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Research Article

ANTIOXIDANTS OF CLOVE (*SYZYGIUM AROMATICUM*) PREVENT METAL INDUCED OXIDATIVE DAMAGE OF BIOMOLECULES

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ABSTRACT

The present study evaluates the antioxidant potential of clove against copper induced lipid peroxidation, protein oxidative modifications and iron induced damage to DNA. 1, 1-diphenyl1-2 picryl hydrazyl free radical scavenging capability of methanol extract of clove is examined. The clove extract exhibited a concentration dependent antioxidant activity on these processes. 56% of DPPH free radicals were scavenged with extract equivalent to 100µg clove. Production of thiobarbituric acid reactive substances as a result of lecithin peroxidation and metal ion induced protein modifications (measured as DTNB reactive groups) was reduced by 50% when clove extract equivalent to 0.26 mg dry weight was included in the assay mixture. Cloves have low riboflavin (1.5µg/g) content but high ascorbate (985.6µg/g) and tocopherol (660.6µg/g) content. HPLC analysis of the extract for phenolics reveals the presence of gallic acid, eugenol and quercetin. The high antioxidant activities observed can be correlated to considerable ascorbate, tocopherol and phenolic content.

Key words: clove, antioxidant properties, polyphenols, tocopherol

INTRODUCTION

Exposure to reactive oxygen species (ROS) is continuous and unavoidable for aerobic organisms. ROS encompass a variety of partially reduced metabolites of oxygen like superoxide, hydrogen peroxide, and hydroxyl radicals, possessing higher reactivities than molecular oxygen[1]. These reactive oxygen radicals are produced intracellularly through a variety of processes as byproducts of normal aerobic metabolism or as second messengers in various signal transduction pathways. The intracellular level may increase by their uptake from exogenous sources, either being taken up directly by cells from the extracellular milieu, or produced as a consequence of the cell's exposure to some environmental insult. Cells have evolved a number of defense systems to combat the accumulation of ROS including both enzymatic and non-enzymatic molecules like glutathione, vitamins A, C, E and flavonoids[2]. Unfortunately, these defense mechanisms are not always adequate to counteract the production of ROS, resulting in the state of oxidative stress. A wide variety of disease processes including atherosclerosis, diabetes, pulmonary fibrosis, neurodegenerative disorders, arthritis have been correlated to the oxidative stress and is believed to be a major factor in aging[3]. ROS can cause severe damage to DNA, protein, and lipids[1,2].

The free-radical reaction of lipid peroxidation is an important issue in the food industry also where manufacturers minimize oxidation in lipid-containing foods by use of antioxidants during the manufacturing process to maintain the nutritional quality of food over a defined shelf life[4]. Because of consumer awareness and reported carcinogeneticity of synthetic antioxidants, for biomedical scientists and food technologists, natural antioxidants has become an area of intense interest. Herbs and spices rich in phytochemicals and polyphenols are well known to have antioxidant properties[5-9] .

Clove (*Syzygium aromaticum*) is a tree belonging to family Myrataceae. Unopened flowering buds are used as spice in food preparations all over the world. Clove oil is traditionally used as antimicrobial, antiviral, anti-inflammatory, antidiabetic and hepatoprotective agent[7,10]. It is also used for relief in inflammatory rheumatism and arthritis. Clove extract is reported to inhibit 5-lipoxygenase activity and leukotriene-4 in human PMNL cells[11]. Clove extract is also reported to affect gene expression in liver cells[12]. Free radical scavenging, metal ion binding and iron reducing properties of steam distilled essential oils[7,13,14], methanol [5,6] and alcohol extracts[15] from various spices including clove have been reported. Steam distilled extract of clove could inhibit the oxidation of cod liver oil[13].The method of extraction, solvent used, temperature and duration of extraction influence the extraction of phytochemicals and polyphenols. In the present study, dried clove buds are extracted with 80% methanol to evaluate the possible protection of biomolecules against oxidative stress and estimate the level of various bioreactive antioxidant compounds.

