ANALYSIS OF ANTIOXIDANT POTENTIAL OF CLOVE EXTRACT

***Dr. Sneha Shende[1], Dr. Harish Kumar[2], Rahul Mehra[3],**

1*Corresponding Author, Assistant professor, Dairy Chemistry Department, College of Dairy Technology, Warud (Pusad), Maharashtra Animal and Fishery Science University Nagpur

[2]Assistant Professor, Amity University Rajasthan, Jaipur,

[3] Ph.D Scholar, Amity University Rajasthan, Jaipur

Abstract

The main investigation of this study is based on to determine the antioxidant potential of clove extract (steam distilled and oleoresin) using DPPH, beta carotene model system and total phenolic content respectively. Total phenoloic content was observed in steam distilled clove extract found compared to its oleoresin. With 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 DPPH method. 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 recovery was also affected by period of refluxing, 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 by using RP-HPLC. The steam distilled clove extract was found to be highest antioxidant activity than its oleoresin counterparts.

1.0 Introduction

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 human intake of clove oil at 2.5 mg/kg body weight for humans (Nagababu and Lakshmaiah, 1992; Anderson *et al.,* 1997). It 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 (Keene *et al.,1998*), besides this, eugenol acetate (> 17%) and β caryophylene (> 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, e.g. digestive stimulation action, anti inflammatory, antimicrobial, anticarcinogenic potential, hypolipidemic, antimutagenic effects (Aaby *et al.,* 2004). Clove has 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, and vitamin A and vitamin C. In addition, humans have used clove oil for centuries, as an anaesthetic 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 anaesthesia 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 particular antioxidant capacities of widely consumed clove extract. The main objective of the present study was to evaluate the antioxidant activities of clove extract of steam distilled and clove oleoresin and their eugenol compound which is source of antioxidant activity. The antioxidant activities of steam distilled and clove oleoresin, were investigated using three antioxidant assays. We also investigated the possibility of antioxidant activities of the main 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.

2 Materials and methods

2.1 Chemicals and reagents:

β-Carotene, linoleic acid, 2,2-diphenyl-1-picrylhydrazyl hydrate free radical (DPPH), Folin–Ciocalteu's reagent and Tween-40 were (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, Singapour), C18 column (5µm, 4.5 × 250 mm, 100 A^0, Phenomener, USA), filtration assembly with Milipore 0.45 µm filter for filtering all reagents, water, syringe filter 0.45 µm for sample filtration.

Clove Extract:

The clove oleoresin was procured from Synthite Industry Ltd., Kerala, India and steam distilled extract of clove was provided by Katyani Exports, Delhi, India.

2.2 Determination of antioxidant capacity

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).

2.2.1 Total phenolic content:

Total phenolic content of clove oleoresin and steam distilled clove extract were analyzed by Folin-Ciocalteu's method (Kahkonen *et al.,* 1999). 400 µl of appropriately diluted sample/gallic acid standard was taken in a test tube. To it added 2000 µl of diluted Folin-Ciocalteu's reagent and mixed with vortex mixer. 1600 µl of sodium carbonate solution was added after 3 min and incubated under dark at room temperature for 30 min. For blank preparation, 400 µl of distilled water was taken instead of sample. The absorbance of the

samples was measured against blank at 765 nm using Spectrophotometer (Specord 700). Quantification was done with respect to the standard curve of gallic acid (400 µl of 10-100 µg/ml) concentration was taken in place of sample for standard curve preparation.The results were expressed as gallic acid equivalents (GAE), milligrammes per gm of spice extract.

2.2.2 β-carotene–linoleic acid model system

The antioxidant activity of solvent extracts was determined according to the procedure of Marco (1968), with minor modification. β-carotene (0.2 mg) in chloroform (0.5 ml) was added to 20 mg of linoleic acid, followed by 200 mg of Tween 40. The chloroform was evaporated at 40^{o}C using nitrogen gas. The resulting solution was immediately diluted with 10 ml of double distilled water and the emulsion formed was mixed well over magnetic stirrer for one minute. The emulsion was further diluted with 40 ml distilled water. Aliquots (4 ml) of this reagent were transferred into different stopper test tubes containing 1 ml of the desired amount of sample extracts in ethanol. The extract of steam distilled and clove oleoresin was taken at the rate of 200 ppm and dissolved in ethanol. Control, consisting of 1 ml of ethanol and 4 ml of emulsion, was prepared. Optical density of all samples was measured immediately (t = 0) and second reading after 15 min, followed by readings at 30 min intervals for 3 h (t = 180). The tubes were placed in water-bath at 50^{0}C while taking in between the readings. Then it was incorporated into a β-carotene–linoleic acid model system independently and the activity was monitored spectrophotometrically at 470 nm. (Specord, 700).

