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CHEMICAL COMPOSITION AND ANTIOXIDANT ACTIVITIES OF THE OLEORESIN FROM THE CLOVE BUDS (SYZYGIUM AROMATICUM)

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ABSTRACT: *The chemical composition and antioxidant activities of the oleoresin from clove buds were investigated in this work. The results showed that 20 compositions of the oleoresin were identified, and eugenol (72.85%) was found to be the main components of the oleoresin, followed by eugenyl acetate (10.54%), β-caryophyllene (4.54%), and d-cadinene (3.88%). The oleoresin from clove buds had the better scavenging capacity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2, 2'-azino-bis (3-ethylbenothiazoline-6-sulfonic acid) diammonium salts (ABTS) radicals, and inhibiting lipid peroxidation reaction in lard emulsion, as well as exhibited different protection effects against DNA damage caused by Fe^{2+} and 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH). And the antioxidant activities of oleoresin showed a dose-response relationship to some degree. However, the oleoresin did not have the protection against protein damage, but had a prooxidant effect on protein oxidative damage.*

KEYWORDS: Chemical composition, Free radical, Antioxidant activity, Oleoresin

INTRODUCTION

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated by many redox processes that normally occur in the metabolism of aerobic cells. However, the excessive amounts of ROS and RNS can attack important biological molecules such as carbohydrates, proteins, lipids, DNA and RNA, which lead to cell death and tissue damage (Alexandrova & Bochev 2005; Alamed *et al*., 2009). In addition, as it is known, the presence of high amounts of polyunsaturated fatty acids such as linoleic and linolenic acids in oils and fats make them more susceptible to oxidation in the course of the time depending on external factors such as such as oxygen, light, high temperatures or trace metals, mainly transition metals such as Fe and Cu (Sikwese & Doudu, 2007). As a result of autoxidation especially which occurs with the effect of oxygen in the air, unpleasant taste and smell that are known as the signs of rancidity in oil occur (Özcan & Arslan, 2011). Dietary antioxidant intake may be an important strategy for inhibiting or delaying the oxidation of susceptible cellular substrates, and is thus relevant to disease prevention in many paradigms.

The flower bud of *Syzygium aromaticum* (L.) Merr. & Perry. (Family Myrtaceae), commonly known as clove, is a well known food flavor for exotic food preparations and a popular remedy for dental disorders, respiratory disorders, headache and soar throat in traditional medicines of Australia, and Asian countries (Gurib-Fakim, 2006; Domaracky *et al.*, 2007). Clove oil has been listed as a 'Generally Regarded As Safe' substance by the United States

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Food and Drug Administration when administered at levels not exceeding 1500 ppm in all food categories (Kildeaa *et al.*, 2004). At present, there are many reports about essential oil from clove buds including their wide range of pharmacological and biological activities such as antioxidant (Özcan *et al.*, 2011; Gülçin & Aboul-Enein, 2012), antibacterial (Hemaiswarya *et al.*, 2009), antiviral, anticarcinogenic and antimutagenic, anesthetic, repellent (Chaieb *et al.*, 2007) and antiprotozoal (Machado *et al.*, 2011) effects. In our previous study, our team members have reported the chemical composition and antioxidant activities of the essential oil from clove buds (Zhang, 2015). However, to the best of our knowledge, clove buds oleoresin obtained by organic solvent extraction almost has not been reported. In the present study, the chemical composition and antioxidant activities of oleoresin from the clove buds toward various oxidative stresses are investigated in order to provide a reference for the comprehensive development and utilization of clove buds.

MATERIALS AND METHODS

Plant Materials and Regents: The dried buds of clove were purchased as commercial products from the local market in 2013. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenothiazoline-6-sulfonic acid) diammonium salts (ABTS), and 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH) were from Sigma (United States). Other chemicals used were all of analytical grade.

Extraction of Oleoresin: The dried clove buds were ground, and the oleoresin was extract by adding a certain mount of petroleum ether (ratio of liquid to solid from 4:1) for 30 min. The extraction was carried out at room temperature and in absence of light and repeated three times. Then, the mixtures were filtered, and the supernatants were pooled, and vacuum-evaporated to dryness at 20 ^{o}C. The oleoresin was obtained as a dark brown transparent liquid and had specific clove aroma and stored in tightly closed dark vials at 4 °C until use.

