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*Original Article*

# Enzymatic and Non-enzymatic Antioxidants of *Calamintha officinalis* Moench Extracts

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## Abstract

*Calamintha officinalis* (COM) is a genus of aromatic herbs, native in north of Iran. Partial purification of aqueous extract from COM was done using ammonium sulfate precipitation followed by dialysis. The total protein content was determined at various purification stages. The highest amount of protein of the extract was found in fraction obtained in 20-40% saturation range. Specific activities of three antioxidant enzymes, peroxidase, catalase, and superoxide dismutase were also determined and compared in each stage. The highest activity of peroxidase, catalase and superoxide dismutase was in fractions 60-80%, 20-40%, and 20-40% respectively. The non-enzymatic antioxidants were evaluated by two different methods i.e. free radical scavenging activity using DPPH and inhibition of lipid peroxidation by the ferric thiocyanate method. It was found that radical scavenging power of COM methanolic extract was 43.99. Furthermore, the inhibition of li pid peroxidation of the extract was decreased every 24 h. Thus, our findings suggested that *C. officinalis* possess potent antioxidant defense machinery both enzymatic and non enzymatic.

**Keywords:** *Calamintha officinalis*, Antioxidants, Peroxidase, Catalase, Superoxide Dismutase

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## Introduction

Antioxidants are a group of secondary metabolites created by aerobic organisms to frustrate oxidative stress initiated by reactive oxygen species (ROS). A number of ROSs such as superoxide anion radicals and the hydroxyl radicals are generated during normal oxidation/reduction reactions as a result of aerobic metabolism [1]. An antioxidant system is naturally present to scavenge such radicals. However, when the balance between creation and elimination of radical is altered due to various disorders, the use of external antioxidants is necessary. The constant use of artificial antioxidants may initiate some health hazards and prompt toxicity level in the body system. Therefore, an alternate approach is required to ease and dominate worries [2]. Nowadays, interest in the discovery of natural antioxidants that can defend oxidative stress has increased significantly. Their application in packed food and medicinal preparations to substitute artificial antioxidants is of prime importance. The use of plants as a source for various medical preparations has obtained enhanced interest [3]. Many plants are able to exhibit antioxidant defense mechanisms including superoxide dismutase (SOD) (EC 1.15.1.1), catalase (CAT) (EC 1.11.1.6), peroxidase (POD) (EC 1.11.1.7) enzymes which work together to detoxify ROS. Medicinal plants may also produce non-enzymatic molecules including ascorbate, glutathione, carotenoids, and anthocyanins which eliminate, counteract and scavenge the ROS in plant systems and protect the main enzymes from ROS [4]. SODs are a group of multimeric metalloenzymes that catalyzes the dissociation of superoxide radicals ($O_2^-$) to hydrogen peroxide ($H_2O_2$) and molecular oxygen ($O_2$) in different parts of cells. Therefore, SOD is the main defense against the damage that is initiated by ROS. CATs and PODs are also significant scavengers of H2O2 in plant cells [4].

*Calamintha officinalis* is a member of *Lamiaceae* family and very similar to the common mint from morphological point of view and aroma characteristics. It is generally used as a substitute for the mints in various beverages [5-7]. The present study was intended to evaluate the activity of various antioxidant enzymes including (POD, CAT, and SOD) in COM extract. Moreover, the role of partial purification of the extract on the activity of enzymes was also evaluated. Our findings revealed significant antioxidant potentials of the enzymes. Since antioxidants act through different mechanisms, in this research we decided to evaluate the effect of the plant extract on radical scavenging and lipid peroxidation through two different methods; 2,2-diphenyl-1- picrylhydrazyl (DPPH) and ferric thiocyanate (FTC).

## Materials and Methods

### *Plant samples*

The plant, COM was collected from north area of Iran (Guilan, Lahijan) during April 2015 The fresh leaves were washed thoroughly with distillated water, blotted thoroughly and dried in oven at 40°C. The leaves were then crushed into small pieces, frozen and used for extraction.

