



New Usage of a Fluorometric Method to Assay Antioxidant Activity in Plant Extracts

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Abstract

In the present study, an oxidative conversion of non-fluorescent dichlorofluorescein (LDCF) to fluorescent dichlorofluorescein (DCF) was used for detection of antioxidant properties of plant extracts. The rate of the reaction was followed by monitoring the formation of DCF as a function of time. The antioxidant assay was carried out for different concentrations of gallic acid, *Salvia mirzayanii*, *Rech. f* & *Esfand Phlomis persica Boiss* crude extracts and their fractions obtained by thin layer chromatography (TLC). The results showed that the fluorometric method could be used to detect lower concentrations of antioxidants. Thus, we were able to identify the antioxidant activity of five fractions obtained by TLC of *Salvia mirzayanii* extract and four fractions in TLC of *Phlomis persica* extract. This method is a good candidate to be used in high throughput screening.

Keywords: Antioxidant activity; Dichlorofluorescein; Fluorometric method; *Phlomis persica*; *Salvia mirzayanii*; Thin layer chromatography.

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1. Introduction

Intracellular defense systems in mammalian cells such as superoxide dismutase, catalase or glutathione peroxidase protect the cells against excessive levels of free radicals. Additional protection can be provided by the addition of compounds such as vitamins A, and E, β -carotene, minerals (selenium, zinc) or proteins (transferrin, ceruloplasmin, and albumin) [1]. These natural antioxidants or other compounds

that can inhibit free radicals are very important in prevention of vascular diseases and cancers [2, 3]. Natural antioxidants are used as food additives and also for applications as nutraceuticals [4].

Innovation of new and potent antioxidant method plays an important role in the recognition of neurodegenerative disorders such as Alzheimer disease (AD) [5, 6]. Unequilibrium between prooxidant and

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antioxidant molecules is often referred to as oxidative stress and cause decline of defense mechanisms in aged organisms [7].

Measuring the production of reactive oxygen species by using the fluorogenic probe [8], 2',7'-dichlorofluorescein diacetate (LDADCF), in which flow-cytometric and spectrofluorometric systems are used, has become a popular method. The advantages of this method are simplicity, high sensitivity, and low cost relative to traditional approaches [8]. The mechanism of this method is via oxidation of dichlorofluorescein diacetate (LDADCF) to a nonfluorescent dichlorofluorescein (LDCF) which is converted to a fluorescent dichlorofluorescein (DCF). In this method, hematin (a heme derivative) is used in the conversion of LDADCF to DCF, which accelerates the effect of hydrogen peroxide. It is thought that hydrogen peroxide could dissociate into a hydroxyl radical, which assists in oxidation of LDCF to DCF [2, 6].

This method has been used for detection of enzyme systems such as glucose oxidase [3, 4] as well as in determination of lipid peroxidation [1, 2]. Also, a method using LDCF was used to detect and evaluate the oxidative burst in neutrophils [5].

In the present study, we designed a new application of fluorometric method for measuring the antioxidant activity. This method was used for determination of antioxidant activities of two Labiatae family plants, *Salvia mirzayanii* (SM) and *Phlomis persica* (PP) fractions which were obtained by TLC.

2. Materials and methods

2.1. Materials

2,7-Dichlorofluorescein acetate (LDADCF), silica gel type G and hematin were obtained from Sigma (ST. Louis, MO, USA). All other reagents were purchased from Merck Chem. Co Darmschtadt, Germany.

2.2. Plant material and extraction

Extraction was performed as reported previously [9; 10]. Aerial parts of SM (5 kg) were extracted with 31.8 L ethanol to give 1.1 kg of concentrated crude extract [9]. Also, aerial parts of PP (3 kg) were extracted with 38.4 L ethanol to give 618.9 g of concentrated crude extract [10].

