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Extraction of Eugenol in Clove Extract and Evaluation of its Antioxidant Activity

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Abstract— *Higher total phenolic content was observed in steam-distilled clove extract compared to its oleoresin. Ensuring antioxidant activity of steam distilled and clove oleoresin using β-carotene–linoleic acid model system was found to be 85.51±0.19% and 77.88±0.15%, respectively, at a level of 200 ppm. The radical-scavenging activity of steam-distilled extract and oleoresin of clove were 88.93±0.23% and 80.84±0.36%, respectively, at the same level of 200ppm by using the DPPH method. Eugenol content in the steam-distilled extract of clove (0.518±0.005mg/ml) was significantly higher than that of clove oleoresin (0.433±0.007mg/ml). Recovery of eugenol content affected by the period of refluxing and clove extract refluxed for 4 hr found to be highest recovery in the steam distilled clove extract (0.763±0.007) than its clove oleoresin (0.635±0.020) with 13.904 min retention time using RP-HPLC. The steam-distilled clove extract was found to have the highest antioxidant activity than its oleoresin counterparts.*

Keywords— ***Antioxidant, β-carotene–linoleic acid, DPPH, eugenol, phenolic content.***

I. INTRODUCTION

India, Madagascar, Sri Lanka, Indonesia, and southern China grow clove (Syzygium aromaticum) (Bhuiyan et al., 2010). Distilling clove flowers, stems, or leaves yields clove oil (Anderson et al., 1997; Mylonasa et al., 2005). The USFDA lists clove oil as "GRAS" (Generally Regarded As Safe) at no more than 1500 ppm in all food categories (Kildeaa et al., 2004). The WHO Expert Committee on Food Additives recommends 2.5 mg/kg body weight of clove oil per day. Kildeaa et al., (2004) found it safe, efficacious, and affordable. Clove extract contains eugenol (4-allyl-2-methoxyphenol), which accounts for 70-90% of its weight (Keene et al., 1998), as well as eugenol acetate (17%) and β caryophylene (12%). Eugenol has various applications, including antioxidant and antibacterial properties (Rajakumar and Rao, 1993). It has more therapeutic features such as digestive stimulation, anti-inflammatory, antibacterial, anticarcinogenic, hypolipidemic, and antimutagenic actions (Aaby et al., 2004). In addition, it possesses anti-vomiting, analgesic, antispasmodic, anti-carminative, kidney-reinforcing, and antiseptic (Liu et al., 1997). It may prevent cancer due to its antioxidant characteristics, according to Lee and Shibamoto (2001). It contains calcium, iron, phosphorus, sodium, potassium, vitamin A, and vitamin C. For decades, clove oil has been used to treat toothaches, headaches, and joint discomfort (Shelef, 1983; Soto and Burhanuddin, 1995). It is used worldwide for food flavouring and dental local anaesthetic (Anderson et al., 1997).

Given the popularity of nutraceuticals and their nutritional benefits, clove extract's antioxidant properties should be assessed. The present work examined the antioxidant activity of steam-distilled clove extract, clove oleoresin, and their antioxidant component, eugenol. Three antioxidant experiments examined steam-distilled and

clove oleoresin antioxidant activity. We also examined the antioxidant activity of the major eugenol in steam-distilled clove extract and clove oleoresin using RP-HPLC.

II. MATERIALS AND METHODS

2.1 Clove Extract:

Clove oleoresin was obtained from Synthite Industry Ltd. in Kerala, India, while the steam-distilled extract was provided by Katyani Exports in Delhi, India.

2.2 Determination of antioxidant capacity: To assess antioxidant potential, total phenolic content, β carotene linoleic acid model system, and radical scavenging assays were used (Prior et al., 2005).

2.3 Total phenolic content

Folin-Ciocalteu's technique was used to determine the total phenolic content of clove oleoresin and steam distilled clove extract (Kahkonen et al., 1999). In a test tube, 400 µl of adequately diluted sample/gallic acid standard was placed. It was combined using a vortex mixer after adding 2000 µl of diluted Folin-Ciocalteu's reagent. After 3 minutes, 1600 µl of sodium carbonate solution was added and incubated at room temperature for 30 minutes in the dark. Instead of sample, 400 µl of distilled water was used for blank preparation. Using a Spectrophotometer (Specord 700), the absorbance of the samples was measured against a blank at 765nm. The concentration of gallic acid (400 µl of 10-100 g/ml) was collected in place of the sample for standard curve preparation and quantified with regard to the standard curve. The outcomes were expressed as gallic acid equivalents (GAE), milligrams per gm of spice extract.

