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In vitro studies on antioxidant activity of lignans isolated from sesame cake extract

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Abstract: The antioxidant activity of compounds isolated from a methanolic extract of commercial sesame cake was studied using a peroxidation model and a radical-scavenging method. Pure compounds were isolated from the extract by preparative high-performance liquid chromatography (HPLC) and identified and confirmed as sesamol, sesamin, sesamolin, sesaminol diglucoside and sesaminol triglucoside by HPLC, infrared, nuclear magnetic resonance and mass spectrometry. When the rate of inhibition of lipid peroxidation and the superoxide radical-scavenging power of the individual compounds were evaluated, the compounds showed antioxidant activity to different extents. The antioxidant activity of compounds by the β-carotene-bleaching assay followed the order sesamol > sesamolin ≥ sesamin > butylated hydroxytoluene (BHT) > sesaminol triglucoside > sesaminol diglucoside. By the thiocyanate method the inhibition of linoleic acid peroxidation shown by sesamol, sesamin, sesamolin, sesaminol triglucoside, sesaminol diglucoside and BHT at 200 mg l^{-1} was 77, 60, 69, 32, 25 and 49% respectively. A concentration–dependent superoxide–scavenging effect was also shown by these compounds. Sesamolin had an appreciable effect at 300 and 500 mg l^{-1}, while the other compounds were more effective at 100 mg l^{-1}. The study also established the occurrence of sesamol in the methanolic extract of defatted sesame cake for the first time.

Keywords: sesame cake extract; sesamol; lignans; antioxidant activity; superoxide radical scavenging power; NBT method

INTRODUCTION

Sesame is an important oil seed crop and India is one of the major producers of sesame in the world.[1] It provides good-quality oil and nutritious food.[2] The oil is used mainly for culinary purposes and also as a carrier ingredient in herbal preparations based on India's traditional health system called Ayurveda. The unusual stability of sesame oil is attributed to the presence of endogenous antioxidants, namely sesamol, sesaminol, sesamin and sesamolin.[3] The reported biological effects of sesame include anti-aging and hypocholesterolaemic effects,[4,5] among others, and sesamin has been shown to be a specific inhibitor of Δ5-desaturase in polyunsaturated fatty acid (PUFA) biosynthesis.[6] According to Kang *et al*,[7] sesamolin inhibits lipid peroxidation in rat liver and kidney and contributes to the antioxidant effects of sesame. Pinoresinol glucosides obtained from aqueous extracts of defatted sesame seed are reported to be useful for preventing lipid peroxidation.[8]

Antioxidants are reported not only to control lipid oxidation in food systems but also to prevent free radical-induced diseases such as cancer, inflammation, atherosclerosis, etc.[9] During our investigation on natural antioxidants from industrial by-products, an antioxidant extract was prepared from sesame cake whose efficacy was evaluated by various *in vitro* methods. The extract was found to be effective in protecting different vegetable oils against oxidative deterioration.[10,11] In the present study the antioxidant potential of pure compounds isolated from sesame cake extract has been evaluated by different *in vitro* methods.

Since different antioxidant compounds have different mechanisms of action,[12] different methods have been used to assess the antioxidant efficacy of pure compounds and extracts. In this study the antioxidant activity has been evaluated *in vitro* by peroxidation methods and a superoxide radical-scavenging assay.

EXPERIMENTAL

Materials and equipment

Sesame cake was purchased from a local market. Standard sesamol, trolox (6-methoxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), linoleic acid, ferrous chloride, nitro blue tetrazolium (NBT) salt, xanthine, xanthine oxidase and β-carotene were obtained from Sigma Chemicals (St Louis, MO, USA). High-performance liquid chromatography (HPLC)-grade solvents and other chemicals of reagent grade

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were obtained from E Merck (Bangalore, India). Sesamin and sesamolin were isolated from sesame oil by the method of Soliman *et al*.[13]

Infrared (IR) spectra were recorded with a Nicolet IR spectrometer 'IMPACT 400D' (Madison, WI, USA). ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were taken using a Bruker ADVANCE DPX-400 instrument (Fallander, Switzerland). Mass spectra were recorded with a Shimadzu QP 5050 spectrometer (Kyoto, Japan) in electron ionisation (EI) mode. The conditions of analysis were: ionisation voltage 70 eV; source/injector temperature 250 °C; interface temperature 270 °C; detector at 1.40 kV.