### *Determination of DPPH˙ radical scavenging activity*

The DPPH method described in 2010 was used with slight modification [8]. In practice, 2.5 g of the powder was mixed with 25 ml of 96% methanol. The solution was placed on a shaker at 80 rpm, 25°C for 48 h. The sample was then filtered using Whatman No. 3 filter paper. Anti-

oxidant activity of the plant extracts was determined on the basis of scavenging activity of the 2, 2-diphenyl-1- picryl-hydraziyl (DPPH˙) radical. The antioxidant reaction was started by adding 0.5 ml of plant extract into a tube containing 3.5 ml of freshly prepared DPPH˙ methanol solution (0.004 g DPPH˙ to 100 ml methanol). After 30 min incubation in dark at room temperature, the absorbance was read at 517 nm by a UV-Visible spectrophotometer. Inhibition percent of DPPH˙ (I%) of the extract was computed by the following formula [8]:

I%=A0-AT /A0× 100

Where A0 is absorbance of control (methanol-water with DPPH˙) and AT represents the absorbance of sample.

***Ferric thiocyanate assay (FTC)***

The assay is based on the ability to reduce ferric ion as the result of oxidation. In practice, five grams of powdered leaves were added to 50 ml of ethanol and mixed thoroughly. It was then placed on ultrasonic bath at room temperature and sonicated constantly for two periods of 5 and 10 minutes. The resulting mixture was shaken using an incubator shaker at 80 rpm, 25°C for 24 hours. The sample was then centrifuged at 3000 rpm for 15 minutes and the supernatant was used for FTC assay. The reaction mixture included 400 µl of ethanol extract, 10 µl of 2.51% linoleic acid in ethanol, 800 µl of 0.02 M phosphate buffer (pH 7.0) and 400 µl of distilled water. The vial containing the solution was placed in oven at 40°C in the dark. This was followed by mixing 10 µl of the solution, 970 µl of 75% (v/v) aqueous ethanol and 10 µl of 30% aqueous ammonium thiocyanate. Ten µl of 0.02 M ferrous chloride in 3.5% hydrochloric acid was later added and the absorbance of the subsequent mixture (red color) was read after 3 minutes at 500 nm every 24 h until the absorbance of the control reached its maximum value. The mixture without the sample was used as control [10].

***Preparation of plant extract for enzyme assay***

The frozen leaves (1g fresh mass) were ground in liquid nitrogen and extracted with a cool extraction buffer 3 ml (50 mM potassium phosphate, pH 7.5). The extract was centrifuged for 30 min at 12,000 rpm at 4°C and the resulting supernatants were used as crude extract.

***Ammonium sulphate precipitation and dialysis***

The crude extract was exposed to ammonium sulphate fractionation and the precipitate in the 20-80% saturation range was centrifuged for 30 min at 9.000xg. The precipitate was dissolved in 2 ml phosphate buffers (pH 7.0, 50 mM) and dialyzed 24 h at 4°C against 1 L above buffer for further use [11].

***Determination of total protein***

Total protein concentration of extract was determined by a colorimetric method initially described by Bradford [12]. The protein concentration is determined by measuring the binding of the dye, Coomassie Brilliant Blue G-250, to the unknown protein solution, in comparison with known standards. Initially, 2.5 ml of Coomassie Blue was added to 50 µl extract and 0.15 M NaCl solution was used as solvent, with 50 µl of 0.15 M NaCl as blank. The reaction mixtures were kept at room temperature for 5 min. Certain concentrations of bovine serum albumin (0-1mg/ml) were used for preparation of standard solutions. The absorbance at 595 nm was read against the blank and the protein concentration of the extract was calculated using the standard curve.

***Zymogram analysis***

Activities of all three enzymes were calculated (POD, SOD, and CAT) using a zymogram method described in [4] with a slight modification. Practically, electrophoresis of the plant extract was performed on a 12% polyacrylamide gel. The resulting gel was then washed three times in 50 mM (pH 7) phosphate buffer and mixture of each substrate solution was used for detection of the appropriate enzyme.

In the case of POD, the gel was immersed in a mixture of 0.1 mM phosphate buffer (pH 6.8), guaiacol (30 mM), $H_2O_2$ (30 mM) and left at room temperature in dark until the formation of reddish-brown bands.

In the case of SOD, the resultant gel from electrophoresis was first immersed in 25 ml of 1.23 mM NBT for 15 min, for a short time. It was then washed and immersed in 30 ml of 100 mM potassium phosphate buffer (pH 7.0) containing 28 mM TEMED and 2.8 ×10-2 mM riboflavin and kept in dark for another 15 min. The gel was washed again and using a white light the photochemical reaction was allowed to start. All the procedures were performed at room temperature.

