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Inhibition of lipid peroxidation by extracts of *Pleurotus ostreatus*

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ABSTRACT

The inhibition of lipid peroxidation by *Pleurotus ostreatus* was established using Thiobarbituric (TBARS) assay. Three solvent extracts of *P. ostreatus* namely, the acetone, dichloromethane and hexane extracts were used. All three extracts showed inhibition of lipid peroxidation. The antioxidant indexes at 1.25 mg/ml concentration of acetone, dichloromethane and hexane were 38%, 43% and 36% respectively. Ergosterol was isolated and characterized from the dichloromethane extract. The antioxidant index of ergosterol at 1.25 mg/ml was 73% when tested using the same method. There exist a possibility that ergosterol can inhibit the peroxidation of common fatty acids present in egg yolk substrate (which was used for TBARS assay). The relative susceptibilities for peroxidation of ergosterol, linoleic acid and linolenic acid were investigated using computational calculations. It was found that the addition of an oxygen molecule to ergosterol is kinetically much more feasible than the addition of the same to linoleic or linolenic acids. The energy barriers for peroxidation with triplet oxygen at HF/3-21 G level of theory for ergosterol and linoleic acid (trans, trans) were 231.85 $kJmol^{-1}$ and 420.52 $kJmol^{-1}$ respectively. It was also found that the former reaction is thermodynamically less favorable compared to the latter. The results from theoretical investigation support the experimental observation of the capability of lipid peroxidation inhibition by ergosterol.

Keywords: Antioxidant activity, basidiomycetes, energy minimization, ergosterol, Oyster mushroom, TBARS assay.

INTRODUCTION

Inhibition of lipid peroxidation is gaining a lot of importance as it is accepted that large number of disorders such as cancer, diabetes, cardiovascular and other degenerative diseases and aging are closely related to the peroxidation reaction in living organisms.(1) As the natural antioxidants present under normal physiological conditions may be insufficient to inhibit these peroxidation reactions, enriching our diet with antioxidants (2) is useful to protect us from these harmful diseases.

Mushrooms are known to contain antioxidant properties. Fruiting bodies of the edible mushrooms *Agrocybe aegerita* (3) and *Lentinus edodes, Volvariella volvacea, Flammulina velutipes, Pleurotus* (4), (5), (6) were shown to possess antioxidant activity. Commercial mushrooms (7), medicinal mushrooms (8), ear mushrooms (9) and mushroom mycelia (10) in Taiwan are known to posses antioxidant activity. A study conducted in Portugal (11) on *Lactarius deliciosus* and *Tricholoma portentosum* showed *Lactarius deliciosus* to have more antioxidant activity and that the activity is concentrated in the cap of the mushroom.

Further, polyphenols (12), hispidins (13), a peptide (14), a pseudo-di-peptide (15), Betulinan A (16), Sterins A and B (17) have been identified as compounds responsible for antioxidant activity in some mushrooms.

Objective of the present study is to evaluate the lipid peroxidation inhibitory property of *Pleurotus ostreatus*

