarase A and B. The manganese superoxide dismutase is more important since it is the first line of defense in $O^{\cdot}_2^-$ detoxification, suggesting a possible rationale for its high sensitivity to maintain the harmless intracellular $O^{\cdot}_2^-$ level (Lu et al., 2005). In general, DPPH and SOD activity assays involve only *in vitro* chemical reactions and are performed under nonphysiological conditions that do not depend on any cellular function. In contrast, the cell-based assay is a measurement for total biological effects that supposed to represent the actually antioxidant activity inside cells that may be affected by many factors, such as bacterial cell viability, membrane permeability of compounds, indirect complexity of cellular function and other unknown factors.

Obviously, our results demonstrated that the effectiveness of tested compounds in antioxidant activity was not exactly similar among these methods. Not surprisingly, two chemical-based methods revealed the results that all tested compounds, except α-tocopherol, had the similar pattern in which antioxidant activity depends on the numbers of hydroxyl groups in their structures.

It was noted that α-tocopherol had no SOD-like activity even it showed high radical scavenging activity (~ 86 %). Such result could be possibly explained by inductive effect of keto group. In case of quercetin, it had a keto group on its molecule which can create an electrophillic center to react with the superoxide anions that lead to its high SOD-like activity (Table 2).

This could not apply to the α-tocopherol which is perhaps due to the lack of keto group on its molecule. However, there is no advanced explanation to point out that why the SOD-like activity was not observed for α-tocopherol. Apparently, there is still no evidence from the literatures to reason such result. Importantly, our experiments indicated that only quercetin and α-tocopherol exerted the highest significant intracellular antioxidant activity tested by bacterial-based assay, whereas caffeic acid, gallic acid and protocatechuic acid showed the highest antioxidant activity determined by DPPH and SOD assays, but only moderate or weak intracellular antioxidant activity. We propose that these discrepancies could be due to chemical structures and ability of compounds for penetrating into biological membranes. The chemical structures of both quercetin and α-tocopherol are planar aromatic compounds that the quercetin is a flavonoid compound composing of coumarin and benzene rings with many hydroxyl groups, while α-tocopherol composes of chroman ring with hydroxyl group and long hydrophobic side chain (Figure 1). These compounds possess high antioxidant activity according to the numbers of their hydroxyl groups that can donate hydrogen radical to scavenge free radicals (Rice-Evans et al., 1996).

In fact, the benzene ring and hydrophobic side chain are nonpolar or lipophillic groups, which allow them to penetrate into biological membranes. As described, it is reasonable to account for these compounds to exert the most potent intracellular antioxidant activity. On the other hand, caffeic acid, gallic acid and protocatechuic acid are polyphenolic compounds containing carboxylic acid group that they are more hydrophilic or polar than the quercetin and α-tocopherol. Taken together, although these compounds exhibit *in vitro* antioxidant activity, they cannot well penetrate into the membranes and scavenge the free radicals when comparing with the quercetin and α-tocopherol. To elucidate this hypothesis, further experiments regarding to membrane permeability are necessary. Using our bacterial-based method, the intracellular antioxidant activity of ethyl acetate extracts from three Thai traditional medicinal plants was investigated and compared with previously reported antioxidant activity using DPPH method. Although, it was not possible to compare these methods in an absolute manner since the DPPH assay is a chemical-based

assay for antioxidant activity that is not physiological relevance as in the case of the cell-based assay. Moreover, plant extracts may not have only affected cell function by antioxidative property but they may also affect cells through non-antioxidative effects. However, this appears to be a benefit of cell-based assays that they are more applicable for investigating the total biological effects of plant extracts to cells. The results demonstrated that the extract of *H. formicarum* Jack. exerted the highest intracellular antioxidant activity followed by *S. acmella* Murr. and *E. prostrata* Linn., in which the latter two plant extracts displayed comparable activity (Figure 5). These experimental findings are in good agreement with our previous study on the DPPH radical scavenging activity of these extracts (Prachayasittikul et al., 2008, 2009; Prachayasittikul, unpublished data). The ethyl acetate extract of *H. formicarum* Jack. exhibited the strongest radical scavenging activity with IC_{50} of 8.40 µg/ml, whereas the ethyl acetate extracts of *S. acmella* Murr. and *E. prostrata* Linn. displayed moderate to weak radical scavenging activity with IC_{50} of 216 and 101.14 µg/ml, respectively.

CONCLUSION

The present study is a preliminary investigation of the potential application of novel bacterial cell-based assay as an alternative screening tool for the intracellular antioxidant activity of both pure compounds and plant extracts. This assay is simple, inexpensive, reproducible and very specific for the intracellular antioxidant activity against superoxide radical that is applicable for estimating the antioxidative effects in terms of biological relevance. An additional advantage of this method is the use of very small sample volumes comparing with chemical-based assays. However, the limitation of our assay may be on the assay duration since the GFP measurement was taken within 8 h. Therefore, further experiments should be pursued in order to improve the quality of assay in case of short assay duration and broader use for development of a high throughput screening method for a large number of antioxidant compounds.

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