2.4 β-carotene–linoleic acid model system

The antioxidant activity of solvent extracts was evaluated using the procedure described by Marco (1968), with slight modifications. β-carotene (0.2 mg) in chloroform (0.5 ml) was mixed with 20 mg linoleic acid and 200 mg Tween 40. Nitrogen gas was used to evaporate the chloroform at 40°C. The resultant solution was immediately diluted with 10 ml of double distilled water, and the emulsion was thoroughly stirred for one minute using a magnetic stirrer. The emulsion was diluted further with 40 mL of distilled water. Aliquots (4 mL) of this reagent were placed into separate stopper test tubes holding 1 mL of the necessary amount of ethanol-based sample extracts. At a rate of 200 ppm, the steam-distilled and oleoresin of clove extract dissolved in ethanol. A control was created using 1 ml of ethanol and 4 ml of emulsion. Optical densities of all samples were tested immediately (t = 0), again after 15 minutes, then every 30 minutes for the next 3 hours (t = 180). In between observations, the tubes were submerged in a water bath set at 50°C. After that, it was independently added to a model system of β-carotene linoleic acid, and the activity was measured spectrophotometrically 700 (Specord) at 470 nm. The following expression was used to assess the extracts antioxidant activity (AA) in terms of the photo-oxidation of beta-carotene. AA is represented by the antioxidant activity, A_o is the initial absorbance of sample, A_t is the absorbance of sample after time t, $A0_o$ is the initial absorbance of control, and $A0_t$ is the absorbance of control after time t respectively.

$$AA = 100\left[1 - \frac{A0 - At}{A00 - A0t}\right]\%$$

2.5 Radical-scavenging activity by DPPH model system

According to the method described by Blois (1958), the extracts of steam-distilled and oleoresin clove were taken at a rate of 200 ppm, dissolved in ethanol, and tested for their capacity to scavenge free radicals in the DPPH system. Test tubes containing 200 ppm of sample extract diluted in ethanol (1 ml) were then filled with 4 ml of a 0.1 mM ethyl acetate solution of DPPH and shaken vigorously. Tubes were left at 27°C for 20 min. Without any additional extract, the control was made as described above, and ethanol was used to adjust the baseline. Optical densities (OD) of the samples were measured spectrophotometrically (Specord 700) at 517 nm. Radical-scavenging activity was expressed as % inhibition percentage.

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

2.6 Extraction and quantification of eugenol from clove extract

The preparation of the sample is the first and most important step. It is necessary to extract the appropriate chemical components from the spice materials in order to proceed with further separation and characterization. 30 mg of clove extracts (steam distilled and oleoresin clove) was taken in a round-bottom flask, 30 ml of ethanol was added to the flask and allowed to reflux. Using a water reflux condenser, the sample was allowed to reflux for varying amounts of time ranging from 2 to 5 hours. Following the process of refluxing, the mixture was evaporated on a water bath until it reached the desired level of dryness. After re-dissolving the resulting solution in 30 mL of ethanol, it was filtered using a PTFE (Polytetrafluoroethylene) syringe filter with a 0.45-m pore size and then immediately injected into an HPLC system in a volume of 20 µL. Quantification was performed based on the standard curve of eugenol concentration in ethanol, which ranged from 0.25 to 1.5 mg/10 ml. At a wavelength

of 280 nm, the analysis was carried out using a C18 column (5 µm, 4.5×250mm, 100 A^0). The mobile phase consisted of methanol and water at a volumetric ratio of 60:40. The temperature of the column was maintained at 30^0C and the flow rate was held constant at 0.8ml/min.