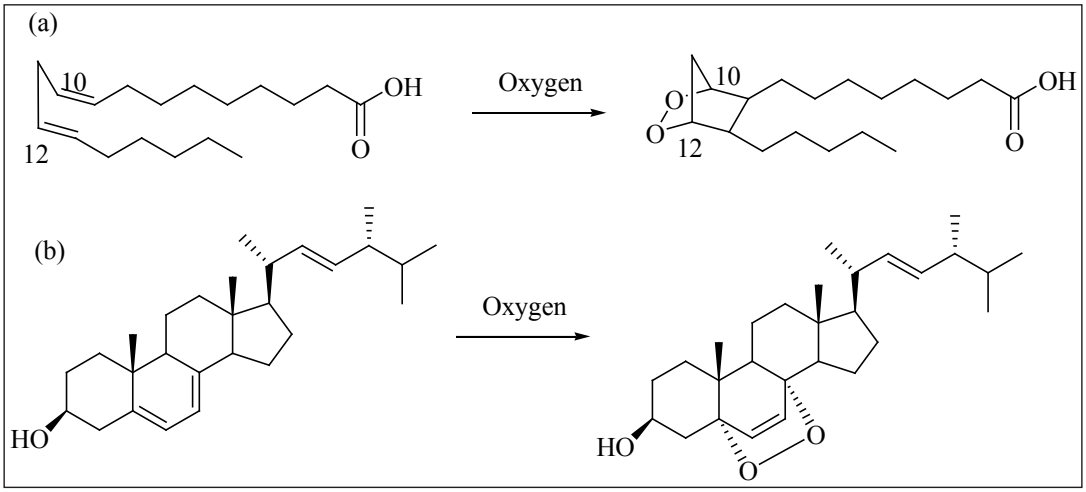

Figure 1: (a) Formation of oxygen adduct with linoleic acid, the adduct breaks down to give malonaldehyde (b) Oxygen addition to ergosterol results ergosterol peroxide.

(Jacqu: Fr.) Kummer using TBARS method and to identify the compound responsible for the said activity.

In the assay of lipid peroxidation, malonaldehyde detected by the TBARS method is known to arise from the oxygen adduct (18) of polyunsaturated fatty acids (Fig. 1a). It is also known that ergosterol which is a common steroid found in fungi (19), too undergoes oxygen addition giving ergosterol peroxide (Fig. 1b). Relative reactivity of linoleic acid, linolenic acid (common polyunsaturated fatty acids in egg york) and ergosterol towards oxygen was investigated by doing *ab initio* computational calculations.

METHOD AND MATERIALS

Preparation of egg yolk

Egg yolk was separated from the albumen and the yolk membrane was removed. 10 % v/v solution was prepared in 1.15 % of KCl. The solution was homogenized for 30 seconds and ultra sonicated for 5 min.

Preparation of Thiobarbituric acid (TBA)

0.8 % (w/v) of TBA solution was prepared using 1 % SDS solution.

Preparation of mushroom extracts

Acetone extract of P. ostreatus *(AE)*

Fresh *P. ostreatus* mushroom (3 kg) was extracted with 3 l of distilled acetone twice using a homogenizer. The solution was filtered and it was evaporated using a rotary evaporator. The crude extract was freeze dried to obtain 65 g of dark brown solid.

Dichloromethane extract of P. ostreatus *(DE)*

The residue after extracting with acetone was again extracted with 1.5 l of distilled dichloromethane for two times and it was filtered. The solvent was evaporated using a rotary evaporator and freeze dried.

Hexane extract of P. ostreatus *(HE)*

The residue after extracting with dichloromethane was again extracted with 1.5 l of distilled hexane twice and the extract was filtered. The solvent was evaporated using a rotary evaporator and freeze dried.

From each extracts 5.00, 2.50, 1.25 and 1.00 mg /ml of test samples were prepared.

TBARS method

Into 4 snap capped vials different concentrations of AE (5.00, 2.50, 1.25 and 1.00 mg/ml concentrations 10 µl each) and egg yolk 50 µl were added. Distilled water (10 µl) was used as the control and ascorbic acid (10 µl from 100 µl/ml solution) was used as the positive control. Acetic acid (20% solution, 150 µl) and 0.8% thiobarbituric acid (TBA, 150 µl) were added to each snap capped vial. Total volume was adjusted to 400 µl by adding distilled water. These mixtures were vortexed for 5 s and kept in a water bath (LCH-110 Lab Thermo Cool, Advantec, Tokyo, Japan) at 95 °C for 60 min. Butanol (1 ml) was added to each tube and vortexed for 5 s. After centrifuging at 1500 g for 5 min, butanol layer was separated. Absorbance values were measured at 532 nm. (20)

This procedure was repeated for extracts DE, and HE extracts. Antioxidant index (AI) was calculated using the following equation.

AI = (1−T/C) × 100

T = absorbance of test sample

C = absorbance of fully oxidized control

All values are based on the Anti-oxidant index whereby the control is completely peroxidized and each drug providing a degree of improvement, indicated as % protection.

In case of DE and HE extracts as the solubility was low in water, 30% dimethylsulfoxide (DMSO) in water was used in preparation of solutions. Therefore 30% DMSO was used as the control in these two experiments.