MATERIAL AND METHODS

The clove *(S. aromaticum*) buds, purchased from the local market, were identified and authenticated at Department of Botany, Kurukshetra University, Kurukshetra, India. Thiobarbituric acid, bovine serum albumin, calf thymus DNA and 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Company, USA. Diphenyl-picrylhydrazyl (DPPH), acetonitrile, gallic acid, Folin-Ciocalteau reagent and methanol were purchased from Hi-media, Mumbai, India. All other chemicals and solvents used were of analytical grade.

Extraction

Clove buds were dried at 50°C in hot air oven till constant weight was attained. Finely powdered clove buds were extracted with 80%

methanol (1g/10ml) in a shaker at room temperature for 4 h. Residue was re-extracted with 80% methanol for 2 h. Extract was pooled, filtered through double layered muslin cloth and centrifuged at 5000g for 5min in order to get clear supernatant. Extract was concentrated in a vacuum evaporator and stored at -20°C for further use. The extract was diluted appropriately for different experiments.

Estimation of antioxidants

Ascorbate was estimated by oxidation of dehydroascorbate followed by coupling with 2,4- dinitrophenylhydrazine to give red colored osazones as described earlier [16]. Clove extract was diluted in 5% metaphosphoric acid in presence of 10% stannous chloride. Equal volume of 2% thiourea in 5% HPO_3 was added and incubated at 37°C for 6 hrs. Contents were shifted to ice bath and 5ml of 85% H_2SO_4 was added slowly. Absorbance was read at 540 nm after 30 min. Ascorbic acid (1-20 µg/ml) was used to prepare calibration curve. Reduced ascorbate was first oxidized by adding bromine water to determine total ascorbic acid content.

Riboflavin content was analyzed by diluting clove extract with 0.2M acetate buffer pH 4.0. Few drops of caprylic alcohol and 3ml of freshly prepared 4% potassium permangnate solution were added. Within 2 min, 3ml of H_2O_2:DW solution (1:1) was added and pH adjusted to 7.0. The solution was filtered and the fluorescence by the filtrate was measured at 530 nm with excitation at 470 nm using fluorescence spectrophotometer. Standard riboflavin (1µg/ml) was used for calibration purpose[17].

Tocopherol was extracted with saturated potassium hydroxide and hexane. Hexane layer was collected and evaporated under nitrogen. The contents were dissolved in ethanol. To one ml of ethanol extract, 0.2ml of 2% bathophenanthroline was added in dark. The contents were mixed thoroughly and 0.2 ml of ferric chloride reagent was added rapidly. After 1 min, 0.2ml of 0.01M phosphoric acid (prepared in alcohol) was mixed and read at 534 nm. Standard DL-tocopherol (1-10 µg) was treated in same way for preparing the calibration curve[9].

HPLC analysis of phenolic compounds

The clove extract was treated with 2N HCl to hydrolyze glycosidic bonds. The extract was dried, dissolved in methanol and was subjected to HPLC for qualitative and quantitative analysis of phenolic contents. The HPLC system (Agilent Technologies Company) was equipped with dual lamp binary system, UV detector, C18 column (i.d. 4.6 mm×150mm, 5µm) and the data was integrated by Agilent Chem Station software. Standards and sample extract were analyzed using the following gradient program (A.100% acetonitrile B. HPLC Grade Water: 0min 5%A;10min 15% A; 20 min 25% A; 30min 35%A; 40min 45%A; 50min 55% A). Flow rate was 0.5 ml/min and injection volume was 10µl. Detection was done at 280 nm[16,17]. Peak area (280 nm) of the sample is an index of the amount of component and the retention time of individual peaks is used to identify polyphenols by comparing with standard polyphenols – eugenol, caffeic acid, ellagic acid, ferulic acid, quercetin and kaempferol.

Measurement of antioxidant activity
Measurement of DPPH radical scavenging activity

Different dilutions of the clove extract were incubated with 1ml of DPPH solution (50 x10 $^{-5}$M) in a final volume of 1.1ml. The decrease in absorbance due to the scavenging of DPPH radicals by the spice extract was recorded at 517 nm. The percentage of remaining DPPH after 5 min with different dilutions of extract was calculated and IC_{50}, the concentration at which 50% of the initial DPPH could be scavenged was noted from the graph[17].

