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Special Article - Role of Antioxidants

Antioxidant Activity Assay of Phenolic Compounds Isolated from *Origanum Onites* L. Aromatic Water by High Performance Liquid Chromatography

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Abstract

A High Performance Liquid Chromatographic (HPLC) method was developed to investigate the antioxidant performance of *Origanum onites* L. aromatic water and its phenolic compounds in terms of the free radical scavenging activity in Fenton reaction. The remaining concentrations of carvacrol, thymol and thymoquinone were measured after the oxidation reaction by HPLC to evaluate the scavenging potential of these compounds for reactive hydroxyl radicals. The main oxidation product of thymoquinone showed the highest scavenging activity in Fenton reaction. The aromatic water of *Origanum onites* L. could be used as a rich source of natural antioxidants with potential applications in pharmaceutical industry.

Keywords: Fenton reaction; Hydroxyl radical; Carvacrol; Thymol; Thymoquinone; HPLC

Introduction

Phenolic compounds such as carvacrol, thymol and thymoquinone have been reported to exhibit a wide range of biological effects, including analgesic, anti-inflammatory, protection of organs against oxidative damage induced by a variety of free radical generating agents [1,2]. Carvacrol and its isomer thymol are one of the main components of the essential oils of *Labiatae (Laminaceae)* members like oregano, thyme and savory [3]. Thymoquinone is the main constituent of Nigella sativa essential oil from most sources [4]. Thymoquinone is also the main oxidation product of carvacrol and thymol. These compounds inhibit lipid peroxidation, thus exerting their effects as antioxidant and free radical scavengers [5]. The scavenging properties of phenolic compounds are generally accepted to be due to aromatic hdyroxyl substituents.

Antioxidants can react by depleting molecular oxygen or decreasing its local concentration, scavenging chain initiating radicals like hydroxyl HO•, alkoxyl RO• or peroxyl ROO• and breaking the chain of radical sequence [6]. The chain-breaking antioxidants are able to scavenge free radicals due to their hydrogen donating ability with subsequent stabilization of the resulting phenoxyl radical [7]. It has been reported that carvacrol, thymol and thymoquinone have free radical scavenging activity and good reducing power [8,9]. The reducing properties of phenolic compounds are generally associated with the presence of reductones, which have been shown to exert protective action of antioxidant due to inhibition of free radical induced chain reaction through the donation of hydrogen atom. The antioxidant ability of carvacrol and thymol and their mixture has been evaluated taking into account their capacity to protect Caco-2 cells against a further exposure to H_2O_2. It was found that carvacrol and thymol possess a strong free radical scavenging ability during oxidative stress [10]. Hydroxyl free radicals are highly reactive and have short half-lives. Furthermore, direct detection of their concentrations in natural systems is inadequate with ESR spectroscopy. Spin-trapping is a chemical reaction that provides an approach to help overcome this problem [11].

Materials and Methods

Chemicals and reagents

Carvacrol, thymol, thymoquinone, catalase and H_2O_2 were purchased from Sigma (Sigma-Aldrich Company, USA). Acetonitrile and methanol were obtained from Merck. $FeSO_4.5H_2O$ was purchased from Riedel-de Haën (Seelze, Germany).

Quantification of oxidation products

The scavenging capacity of carvacrol, thymol and thymoquinone to hidroxyl radical was followed and remaining concentrations of compounds after oxidation were quantified by HPLC. The reaction mixture contained the following reagents for carvacrol and thymol separately: 400µM carvacrol, 400µM thymol, 200µM H_2O_2 and 100µM $FeSO_4.5H_2O$ in water. The reaction mixture contained 40µM thymoquinone, 20µM H_2O_2 and 10µM $FeSO_4.5H_2O$ to monitor the thymoquinone Fenton reaction. Because of the high absorption, the concentration of reaction mixture for thymoquinone is ten times lower than carvacrol and thymol. The reaction solutions were incubated at 37°C for each phenolic compound. The Fenton reaction was stopped by adding 50µL 0.6mg/mL catalase. The quantification of compounds was performed by HPLC at wavelength 273nm for standard carvacrol and thymol and 254nm for thymoquinone. Peaks were identified based on comparison of retention times and UV spectra with standards of carvacrol, thymol and thymoquinone.