Purification of Dichloromethane extract

Silica gel (30 g, TLC grade silica gel 60 GF_{254}, Merck KGaA, Darmstadt, Germany) and gypsum (12 g) were mixed well and 85 ml of distilled water (0–10 ^{0}C) was added and stirred until a clear slurry is obtained. This was carefully poured onto a chromatotorn plate and allowed to set for 24 hrs. After cutting the rough surface it was used to purify the dichloromethane fraction. This fraction dissolved in minimum amount of dichloromethane was loaded onto the chromatotorn and eluted with hexane. Thereafter, solvents with increasing concentrations of CH_2Cl_2 were used until 100% CH_2Cl_2 is used as the mobile phase. Fraction having 4:6 mixture of hexane:CH_2Cl_2 gave a white crystalline compound. NMR spectra of this compound was recorded.

The lipid peroxidation inhibition ability of this compound was also evaluated using the TBARs assay. The solvent used in the preparation of ergosterol was 30% DMSO in water.

Computational details

Thermodynamic parameters for lipids (ergosterol, linoleic acid, linolenic acid and their oxygen adducts) was calculated by fully optimizing the geometries of lipids and the corresponding oxygen adducts at B3LYP/6-31G level of theory. It was verified that they represent true minima by the absence of negative frequencies. The energy barriers for reactions were calculated at HF/3-21G level of theory by optimizing the reactants and the transition states. All the above calculations were performed in a PC (3GHz Pentium processor and 2GB of RAM, Windows XP operating system) using the calculation package Gaussian 98 [21]. In the geometry optimization of linoleic acid the double bonds in both *trans*, both *cis* and *cis* & *trans* forms were considered. In the case of linolenic acid the three double bonds in all *trans* was taken into consideration.

The addition of oxygen to these molecules was modeled by placing an oxygen molecule (singlet and triplet oxygen) near the two double bonds undergoing oxygen addition and by fully optimizing the geometry of the adduct without any constraints. The barrier height for oxygen addition to each compound was obtained by taking the energy difference between the optimized molecule and the transition state geometry for oxygen addition. In the case of linolenic acid which has three double bonds, oxygen addition can take place at the two double bonds closer to the ω-3 side or to the inner double bonds. The relative magnitudes of energy barriers for these additions were also calculated.

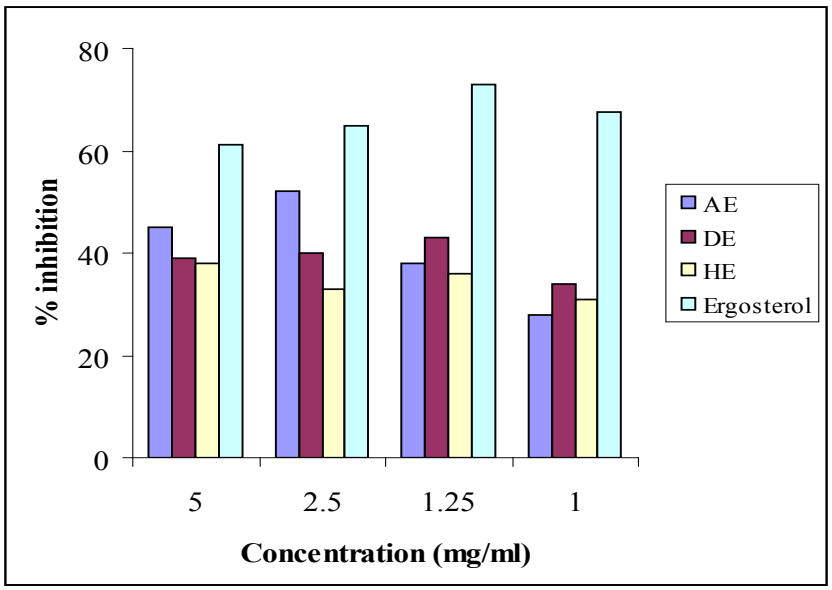

Figure 2: Anti-oxidant index of three extracts of *Pleurotus ostreatus*

RESULTS

TBARS method

The inhibition of lipid peroxidation by the three extracts **AE**, **DE**, **HE** and ergosterol is shown in Fig. 2. The positive control, Ascorbic acid showed 53 % lipid peroxidation inhibition activity. The extract **AE** showed the highest antioxidant index at 2.5 mg/ml concentration. On the other hand, the extract **DE** and ergosterol showed the highest antioxidant index at 1.25 mg/ml concentration.

