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Evaluation of antioxidant potential of clove extracts

Sneha Shende
College of Dairy Technology, Warud (Pusad), Maharashtra 445204
Email: snehashendecdtw@gmail.com

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Abstract

The antioxidant activity of steam distilled clove extract and clove oleoresin was assessed using β-carotene–linoleic acid model system, and was found to be 85.51±0.19% and 77.88±0.15%, respectively. DPPH free radical-scavenging activity of steam distilled extract and oleoresin of clove were 88.93±0.23% and 80.84±0.36%, respectively. Eugenol content in steam distilled extract of clove (0.518±0.005mg/ml) was significantly higher than that of clove oleoresin (0.433±0.007mg/ml). Eugenol content was also affected by time period of refluxing. Highest recovery of steam distilled clove extract was obtained by refluxing with 90 % ethanol for 4 hr (0.763±0.007). The steam distilled clove extract was found to possess higher antioxidant activity than the oleoresin.

Keywords: Clove extracts, steam distilled, eugenol, oleoresin

Clove (*Syzygium aromaticum*) is widely cultivated in India, Madagascar, Sri Lanka, Indonesia and southern China (Bhuiyan *et al.,* 2010). Clove oil is obtained by distillation of the flowers, stems or leaves of the clove plant (Anderson *et al.,* 1997; Mylonasa *et al.,* 2005). Clove oil has been listed as a ''GRAS'' (Generally Regarded as Safe) substance by the USFDA (United States Food and Drug Administration) at levels not exceeding 1500 ppm in all food categories (Kildeaa *et al.,* 2004). Additionally, the World Health Organisation (WHO) Expert Committee on Food Additives has established the acceptable daily intake of clove oil at 2.5 mg/kg body weight for humans (Nagababu and Lakshmaiah, 1992; Anderson *et al.,* 1997). It is considered as safe, effective, and relatively inexpensive (Kildeaa *et al.,* 2004). Its active ingredient is eugenol (4-allyl-2-methoxyphenol) which makes up 70 to 90% by weight. Besides this, eugenol acetate (>

17%) and β caryophyllene (> 12%) are also present in clove extract. Eugenol is used in a variety of different applications, *e.g.* as an antioxidant and antimicrobial (Rajakumar and Rao, 1993). They have been recognized to have medicinal properties and beneficial impact on health, *viz.,* digestive stimulation action, anti-inflammatory, antimicrobial, anticarcinogenic potential, hypolipidemic, antimutagenic effects (Aaby *et al.,* 2004). Clove has been reported to have several therapeutic effects, including anti-vomiting, analgesic, antispasmodic, anti-carminative, kidney reinforcement and antiseptic effect (Liu *et al.,* 1997). Lee and Shibamoto (2001), reported that it might also be used as an anti-carcinogenic agent due to its antioxidant properties and suggested as a potential chemo preventative agent. It is enriched with minerals such as calcium, iron, phosphorus, sodium, potassium, vitamin A and vitamin C. In addition, humans have used clove oil for centuries, as an anesthetics for toothaches, headaches and joint pain (Shelef, 1983; Soto and Burhanuddin, 1995). It is used throughout the world for applications ranging from food flavouring to local anesthesia in dentistry profession (Anderson *et al.,* 1997).

Considering the great interest in nutraceuticals and their dietary contribution to human health, it is important to evaluate the antioxidant capacities of widely consumed clove extract. The main objective of the present study was to evaluate the antioxidant activities of steam distilled clove extract and clove oleoresin and their eugenol content which is the source of antioxidant activity. The antioxidant activities of steam distilled extract and clove oleoresin, were investigated using three antioxidant assays. We also investigated the possibility of antioxidant activities of the eugenol present in steam distilled clove extract and clove oleoresin by RP-HPLC and evaluated the contribution of eugenol in the antioxidant capacity of clove extracts.