HPLC-DAD conditions

An Agilent Technologies 1200 HPLC system (Waldbronn, Germany), consisting of a vacuum degasser, binary pump, autosampler and a diod-array detector, was used for determination of phenolic compounds. Chromatographic separations were carried out

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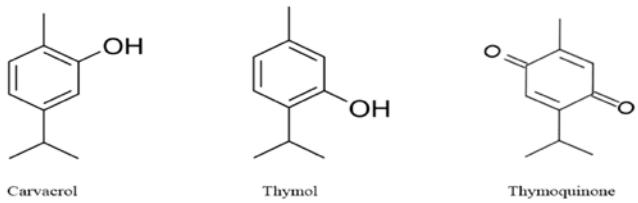

Figure 1: The chemical structures of carvacrol, thymol and thymoquinone.

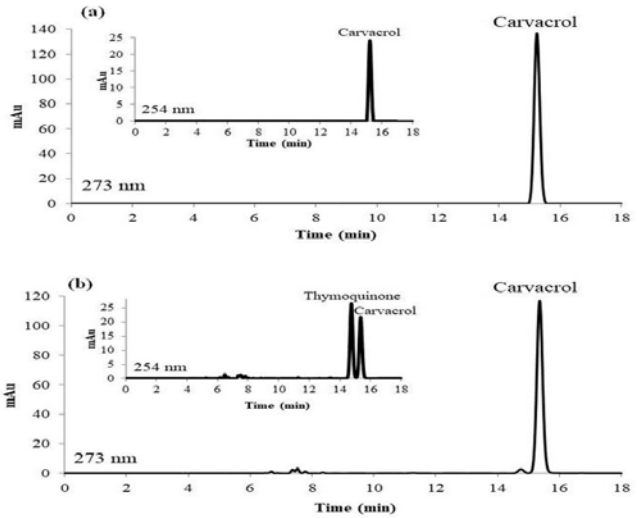

Figure 2: (a) HPLC chromatogram of carvacrol at the beginning of the oxidation reaction. (b) HPLC chromatogram of carvacrol after 10 min of oxidation reaction. Reaction conditions: 400μM carvacrol; 200μM H_2O_2; 100μM Fe^{2+}; temperature 37°C; wavelength 254 and 273nm.

using an XBridge C18 (4.6×250 mm, 3.5μm) column from Waters. Mobile phase consists of water and acetonitrile (58:42, v/v). Total run time is 18 min. Flow rate was 0.5mL/min and injection volume was 20μL. Data acquisition and preprocessing was done with Chemstation for LC (Agilent Technologies).

Results and Discussion

Hydroxyl radical is a highly aggressive radical species resulted from Fenton-type reactions, responsible for the oxidative damage of most biomolecules. Antioxidants prevent hydroxyl radical occurrence and act as radical scavenger in antioxidative defense systems. Antioxidant compounds present in those systems maintain an important balance between the formation of radicals and their removal. The phenolic compounds can scavenge free radicals by rapid donation of the hydrogen atom to radical form. In this study, the hydroxyl radical scavenging reaction was based on the oxidation of phenolic compounds with Fenton reaction at 37°C. The scavenging of hydroxyl radical was followed by HPLC measurements of carvacrol, thymol and thymoquinone during the oxidation reactions. The chemical structures of these compounds are shown in Figure 1.

The Fenton reactions for carvacrol (Figure 2a) and thymol (Figure 3a) were followed at 400μM initial concentrations. HPLC measurements revealed that carvacrol and thymol transformed to thymoquinone by catalytic transformation after 10 min oxidation (Figures 2b & 3b). It has been reported that oxidation of carvacrol