GC-MS Analysis: The analysis of the oleoresin were performed using a Hewlett-Packard 5890 II GC, equipped with a HP-5 MS capillary column (30 m×0.25 mm; film thickness, 25 μm) and a HP 5972 mass selective detector for the separation. The mass selective detector was operated in electron-impact ionization (EI) mode with a mass scan range from *m*/*z* 30 to 500 at 70 eV. Helium was the carrier gas at a flow rate of 1 mL min^{-1}. Injector and MS transfer line temperatures were set at 250 and 200 °C, respectively. The oven temperature was programmed from 40 °C for 2 min, raised to 180 °C at a rate of 3 °C/min, held isothermal for 2 min, and finally raised to 250 °C for 5 min. A sample of 0.1 μL of the oleoresin were injected manually using a 1:50 split ratio. The components were identified by comparing their GC retention indices, NIST mass spectral search program, and mass spectra with publish data.

DPPH Radicals Scavenging Activity Assay: The DPPH radical scavenging activity was determined according to the method of Xu et al. (2012). The scavenging rate on DPPH

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radicals was calculated according to the formula, scavenging rate (%) = [(Ao-A_1)/Ao]×100, where Ao is the absorbance of the control solution, A_1 is the absorbance in the presence of samples in DPPH solution. The scavenging activity of the sample on DPPH radicals was expressed by IC_{50} value. IC_{50} value is the effective concentration at which DPPH radicals are scavenged by 50% and is obtained by interpolation from regression analysis.

ABTS Free Radicals Scavenging Activity Assay: ABTS was incubated with potassium persulfate in order to produce the free radical cation. In brief, ABTS was dissolved in deionized water to make a 7 mmol/L concentration solution. ABTS was produced by mixing ABTS stock solution with 2.45 mmol/L potassium persulfate (final concentration) and the mixture was allowed to stand in the dark at room temperature for 12-16 h before use. In our study, the ABTS solution was diluted with methanol, to an absorbance of 0.70 (±0.02) at 734 nm. After addition of 3.8 mL of diluted ABTS to 100 µL of extracts, the absorbance reading was taken exactly 6 min after initial mixing. The scavenging rate and IC_{50} value were calculated using the equation described for DPPH assay.

Antioxidant Activities in Lard Emulsion: The antioxidative effects of the oleoresin on lipid peroxidation were evaluated according to the method of Kamkar et al. (2010) with some modifications. Each emulsion sample (15 mL) was transferred to a series of capped glass test tubes. Then, oleoresin and BHT were added to the test tubes and put in a dark oven at 70 °C respectively. The stability of emulsion to oxidation was evaluated 5 times over an 11-day period by analyzing the peroxide values (PVs) measured by AOCS cd 8-53 Official Method. POVs (meq of oxygen/kg) was calculated using the following formula: peroxide value = 1000S×N/W. In this formula, S is the volume of sodium thiosulphate solution (blank corrected) in mL; N is the normality of sodium thiosulphate solution (0.02 N) and W is the weight of oleoresin sample (gram).

DNA Damage Protective Effect Assay:

Protection of DNA from oxidative damage of Fe^{2+}. The ability of samples to protect supercoiled pBR322 plasmid DNA against Fe^{2+} and H_2O_2 was estimated with the DNA nicking assay as described by Xu et al. (2012).

Protection of DNA from oxidative damage of AAPH. The ability of samples to protect supercoiled pBR322 plasmid DNA against AAPH was measured following the method described by Zhang and Omaye (2001) with some modifications. To assay inhibition of DNA damage induced by oleoresin, 0.5µg of pBR322 DNA was incubated with various concentrations of compounds and 2 µL of 25 mM AAPH in PBS for 30 min at 37 ^oC. Then the samples were electrophoresed on 0.8% agarose gel containing 0.5 µg/mL ethidium bromide and the DNA was photographed under ultraviolet light.

Determination of Protein Oxidation: Protein oxidation was assayed as described by Xiang et al. (2013) with minor modifications. Briefly, 2 µL 3mg/mL of bovine serum albumin (BSA) in PBS was mixed with 2 µL of 0.5 mM $CuSO_4$ solution and 2 µL of 10 mM H_2O_2 solution

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and inhibited by various concentrations of test samples. After incubation for 2 h at 37 °C, the samples were then assayed with normal SDS–PAGE.

Statistical Analysis: All experiments were conducted three times independently and the data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) and Duncan's multiple range tests were carried out to determine significant differences ($p < 0.05$) between the means.

RESULTS AND DISCUSSION

GC-MS Analysis: The chemical compositions of the oleoresin from clove buds are analyzed by GC-MS, and the results are presented in Table 1. In total, 20 components in oleoresin were identified, representing 95.06% of the total amount. The eugenol (72.85%) was the major component of the oleoresin, followed by eugenyl acetate (10.54%), β-caryophyllene (4.54%), and d-cadinene (3.88%). Similar to our finds, some studies reported that eugenol was the main component of clove essential oil, but its content was different. For example, Tang et al. (2008) identified 19 components in the clove essential oil and the content of eugenol was 62.21 %; Guan et al. (2007) compared clove essential oil extracted with supercritical carbon dioxide and other three extraction methods, and found that there were differences in the components and content of oleoresin of clove buds by various methods, but the content of eugenol was more than 50%; Jirovetz et al. (2006) identified 23 components of clove leaf oil and found that eugenol (76.8%) was the main component, followed by β-caryophyllene (17.4%), α-humulene (2.1%), and eugenyl acetate (1.2%).