β-Carotene, linoleic acid, 2,2-diphenyl-1-picrylhydrazyl hydrate free radical (DPPH), Folin–Ciocalteu's reagent and Tween-40 (Sigma Chemical Co., USA). organic solvents, namely, chloroform and gallic acid (Loba Chemie Pvt. Ltd., Mumbai, India) and ethyl acetate (RFCL Ltd, New Delhi, India) were used. Sodium carbonate was obtained from Qualigens fine chemicals, Mumbai, India.Vacuum pump (Millipore, India), The reverse phase HPLC system (Waters, 515, Singapore), C18 column (5 μm, 4.5 × 250 mm, 100 A^0, Phenomener, USA), and filtration assembly with Millipore 0.45 μm filter for filtering all reagents, water, syringe filter 0.45 μm for sample filtration were used in the study. The clove oleoresin was procured from Synthite Industry Ltd., Kerala, India and steam distilled extract of clove was provided by Katyani Exports, Delhi, India.

Antioxidant capacity was determined by total phenolic content, β carotene linoleic acid model system and radical scavenging assays belonging to different mechanisms because multiple reactions are involved in clove extract (Prior *et al*., 2005). Total phenolic content of clove oleoresin and steam distilled clove extract was analyzed by Folin-Ciocalteu's method (Kahkonen *et al.,* 1999). Quantification was done with respect to the

standard curve of gallic acid (400 µl of 10-100 µg/ml). The results were expressed as gallic acid equivalents (GAE), mg per g of spice extract.

The antioxidant activity of solvent extracts was determined according to the procedure of Marco (1968), with minor modification. The activity was measured spectrophotometrically at 470 nm (Specord, 700). The antioxidant activity (AA) of the extracts was calculated using the following expression in terms of photo-oxidation of β - carotene.

$$AA = 100\left[1 - \frac{A_0 - A_t}{AC_0 - AC_t}\right]\%$$

Where, AA is the antioxidant activity, A_o is the initial absorbance of sample, A_t is the absorbance of sample after time t, AC_o is the initial absorbance of control, AC_t is the absorbance of control after time t.

Radical-scavenging activity by DPPH model system

The steam distilled extract and clove oleoresin (200 ppm) were dissolved in ethanol and evaluated for their radical scavenging activity in the DPPH system according to the procedure of Blois (1958). The optical densities (OD) of the samples were measured spectrophotometrically at 517 nm (Specord 700). Radical-scavenging activity was expressed as % inhibition and was calculated using the following formula

$$\% \text{ Radical scavenging activity} = \frac{\text{Control OD-Sample OD}}{\text{Control OD}} \times 100$$

Extraction and quantification of eugenol from clove extract

Clove extracts (steam distilled and clove oleoresin, 30 g each) were taken in round bottom flask and refluxed with 30 ml of ethanol. The sample was refluxed for different time interval from 2 to 5 hr using water reflux condenser. After refluxing, evaporation of the mixture was carried out on water bath till dryness. The residue obtained was re-dissolved with 30 ml ethanol and filtered through PTFE (polytetrafluoroethylene) syringe filter (0.45 µm) and 20 µL was directly injected into HPLC system. Quantification was done with respect to the standard curve of eugenol (0.25 -1.5 mg/10 ml) in ethanol. The analysis was performed in C18 column (5 µm, 4.5×250 mm, 100 A^0) with isocratic flow of mobile phase (methanol and water in the ratio 60:40 v/v) at 280 nm. The flow rate was maintained at 0.8 ml/min, and column temperature was set at 30^0C.

Total phenols

Total phenolic content of steam distilled clove extracts and clove oleoresin varied widely and the results are presented in Table 1. The total phenolic content of oleoresin and steam distilled clove extract were found to be 177.039±0.35 mg and 256.506±0.45 mg GAE/g, respectively. Steam distilled clove extract showed significantly higher ($P< 0.05$) amount of phenolic compounds as compared to its oleoresin counterpart. This difference was likely to be due to the presence of different hydrophilic and lipophilic (flavonoids, terpenoids, carotenoids, phytoestrogens) antioxidant compounds in various extracts. Hydrophilic and lipophilic antioxidant activity of loquat fruits were evaluated by Zhou *et al.* (2011) and they reported that the