2.3. TLC separation

One mm thick silica gel G plates were prepared and were activated during 30 min at 110 °C and cooled to room temperature. Extracts of SM and PP were applied several times in a narrow band to obtain sufficient concentration at 2 cm distance from the bottom of the plate. Three different elution mixtures [11] with variable polarities containing (toluene-acetone 8: 2), (toluene-chloroform-acetone 40:25:35) and (n-butanol-glacial acetic acid -water 50:10:40) were used. The elution time of 2 h was applied. The plates were then dried at room temperature and the bands were detected by fluorescent light. The best separation was found to occur in the most non-polar solvent in which some distinct fluorescent bands were detected. The fluorescent bands and areas in between were

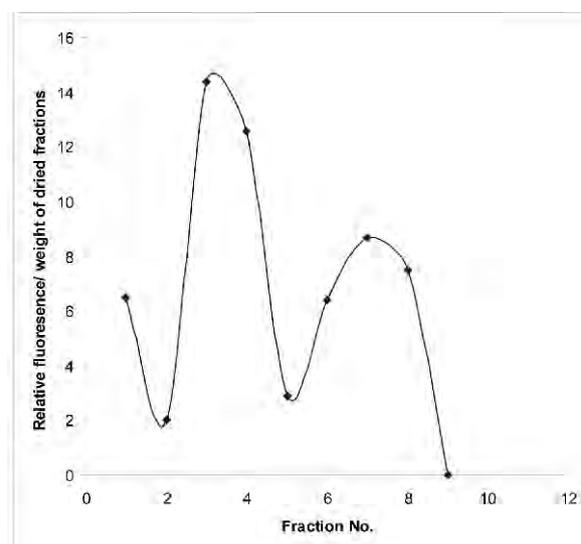


Figure 1. Antioxidant properties of fractions obtained in TLC using fluorometric method of evaluation. The values were calculated per equal weights of fractions to eliminate the weight factor.

assigned as F1-F8 for SM and F1-F16 for PP (Figures not showed). The bands were separately marked and scraped of the plates and were eluted with similar elution solvent. Each fraction was obtained by centrifugation at 3000 rpm to remove the solid gel. The supernatants were separated and the gels were washed twice with the same solvent. Each fraction was then dried and weighed. The weights of antioxidant fractions of SM were as follows: F3=14.40 mg, F4=12.6 mg, F6=6.4 mg, F7=8.7 mg and F8=7.5 mg.

The weights of antioxidant fractions of PP TLC were as follows: F2=5.41 mg, F3=15 mg, F5=4.67 mg and F16=14 mg.

2.4. Dichlorofluorescein solution

Stock solution of LDADCF (1 mM) was made in ethanol and stored in dark. LDADCF is stable for months under this condition [12]. Activation of LDADCF to LDCF for assay required dilution of 1:4 V/V of ethanol and 0.01 N sodium hydroxide. The mixture was allowed to stand at room temperature for 30

min. LDCF has a high rate of auto-oxidation, so it is required to be prepared each day.

2.5. Hematin solution

The hematin solution (0.01 mg/ml) was prepared by dissolving 1 mg of hematin in 0.5 ml of 0.2 N NaOH and then diluted to 100 mL with 50 mL of 0.05 mol/L of tris-HCl buffer. This solution was made freshly each day.

2.6. H_2O_2 solution

100 μ l solution of concentrated hydrogen peroxide was diluted to 10 ml and 100 μ l of this solution is diluted again to 100 ml with distilled water. The reaction mixture contained 7 ml of hematin solution which was mixed with 50 ml of tris buffer (0.05 M, pH=8). After boiling for 5 to 6 min, 0.10 μ l of this solution was added to 20 μ l of H_2O_2 followed by addition of 3 ml of tris buffer.

Fluorescence of solutions containing SM extract or fractions was assayed for antioxidant activity in a single beam fluorimeter, (FP 2600 Jasco Corporation,