III. RESULTS AND DISCUSSION

3.1 Total Phenolic Content by Folin-Ciocalteu method

The Folin–Ciocalteu method (Kahkone et al., 1999) was used to measure the total amount of phenolic substance. A standard curve of gallic acid (ranging from 10 to 100 µg/ml) was prepared to evaluate the overall phenolic content of oleoresin and steam-distilled clove extracts and results were expressed as mg gallic acid equivalents (GAE) per gram of spice extract. Table 1 displayed the results that oleoresin had a GAE of 177.039±0.35mg and the steam-distilled clove extract had a GAE of 256.506±0.45mg/gm respectively. The reason for this difference was probably because different extracts contained different antioxidant chemicals that bind to water and fat (flavonoids, terpenoids, carotenoids, phytoestrogens). Zhou et al. (2011) looked at the hyrophillic and lipophillic antioxidant activity of loquat fruits and found that the overall antioxidant activity and phenolic content were linked to hydrophilic antioxidant compounds in a good way. Vicas et al. (2009) found similar results by using FRAP (ferric tripyridyltriazine complex) of mistletoe (Visum album) had about 100 times less lipophillic antioxidant activity (LAA) than hydrophilic antioxidant activity (HAA). Also, HAA was linked to higher levels of total phenolics in the leaves (R2=0.9363) and stems (R2=7337) of mistletoe (Visum album). In 2005, Shan et al. said that clove bud's main phenolic substances are phenolic acids (gallic acid), flavonolglucosides, tannin, and phenolic volatile oils (eugenol and acetyl eugenol).The phenolic chemical content of clove extract that was made by steam distillation was higher. It also depends on the method used to separate the oil and how well the different parts of the oil dissolve (Reverchon, 1997; Mostafa et al., 2004). A study by Ozka et al. (2012) found that a steam-distilled extract of the Rosa damascena flower had a high phenolic content.

Table 1. Total phenolic content of steam distilled and clove oleoresin

Samples	Total phenolic content (mg of GAE/gm)
Clove Oleoresin	177.039±0.35[b]
Steam distilled	256.506±0.45[a]

Data are presented as means ± SEM (n=3).

a-bMeans with different lowercase superscripts letters are significantly different (P < 0.05) from each other.

3.2 Antioxidant activity by β-carotene–linoleic acid model system

Table 2 shows the antioxidant activity of clove extract (steam distilled and oleoresin) at 200 ppm using β-carotene-linoleic acid linked oxidation model system (Marco, 1968). At a concentration of 200 ppm, the antioxidant activity of steam distilled clove extract (84.950±0.23%) was substantially (P>0.05) higher than that of oleoresin (77.886±0.31%). This difference can be attributed to the difference in total phenolic content of steam distilled clove extract and clove oleoresin, which is dependent on extraction method and solubility of oil components. This is consistent with the findings of Mostafa et al., (2004), who found that super critical fluid (SFE) extraction increases the yield of phenolic compounds due to an increase in the solubility of oil components.

Table 2. Antioxidant activity of steam distilled and clove oleoresin

Samples	Antioxidant activities (%) at 200ppm
Clove oleoresin	77.886±0.15[b]
Steam distilled clove extract	85.510±0.19[a]

Data are presented as means ± SEM (n=3).

a-bMeans with different lowercase superscripts letters are significantly different (P < 0.05) from each other.

3.3 Radical-scavenging activity by DPPH assay

The radical-scavenging activity of clove extracts (steam distilled and oleoresin) was tested in the DPPH system at 200 ppm and the findings are shown in Table 3. Steam distilled clove extract and its oleoresin were shown to have radical-scavenging activities of 88.935±0.23% and 80.841±0.36%, respectively. The radical-scavenging potential of steam distilled clove extract was found to be considerably (P>0.05) higher than that of its oleoresin counterpart. This disparity in radical-scavenging (antioxidant) activity could be attributed to differing hydrophilic and lipophillic antioxidant chemicals. Another reason could be that the total phenolic content of steam distilled clove extract and clove oleoresin differs. The results of the radical-scavenging (antioxidant) assay were similar with the data obtained from the total phenolic

content and the β-carotene-linoleic acid model system. Our findings suggested that there was a positive relationship between phenolic content and antioxidant activity, since the higher activity of the steam distilled clove extract may be related to its higher phenolic content.

Table 3: Radical-scavenging activities of clove extracts (steam distilled and oleoresin) as expressed in % inhibition

Samples	% Inhibition
Clove oleoresin	80.841±0.36[b]
Steam distilled clove extract	88.935±0.23[a]

Data are presented as means ± SEM (n=3).

a-bMeans with different lowercase superscripts letters are significantly different (P < 0.05) from each other.