For visualizing catalase activity, following after electrophoresis, the gel was incubated in 50 mM potassium phosphate buffer (pH 7.0) for 15 min and then left in 0.03% $H_2O_2$ solution for 10 min. The gel was washed twice with water and left in a mixture of freshly prepared 2% potassium ferricyanide and 2% ferric chloride (1:1). The catalase in gel was visualized yellow in a greenish-blue background [4].

***Assay of peroxidase activity***

The activities of antioxidant enzymes in COM extract were assayed quantitatively after detection by zymogram. The activity of POD was measured using guaiacol and hydrogen peroxide as substrates. In practice, the enzymatic reaction was performed by mixing 1.5 ml of 0.1 M phosphate buffer (pH 6.8) which contained 30 mM guaiacol and 30 mM $H_2O_2$ with 0.15 ml of enzyme extract. Alternations in optical density (OD) of reaction mixture at 470 nm were determined every 20 sec. A unit of peroxidase activity was expressed as the change in absorbance per min and specific activity as enzymes units per mg soluble protein [13].

***Assay of superoxide radical scavenging activity (SOD)***

To start the reaction, 100 µL enzyme extract and 3 ml reaction mixture were mixed together. The reaction mixture contained 50 Mm potassium phosphate buffer (pH 7.8), 13 mM methionine, 2µM riboflavin, 0.1 mM EDTA and 75 µM NBT. A blank was comprised of the reaction mixture but without enzyme and NBT. The tubes containing solutions were subjected to 400 W (4×100 W bulbs) for 15 minutes and the absorbance was instantly read at 560 nm (Siavoshi and Laware, 2013). The percent of scavenging the superoxide radical was then calculated using the following equation:

% scavenging = (1−Ae /A0) ×100

Where A0 is the absorbance of control, and Ae is absorbance of sample [14].

***Assay of catalase activity***

The CAT reaction mixture (3 ml) was composed of 50 mM phosphate buffer (pH 7.0), 15 mM $H_2O_2$ and 0.1 ml enzyme extract. The reaction was started as soon as the enzyme extract was added. Alterations in absorbance of the reaction mixture at 240 nm were recorded every 20 s. One unit of CAT activity was defined as an absorbance change of 0.01 unit min-1 [13]. The specific activity of the enzyme was reported as units per mg protein.

**Results**

***DPPH radical scavenging activity***

This assay is known to give reliable information concerning the antioxidant ability of the tested compounds. The principle behind this assay is the color change of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) solution from purple to yellow, when the radical is quenched by an antioxidant. The calculated radical scavenging for COM methanolic extract was 43.99 percent. The result of experiment showed that COM extract had a comparably significant radical scavenging activity suggesting the plant as a source of fare antioxidants.

***Ferric thiocyanate assay (FTC)***

The FTC assay was used to determine the amount of peroxide produced at the primary stage of linoleic acid peroxidation. In principle, peroxide reacts with ferrous chloride ($FeCl_2$) to create a reddish ferric chloride ($FeCl_3$) that yields ferric thiocyanate by ammonium thiocyanate. Low absorbance value is due to the high inhibition percentage of lipid peroxidation. As shown in Table 1, the absorbance of COM ethanolic extract gradually decreased up to 4 days and a slight increase was observed at day 5. Inhibition of lipid peroxidation was decreased to about half, every 24 hours so that the inhibition was 12.48% in the first day, 6% in the 2nd day, 3.15% in the 3rd day, 1.77 in the 4th day, and approached zero by 5th day.

***Total protein assay***

Determination of total protein concentration is necessary to assess the specific activity of antioxidant enzymes. Table 2 presents total protein concentration in COM crude extract measured as 0.25 mg/ml. Total protein concentration was maximum (0.33 mg/ml) in fraction obtained at percentage saturation of 20 to 40 and most of the proteins precipitated in this percentage. Therefore, saturation percent of 20 to 40 was the optimum to precipitate maximum proteins of the extract. However, total protein concentration of the extract decreased in upper levels of saturation. It was, therefore, found that at saturation percent of 40 to 60 and 60 to 80, the total protein concentrations were 0.2 mg/ml and 0.14 mg/ml respectively.