Table 1. Chemical composition of oleoresin from clove buds

Compound	Peak area (%)	Compound	Peak area (%)
2-Carene	0.21	Eugenol	72.85
α-Pinene	0.08	β-Caryophyllene	4.54
Eucalyptol	0.29	Globulol	0.16
Methyl salicylate	0.26	(-)-b-Cadinene	0.04
Cinnamaldehyde	0.06	(-)-g- Cadinene	0.08
α-cubebene	0.08	Germacrene	0.74
Anethol	0.12	d-Cadinene	3.88
α-Muurolene	0.01	trans-Squalene	0.12
α-Copaene	0.43	Ledol	0.23
Eugenyl acetate	10.54	Cedrene	0.34

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DPPH Radicals Scavenging Capacity: The scavenging capacity assayed herein on DPPH radicals is shown in Table 2. The IC_{50} values of oleoresin on DPPH radicals was 525.4 μg/mL, and the scavenging rates of oleoresin on DPPH radical increased by 85.4% from 34.1% to 63.23% with the increase of its concentrations, showing a concentration-dependent scavenging of the DPPH radicals at certain concentrations, which was also consistent with the previous reports (Jirovetz *et al.*, 2006; Gülçin *et al.*, 2012).

Table 2. Radicals scavenging capacity of oleoresin

	Scavenging rate (%)at different concentrations (μg/mL)					Regression equation	IC_{50} (μg/mL)
DPPH	100	250	500	750	1000	y=0.6332x+0.1673 R^2=0.9944	525.4±2.7
	34.10	38.72	49.02	58.61	63.23		
ABTS	10	25	50	75	100	y=5.2821x+0.0462 R^2=0.9927	85.9±0.7
	21.40	27.29	31.32	38.29	57.98		

Values represent means of three independent replicates ± SD. R^2 refer to the regression coefficients. Different letters within a row indicate statistically significant differences between the means ($p < 0.05$) for different concentrations oleoresin.

ABTS Radicals Scavenging Capacity: The scavenging capacity of oleoresin from clove buds on ABTS radicals is shown in Table 2. The profile of scavenging capacity of oleoresin on ABTS was similar to the result of the scavenging capacity on DPPH radicals. Somewhat differently, the IC_{50} value on scavenging ABTS radicals was 85.9 μg/mL. Similarly, ABTS radicals scavenging capacity of oleoresin increased dose-dependently at concentrations ranging from 10 to 100 μg/mL, which may be attributable to its hydrogen-donating ability. These differences in data between DPPH and ABTS assays were likely due to different experimental conditions.

Antioxidant Activities in Lard Emulsion: Figure 1 showed the changes in POVs during the storage of lard emulsion over an 11-day period in the presence of various concentrations of oleoresin from clove buds. As shown in Figure 1, the POVs of lard emulsion increased gradually within 2 days after treatment. Thereafter, the POVs of control significantly and rapidly increased, and were much higher than the treated samples. These results indicated that the oleoresin from clove buds had inhibiting lipid peroxidation reaction, and the antioxidative effects depend on its concentration and action period.

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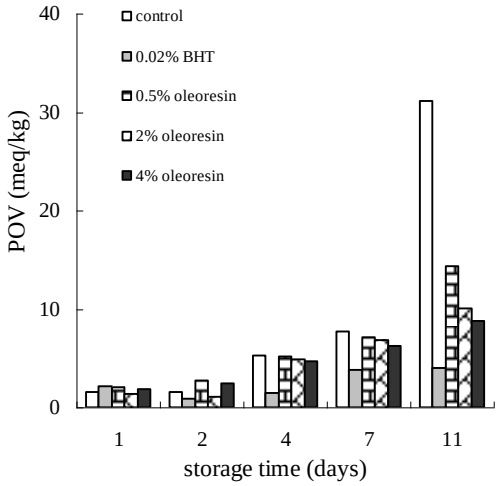

Figure 1. Effects of oleoresin and BHT on POV of lard emulsion. Data are expressed as the mean values of three independent replicates ± SD. Different letters indicate statistically significant differences between the means ($p < 0.05$) for different treatments at the same treatment time.