Purification of Dichloromethane extract

The white solid obtained from the purification by chromatotron was identified as ergosterol based on the comparison of ^{13}C NMR data to the literature (22) reported values.

Computational details

As expected the results of the geometry optimization indicated that the lowest energy structures of linoleic acid and linolenic acid are the ones with all double bonds in *trans* geometry. Frequency calculations performed for the optimized geometries of stable species gave no negative frequencies indicating that these were true minima. Similar observations were made for ergosterol as well.

In the case of oxygen adducts, it was verified that the structures represent true minima by the absence of negative frequencies.

The transition state geometries for oxygen addition to ergosterol and linoleic acid are shown in Fig. 3. Energy differences between all other fatty acids and corresponding transition states (energy barriers) are given in Table 1. The Gibbs free energy changes for peroxidation with triplet and singlet oxygen were also calculated and are also given in Table 1.

The energy barriers (ΔE) for peroxidation of ergosterol and linoleic acid (trans, trans) were 231.85 $kJmol^{-1}$ and 420.52 $kJmol^{-1}$ respectively when calculated using transition state optimization at HF/3-21 G level of theory. This shows that there exists a clear difference in reactivities of ergosterol and linoleic acid. The reason to have a larger energy barrier for linear fatty acid is that the molecule needs to be bend for a five membered carbon ring along the reaction path. This structure is associated with a large loss of entropy and cause high bond strech compared to the transition state structure of ergosterol.

DISCUSSION AND CONCLUSION

In the present study inhibition of lipid peroxidation ability of *Pleurotus ostreatus* extracts was scientifically evaluated. At most concentrations, either the acetone extract (AE) or dichloromethane extract (DE) showed the highest activity. It is reported in the literature (23) that Pleuran which is a beta-glucan isolated from *P. ostreatus* had shown antioxidant activity. Hence the possibility exists that the presence of lipid proxidation activity in extract AE may be due to these beta-glucans. One can reason out the lipid peroxidation ability of extract DE as follows. Ergosterol is known to undergo (24) facile reactions with singlet oxygen yielding a variety of oxygen adducts. The major product is (22E)-5α,8α-epidioxyergosta-6,22-dien-3β-ol. If a similar reaction can takes place between ergosterol and triplet oxygen, peroxidation of other lipids could be minimized. Isolation and characterization of ergosterol from the extract DE gave preliminary evidence supporting this argument. The mechanism in which ergosterol present in *P. ostreatus* brings about the inhibition of lipid peroxidation activity can be explained using the results of our computational calculations as follows.

In the TBARS assay, the lipid substrate used was egg yolk. It is known that linoleic acid and linolenic acid are two of the polyunsaturated fatty acids present in this substrate. Upon reaction with oxygen these fatty acids produce malonaldehyde which reacts with TBA producing a pink

Table 1: Thermodynamic parameters for peroxidation.

Molecule (double bond geometry:Position of oxygen addition)	Kinetic/Thermodynamic parameters (kJ/mol)			
	$\Delta E^{\#}_{triplet}$	$\Delta E^{\#}_{singlet}$	$\Delta G_{triplet}$	$\Delta G_{singlet}$
Ergosterol	231.85	16.81	- 3.80	-171.28
Linoleic acid (*trans, trans*)	427.39	212.34	- 33.04	-200.52
Linoleic acid (*trans, cis*)	420.52	198.45	- 44.01	-211.48
Linoleic acid (*cis, cis*)	413.49	205.47	- 53.51	-220.98
Linolenic acid (*trans, trans, trans*:10, 12)	396.15	181.10	-35.10	-202.58
Linolenic acid (*trans, trans, trans*: 13, 15)	399.12	184.07	-35.62	-203.11

For the transition state

$\Delta G_{triplet}$: Gibbs free energy change for the reaction with triplet oxygen

$\Delta G_{singlet}$: Gibbs free energy change for the reaction with singlet oxygen