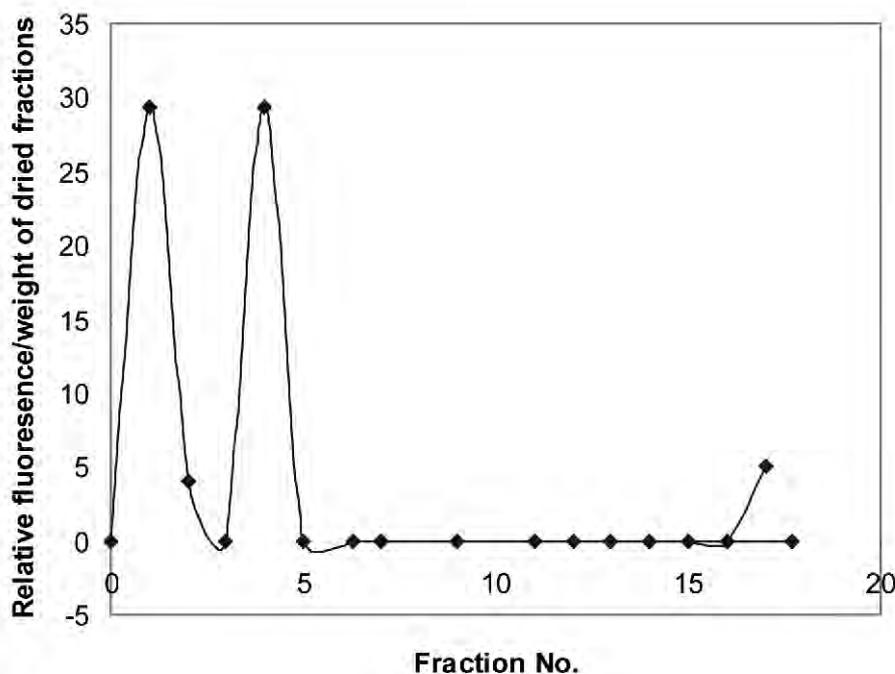


Figure 2. Antioxidant properties of *Phlomis persica* fractions obtained in TLC using fluorometric method of evaluation. The values were calculated per equal weights of fractions to eliminate the weight factor.

Tokyo, Japan). The reaction was started by addition of 20 μl of DCF in a cuvette. The excitation wavelength was fixed at 496 nm and the emission at 521.6 nm. Both of excitation and emission slit were in 5 nm band width. The time course of the reaction depicting the evolution of fluorescence as a function of time could be used as a measuring of potency of the oxidation reaction. This base reaction was lowered in intensity when coupled with our antioxidant reagents.

We employed this effect in evaluating the antioxidant properties of fractions obtained from TLC separation of SM and PP extracts. 20 μl of crude extracts (or fractions) were used in this evaluation and the results compared with those of gallic acid.

2.7. Statistical analysis

Means \pm SD were calculated. The data were analyzed for statistical significance using one way ANOVA followed by Tukey post test. P values less than 0.05 were considered significant.

3. Results

3.1. TLC results

Five antioxidant bands obtained in TLC of SM with Rf's of 0.49, 0.55, 0.61, 0.77, 0.83 and 0.88 cm were detected. The fractions which obtained were dried and weighed separately and their antioxidant values were determined. The results are depicted in Figure 1. From the antioxidant plots versus fraction number (Figure 1), it was evident that fractions 3, 4, 6 and 7, 8 had antioxidant activities. Four antioxidant bands in TLC of PP were observed. Figure 2 shows that PP fractions 2, 3, 5 and 16 contained antioxidant activities with Rf's 0.083, 0.125, 0.167 and 1 cm, respectively.

3.2. Fluorometric results

Figure 3 shows the fluorometric assay of gallic acid in the range of 0.001-1 $\mu\text{g/ml}$. The relative fluorescence of 1 $\mu\text{g/ml}$ of gallic acid was less than other concentrations of gallic acid ($p < 0.001$). The oxidation reaction with no antioxidant (base reaction) attains the highest