Extraction

Sesame cake was dried in an air oven at ≤60 °C and powdered in a Domestic plus, domestic mixer (Mumbai, India) to pass through a 300 μm sieve. An approximately 100 g sample was initially defatted with hexane (1.5 l) in a Soxhlet extractor for 12 h. The defatted cake was further extracted with methanol (1.5 l) for 16 h. The methanolic extract was filtered through Whatman No 1 filter paper and the solvent was removed under vaccum in a Heidolph rotary flash evaporator model LABOROTA 4000 (Schwabach, Germany) (temperature ~45 °C). The residue was weighed, redissolved in methanol and stored under refrigeration until further analysis.

Isolation and characterisation

The extract was initially analysed for lignan profile and content using an analytical high-performance liquid chromatograph (HPLC) (LC-10, Shimazdu binary system) fitted with an LC-10 AD pump, a 7125 rheodyne injector with 20 μl sample loop, an SPD-10A UV-vis detector and a C-R7Ae Plus integrator and equipped with a μ-Bondapak C_{18} column (4.6 mm id × 250 mm; Waters, Milford, MA, USA). The solvent system used was methanol/water (70:30 v/v) at a flow rate of 1 ml min^{-1}. The wavelength of detection of sesamol and sesame lignans was set at 290 nm as reported by other workers.[1,14] Suitably diluted sample solutions (20 μl) were injected into the HPLC for analysis. Analyses were carried out in triplicate at a flow rate of 1 ml min^{-1} with the UV detector set at 290 nm.

Individual compounds were isolated by preparative HPLC (Shimadzu LC-8A) using an ODS column (250 mm × 20 mm id; Shimadzu). The solvent system used was methanol/water (70:30 v/v) at a flow rate of 10 ml min^{-1}. The fraction corresponding to each main peak was collected while eluting from the column and lyophilised to yield the pure compound. For identification of compounds, IR, NMR and mass spectrometry (MS) techniques were employed.

Antioxidant assays

β-Carotene-bleaching method

The β-carotene-bleaching method of Hidalgo *et al*[15] was used to evaluate the antioxidant activity of individual compounds, ie sesamol, sesamin, sesamolin, sesaminol diglucoside and sesaminol triglucoside, separated by preparative HPLC. β-Carotene (0.2 mg), linoleic acid (20 mg) and Tween 20 (200 mg) were mixed in 0.5 ml of chloroform. The solvent was subsequently removed at 40 °C in a vacuum evaporator and the mixture was diluted with 50 ml of triply distilled, oxygenated water. Aliquots (4 ml) of this emulsion were transferred into test tubes, to which were then added 0.2 ml aliquots of test samples in ethanol. Butylated hydroxytoluene (BHT) was used for comparative purposes. A control containing 0.2 ml of ethanol and 4 ml of emulsion was also used. The test tubes were covered with aluminium foil and placed in a water bath at 50 °C. The absorbance at 470 nm was recorded with a UV-vis spectrophotometer (UV-1601 Shimadzu, Kyoto, Japan) at intervals of 30 min until the colour of β-carotene had disappeared from the control tubes. The above mixture without β-carotene served as blank. All determinations were carried out in triplicate. Antioxidant activity (A_A) was expressed as percentage inhibition relative to the control using the equation

$$A_A(\%) = 100[1 - (A_0 - A_t)/(A_0^0 - A_t^0)]$$

where A_0 and A_0^0 are the absorbance values measured at zero time of incubation for the test sample and control respectively and A_t and A_t^0 are the corresponding values at the end of the reaction time.

Linoleic acid emulsion system/thiocyanate method

The antioxidant activity of fractions was further evaluated by the thiocyanate method.[16] Linoleic acid emulsion was prepared by mixing linoleic acid (0.28 g), Tween 20 (0.28 g) and phosphate buffer (50 ml, 0.2 M, pH 7.0). Test samples were prepared in methanol/water (6:4 v/v). Different test samples (0.5 ml each) were mixed with 2.5 ml of linoleic acid emulsion and 2.5 ml of phosphate buffer (0.2 M, pH 7.0) and incubated at 37 °C for 120 h (5 days). The mixture prepared as above without any test sample served as control. Aliquots (0.1 ml) were drawn from the incubation mixture at intervals of 24 h and mixed with 5.0 ml of 750 g l^{-1} ethanol, 0.1 ml of 300 g l^{-1} ammonium thiocyanate and 0.1 ml of 20 mM ferrous chloride in 35 g l^{-1} HCl. After precisely 3 min the absorbance at 500 nm was recorded. Antioxidant activity was expressed as

$$\text{antioxidant activity (\%)} = 100 - \frac{\text{increase in absorbance of sample}}{\text{increase in absorbance of control}} \times 100$$

