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ORIGINAL PAPER

Determination of antioxidant activity using oxidative damage to plasmid DNA – pursuit of solvent optimization

Jakub Treml*, Karel Šmejkal, Jan Hošek, Milan Žemlička

Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Palackého tř. 1/3, 612 42 Brno, Czech Republic

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Oxidative stress plays a key role in the pathophysiology of many diseases. Hydroxyl radical is the oxidative species most commonly causing damage to cells. The aim of this work was to optimize the method for antioxidant activity determination on a model lipophilic geranylated flavanone, diplacone. This method uses protection of plasmid DNA from oxidation by a hydroxyl radical generated by the Fenton reaction involving oxidation of metal ions using H_2O_2 and ascorbate. The method was optimized for lipophilic compounds using several solvents and co-solvents. It was found that (2-hydroxypropyl)-β-cyclodextrin (0.1 mass % aq. sol.) is the best co-solvent for our model lipophilic compound to measure the antioxidant activity by the method presented. Other solvents, namely dimethyl sulfoxide, Cremophor EL® (0.1 mass % aq. sol.), ethanol, and methanol, were not suitable for the determination of the antioxidant activity by the method described. Tween 80 (0.1 mass % aq. sol.) and a mixture of 10 vol. % ethanol and 9 mass % bovine serum albumin (aq. sol.) significantly decreased the antioxidant activity of the model lipophilic compound and thus were not suitable for this method.

Keywords: antioxidant activity, plasmid DNA, Fenton reaction, solubility, diplacone

Introduction

Aerobic organisms take advantage of oxidative phosphorylation and therefore gain much more energy from one molecule of glucose compared to anaerobic organisms. The price paid is the production of reactive oxygen species (ROS). ROS consist of various small molecules derived from oxygen, including oxygen radicals (such as hydroxyl radical $^{\bullet}OH$) and certain non-radicals (such as hydrogen peroxide; H_2O_2). Cells use numerous antioxidant enzymes and defensive molecules to avoid the overproduction of ROS; however, if the production of ROS exceeds the capacity of the cellular antioxidant system, the cell has to face a state called oxidative stress. Oxidative stress plays a key role in the pathophysiology of many diseases such as neurodegeneration, cardiovascular diseases, and cancer (Ma, 2010).

The method presented for antioxidant activity determination is based on the generation of hydroxyl radicals which then cause oxidative damage to plasmid DNA. The hydroxyl radical can be produced by the Fenton reaction Eq. (1). Originally, the metal ion reacting with H_2O_2 was iron but also copper reacts with H_2O_2 (Que et al., 1980). Ascorbate is an excellent antioxidant; however, it also shows a pro oxidant activity due to its reducing character, Eq. (2) (Chiou, 1983). Chiou (1984) reported that H_2O_2 alone (without metal ions) does not show any visible cleavage of DNA, which makes it an important intermediate in the reaction.

There are various ways of DNA damage; it can be fragmented (single- or double-strand breaks) or cross-linked (intra- or inter-strand). The hydroxyl radical also causes base oxidation (e.g., formation of 8-hydroxyguanine or thymine glycol). If not repaired, these DNA alterations lead to mutations or cell death (Cooke et al., 2006). The production and effects of hy-

*Corresponding author, e-mail: jakub.treml@gmail.com

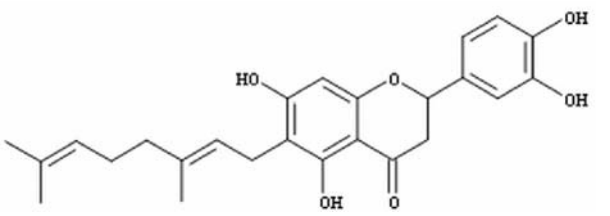

Fig. 1. Structure of diplacone.

droxyl radicals used in this method are summarized in the following reactions:

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + {}^{\bullet}OH + OH^{-} \quad (1)$$

$$Cu^{2+} + \text{ascorbate} \rightarrow Cu^{+} + \text{dehydroascorbate} \quad (2)$$

$${}^{\bullet}OH + \text{DNA} \rightarrow \text{product of DNA damage} + OH^{-} \quad (3)$$

The detection of DNA breakage caused by oxidative damage has been described by Sagripanti and Kraemer (1989). Plasmid DNA consists of a circular double strand in a native supercoiled conformation, also known as covalently closed circular (CCC) conformation. The first step of degradation after the attack by the hydroxyl radical is the formation of an open circular form (OC) with one broken strand. In the second step, both strands are cut resulting in the linear form (L) of plasmid DNA. All forms are made visible by electrophoretic separation (Sagripanti & Kraemer, 1989).