2.2.3 Radical-scavenging activity by DPPH model system

The extracts of steam distilled and clove oleoresin were taken at the rate of 200 ppm, dissolved in ethanol and evaluated for their radical scavenging activity in the DPPH system according to the procedure of Blois (1958). 200 ppm of sample extract dissolved in ethanol (1 ml) were taken in test tubes and add 4 ml of 0.1 mM ethyl acetate solution of DPPH to these tubes and shaken vigorously. The tubes were allowed to stand at 27^{0}C for 20 min. The control was prepared, as above without any added extract and ethanol was used for the baseline correction. Optical densities (OD) of the samples were measured spectrophotometrically at 517 nm (Specord 700).

2.3 Extraction and quantification of eugenol in clove extract using HPLC

Sample preparation is the crucial first step in analysis of spice extract, because it is necessary to extract the desired chemical components from the spice materials for further separation and characterization. 30 mg of clove extracts (steam distilled and oleoresin clove) 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 by using water reflux condenser. After refluxing, evaporation of the mixture was carried out on water bath till dryness. The obtained solution was redissolved with 30 ml ethanol and filtered through PTFE (Polytetrafluoroethylene) syringe filter 0.45 µm and directly injected 20 µL into HPLC system. Quantification was done with respect to the standard curve of eugenol (0.25 -1.5 mg/10ml) concentration in ethanol was carried out as above said. 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.

3 Results and discussion

3.1 Total Phenolic Content by Folin-Ciocalteu method

The amount of total phenolic content was measured by Folin–Ciocalteu method (Kahkonen *et al.*, 1999). The computation of total phenolic content of oleoresin and steam distilled clove extracts was carried out by using standard curve of gallic acid (ranging from 10-100 µg/ml) and results were expressed as mg gallic acid equivalents (GAE) per gm of spice extract. The oleoresin and steam distilled clove extract were found to be 177.039±0.35 mg and 256.506±0.45 mg GAE/gm, 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 due to the presence of different hydrophilic and lipophilic (flavonoids, terpenoids, carotenoids, phytoestrogens) antioxidant compounds in various extracts. Hyrophillic and lipophillic 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 lipophillic 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 oleoresin are hydrophobic in nature and contains lypophillic compounds including various terpenoids. An oleoresin is obtained by using efficient solvent which refers to low concentration of phenolics due to the presence of other nonphenolics (lipids, mainly mixture of terpenes) compounds. According to Shan *et al.*, (2005) the major phenolic compounds of clove bud are phenolic acids (gallic acid), flavonol glucosides, tannin and phenolic volatile oils (eugenol and acetyl eugenol). Clove extract obtained by steam distillation, contained higher amount of phenolic compound. It is 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, 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).

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

The antioxidant activity of clove extract (steam distilled and oleoresin) were evaluated at 200 ppm using the β-carotene–linoleic acid coupled oxidation model system (Marco, 1968). 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 oil components, this is in alignment 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.

3.3 Radical-scavenging activity by DPPH assay

The radical-scavenging activity of clove extracts (steam distilled and oleoresin) was evaluated at 200 ppm in the DPPH system. 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) activities might be due to various hydrophilic and lipophillic, 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 hydroperoxide into free radicals (Pokorny *et al.,* 2001; Pitchaon *et al.,* 2007). DPPH has been widely used to evaluate the free radical scavenging activity of various antioxidant substance (Ozcelik *et al.,* 2003). It could be inferred from our results that there was a positive correlation between phenolic content and antioxidant activity, as the higher activity of the steam distilled clove extract could be attributed due to the higher content of phenolic compounds

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 phenolic compound of clove, possesses many medicinal properties including antioxidant activity. Eugenol was determined using HPLC method developed by Yun *et al.,* (2010) with some minor modification. To improve the sensitivity and selectivity for separation of eugenol content, optimization of extraction method was undertaken for quantitative analysis.