Superoxide scavenging by NBT method

The superoxide-scavenging ability of compounds was studied by the xanthine/xanthine oxidase/NBT method according to Sur *et al*.[17] The assay mixture consisted of 1 ml of sodium carbonate/ethylene diamine tetraacetic acid (EDTA) buffer (carbonate buffer 50 mM, pH 10.2 containing EDTA), 50 μl of

xanthine (8.7 mg ml^{-1}), 20 µl of test solution and 10 µl of NBT (6 mg ml^{-1}). The reaction was initiated by the addition of 20 µl of xanthine oxidase (10 mg ml^{-1}). The reduction of NBT to formazan complex was followed for about 12 min at 560 nm. The percentage of NBT reduction was calculated as follows:

$$\%\text{of NBT reduction} = \frac{\text{rate of change in absorbance of sample}}{\text{rate of change in absorbance of control}} \times 100$$

$$\text{radical-scavenging ability} = 100 - \%\text{ of NBT reduction}$$

Statistical analysis

All analyses were carried out in triplicate with duplicate sets of samples. The mean of these values was taken.

RESULTS AND DISCUSSION

The methanolic extract of sesame cake was initially tested for antioxidant activity by the radical-scavenging assay using the diphenyl picrylhydrazyl (DPPH) radical[18] and the results showed high radical-scavenging activity for the extract.[10] The Schaal oven test[10,11] with three different vegetable oils, namely soybean, sunflower and safflower oils, also showed the ability of sesame cake extract to protect vegetable oils against oxidation at low concentration compared with that of BHT at 200 mg l^{-1}. The extract was analysed by analytical HPLC using a reverse phase C_{18} column and the peaks were identified as sesamol, sesamin, sesamolin, sesaminol diglucoside and sesaminol triglucoside by comparison of retention times of sesamol (Sigma), sesamin and sesamolin (isolated from sesame oil by the method of Soliman *et al*[13]). Glucosides were initially compared for their sequence of elution based on a previous report.[19] The compounds present in sesame cake extract included sesamol, sesamin, sesamolin, sesaminol diglucoside and sesaminol triglucoside. Preparative HPLC was carried out based on the optimised HPLC conditions of the analytical run. The collected fractions were concentrated and freeze-dried. The purity of the separated compounds was checked by analytical HPLC to see whether each compound eluted as single peak. Identification of pure compounds was confirmed by comparison of IR, NMR and MS data with those published previously.[14,20] The spectroscopic data for glucosides were compared with those of Katsuzaki *et al*.[20]

The individual compounds obtained from collected fractions by preparative HPLC were assayed for antioxidant activity by various *in vitro* methods. All compounds were tested at the same concentrations of 100 and 200 mg l^{-1}. BHT was used for comparative purposes. The maximum permissible level for BHT is 200 mg l^{-1}, hence it was used for the analysis at this concentration. The results are presented in Table 1. The activity follows the order sesamol > sesamolin > sesamin > BHT > sesaminol triglucoside > sesaminol diglucoside at 200 mg l^{-1}. The antioxidant activity (A_A) in terms of percentage inhibition for sesamol, sesamin, sesamolin, sesaminol triglucoside, sesaminol diglucoside and BHT after 2 h ($t = 120$ min) was 76, 59, 65, 30, 22 and 48% respectively at 200 mg l^{-1}. The activity values for the compounds at 100 mg l^{-1} are also given in Table 1. Induction of lipid oxidation is considered as a measure of antioxidant activity. In the β-carotene-bleaching method the oxidation of β-carotene takes place in the presence of linoleic acid. As a result, the absorbance values at 470 nm decreased. The presence of antioxidants hinders the oxidation of β-carotene. In the present study, using β-carotene bleaching, the activity shown by sesamin was slightly higher than that of sesamolin at 100 mg l^{-1} and lower at 200 mg l^{-1}. BHT showed higher antioxidant activity than that of glucosides and lower than that of lignans. Shyu and Hwang[19] also reported lower activity of the glucosides isolated from sesame meal extract by the low-density lipoprotein (LDL) oxidation method. In the present study also the activity of glucosides is lower than that of lignans and BHT.