Measurement of copper induced egg lecithin peroxidation Inhibition

Lipid peroxidation inhibition was monitored as the amount of malonaldehyde produced by copper induced egg lecithin peroxidation [17,18]. Different dilutions of the clove extract were added to the reaction mixture containing 2.5 mM lecithin and 250 mM $CuCl_2$ in 50 mM Tris-HCl buffer (pH 7.4) in a total volume of 1ml. After incubation at 37° C for 15 min, thiobarbituric acid reacting substances (TBARS) were monitored by adding 2ml of TBA reagent containing 0.37% thiobarbituric acid (TBA), 15% trichloroacetic acid (TCA), 0.04% butylated hydroxyl toluene (BHT) and 2% ethanol. Mixture was heated at 100°C for 15 min, centrifuged at 3000g for 10 min and the absorbance of supernatant was recorded at 535 nm as an index of malonaldehyde concentration. The graph plotted is used to note IC_{50}, the concentration inhibiting 50% of peroxidation.

Measurement of inhibition of copper induced protein oxidative modification

Oxidative modification in albumin was induced by copper in presence and absence of different dilutions of clove extract[16]. In brief, 0.3 ml of the reaction mixture containing albumin (10mg/ml), 100 mM $CuCl_2$ in 50 mM Tris–HCl buffer (pH 7.4) was incubated at 37°C for 2 hr in absence and presence of different concentrations of extract. After incubation, 1.6 ml of 0.125 M phosphate buffer (pH 8.0) containing 12.5mM EDTA plus 10.0 M urea and 0.1ml of 50mM phosphate buffer (pH 7.0) containing 10 mM 5,5-dithio-bis(2-nitrobenzoic acid) was added to the reaction mixture. The absorbance was recorded at 412 nm as an index of cysteine-SH residues. Percent inhibitory ratio was calculated as follows:

$$\% \text{ inhibition} = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$$

Measurement of antioxidant activity against oxidative damage to DNA

Hydroxyl radicals generated by Fenton's reaction were used to induce oxidative damage to DNA[16]. The reaction mixture (9 µl) contained 3µg of calf thymus DNA in 20.0 mM phosphate buffer saline (pH 7.4) and different concentrations of extract (0.5, 1.0, 1.5 and 2.0µg) was preincubated for 15 min at ambient temperature. The oxidation was induced by incubating DNA with 1.0 mM $FeSO_4$ and 10.0 mM ascorbic acid for 1 h at 37°C. The reaction was terminated by the addition of loading buffer (xylene cyanol, 0.25%; bromophenol blue, 0.25% and glycerol 30%).The mixture was subjected to gel electrophoresis in 1.5% agarose-TAE buffer system and run at 60 V. DNA was visualized and photographed by using UV- transilluminator (Genei) and Chemidoc (Biorad) system to assess the damage and protection.

RESULTS AND DISCUSSION

In biological systems, ROS harm the structural and functional machinery of the cells by causing oxidation of biomolecules. Double bonds in the biomolecules especially lipids are susceptible to the attack by ROS. The situation further worsens with the propagation of free radicals. Antioxidants are the chemical moieties which eliminate the ROS by inhibiting the production and propagation reactions when present in small amounts. Ascorbate, riboflavin, tocopherol and polyphenols present in plants act as efficient antioxidants since they have redox potential high enough to eliminate ROS and terminate the chain reactions. The enzymatic reducing system of the cells can reduce these phytochemicals to reproduce their reduced state [1,2]. Polyphenols and tocopherols are the primary antioxidants while ascorbic acid may regenerate the oxidized primary antioxidants by reduction. Polyphenols have metal ion chelating character due to the presence of various hydroxyl