3.4.1 Optimization of eugenol extraction using two different solvent:

In order to obtain the chromatograms with better resolution of adjacent peaks within short time, the extraction procedure of eugenol content was standardized and efficiency of different extraction solvents from clove extracts (steam distilled and oleoresin) were estimated. 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 that 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. The results are presented in figure 1

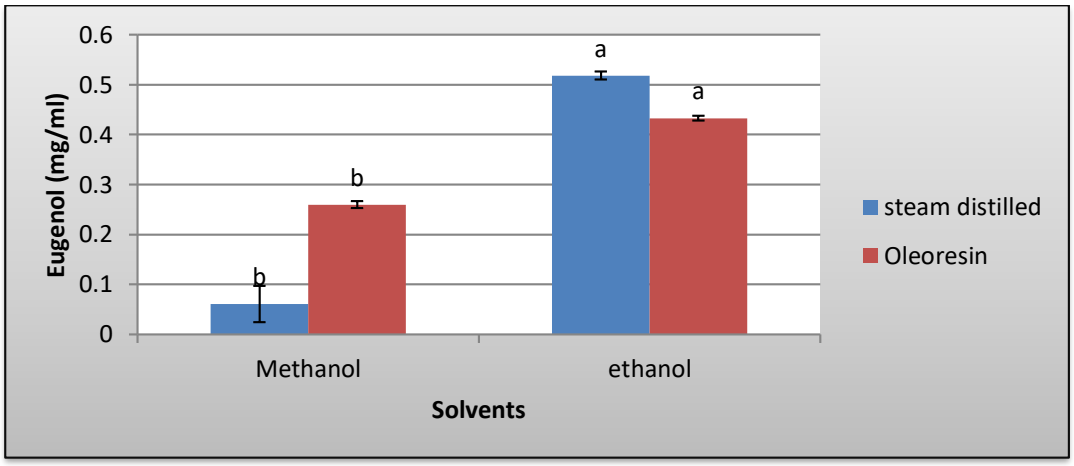

Figure 1: Two different solvent used to estimate eugenol content from clove extracts (oleoresin and steam distilled) as expressed in (mg/ml): The mean changes between the samples were analyzed by one-way ANOVA. a-b Means with different lowercase superscripts letters are significantly different ($P < 0.05$) from each other. Error bars show the variations of three determinations in terms of standard error of mean.

4 Conclusion:

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

5 Acknowledgements

This study is a part of the NAIP project, financially supported by the Ministry of Agriculture (government of India). Thanks to NDRI, Karnal, for providing the cream for this study. I also acknowledge the Katayni Export, New Delhi, who provided steam distilled clove extract.

REFERENCE

Aaby, K.; Hvattum, E. and Skrede, G. (2004). Analysis of flavonoids and other phenolic compounds using high-performance liquid chromatography with coulometric array detection: Relationship to antioxidant activity. *Journal of the Agricultural and Food Chemistry*, **52**:4595–4603.

Anderson, W. G.; McKinley, R. S. and Colavecchia, M. (1997). The use of clove oil as an anaesthetic for rainbow trout and its effects on swimming performance. *North American Journal of Fisheries Management*, **17(2)**:301–307.

Arnao, M. B.; Cano, A. and Acosta, M. (2001). The hydrophilic and lipophilic contribution to total antioxidant activity. *Food Chemistry*, **73**:239-244.

Bhuiyan, I. M.; Begum, J. and Akter, F. (2010). Constituents of the essential oil from leaves and buds of clove (*Syzigium caryophyllatum* (L.) Alston). *African Journal of Plant Science*, **4(11)**: 451-454.

Blois, M. S. (2001). Antioxidant determination by the use of stable free radical. *Nature*, **181**:1199–1200.

Jayaprakasha, G. K.; Singh, R. P. and Sakariah, K. K. (2001). Antioxidant activity of grape seed (*Vitis vinefera*) extracts on peroxidation models in vitro. *Food Chemistry*, **73**:285–290.

Kahkonen, M. P.; Hopia, A. I.; Vuorela, H. J.; Rauha, J.; Pihlaja, K.; Kujala, S. T. and Heinonen, M. (1999). Antioxidant activity of plant extracts containing phenolic compounds. *Journal of Agriculture and Food Chemistry*, **47**:3954-3962.

Kildeaa, M. A.; Allanb, G. L. and Kearney, R. E. (2004). Accumulation and clearance of the anaesthetics clove oil and AQUI-S_ from the edible tissue of silver perch (Bidyanus bidyanus). *Aquaculture*, **232**:265–277.

Lee, K. G. and Shibamoto, T. (2001). Antioxidant property of aroma extract isolated from clove buds. *Food Chemistry*, **74(4)**:443-448.