A huge advantage of this method is the use of a naturally occurring hydroxyl radical and DNA to display the oxidative damage and the possible positive protective effect of an antioxidant compound. Compared to other in vitro methods used to determine antioxidant activity, this method is one step closer to in vivo conditions (Zima et al., 2010). This method was originally designed for aqueous conditions and the testing of water-soluble compounds; therefore, it had to be adopted with some changes and modifications. For lipophilic compounds and extracts, dimethyl sulfoxide (DMSO) was used as a suitable solvent. Furthermore, $CuSO_4$ was used instead of $FeSO_4$, due to its greater stability during storage. To optimize the reaction conditions, various concentrations of H_2O_2 and $CuSO_4$ were tested.

The aim of this work was to optimize the method for lipophilic water-insoluble compounds testing using several solvents and co-solvents. DMSO, Tween 80 (0.1 mass % aq. sol.), a mixture of 10 vol. % ethanol and 9 mass % bovine serum albumin (BSA) aq. sol., Cremophor EL® (0.1 mass % aq. sol.), (2-hydroxypropyl)-β-cyclodextrin (HPβ-CD) (0.1 mass % aq. sol.), ethanol, and methanol were tested. Diplacone, a geranylated flavanone (Fig. 1) obtained from the flowers and fruits of the plant *Paulownia tomentosa* (Thunb.) Steud. (Scrophulariaceae) (Jiang et al., 2004; Šmejkal et al., 2007), was used as the model lipophilic compound with proven in vitro antiradical activity (Šmejkal et al., 2007).

Experimental

General

For the isolation of pUC19 plasmid DNA, *Escherichia coli* TOP 10F' strain transfected with this plasmid was cultivated. The cultivation was performed overnight in Difco™ LB Broth, Miller medium (BD Diagnostics, France). After the cultivation, plasmid DNA was isolated using the isolation kit QIAprep Spin Miniprep (Qiagen, Germany). The concentration and purity of the isolated plasmid DNA were evaluated using a BioPhotometer spectrophotometer (Eppendorf, Germany).

Plasmid DNA was analyzed using gel electrophoresis separation on 0.8 % agarose (voltage 5 V cm^{-1} of gel, 45 min) penetrated by ethidium bromide (0.15 μg mL^{-1}) for visualization. After the complete run, pictures of the gel were taken using UV detection (λ = 312 nm; a model Transluminator EBW-20 Ultra-Lum Tech, USA). Intensities of the individual plasmid band conformations were calculated using the AlphaEasy FC 4.0.0 software (Alpha Innotech, USA) for densitometric analysis and described as the area under the curve (AUC).

Material

Agarose (Amresco, USA), ethidium bromide (Sigma–Aldrich, Germany), TE buffer (Amresco), H_2O_2 30 % (Penta, Czech Republic), ascorbate (Lachema, Czech Republic), $CuSO_4 \cdot 5H_2O$ (Lachema) were used in this study.

Diplacone (isolated from *Paulownia tomentosa*, as described by Šmejkal et al. (2007)), DMSO (Sigma–Aldrich), Tween 80 (Lachema), Cremophor EL® (Lachema), HPβ-CD (Roquette Pharma, France), ethanol (Amresco), methanol (Penta) were used as solvents and co-solvents.

Determination of antioxidant activity

The reaction was performed in PCR tubes (Axygen, USA). Total volume of the reaction mixture was 20 μL. The reaction was carried out with 300 ng of plasmid DNA in TE buffer. First, diplacone (dissolved in the chosen test solvent and co-solvent to reach the final concentrations of 20 μM and 100 μM) was added. Finally, the Fenton solution consisting of H_2O_2, ascorbate, and $CuSO_4 \cdot 5H_2O$ was added. The reaction conditions were optimized prior to the antioxidant activity determination. For H_2O_2, the tested concentrations were: 660 μM, 264 μM, and 52.8 μM,

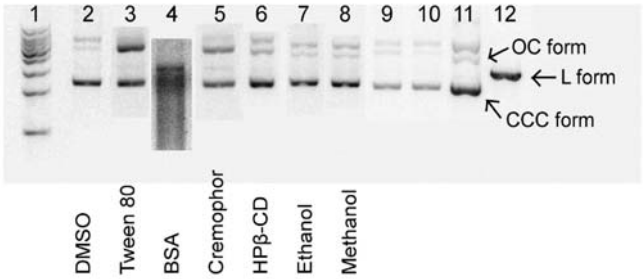

Fig. 2. Gel electrophoresis: diplacone, 100 μM, dissolved in solvents and co-solvents; 1 – ladder 1 kbp, 2–8 – plasmid DNA + diplacone + H_2O_2 + $CuSO_4$ + ascorbate; 9 – plasmid DNA + H_2O_2 + $CuSO_4$ + ascorbate (positive control); 10 – plasmid DNA + H_2O (negative control); 11 – plasmid DNA; 12 – plasmid DNA (L form only).