Protection of DNA Damage: To assay the potential of the oleoresin to prevent DNA damage, oxidative DNA strand breakage induced by AAPH and Fe^{2+} were measured with pBR322 DNA. As shown in Figure 2, the damage of plasmid DNA produces a relaxed open circular form and further a linear double-stranded DNA molecule. The plasmid DNA was mainly of the supercoiled form in the absence of Fe^{2+} and H_2O_2 (Figure 2, Lane 1). During the addition of Fe^{2+} and H_2O_2, the supercoiled form of DNA converted into the open circular and linear forms (Figure 2, Lane 2). From the gel analysis, the protection effects against DNA damage offered against DNA damage by oleoresin was concentration dependent, but a lower concentration of oleoresin did not seem to have the protection effects (Figure 2, Lane 3, 4). The results indicated that the oleoresin might prevent the reaction of Fe ions with H_2O_2, on the other hand, and it probably quenched hydroxy radicals by donating hydrogen-atom or electron, and therefore protecting the supercoiled plasmid DNA from hydroxy radicals dependent strand breaks (Singh *et al.*, 2009).

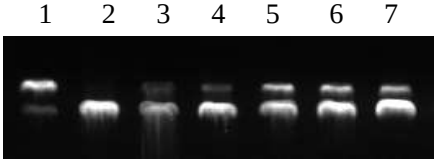

Figure 2. The protective effect of the oleoresin to DNA damage caused by Fe^{2+}. Lane 1, the native DNA; Lane 2, the DNA treated with 1 mM $FeSO_4$ and 1 mM H_2O_2; Lane 3-7, the DNA treated with 15.62, 31.25, 62.5, 125, and 250 mg/mL oleoresin, respectively.

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As shown in Figure 3, similar results were found in protective effect assay of DNA from oxidative damage of AAPH. The protection offered against DNA damage by oleoresin was also concentration dependent, and the oleoresin exhibited a stronger protective effect against DNA damage caused by AAPH with a lower completely prevented concentration of 3.9 mg/mL (Figure 3, lane 3). The differences in protective effect of oleoresin against DNA damage caused by Fe^{2+} and AAPH may come from different determination method (Dizdaroglu *et al.*, 2002).

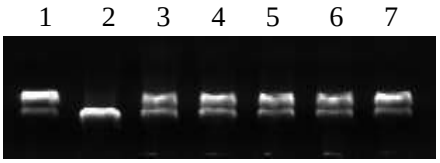

Figure 3. The protective effect of the oleoresin to DNA damage caused by AAPH. Lane 1, the native DNA; Lane 2, the DNA treated with AAPH; Lane 3-7, the DNA treated with 3.9, 7.81, 15.62, 31.25, and 62.5 mg/mL oleoresin, respectively.

Inhibition of Protein Oxidation: The protection effect against protein oxidative damage was determined by the oxidation of BSA initiated by Cu^{2+} and the results are shown in Figure 4. The results showed not only non-protective effect against oxidation of BSA but also a prooxidant effect, which was augmented by increasing concentrations of the oleoresin. Similar to our findings, Xiang et al. (2013) reported that carnosic acid had the potential to prevent BSA oxidant damage in low concentrations but showed a prooxidant effect against BSA when the concentrations of carnosic acid were higher and the effect was concentration related. Some researches (Hayakawa *et al.*, 2004; Halliwell, 2008; Wu *et al.*, 2014) reported that polyphenol have prooxidant effect with the existence of mental ions. Nakagawa et al. (2007) suggested that Cu(II) ions could convert (+)-catechin from an antioxidant to a prooxidant in protein oxidation. According to our results of chemical composition analysis, eugenol that was the major composition of the oleoresin, as a phenolic compound, may be the most possible reason of producing prooxidant effect.

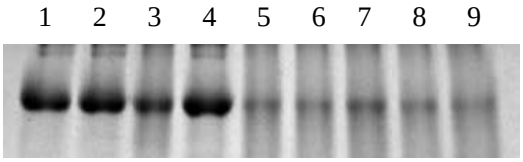

Figure 4. The inhibitions of the oleoresin against BSA oxidative damage. Lane1, the native BSA; lane 2, the BSA and ethanol; lane 3, the BSA treated with 0.5 mM Cu^{2+}; lane 4, the BSA and oleoresin; lane 5-9, the BSA treated with 0.5 mM Cu^{2+} and 0.625, 1.25, 2.5, 5, 10 mg/mL oleoresin, respectively.

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CONCLUSIONS

In conclusion, this work showed that eugenol was the main component in oleoresin from clove buds, and the oleoresin exhibited different antioxidant activities in vitro. The oleoresin from clove buds had the better scavenging capacity against DPPH and ABTS radicals, and inhibiting lipid peroxidation reaction in lard emulsion during storage, as well as it exhibited different protection effects against DNA damage caused by Fe^{2+} and AAPH. And all the antioxidant activities of oleoresin from clove buds showed a dose-response relationship to some degree. However, the oleoresin had a prooxidant effect on protein oxidative damage.

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