radicals and can contribute to the antioxidant property by binding the metal ions which can induce oxidation reactions. Polyphenols have one or more benzene rings with π electron cloud which makes them suitable as antioxidants[2]. To identify and determine the level of possible antioxidants, the methanol extract of clove was analyzed for the presence of phytochemicals, known to have antioxidant activity. The clove extract had considerable ascorbate and tocopherols (Table 1). The content of total ascorbate was 985.6µg and the tocopherol was 660.6µg/ g dry weight, where as riboflavin was only 1.5µg/ g dry seeds. We have reported high polyphenol content (11.25 mg GAE/g dry weight) in 80% methanol extract of clove in our earlier work[19]. High polyphenol extraction from cloves using water and ethanol as solvents has been reported[20]. Comparable amount (160-171mg/100g) of polyphenols could be extracted with methanol and water[21]. Total polyphenol extract with methanol here is higher than reported with other solvents. Qualitative and quantitative HPLC analysis of the phenolic compounds shows the presence of quercetin (445.865 µg/g), eugenol (226.473 µg/g) and gallic acid (89.648µg/g) in methanol clove extract (Figure 1). Gallic acid, eugenol, eugenyl acetate and their glucuronoid derivatives were identified as the major phenolics while quercetin and kaempferol were present in small amounts in methanol extract of clove[5]. Number of hydroxyl groups and benzene rings in the polyphenols and the density of π electron cloud is an index of antioxidant potential of the polyphenols. Hence gallic acid, among the simple phenolics and quercetin among the flavanols are the most potent antioxidants[1]. Methanol extract of clove has high amounts of ascorbate, tocopherol and polyphenols including quercetin, eugenol and gallic acid indicating high potential of clove bud extract as an antioxidant.

Oxidation of biomolecules lipids and proteins can damage the membranes and disturb the integrity of cells. ROS can damage DNA to cause mutations or influence the expression of genes. Antioxidants reduce the damage caused by ROS such as hydroxyl or superoxide radicals by scavenging free radicals, inhibiting ROS production, donating electrons or hydrogen to terminate chain reactions or chelating metal ions. The mode of action of natural antioxidants may be varied, may involve either of these mechanisms or multiple mechanism of actions simultaneously. Synergism between various antioxidants has been documented[22]. Antioxidant activity of clove extract was examined by DPPH free radical scavenging. Protection of lecithin and BSA against copper induced oxidation and inhibition of peroxide induced damage to DNA by clove extract was examined.

DPPH is a stable free radical which can absorb an electron or hydrogen to become a stable diamagnetic molecule. Scavenging of these free radicals by the antioxidants in clove extract was observed. Clove extract exhibited a concentration dependent elimination of DPPH free radicals (Figure 2). Clove extract equivalent to 200µg dry weight caused scavenging of 92% of the free radicals. IC_{50} of the spice is 82µg for DPPH free radical scavenging activity. Free radical scavenging by the clove extract indicates that antioxidants in clove are efficient electron or hydrogen donors. Free radical scavenging activity is positively correlated to the polyphenol content of various spices[5]. 85% and 56% DPPH free radical scavenging by aqueous and methanol extracts of clove is reported[21]. Essential oils from clove rich in tocopherol are reported to scavenge 70% free radicals[7]. Polyphenols from other spices are also reported to have DPPH free radical scavenging activity[6,9,16-18]. Almost complete free radical scavenging and a lower IC_{50} observed here may be due to synergetic effect of ascorbate, tocopherols and polyphenols in the extract.

Lipid oxidation brings about chemical changes causing spoiling the fats and fatty acids of food preparations. Membrane lipids of cells have polyunsaturated fatty acids so are more susceptible to oxidation. Damage to the membrane lipids can affect permeability and hence various processes related to membrane integrity such as apoptosis, necrosis, autogenesis and carcinogenesis[2,3]. Metal ions such as iron and copper can induce oxidation of lipids leading to the production of peroxy radicals, which can propagate the chain reactions and accelerate lipid oxidation and damage the cells. Thiobarbituric acid reactive substances produced by copper induced oxidation of lecithin in presence and absence of different dilutions of clove extract were determined (Figure 3). In controls, 18.77±0.318 nmoles of MDA was produced and the production of MDA was reduced to 1.07±0.68 nmoles in presence of the extract equivalent to 0.5 mg of the clove. Clove extract could protect the lipids against oxidative damage with IC_{50} 0.26 mg dry weight. These results indicate that antioxidants from clove are preventing the oxidation of lipids induced by metals efficiently either by chelating metal ions and/or by inhibiting the propagation reactions being hydrogen/electron donor. Lipid oxidation was completely inhibited in presence of ethanolic clove extract[15] with IC_{50} 0.28 mg. Aroma extracts of clove inhibited 93% lipid peroxidation with 160µg/ml extract[13]. Aqueous and ethanol extracts of clove have shown lipid oxidation inhibition comparable to butylated hydroxytoluene[20].