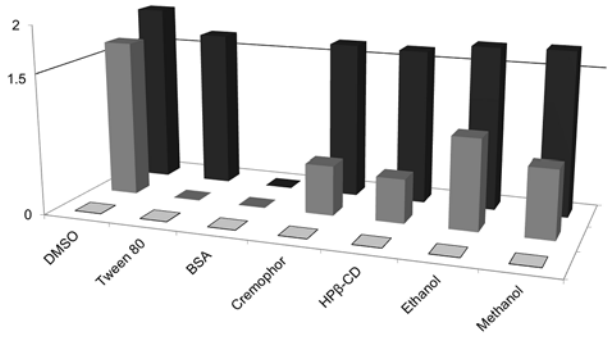

Fig. 3. Optimization of $CuSO_4$ concentration; □ – 330 μM, ■ – 248 μM, ■ – 165 μM.

and for $CuSO_4$: 330 μM, 248 μM, and 165 μM. All experiments carried out to determine the antioxidant activity were performed with a Fenton solution containing H_2O_2 (660 μM), ascorbate (830 μM), and $CuSO_4 \cdot 5H_2O$ (165 μM).

The reaction mixture was incubated at 37 °C for 1 h. Then, the samples were analyzed using gel electrophoresis (as described above). Plasmid DNA was separated in the gel as shown in Fig. 2. The OC form is the slowest conformation in the gel and it thus occurs at the top of the gel. The next conformation is the L form, followed by the CCC form. All reactions were carried out in triplicate. The corresponding pure solvents were used instead of the diplacone solution as positive controls. The negative control contained only water instead of the Fenton solution.

After the run, the gel was analyzed and the intensities of the individual bands were measured. The intensity of each band corresponded to the amount of DNA and was expressed as the percentage of the area under the curve (AUC). A ratio Eq. (4) expressing the amount of intact DNA divided by the total amount of DNA was calculated for each sample. Both strands in the CCC DNA were intact; thus, the percentage was multiplied by 2. To cover the possible influence of solvents and co-solvents on the reaction, the damage index (D_i), dividing the ratio of the sample by the ratio of the positive control (Eq. (5)), was calculated:

$$\mathrm{Ratio}_{\mathrm{sample}} = \frac{\%_{\mathrm{OC}} + (2 \times \%_{\mathrm{CCC}})}{100\ \%} \tag{4}$$

$$D_i = \frac{\mathrm{Ratio}_{\mathrm{sample}}}{\mathrm{Ratio}_{\mathrm{possitive\ control}}} \tag{5}$$

Results and discussion

Suitable concentration of $CuSO_4$ for each solvent and co-solvent was determined. The optimal combination of concentrations should lead to the damage of half of the DNA (≈ 50 % of the CCC form). In other words, the optimal ratio Eq. (4) value is approximately 1.5. After the electrophoretic separation and visualization, the ratio representing the amount of intact strands divided by the total amount of DNA in each sample was calculated.

The concentration of H_2O_2 was adjusted from 660 μM to 264 μM and then to 52.8 μM, but this did not lead to the optimal ratio whereas adjusting the concentration of $CuSO_4$ from 330 μM to 248 μM and 165 μM led to a ratio closer to 1.5. Results of the $CuSO_4$ concentration optimization are shown in Fig. 3. After the adjustment of the optimal combination of concentrations, 660 μM for H_2O_2 and 165 μM for $CuSO_4$, the modified method for antioxidant activity assay was applied for diplacone dissolved in different solvents and co-solvents at concentrations of 20 μM and 100 μM. Not all pictures of the gels are presented and only the results of diplacone dissolved in solvents and co-solvents at the concentration of 100 μM are shown in Fig. 2.

D_i was calculated as the ratio of the sample divided by the ratio of the corresponding solvent or co-solvent. This calculation is necessary due to the possible antioxidant or pro-oxidant activity of the solvents. Fig. 4 shows the damage indexes of diplacone at the concentrations of 20 μM and 100 μM dissolved in all solvents and co-solvents used. ANOVA statistical analysis was performed followed by a post-hoc Tuckey's test of damage indexes.