Table 1. Antioxidant activity (%) of compounds isolated from sesame cake extract and butylated hydroxytoluene (BHT) by β-carotene-bleaching method

	Concentration (mg l^{-1})	
Compound	100	200
Sesamol	61 ± 1.76	76 ± 1.04
Sesamin	50 ± 2.52	59 ± 2.26
Sesamolin	47 ± 1.85	65 ± 1.75
Sesaminol triglucoside	26 ± 1.76	30 ± 1.93
Sesaminol diglucoside	17 ± 2.69	22 ± 2.32
BHT	32 ± 3.51	48 ± 1.53

An aqueous alcoholic extract of sesame seed has previously been reported[20] to show antioxidant activity. Antioxidant activity of lignans and lignan glucosides in defatted sesame meal extracted using 800 g l^{-1} methanol was reported by Shyu and Hwang.[19] In that study, compounds extracted and identified were sesamin, sesamolin, sesaminol diglucoside and sesaminol triglucoside. The presence of sesamol has not so far been reported in any of the sesame extracts studied earlier. In sesame oil it is reported to be present in trace amounts (≤50 mg l^{-1}).[1] However, in our study the extract of sesame cake with pure methanol was found to contain sesamol in appreciable amounts (>1 g kg^{-1} on an extract weight basis), whereas in aqueous alcoholic extract or aqueous extract no sesamol was detected.[10] The presence of sesamol was confirmed by HPLC, IR, NMR and MS data obtained for the corresponding fraction eluted from preparative HPLC. In our study,[10] sesame seed extracts with methanol and ethanol were also found to contain sesamol in measurable quantities. This is

the first report regarding the presence of sesamol in sesame cake extract.

Kang *et al*[21] have reported that when defatted sesame flour was fed to rabbits with induced hypercholesterolaemia, sesaminol glucosides could not protect against the condition, but could reduce the oxidative stress in cholesterol-fed rabbits owing to the antioxidant activity of sesaminol. However, sesamin also exhibits hypocholesterolaemic effects.[22] It retards cholesterol absorption from the gut as well as affecting cholesterol synthesis by controlling hydroxymethyl glutaryl-CoA reductase activity. In another study, Kang *et al*[7] reported *in vivo* antioxidant effects of sesamolin, which is present in sesame oil. The study suggests that sesamolin is metabolised to sesamol and sesaminol under *in vivo* conditions and that these compounds strongly inhibit lipid peroxidation.

Real food systems generally consist of multiple phases in which lipid and water coexist with some emulsifier. Hence an antioxidant assay using a heterogeneous system such as an oil-in-water emulsion is required. Autoxidation of linoleic acid in ethanol buffer is one of the model systems for such evaluation, satisfying the above conditions.[23] The linoleic acid emulsion system/thiocyanate method has been used here for evaluation under the above conditions. The results are shown in Fig 1. During peroxidation of linoleic acid at 37 °C in an incubator the absorbance values increased owing to the oxidation products, which react to form ferric thiocyanate, the colour of red blood.[24] Antioxidants can hinder the oxidation and, consequently, the increase in absorbance will be less. The inhibition exhibited by sesamol, sesamin, sesamolin, sesaminol triglucoside, sesaminol diglucoside and BHT was 77, 60, 69, 32, 25 and 49% respectively. The inhibition rate of compounds by the thiocyanate method supports the results of the β-carotene method.

The superoxide radical-scavenging effect of individual compounds isolated from sesame cake extract by the NBT assay using xanthine/xanthine oxidase is shown in Fig 2. Different concentrations of the antioxidant compounds were tried and the effect has been presented in Fig 2. At 50 mg l^{-1}, sesamol, sesamin and sesaminol diglucoside showed 48.4, 29.4 and 13.0% inhibition respectively and trolox showed 35% inhibition. Sesamolin had no effect at 50 and 100 mg l^{-1}, but an appreciable effect was seen at 300 and 500 mg l^{-1}. Sesaminol triglucoside also showed no effect at 50 mg l^{-1}. Sesamin and sesaminol triglucoside showed approximately 84% inhibition at 100 mg l^{-1}.

Superoxide is a biologically important substance which can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals. The highly reactive OH radicals can cause oxidative damage to DNA, lipids and proteins. Various strands of evidence have shown that superoxide radical anions ($O_2^{-\bullet}$) are generated in living cells by single-electron reduction of oxygen under physiological conditions. Superoxide radicals contribute to the pathological processes of many diseases.[25]

Xanthine oxidase is a key enzyme that catalyses the oxidation of xanthine or hypoxanthine to uric acid. During the catalytic oxidation, molecular oxygen acts as an electron acceptor, promoting superoxide radicals and H_2O_2. Consequently, xanthine oxidase is considered to be an important biological source of superoxide radicals.[26]