***Peroxidase activity***

Specific activity of POD in purified and crude extract of COM leaves was determined. The results are shown in Table 2. Specific activity of POD in the crude extract was 389.18 U/min/mg. However, specific activity of POD in extract decreased during purification, so that POD activity in percentage saturation of 0 to 20, 20 to 40, 40 to 60 and 60 to 80 were 12.38, 22.19, 15.6 and 28.84 U/mg respectively. The minimum specific activity of POD obtained in 0-20% saturation range and the maximum value was at 60-80% saturation range. It can be seen that, as the ammonium sulphate salt concentration increased, the specific activity of POD was reduced in COM extract. Also, most of the peroxidase protein precipitates out when saturation reached 60-80% w/v.

***Activity of catalase***

Specific activity of CAT in purified and crude extract of COM leaves was determined. The results are also presented in Table 2. It was found that the specific activity of CAT in crude extract of COM leaves was 97 U/mg. Unlike POD, the activity of CAT increased generally during purification, so that CAT activity in percentage saturation of 0 to 20, 20 to 40, 40 to 60 and 60 to 80 were150, 262.5, 180 and 128.57 U/mg respectively. The minimum specific activity of CAT obtained in 0–20% saturation range and the maximum was obtained in 20-40% saturation range. It can be concluded that the precipitation process results in enhancing specific activity of catalase in COM extract because most of the catalases precipitates in percentage saturation of 20 to 40 of ammonium sulphate.

***Superoxide radical scavenging activity***

Scavenging activity of SOD in purified and crude extract of COM leaves was determined by spectrophotometric method. As the results in Table 2 indicate, scavenging activity of SOD in the crude extract was 89.8%. A significant alteration in the activity of SOD was not observed during purification, so that the fraction obtained in 20-40% saturation range showed almost the same amount of activity with the of 89.2%. The minimum scavenging activity of CAT obtained in 0-20% saturation range and the maximum obtained in 20-40% saturation range. It was, therefore, concluded that percentage saturation of 20 to 40 is optimum for purification of this enzyme as most of the enzymes precipitates in this saturation percentage.

**Table1**. Inhibition of lipid peroxidation assessed by FTC method.

| | Day1 | Day2 | Day3 | Day4 | Day5 |
|---|---|---|---|---|---|
| **Absorbance ($A_{Sample}$)** | 0.547 | 0.509 | 0.500 | 0.480 | 0.485 |
| **Inhibition (%)** | 12.48 | 6.0 | 3.15 | 1.77 | 0 |

***Zymogram studies***

Based on our literature investigation, COM has not been investigated previously for POD and CAT and SOD through zymography analysis by other researchers.

The zymogram test of COM extract for POD was carried out after concentrating with ammonium sulphate and dialysis. The results showed existence of brown color bands in an elucidative ground in gel (Fig. 1a). A pale bond was observed in saturation percent of 0 to 20 showing the lowest POD activity in the fraction obtained in percentage saturation of 0 to 20. However, intensive bands in percentages saturation of 20 to 40, 40 to 60 and 60 to 80 were observed. The intensity of brown band was highest at 40-60% indicating the highest activity of peroxidase in this fraction.

Zymogram of CAT was also carried out after concentrating with ammonium sulphate and dialysis. The results

showed existence of green color bands in a yellow background in gel (Fig. 1b). A pale green band was observed in percentage saturation of 0 to 20 indicating the lowest CAT activity at this range. However, intensive bands in percentages saturation of 20 to 40, 40 to 60, and 60 to 80 were observed. The most intensive band for catalase activity was observed in saturations of 40- 60%. Therefore, we concluded this range of saturation was optimum for precipitation of CAT.

The result of zymogram test for SOD showed existence of yellow color bands in a dark violet background of the gel (Fig. 1c). Similar to POD and CAT, a pale band in percentage saturation of 0 to 20 was observed.

However, the bonds at 20-80% saturation were completely obvious indicating that the activity of SOD in these fractions is considerable with the darkest band at 20-40% range.