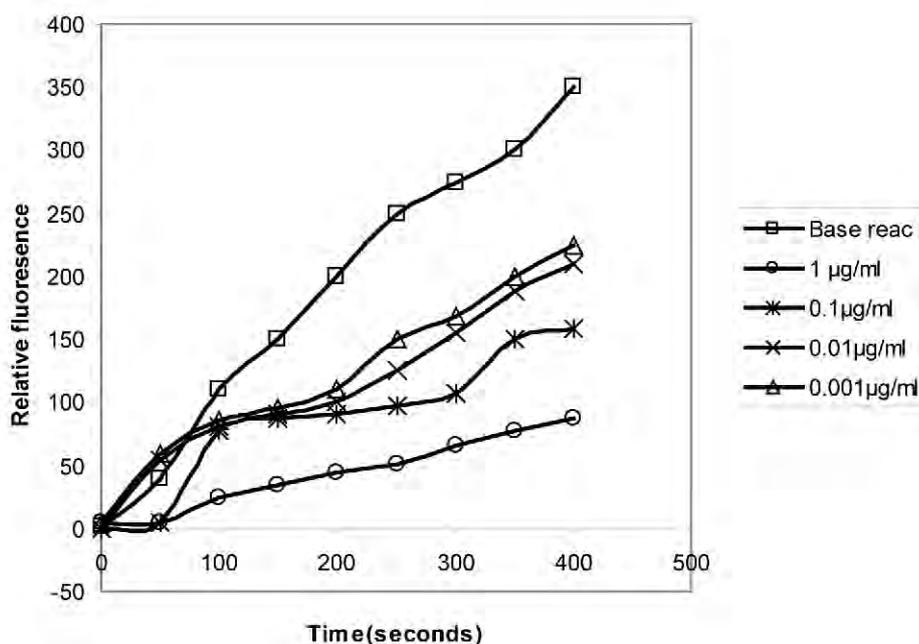


Figure 3. Usage of different concentration of gallic acid to detect the limitation of concentration in fluorometric method. The steepest line was the base reaction that was lowered by the effect of several concentrations of gallic acid. 0.001 $\mu\text{g/ml}$ of gallic acid was shown to be detectable.

level of fluorescent (the uppermost curve, Figure 3). In the fluorometric method, the differences in slopes with respect to the base line could be considered. Based on this treatment, we measured the antioxidant properties of crude extract and fractions obtained from TLC of SM and PP (Figures 2, 3). The apparent values of antioxidant activities for crude extract and each fraction was divided by their weights to obtain the values of antioxidant per mg weight of each fraction. These values in fractions number 6, 7 and 8 of SM (Figure 1) showed the highest antioxidant properties (Figure 2). A significant difference between the antioxidant activities of SM fractions and the concentrations of 2.5 and 1.25 $\mu\text{g/ml}$ of gallic acid ($p < 0.001$) was observed. This significant difference was also observed between antioxidant activities of PP fractions and the 2.5, 1.25 $\mu\text{g/ml}$ concentrations of gallic acid, ($p < 0.001$). In other words, the relative fluorescence of PP fractions was higher than gallic acid.

3. Discussion

The levels of ROS in cell systems can be measured using DCF fluorescence which is an excellent tool [12, 13]. Also, the preventive effect of antioxidant compounds on H_2O_2 [14] (which induces cell injury) was reported using DCF [15-17].

Based on these results, we apply new usage of a fluorometric method for detection of antioxidant activity of plant extracts. In this research, the conversion of LDCF to DCF was used for detecting antioxidant properties of antioxidant compounds (i.e. antioxidant plant extract). By using this method, the antioxidant activity of gallic acid in the range of 0.001-1 $\mu\text{g/ml}$ was detected which showed the sensitivity of this fluorometric method. The antioxidant activities of gallic acid were higher than the TLC fractions of SM and PP extracts ($p < 0.001$, Figures 3, 4, 5). It means that even low antioxidant activities can be determined by this method. In our study, as the concentration of antioxidants increased the slopes of the fluorescent emission as a