No significant differences were found when the damage index of DMSO was compared with those of Cremophor EL®, HPβ-CD, ethanol, and methanol. Thus, these solvents and co-solvents are suitable for the presented method of antioxidant activity determination. However, a significant difference ($p < 0.05$) between the damage indexes of DMSO and Tween 80 was found for diplacone at the concentration of 100 μM. The latter was lower by 6.8 percentage points. There was also a significant difference between the damage index of BSA when compared with those of other solvents and co-solvents ($p < 0.001$). These differences imply that Tween 80 and BSA are not suitable co-

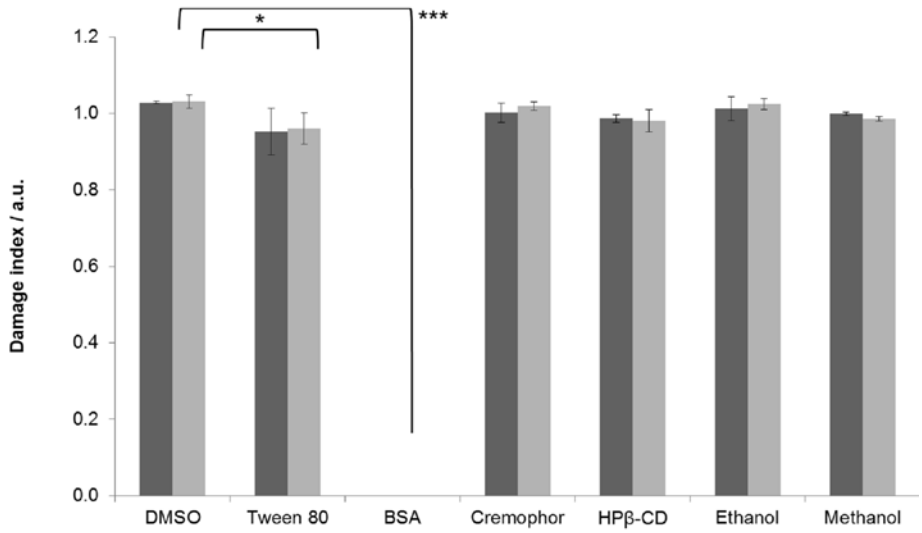

Fig. 4. Damage indexes of 20 μM (■) and 100 μM (■) diplacone dissolved in solvents and co-solvents (*** – $p < 0.001$, * – $p < 0.05$).

solvents for the presented method of antioxidant activity determination. Another reason for the exclusion of BSA is that it seriously interfered with the electrophoretic detection.

DMSO is known to possess some antioxidant properties. For example, Bektaşoğlu et al. (2006) proved the hydroxyl radical scavenging activity of DMSO using a modified cupric reducing antioxidant capacity (CUPRAC) method. Kishioshka et al. (2007) demonstrated the ability of DMSO to protect liver from acute oxidative hepatitis caused by thioacetamide. In this case, DMSO also functioned as a radical scavenger. DMSO is widely used as a solvent for water-insoluble substances in biological studies. The DMSO molecule contains one highly polar domain and two nonpolar methyl groups which make it a very efficient solvent for water-insoluble compounds.

Santos et al. (2003) described several effects of DMSO on cellular processes and systemic side effects in vivo, e.g., nausea, vomiting, hemolysis, hypertension, and pulmonary edema. Therefore, although it is well known that the dissolving ability of DMSO is excellent and its negative effects on cellular processes do not interfere with the results of the presented method for antioxidant activity determination, we decided to avoid the use of DMSO and to find a less harmful solvent for future testing of lipophilic antioxidants.

Tween 80, a synonym for polysorbate 80, is a nonionic surfactant used in food and pharmaceutical preparations. There is no evidence in literature suggesting that Tween 80 itself has any antioxidant activity. Nevertheless, Tween 80 increases the cytotoxicity of hydrogen peroxide under in vitro conditions and thus may increase the susceptibility of cells to oxidative stress (Tatsuishi et al., 2005).

According to the results of the National Toxicology Program (1992), toxicity of Tween 80 is low. As mentioned above, when diplacone was dissolved in Tween 80 at the concentration of 100 μM, its antioxidant activity was significantly lower ($p < 0.05$) than when DMSO was the solvent (Fig. 4). The probable reason is that Tween 80 interferes with the chelation of Cu^{2+} caused by diplacone. Thus, Tween 80 was found to be an unsuitable co-solvent for the presented method.