phenolic content and total antioxidant activity were positively related with hydrophilic antioxidant compounds. Similar results were reported by Vicas *et al.* (2009) where the antioxidant capacity was measured by FRAP (ferric tripyridyltriazine complex) method. They reported that lipophilic antioxidant activity (LAA) of mistletoe (*Visum album*) was about 100 times lower as compared to hydrophilic antioxidant activity (HAA). HAA was also positively correlated with total phenolic concentration from leaves (R^2=0.9363) and stems (R^2= 7337) of mistletoe (*Visum album*). Arnao *et al.,* (2011) reported that oleoresins are hydrophobic in nature and contain lypophillic compounds including various terpenoids. According to Shan *et al.,* (2005) the major phenolic compounds of clove bud are phenolic acids (gallic acid), flavonolglucosides, tannin and phenolic volatile oils (eugenol and acetyl eugenol). Clove extract obtained by steam distillation, contained higher amount of phenolic compound. It was also dependent upon type of extraction method and solubility of oil components (Reverchon,1997; Mostafa *et al.,* 2004). High phenolic content of steam distilled extract of *Rosa damascena* flower was reported by Ozkan *et al.,* (2012). The phenolic compounds depend on the number of phenolic groups; and respond differently to the Folin-Ciocalteu reagent (Singleton *et al.,* 1999). Polyphenolic compounds are commonly found in both edible and inedible plants and they have been reported to have multiple biological effects, including antioxidant activities (Kahkonen *et al.,* 1999).

Antioxidant activity

The antioxidant activity of clove extract

(steam distilled and oleoresin) was evaluated at 200 ppm using the β-carotene–linoleic acid coupled oxidation model system (Marco, 1968) and the results are presented in Table 1. The antioxidant activity of steam distilled clove extract (84.950±0.23%) was significantly ($P < 0.05$) higher than that exhibited by oleoresin (77.886±0.31%) at a concentration of 200 ppm. This difference may be attributed to the difference in total phenolic content of the steam distilled clove extract and clove oleoresin which is dependent on extraction method and solubility of the components. This is in agreement with findings of Mostafa *et al.,* (2004) who reported super critical fluid (SFE) extraction enhances the yield of phenolic compounds due to increase in the solubility of the oil components.

The mechanism of bleaching of β-carotene is a free radical-mediated phenomenon, resulting from the hydro peroxide formed from linoleic acid. In this model system, β-carotene undergoes rapid discoloration in the absence of an antioxidant. The linoleic acid free radical formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups attacks the highly unsaturated β-carotene molecules (Jayaprakasha *et al.,* 2001). As β-carotene molecules lose their double bonds by oxidation and the compound loses its chromophore and characteristic orange colour, which was monitored spectrophotometrically. In the present study, it was observed that, presence of different

isolates of clove could hinder the extent of β-carotene bleaching by neutralizing the linoleate free radical and other free radicals formed in the system.

Table 1. Total phenolic content, antioxidant activity using β carotene LA model and Radical-scavenging activities of steam distilled and clove oleoresin

Sample	Total phenols (mg of GAE/g)	Antioxidant activity	Radical scavenging activity (% inhibition)
Clove oleoresin	177.039±0.35[b]	77.886±0.15[b]	80.841±0.36[b]
Steam distilled clove extract	256.506±0.45[a]	85.510±0.19[a]	88.935±0.23[a]

Data are presented as means ± SEM (n=3). a-b Means with different superscript alphabets are significantly different ($P < 0.05$).

Radical-scavenging activity by DPPH assay

The radical-scavenging activity of clove extracts (steam distilled and oleoresin at 200 ppm) was evaluated in the DPPH system and the results are presented in Table1. The radical-scavenging activity of steam distilled clove extract and its oleoresin was found to be 88.935±0.23% and 80.841±0.36%, respectively. Results revealed that, radical-scavenging potential of steam distilled clove extract was significantly ($P<0.05$) higher than its oleoresin counterpart. This difference between radical-scavenging (antioxidant) activity might be due to various hydrophilic and lipophilic antioxidant compounds. Another reason may be due to difference in total phenolic content of the steam distilled clove extract and clove oleoresin. The antioxidant activity of phenol was mainly due to their redox properties, which allowed them to act as reducing agents, hydrogen donators and singlet oxygen quenchers. In addition, they have a potential metal chelation activity (Rice *et al.,* 1995). They exhibited antioxidant activity by inactivating lipid free radicals or preventing decomposition of hydro peroxide into free radicals (Pokorny *et al.,* 2001; Pitchaon *et al.,* 2007). Our results indicate a positive correlation between phenolic content and antioxidant activity, as the higher activity of the steam distilled clove extract could be attributed to the higher content of phenolic compounds.