3.4 Quantitative analysis of eugenol content in clove extracts (oleoresin and steam distilled) by RP-HPLC.

Eugenol (4-allyl-2-methoxyphenol), a well-known clove phenolic component, has numerous therapeutic effects, including antioxidant action. Eugenol was measured using a modified HPLC method established by Yun et al., (2010). The extraction process was optimized for quantitative analysis to improve the sensitivity and selectivity for separation of eugenol content.

3.4.1 Optimization of eugenol extraction using two different solvent

The extraction technique of eugenol content was standardized, and the efficiency of several extraction solvents from clove extracts (steam distilled and oleoresin) were assessed in order to get chromatograms with superior resolution of neighboring peaks in a short period. The eugenol content of steam distilled clove extract and clove oleoresin was extracted using two different solvents, methanol (absolute) and ethanol (95%). The results showed that ethanol extraction yielded more eugenol than methanol extraction. As a result, ethanol was chosen as the extraction solvent for determining eugenol. Also, for industrial uses, ethanol is probably preferable to methanol since the resulting solvent residues are less hazardous. The results are shown in Fig 1.

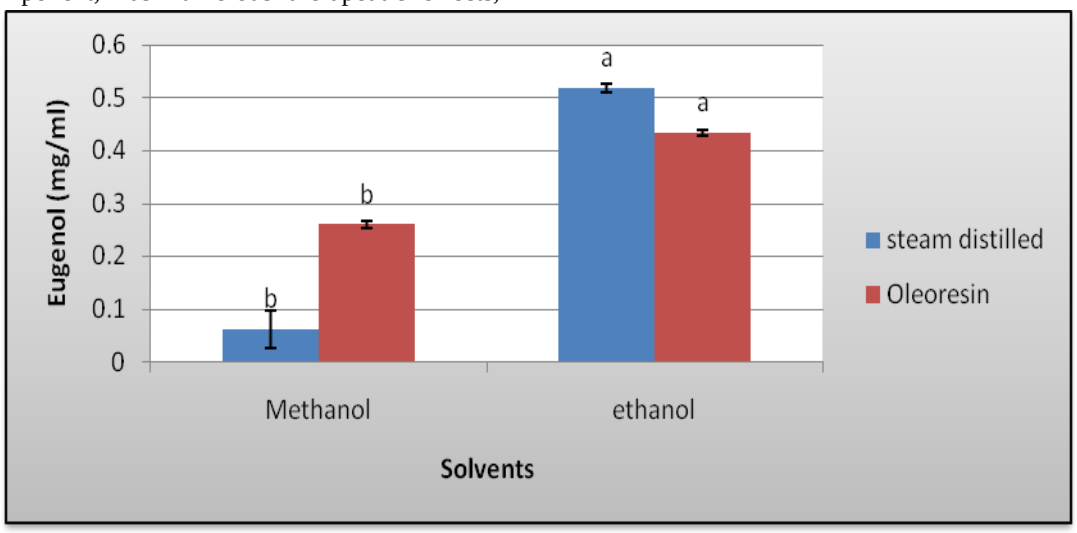
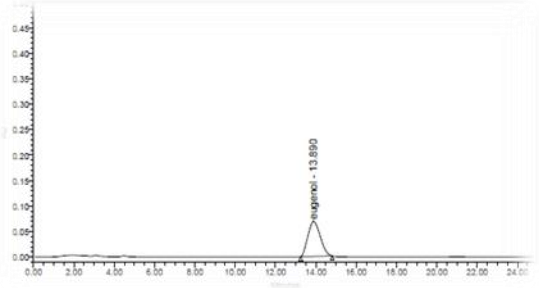
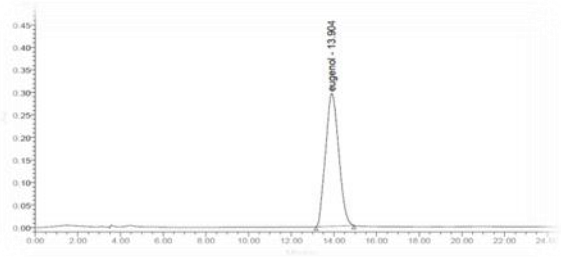


Fig.1: Two different solvent used to estimate eugenol content from clove extracts (oleoresin and steam distilled) as expressed in (mg/ml)

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. Error bars show the variations of three determinations in terms of standard error of mean.