BSA is the most abundant serum protein with the molecular mass of 66 kDa. The role of albumin in ligand binding and free radical-trapping activities has been well described (Roche et al., 2008). Fukuzawa et al. (2005) demonstrated the inhibition of lipid peroxidation by BSA. This effect is caused by the ability of BSA to bind with iron chelates and keep them away from lipid membranes.

Because of the significant difference between the damage index of BSA and those of other solvents and co-solvents ($p < 0.001$), BSA was identified as an unsuitable co-solvent. BSA was not able to dissolve diplacone and thus, nothing prevented the DNA damage.

Cremophor EL® (or polyoxyethyleneglycerol triricinoleate 35) has been used as a co-solvent for hydrophobic drugs in pharmaceutical preparations. Cremophor EL® is considered to be relatively non-toxic; however, several reports suggest side effects such as anaphylactoid hypersensitivity, axonal degeneration, and demyelination. Cremophor EL® is also known to increase oxidative stress and lipid peroxidation in cells (Gelderblom et al., 2001; Gutiérrez et al., 2006). Similarly, Iwase et al. (2004) provided results suggesting that Cremophor EL® increases the susceptibility of thymocytes to oxidative stress caused by hydrogen peroxide.

Cremophor EL® showed good ability to dissolve diplacone. There was no significant difference ($p <$

0.05) between the antioxidant activity of diplacone dissolved in DMSO and that dissolved in Cremophor EL (Fig. 4). Cremophor EL® can be used in the method presented, but for future testing of lipophilic antioxidants, the use of Cremophor EL® is questionable because of the side effects occurring in vivo, similarly to DMSO.

Cyclodextrins (CD) are cyclic oligosaccharides composed of glucopyranose units which can be presented as truncated cone structures with hydrophobic cavities. The cavities form inclusion complexes with a variety of molecules. Cyclodextrins are widely used in pharmaceutical applications to modify the solubility of poorly water-soluble compounds. Hydroxypropyl-β-CD is now used instead of β-CD because it is more soluble in water and has a relatively low nephrotoxicity (Lu et al., 2009). Calabrò et al. (2004) tested β-CD for antioxidant activity and proved the protective effect of β-CD against lipid peroxidation, probably resulting from the chelation of Fe^{2+}. HPβ-CD showed good ability to dissolve diplacone. There is no significant difference ($p < 0.05$) between the antioxidant activity of diplacone dissolved in DMSO and that dissolved in HPβ-CD (Fig. 4). Gould and Scott (2005) proved very low toxicity of HPβ-CD, which makes it a good co-solvent for lipophilic compounds in the method presented and also for further in vivo testing of lipophilic compounds.

Ethanol is widely used as a solvent in laboratory work. Koch et al. (2004) reviewed the factors leading to alcoholic liver disease. Many experiments proved that ethanol increases oxidative stress in hepatocytes. Ethanol-inducible cytochrome P_{450} (CYP2E1), present in microsomes, produces ROS. Electron paramagnetic resonance spectroscopy has also shown free radicals derived from ethanol itself. Moreover, the consumption of ethanol is connected with the dysfunction of the central nervous system. Loureiro et al. (2011) described ROS formation and cytoskeleton disruption in C6 glioma cells after their exposure to ethanol. Ethanol is capable of dissolving diplacone and it is a suitable solvent for the method presented, as it is evident from the similarity of the damage indexes of diplacone dissolved in ethanol and that dissolved in DMSO (Fig. 4). However, similarly to DMSO and Cremophor EL®, its usage is questionable due to its toxic effects on cells.

Methanol is also used as a solvent and cleanser. After ingestion, it causes metabolic acidosis and severe clinical problems such as blindness, serious neurological problems, or even death. Methanol intoxication is associated with mitochondrial damage and microsomal proliferation resulting in higher production of ROS, which, together with the generation of formaldehyde, leads to increased lipid peroxidation increasing the toxic impact of methanol (Parthasarathy et al., 2006). Similarly to ethanol, methanol shows the ability to dissolve diplacone, but because of its reported toxicity, its future usage in the lipophilic antioxidants testing is doubtful.

Conclusions

This study shows that HPβ-CD is the best co-solvent of the model lipophilic compound, diplacone, in the presented method for antioxidant activity determination. Also other solvents and co-solvents tested, namely DMSO, Cremophor EL®, ethanol, and methanol, do not interfere with the method described but their further usage, especially for in vivo testing, is problematic due to the reported side effects and possible toxicity.

Tween 80 and BSA significantly decreased the antioxidant activity of the model lipophilic compound and were thus found not to be suitable for the dissolving of the lipophilic compounds assayed by the method presented.

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