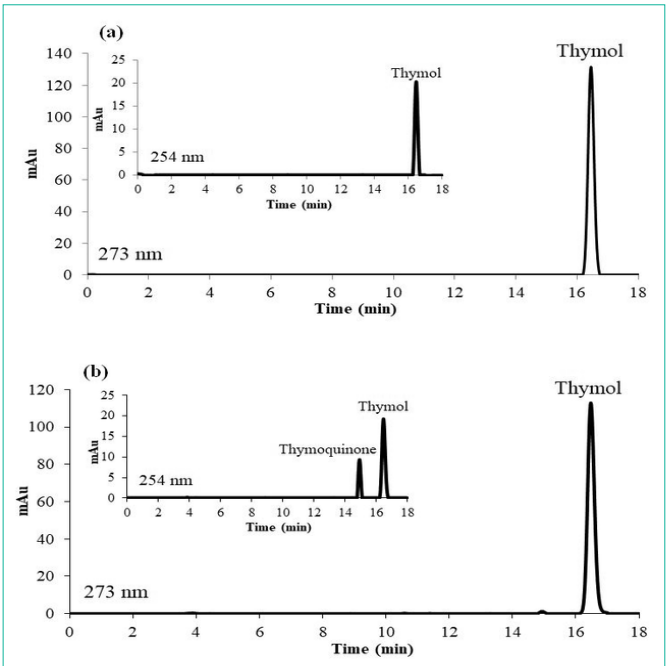

Figure 3: (a) HPLC chromatogram of thymol at the beginning of the oxidation reaction. (b) HPLC chromatogram of thymol after 10 min of oxidation reaction. Reaction conditions: 400μM thymol; 200μM H_2O_2; 100μM Fe^{2+}; temperature 37°C; wavelength 254 and 273nm.

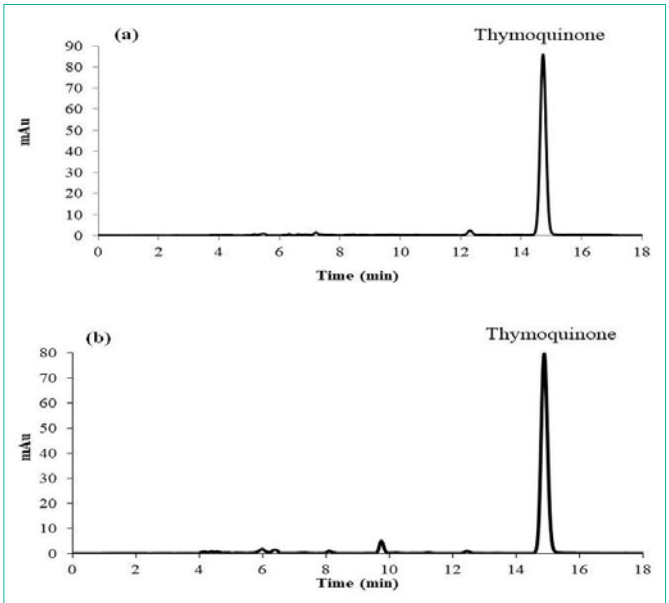

Figure 4: (a) HPLC chromatogram of thymoquinone at the beginning of the oxidation reaction. (b) HPLC chromatogram of thymoquinone after 10 min of oxidation reaction. Reaction conditions: 40μM thymoquinone; 20μM H_2O_2; 10μM Fe^{2+}; temperature 37°C; wavelength 254nm.

and thymol in the presence H_2O_2 with different catalysts yielded thymoquinone as a main product [12].

The oxidation reaction of thymoquinone was followed at 40μM initial concentration (Figure 4a). Thymoquinone reaction was performed at a concentration 10-fold lower than other compounds because of the higher molar absorptivity. The molar absorptivity

coefficient of thymoquinone (20266L $mol^{-1}cm^{-1}$) is 26 times higher than carvacrol (781L $mol^{-1}cm^{-1}$) and 33 times higher than thymol (621L $mol^{-1}cm^{-1}$) at 254nm and approximately 3 times higher at 273nm (2031L $mol^{-1}cm^{-1}$). Thymoquinone was also transformed into small-unidentified compounds during the oxidation reaction (Figure 4b).

The increase in the absorbance for carvacrol and thymol revealed that thymoquinone and other compounds developed during the oxidation process could have remarkable antioxidant abilities and probably play a crucial antioxidative role. These results are in agreement with the literature [13]. According to Jukic and Milos, when exposed to light for five days, thymoquinone is gradually converted to dithymoquinone, thymohydroquinone and some other derivatives [14]. Kruk et al. have shown by two different methods of measurement of the chemiluminescence intensity and bleaching of p-nitrosodimethylvaniline that even small quantities of dithymoquinone and thymohydroquinone have significant antioxidant properties [15]. Antioxidant activities of these three phenolic compounds were determined by the ABTS radical scavenging method as described in our previous work [16]. The antioxidant power decreased in the order: Thymoquinone > thymol > carvacrol.

The scavenging activities of carvacrol, thymol and thymoquinone were examined by measuring the remaining concentrations of phenolic compounds after 10 min oxidation by HPLC. The concentrations of phenolic compounds were determined in the linear range 50-500μM for carvacrol and thymol, 4-100μM for thymoquinone at five concentration levels. Calibration plots with correlation coefficient $R2 \geq 0.998$ were obtained by reporting peak areas as a function of phenolic compounds concentrations. All the compounds were measured above the quantification level.