Eugenol content

Two different solvents i.e. methanol (absolute) and ethanol (95%) were used for extraction of eugenol content from steam distilled clove extract and clove oleoresin. The results revealed that ethanol yield higher eugenol than the extraction using methanol. Therefore, ethanol was preferred as extraction solvent for determination of eugenol. Also for industrial purposes ethanol is probably better than methanol as eventual solvent residues would be less toxic. Eugenol content of oleoresin and steam distilled clove extract was calculated using standard curve of eugenol (ranging from 0.25-1.5 ppm). The retention time for eugenol was observed as 13.904±0.5 min. It was observed that, eugenol

content increased with increasing refluxing time from 2 h to 4 h, while increasing the refluxing time to 5 h, decreased the eugenol content. The quantitative HPLC result showed that, 4 h extraction time had the highest eugenol content in the steam distilled clove extract (0.763±0.007) than its clove oleoresin (0.635±0.020) as shown in Fig. 2. Besides extraction solvent and the method, environment factor may also account for this difference in activity of the extract. Selected solvents such as 95% ethanol was used because it was compatible for separation of eugenol by using reversed-phase HPLC.

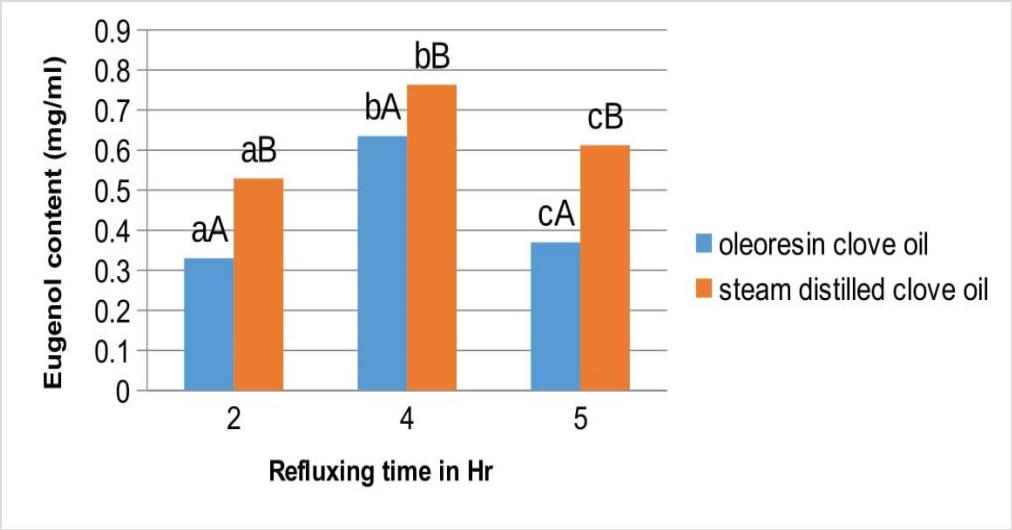

Fig. 1. Eugenol content as influenced by refluxing time of clove extracts (steam distilled and oleoresin)

The mean changes between the samples were analyzed by one-way ANOVA. a-b Means with different lowercase superscripts letters are significantly different ($P < 0.05$) from each other. A-B Means with different uppercase superscripts letters are significantly different ($P < 0.05$) from each other.

In conclusion, the phenolic content in steam distilled clove extract was significantly higher than its clove oleoresin. Positive correlation was observed between total phenolic content, antioxidant potential and free radical-scavenging activity for the clove oleoresin as well as steam distilled extract of clove**.** The eugenol content was found to be significantly higher in steam distilled clove extract as compared to its oleoresin counterparts.

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