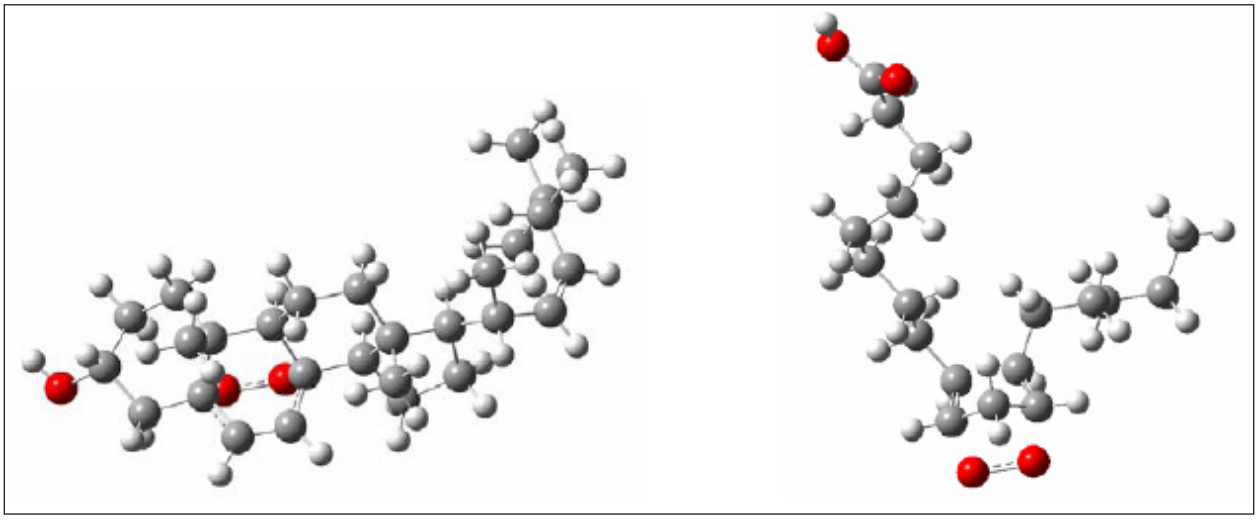

Figure 3: The structures of transition states of ergosterol and linoleic acid

color. Our results indicate that dicholomethane extract (DE) of *P. ostreatus* contains ergosterol. On the other hand the addition of DE to egg yolk substrate inhibits lipid peroxidation as indicated by the low color production with TBA. If ergosterol can react faster with oxygen than the polyunsaturated fatty acids present in egg yolk, one could speculate that ergosterol is an active ingredient responsible for the inhibition of lipid peroxidation. Computational modeling may provide an insight to understand this speculation. The thermodynamic feasibility of a reaction can be obtained by the Gibbs free energy change while the kinetic feasibility is given by the activation energy. Even though a reaction is highly thermodynamically feasible, a large activation energy barrier hinders product formation. Developments in computational chemistry enable one to estimate both these parameters.

According to the results of our computational calculations, the smallest energy barrier for oxygen addition was observed for ergosterol. However the Gibbs free energy change for the oxygen adduct formation with ergosterol is the least negative compared to the formation of oxygen adducts with linoleic and linolenic acid. On the other hand activation energy barriers for oxygen adduct formation of linoleic and linolenic acids are significantly higher than that of ergosterol. This difference in energy barrier corresponds to a much higher rate in the ergosterol oxygen addition reaction compared to the same reaction with linoleic and linolenic acids.

These results help us to conclude that ergosterol present in the extract DE can bring about the inhibition of lipid peroxidation. At this juncture we wonder about the validity of the literature reported data (25) stating that 5,8-epidioxy-ergosta-6,22-dien-3-ol as one of the antioxidant isolated from *Agrocybe aegerita*. How ever the same study states that ergosterol too has antioxidant activity and our study supports that evidence.

Finally we conclude that the freeze dried AE or DE extracts from *P. ostreatus* has lipid peroxidation inhibitory activity. As *P. ostreatus* is an edible mushroom, the freeze dried mushroom powder it self may be useful as a dietary supplements having antioxidant activities.

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