**Table 2**. Total protein content and enzyme activity during saturation of COM extract.

| Purification step | Specific activity of POD (U/min/mg) | Specific activity of CAT(U/min/mg) | Scavenging activity of SOD (%) | Total protein content (mg/ml) |
|---|---|---|---|---|
| Crude extract | 389.18 | 97 | 89.8 | 0.25 |
| Extract in 0-20% saturation | 12.38 | 150 | 7 | 0.17 |
| Extract in 20-40% saturation | 22.19 | 262.5 | 89.2 | 0.33 |
| Extract in 40-60% saturation | 15.6 | 180 | 47 | 0.2 |
| Extract in 60-80% saturation | 28.84 | 128.57 | 18 | 0.14 |

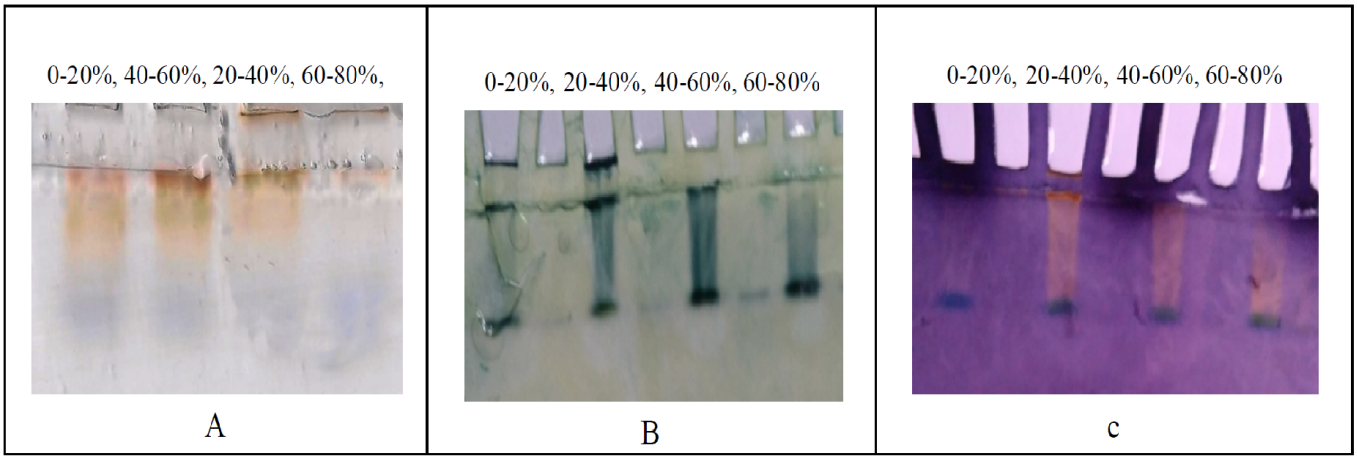


**Figure 1.** Zymogram of a) POD, b) CAT and c) SOD from *Calamintha officinal* moench methanolic extract.

**Discussion**

Complicated antioxidant systems are very vital for protecting cellular membranes and organelles from the injurious effects of active oxygen species. Plants with high amounts of antioxidants, either constitutive or induced, possess more resistance to the oxidative injury. This type of protection comprises both enzymatic and non-enzymatic antioxidants [13].

The specific activity of POD was evaluated in both crude and purified extracts (Table 2). As the highest activity of peroxidase (28.84 U/min/mg protein) was observed in 60-80% saturation, this percent is recommended for purification of this enzyme. In support of our finding, other researchers have extracted POD from cabbage leaves and purified the enzyme partially by ammonium sulphate 80% and dialysis.

Based on their results, total protein of the purified extract was 359.56 mg and the specific activity of the enzyme was 1.502 U/mg. Similar to the present research, a group of researchers extracted POD from Turkish black radish (*Raphanus sativus* L.) and partially purified the enzyme by ammonium sulphate10-80% and dialysis. They also used zymography for detection of the purified active enzyme.

It was reported that the specific activity of POD on guaicol as substrate in crude extract was 24158.4 U/mg and in purified extract it was 42670.5 U/mg. Using Bradford method, they measured total protein in crude and purified extract as 9.09 mg and 1.72mg respectively [15].

Peroxidase has recently been partially purified from wheat bran by ammonium sulphate and dialysis [16]. They precipitated the enzyme in crude extract with saturation ratios 30% and 80%. Total protein of the crude extract was 3.1mg and that of purified extract 3.04mg. Specific activity of POD in crude extract was found to be100 U/mg and in purified extract 240.1 U/mg.