In biological systems proteins perform various important functions such as enzymes, hormones, components of immune system or transcriptional factors. Oxidative change of the cysteine groups to cystine can lead to modifications in the three dimensional structure of the proteins and hence their activity. Deleterious impact of oxidative stress in biological systems is related to the damage of proteins, enzymes and various transcriptional factors like NF-kB and AP-1[1,2,12]. Protection of BSA against oxidative modifications induced by incubation with copper ions in presence of clove extract was determined (Figure 3). Oxidative modifications in BSA were inhibited by 92% in presence of extract equivalent to 0.5mg of clove. Presence of clove extract equivalent to 0.26 mg dry weight is enough to inhibit the metal induced protein oxidation up to 50%. IC_{50} for lipid and protein oxidation is similar indicating a common mechanism of protection of these biomolecules. A higher concentration of clove extract is required to cause 50% inhibition of lipid or protein oxidation as compared to free radical scavenging indicating that metal binding is contributing towards protection of biomolecules along with hydrogen/electron donor characteristic. Metal ion chelating activity of methanol extract of various herbs and spices has been reported [6,14,20]. Metal ion chelation by the plant extract reduces the concentration of the oxidation catalyzing transition metal ions and can contribute significantly to antioxidant activity.

Nucleotide bases are oxidized by ROS generated in the cells. ROS can oxidize purine base guanosine to hydroxyl-2-deoxyguanosine and can modify pyrimidine thymine to thymine glycol. Prolonged exposure to oxidative stress can cause mutations and can influence the binding of transcriptional factors to DNA[1,2]. Oxidative stress generated by Fenton`s reaction causes breaks in calf thymus DNA and damaged DNA moves to a greater extent in the gel (Figure 4). Protection of DNA against damage by the clove extract was examined by the movement of DNA in gel. Incubation of DNA with extract equivalent to 0.5 µg and 1.0 µg clove could prevent the damage. The results in fig 4 show that the antioxidant formulation from clove buds can efficiently quench hydroxyl radicals from the reaction mixture and protect DNA against oxidative damage.

Table 1: **Concentration of various antioxidant compounds in clove extract**

Antioxidant	Amount (µg/g dry wt.)
Total Ascorbate	985.6±4.62
Reduced Ascorbate	140.8±2.63
Oxidized Ascorbate	844.8±2.23
Riboflavin	1.5±0.04
Tocopherol	660.6±4.54
Gallic acid	89.648
Eugenol	226.473
Quercetin	445.865

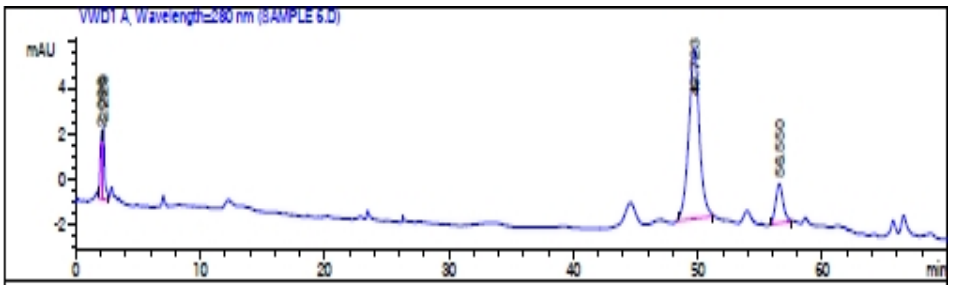

Figure 1: HPLC analysis of the clove extract; peak at retention time 2.229, 49.723 and 56.550 min are identified as gallic acid, eugenol and quercetin respectively