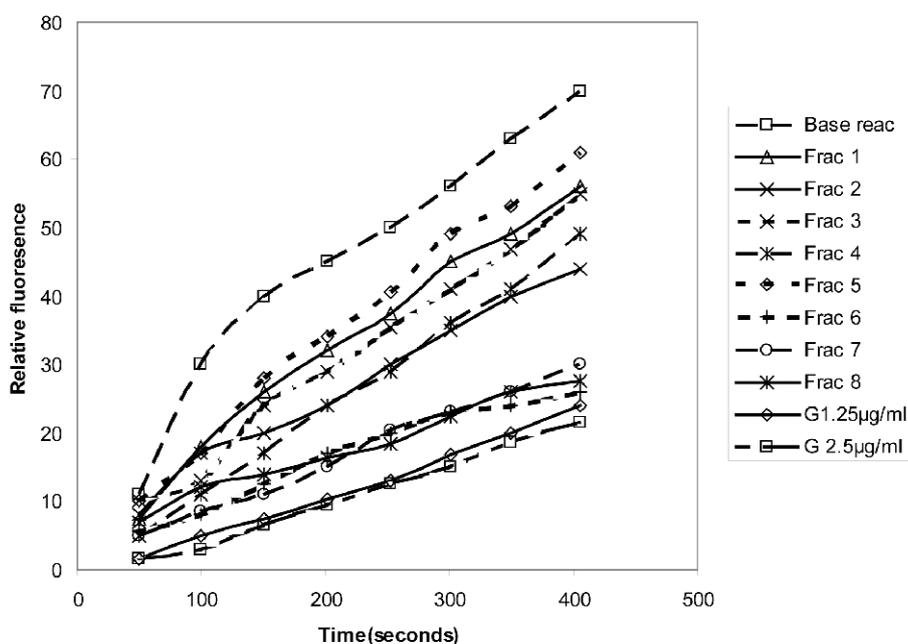


Figure 4. Fluorometric determination of antioxidant properties of fractions obtained from TLC of *Salvia mirzayanii* extract was compared with gallic acid.

function of time decreased, which indicates a decrease in the rate of oxidation or an increase in the antioxidant effect. Thus, to evaluate the antioxidant properties of compounds, the differences in slopes with respect to the base line could be considered.

On the basis of our results, it is noteworthy to mention that in the fluorometric method the changes of emission as a function of time were also proportional to the concentrations of SM, PP extracts and gallic acid (as a standard). A more significant sensitivity was observed in the fluorometric method used for detecting antioxidant properties of plant extracts. This method enables us to carry out our determinations with less amounts of extract fractions obtained by TLC.

It is noteworthy that the initial tests with gallic acid in our experiments indicated that the concentrations lower than 1 $\mu\text{g/ml}$ and as low as 0.001 $\mu\text{g/ml}$ could be assayed (Figure 3). There is a significant difference between relative fluorescence of these gallic acid concentrations ($p < 0.001$).

It was interesting that the antioxidant activity of PP crude extract using fluorometric

method was higher than SM crude extract (Figure 4, 5). This result was confirmed by other method which measured the antioxidant activity [10]. In other researches, it was reported that the radical scavenging activity and lipid peroxidation inhibition of PP crude extract were higher than SM crude extract [10].

5. Conclusion

We believe that fluorometric assay is significantly a sensitive method and has other preferences such as rapidity and ability to assay antioxidant potentials of complex and crude preparations. Also, by using this technique in several antioxidant experiments, more insights into the mechanism of antioxidant reaction become available.

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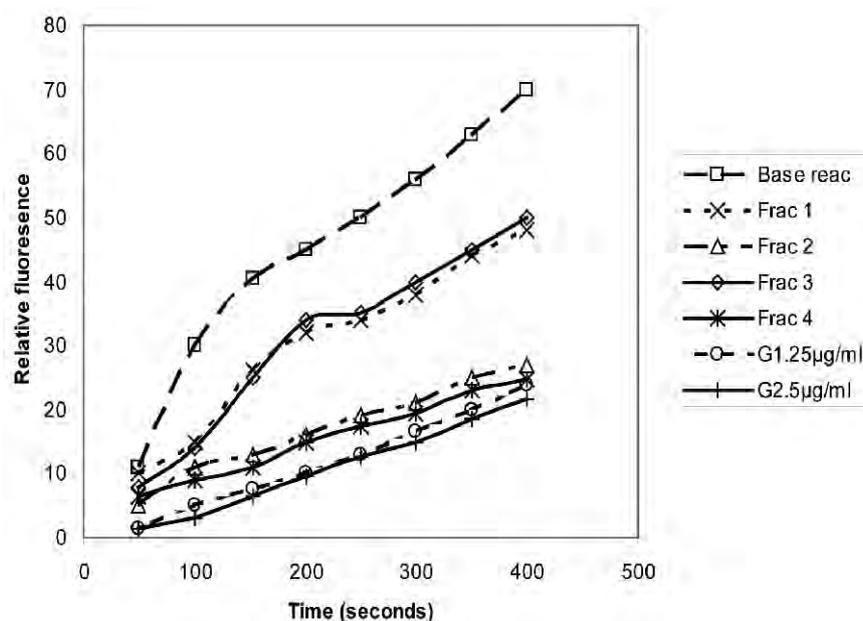


Figure 5. Fluorometric determination of antioxidant properties of fractions obtained from TLC of *Phlomis persica* extract was compared with gallic acid.

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