A balanced amount of H2O2 is very vital for the cells, as its increase leads to cell death. An appropriate control of H2O2 concentration is mainly directed by CAT, which decomposes $H_2O_2$ to non-toxic levels. It is, therefore, suggested that higher activity of CAT could play an important

role in the safety of the plants from injuring effect of $H_2O_2$ [13]. In the present study, the specific activity of CAT in the crude and purified extracts of the plant was compared. It was found that the specific activity of CAT in 20-40% saturation fraction reached its highest value (262.5 U/min/mg of protein) as compared to other fractions. Similar to our investigation, some other researchers have determined catalase activity in various plants. For example, catalase has been extracted and purified from potato crude extract by 40% (w/v) ammonium sulfate saturation [3]. Total protein content extracted from different tissues of *Triticum aestivum*. This has been followed by partially purification and identification of POD, CAT and SOD of the extract by Zymography analysis [4].

Superoxide is a radical which contains oxygen, a weak oxidant, but can create stronger species, including singlet oxygen and hydroxyl radicals, triggering lipid peroxidation [14]. In the present study, the activity of SOD in crude and purified extracts of the plant was evaluated. It was found that the crude extract of test plant had a high superoxide radical scavenging activity (89.8%). A number of research groups have investigated the activity of SOD in plants. A research in 2008 has reported isolation of SODs from the crude enzyme extract of wheat seedlings after heat treatment, ammonium sulfate fractionation, anionic exchange chromatography and gel permeation chromatography [7]. They then followed and observed active SODs by zymogram staining. In 2014, a research team have extracted proteins from the roots of *Stemona tuberosa* and precipitated them with 90% ammonium sulfate. They measured activity of SOD by native polyacrylamide gel electrophoresis and zymogram [5]. At the same year, other scientists extracted SOD from sardinelle (*Sardinella aurita*) viscera and purified the enzyme by the three-step procedure consisting of the heating at 65◦C for 15 min, precipitation with ammonium sulphate (30–60%, w/v) and Sephadex G-100 gel filtration. They have reported a 7.17-fold increase in specific activity of SOD after the last purification step [17]. Superoxide dismutase has also been extracted from *Stemona tuberosa* and purified partially by ammonium sulphate in percentage saturation 90 and dialysis. The isoforms of the purified SOD has been observed using two-dimensional-gel electrophoresis (2D-GE) and zymography [6].

DPPH is a constant free radical which inhibition was estimated to indicate the possible antioxidant component from plant resources [2]. The presence of three aromatic rings in DPPH structure causes the high stability of the molecule with a maximum absorbance at 517nm. Therefore, the molecules capable of quenching the DPPH radical can cause a reduction in the absorbance at this wavelength. Thus, DPPH test could be regarded as a suitable method for assessment of samples in inhibition of free radicals [18]. It offers a fairly precise and repeatable result for evaluation of antioxidant potential [2]. In our present study, the methanolic extract of COM was found to possess a significant inhibitory activity of DPPH radical (43.99%). Reduction in the absorbance of DPPH radical made by phenolic components is the result of reaction between antioxidant molecules and radicals. This leads to scavenging of the radical through hydrogen donation and could be detected by change of color from purple to yellow. We, therefore, suggested that the methanolic extract of COM contains phenolic compounds.

Linoleic acid is an unsaturated fatty acid that can simply be peroxidased and creates several compounds including aldehydes. The extent of peroxidation can be obtained by measuring the absorbance of these components. Therefore, when a compound prevents lipid peroxidation, it reduces absorbance in a specific wavelength. Lipid peroxidation can, however, be stopped by adding natural or artificial antioxidant materials [18].

The extract considerably reduced the absorbance and showed inhibitory activity against linoleic acid peroxidation. For evaluating the effect of the storage time on inhibition of lipid peroxidation, the test was repeated every 24 h after the first experiment.

## Conclusion

The present study has demonstrated that the test plant possess potent antioxidant defense machinery, and can be used a resource of natural antioxidant, on the other hand, aqueous extract of the plant can be used as alternative for antioxidant enzymes including POD, CAT, SOD. Overall, from the results obtained from various investigations in our present study, it can be concluded that the extract of *C. officinalis* possess strong antioxidant activity. Further detailed investigation of phytochemical components and associated chemical studies are recommended to nominate this medicinal plant for use in medicinal and pharmaceutical industries.

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