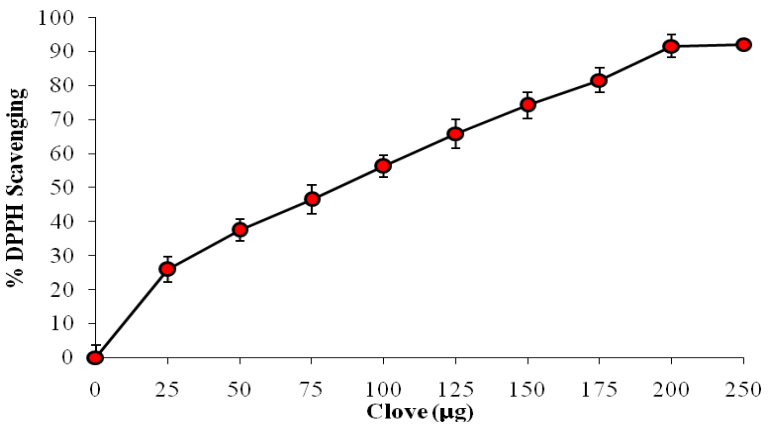

Figure 2: DPPH free radical scavenging in presence of clove extract

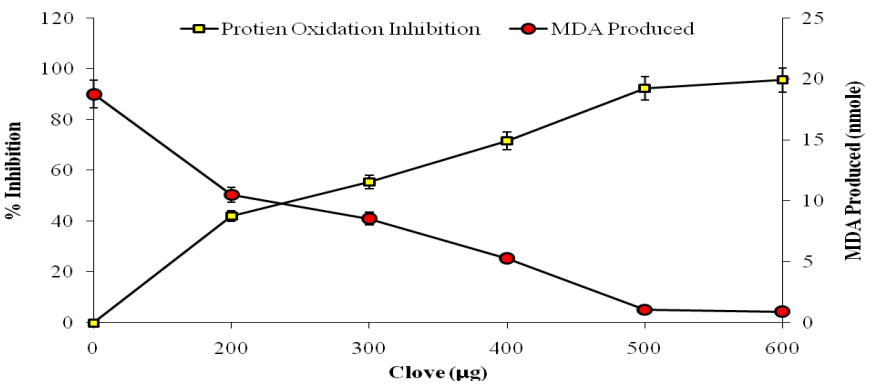

Figure 3: Protein oxidation and malonaldehyde produced in presence of clove extract

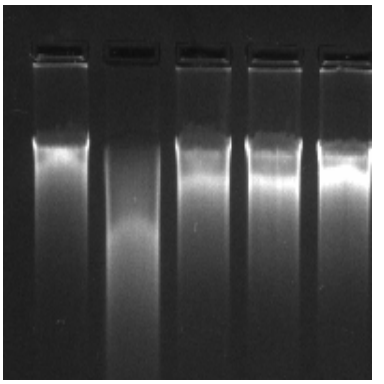

Figure 4: **Protection of DNA against H_2O_2 induced damage by clove extract. Lane 1-control DNA (1.5µg); Lane 2-DNA+Fenton's reagent with ascorbic acid; Lane 3- clove (0.5µg)+DNA+Fenton's reagent with ascorbic acid; Lane 4- clove (1.0µg) + DNA+Fenton's reagent with ascorbic acid; Lane5- clove (1.5µg)+DNA+Fenton's reagent with ascorbic acid**

CONCLUSION

The present study revealed that clove bud is a rich source of natural antioxidant principle which could be extracted efficiently with methanol. Methanol extract containing phenolic compounds; quercetin, eugenol, gallic acid along with considerable amount of tocopherol and ascorbic acid, exhibited good free radical scavenging property and could protect biomolecules against metal ion induced *in vitro* oxidation and peroxidation. Potential use of clove as an antioxidant neutraceutical and as food preservative to improve shelf life along with providing taste, flavor and aroma needs to be explored further.

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