The peroxidation methods evaluated the percentage inhibition of lipid oxidation by antioxidants, and

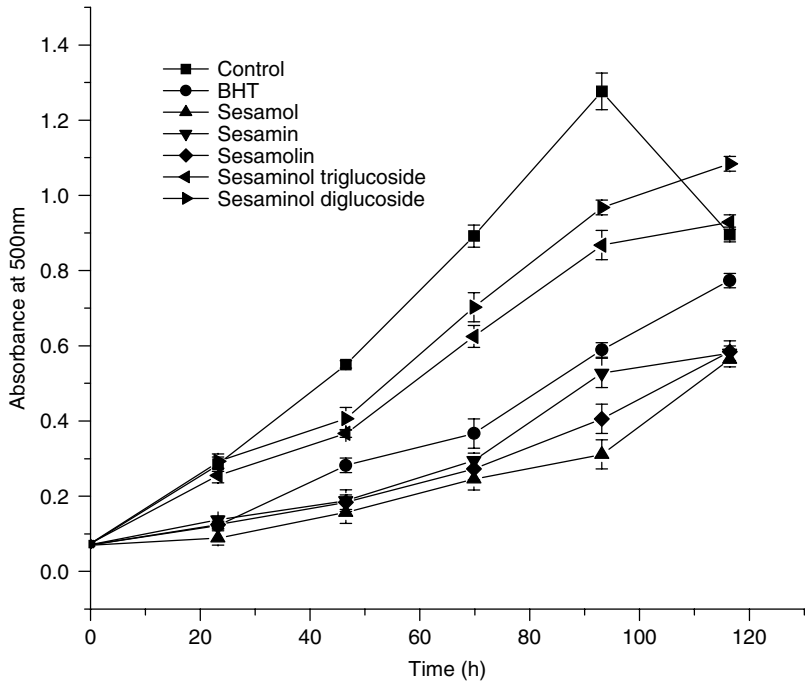

Figure 1. Antioxidant activity of compounds isolated from sesame cake extract and butylated hydroxytoluene (BHT) at 200 mg l^{-1} by linoleic acid emulsion system/thiocyanate method.

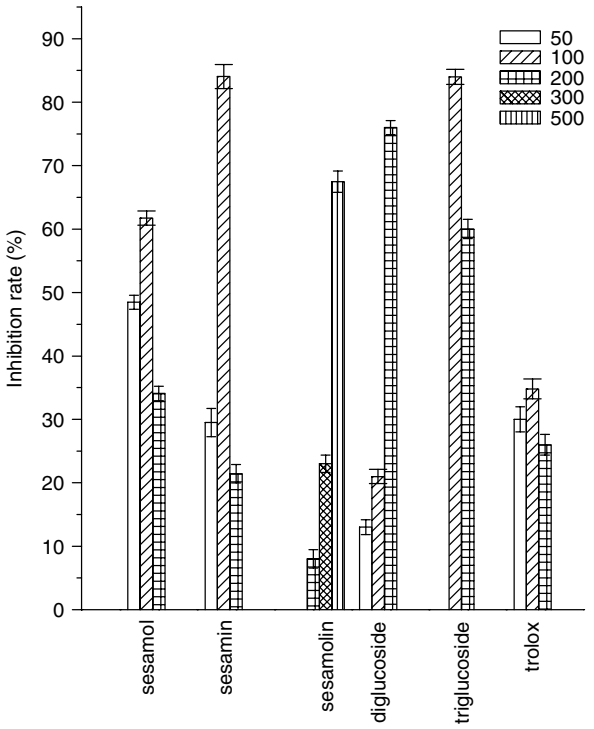

Figure 2. Superoxide radical-scavenging activity of compounds isolated from sesame cake extract and trolox by nitro blue tetrazolium (NBT) method. Concentrations are in mg l^{-1}.

this model system is closer to food systems and membrane lipids. Superoxide radical ($O_2^{-\bullet}$) is a biologically important radical and its increased generation is implicated in many human diseases, including cancer.[26] The superoxide radical-scavenging power of isolated compounds was also evaluated to get an idea of their effects on biological systems. The present paper establishes the antioxidant and superoxide radical-scavenging effects of individual antioxidant compounds, namely sesamol and the difuranoid lignans and lignan glycosides, present in defatted sesame cake extract. All the compounds show antioxidant effects, but the order of activity is different in different model systems and the activity depends on the concentration of the compound. The study complements the authors' previous work on sesame cake extract as a natural antioxidant substitute for protecting vegetable oils.[10,11]

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