Liu, H. and White, P. J. (1992). Oxidative stability of soybean oils with altered fatty acid compositions. *Journal of American Oil Chemists Societ*, **69**:528–532

Marco, G. J. (1968). A Rapid method for Evaluation of Antioxidants. *Journal of American Oil Chemists Society*, **45**:594-596.

Mostafa, K.; Yadollah, Y.; Fatemeh, S. and Naader, B. (2004). Comparison of essential oil composition of Carum copticum obtained by supercritical carbon dioxide extraction and hydrodistillation methods. *Food Chemistry*, **86**:587–591.

Mylonasa, C. C.; Cardinalettia, T. G.; Sigelakia, I. and Polzonetti-Magni, A. (2005). Comparative efficacy of clove oil and 2-phenoxyethanol as anesthetics in the aquaculture of European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) at different temperatures. *Aquaculture*, **246**:467–481.

Nagababu, E. and Lakshmaiah, N. (1992). Inhibitory effect of eugenol on non-enzymatic lipid peroxidation in rat liver mitochondria. *Biochemical Pharmacology*, **43:**2393–2400.

Ozcelik, B.; Lee, J. H. and Min, D. B. (2003). Effects of light, oxygen, and pH on the absorbance of 2, 2-diphenyl-1-picrylhydrazyl (DPPH). *Journal of Food Science*, **68**:487-490.

Ozkan, G.; Sagdic, O.; Baydar, N. G. and Badyar, H. (2004). Antioxidant and Antibacterial Activities of Rosa Damascena Flower Extracts. *Food Science and Technology International*, **10(4)**:277-281

Pitchaon, M.; Suttajit, M. and Pongsawatmani, R. (2007). Assessment of phenolic content and free radical scavenging capacity of some Thai indigenous plants. *Food Chemistry*, **100**:1409-1418.

Prior, X. and Wu, K. (2005). Standardized method for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agriculture Food Chemistry*, **53**:4290-4302

Pokorny, J. and Korczak, J. (2001). Preparation of natural antioxidants. In J. Pokorny, N. Yanishlieva, & M. H. Gordon (Eds.), Antioxidants in food – practical application, Cambridge: Woodhead Publishing Ltd. pp. 311–330.

Rajakumar, D. V. and Rao, M. N. A. (1993). Dehydrozingerone and isoeugenol as inhibitors of lipid peroxidation and as free radical scavengers. *Biochemistry Pharmacology*, **46**:2067–2072.

Reverchon, E. (1997). Supercritical fluid extraction and fractionation of essential oils and related products. *Journal of Supercritical fluids*, **10**:1–37.

Rice-Evans, C. A.; Miller, N. J.; Bolwell, P. M.; and Pridham, J. B. (1995). The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radical Research*, **22***:*375-383.

Shan, B.; Yizhong. Z.; Sun, M. and Corke, H. (2005). Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents. Department of Botany and Department of Zoology, The University of Hong Kong, Pokfulam Road, Hong Kong. *Journal of Agriculture and Food Chemistry*, **53(20):**7749–7759.

Shelef, L. A. (1983). Antimicrobial effects of spices. *Journal of Food Safety*, **6**:29– 44**.**

Singleton, V.; Orthofer, R. and Lamuela-Raventos, R. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin Ciocalteu reagent. In L. Packer (Ed.). Oxidants and antioxidants, part A, methods in enzymology, New York; Academic press, **299**:152-178.

Soares, J. R.; Dinis, T. C. P.; Cunha, A. P. and Almeida, L. M. (1997) Antioxidant activities of some extracts of *Thymus zygis*. *Free Radical Research* **26**:469-478.

Soto, C.G. and Burhanuddin, C.G. (1995) Clove oil as a fish anaesthetic for measuring length and weight of rabbit fish (*Siganus lineatus*). *Aquaculture* **136**:149–152.

Vicas, S.; Prokisch, J.; Rugina, D. and Socaciu, C. (2009). Hydrophilic and lipophilic antioxidant activity of Mistletoe (*Viscum album*) as determined by FRAP method. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, **37(2)**:112-116.

Yun,J. (2010). Quantitative analysis of eugenol in clove extract by a validated HPLC method. *Journal of AOAC international*, **93(6)**:1806-1810

Zhou, C.; Li, X.; Xu, C.; Sun, C. and Chen, K. (2011). Hydrophilic and lipophilic antioxidant activity of loquat fruits. *Journal of Food Biochemistry*, DOI: 10.1111/j.1745-4514.2011.00574.x