The remaining concentrations of carvacrol and thymol were 356.3±0.9 and 341.3±1.8μM from 400μM initial concentrations. Thymoquinone remained 22.4±0.1μM from 40μM initial concentration. Initial concentration of thymoquinone and Fenton reagent were 10 times lower than other phenolic compounds due to high molar absorptivity coefficient of thymoquinone. Thymoquinone is also an oxidation product of carvacrol and thymol. After 10min oxidation, 6.5 and 10μM of thymoquinone was produced from carvacrol and thymol, respectively. This indicates that the hydroxyl radicals are previously scavenged by thymoquinone produced during the oxidation reactions of carvacrol and thymol. Then, the rest of the hydroxyl radicals were scavenged by carvacrol and thymol at 400μM initial concentrations. The decrease in the concentration of phenolic compounds during the Fenton reaction confirms that these compounds are able to scavenge HO• directly. The scavenging property seems to be more pronounced for thymoquinone, whose ability to scavenge HO• is higher when compared to that of the carvacrol and thymol.

Stable DPPH• radicals have been used to evaluate the free radical scavenging ability of oregano extracts containing high content of carvacrol, thymol and thymoquinone (Stamenic et al.; Kulisic et al.). The single electron transfer reactions from phenolic compounds to DPPH• has been monitored by recording the decay of the DPPH• ESR signal. The IC50 value is also a parameter widely used to measure the free radical scavenging activity [17]. A smaller IC50 value corresponds to a higher antioxidant activity. In the case of the investigated phenolic compounds in our study, the free radical scavenging ability was evaluated by measuring the remaining concentrations of phenolic compounds after oxidation reaction by chromatographic technique. The antioxidant activity values of 400μM carvacrol, thymol and thymoquinone were 0.22, 0.28 and 0.30 mg Trolox/mL, respectively. The antioxidant activity of phenolic compounds is mainly due to their simultaneous hydrogen atom donation to free radicals, electron transfer and metal chelating [18]. The antioxidant activity of the investigated compounds on HO• is suggested to take place via hydrogen atom donation. The decrease in the concentrations of phenolic compounds corresponds to the scavenging of HO•. Complete elimination of hydroxyl radicals was achieved with the concentration 356.3±0.9μM of carvacrol, 341.3±1.8μM of thymol and 22.4±0.1μM of thymoquinone.

The developed Fenton reaction was applied to *Origanum onites* L. plant material due to its main constituents of carvacrol compound. Some other Origanum species such as Origanum vulgare L. also contains thymol together with carvacrol [19]. Carvacrol was determined in aromatic water of *Origanum onites* L. plant. Essential oil of *Origanum onites* L. also contains almost mainly carvacrol, but aromatic water was used for Fenton reaction in this study because of its consumption for food purposes. Aromatic water (40mL) was obtained from 40g of dried *Origanum onites* L. plant material using Clevenger apparatus. The amount of carvacrol was found as 8.73±0.12mM in aromatic water. The Fenton reaction was applied to aromatic water by diluting 400μM of carvacrol and the reaction was followed as carvacrol compound. The remaining concentration of carvacrol is 330.0±1.2μM after 15min reaction period. Carvacrol containing aromatic water oxidized higher than the standard carvacrol as described above. The formation of thymoquinone was also followed in Fenton reaction together with carvacrol oxidation. The concentration of thymoquinone was found as 15.1±0.1μM after 15min reaction period. Application of Fenton reaction to real sample revealed that carvacrol was more oxidized and thymoquinone formed higher concentration when compared with standard carvacrol.

Conclusion

Antioxidant ability of carvacrol, thymol and thymoquinone was investigated in Fenton reaction at 37°C. The chromatographic methods can provide more detailed information about the reactivity of these phenolic compounds with free radicals. It was demonstrated that the decrease in concentrations of carvacrol, thymol and thymoquinone detected by HPLC shows the scavenging of free radicals generated during the oxidation reaction. Chromatographic results revealed that thymoquinone is an oxidation product of carvacrol and thymol. It has also the highest antioxidant potential when compared with carvacrol and thymol. The present results show that carvacrol, thymol and thymoquinone can serve as effective scavengers of reactive free radicals. Similar results were observed with the application of the method to real sample of aromatic water obtained from *Origanum onites* L.

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