Eugenol obtained by using Methanol



Eugenol obtained by using ethanol

3.4.2 Optimization of eugenol extraction using two different solvent different time interval of refluxing period

To establish the ideal extraction time for eugenol content, several refluxing times at different intervals such as 2 hr, 4 hr, and 5 hr were tried in terms of eugenol content extraction. The steam distilled clove extract and oleoresin were refluxed with each solvent for 2 to 5 hours using a water reflux condenser. Following the refluxing, the samples were evaporated on a water bath until dry. The resulting solution was redissolved with the solvent and filtered through a 0.45m PTFE syringe filter before being injected directly into the HPLC apparatus. The analysis was carried out at a flow rate of 0.8ml/min at a column temperature of 30°C. The eugenol concentration of oleoresin and steam distilled clove extract was determined using a eugenol standard curve (range from 0.25 to 1.5 ppm). Eugenol had a retention time of 13.904 ± 0.5 minutes. It was discovered that raising the refluxing time from 2 hours to 4 hours increased the eugenol content, whereas increasing the refluxing time to 5 hours lowered the eugenol content. As shown in fig 2, the quantitative HPLC results demonstrate that the 4 hr extraction time has the highest value of eugenol concentration in the steam distilled clove extract (0.763±0.007) than its clove oleoresin (0.635±0.020). Aside from the solvent and method of extraction, environmental factors and different species may also account for the variance in extract activity. Fig 2 shows that the eugenol concentration of steam distilled clove extract was found to be higher than that of its oleoresin counterparts. Selected solvents, such as 95% ethanol, were employed because they were stable and compatible with reversed-phase HPLC separation of eugenol content.

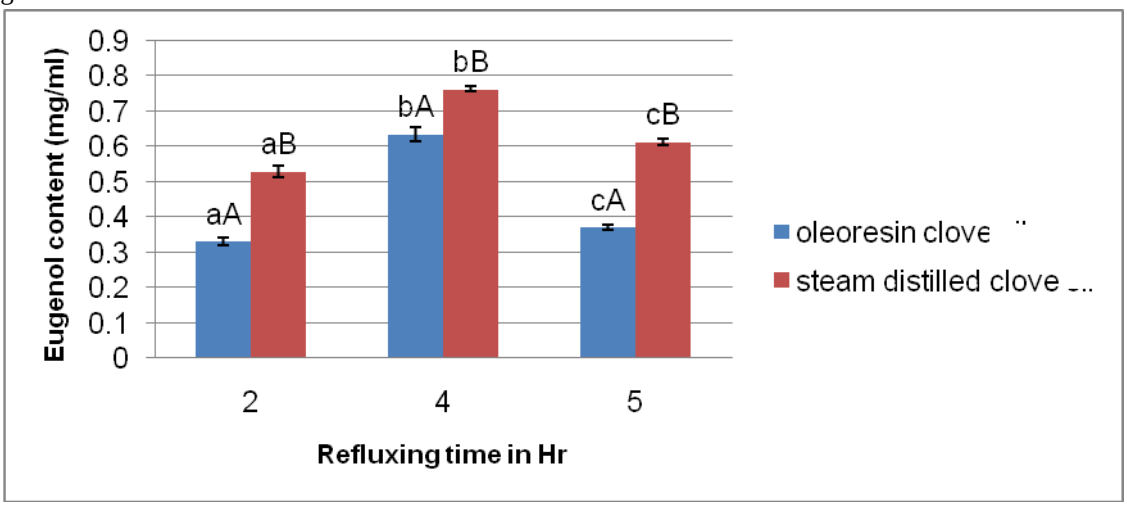


Fig.2. Eugenol content obtained at different time interval of refluxing (mg/ml) of clove extracts (steam distilled and oleoresin)

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. Error bars show the variations of three determinations in terms of standard error of mean

IV. CONCLUSION

Steam distilled clove extract has a much higher phenolic concentration than clove oleoresin. There was also a favourable association between total phenolic content, antioxidant potential, and free radical-scavenging activity for clove oleoresin and steam distilled clove extract. The measurement of eugenol content in steam distilled clove extract was found to be substantially greater than in oleoresin analogues.

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