RESEARCH ARTICLE

Antioxidant effect of ethanolic extract of *Pleurotus Ostreatus* on 4-hydroxy-2-nonenal (HNE) and glutathione (GSH) level in lung rats exposed to cigarette smoke [version 1; peer review: 2 approved with reservations]

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Abstract

Background: Cigarette smoke can trigger oxidative stress. An alternative to overcome the harmful effects of cigarette smoke is through antioxidants. White oyster mushrooms (*Pleurotus Ostreatus*) are a source of exogenous antioxidants because many contain active compounds for potential antioxidants such as phenol and flavonoid compounds. The purpose of this study was to analyze the effect of ethanolic extract of *Pleurotus ostreatus* on 4-hydroxy-2-nonenal (HNE) and glutathione (GSH), as well as to analyze their correlation in the lung of Wistar male rats exposed to cigarette smoke.

Methods: The study was a preclinical experiment conducted on 24 rats divided into four groups. The treatment was carried out for 42 days and antioxidant effects were assessed through levels of HNE and GSH in rat lungs. Groups were divided as follows: I- normal control, II- negative control, III and IV exposed to cigarette smoke for 60 minutes/day. Group III (treatment group) was treated with ethanolic extract of *Pleurotus ostreatus* at 250 mg/kg BW rat/day, and group IV (comparison group) was treated with N-acetyl cysteine 600 mg /day. Data analysis used was one-way ANOVA and Kruskal Wallis test, and Spearman rank correlation coefficient test.

Results: The results showed that the group receiving ethanolic extract of *Pleurotus ostreatus* had HNE levels of (44,18 ± 2,09 pg/mL) and GSH (0,04 ± 0,00 pg/mL) protein. This extract significantly increased GSH levels and inhibited the increase of HNE levels. Results of GSH (p ≤ 0.01) showed significant results using one-way ANOVA.

Conclusions: The ethanolic extract of *P. ostreatus* can prevent lipid

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peroxidation and decrease endogenous antioxidant levels in lung cells exposed to cigarette smoke. Ethanol extract of *Pleurotus ostreatus* has good antioxidant potential.

Keywords

Antioxidants, cigarette smoke, ethanol extract, lung rats, *Pleurotus ostreatus*

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Introduction

Cigarette smoke is a mixture of more than 4,700 chemical components (nicotine, tar, benzene, carbon monoxide, etc.), reactive oxygen species (ROS), and a large source of free radicals that are divided into the gas phase and tar phase.^{1–3} Free radicals can cause oxidative stress, and cause damage to the important biomolecular (lipid, sugar, protein, DNA), membrane dysfunction, protein modifications, DNA damage, and enzyme inactivation. The effects of cigarette smoke on the body are influenced by many variables, including the dose and type of tobacco used, the route of administration, duration or duration of exposure, and other factors that may be present during stimulation. Inactive smokers and passive smokers, cigarette smoke causes a rapid dissolution of toxins in the mouth or respiratory epithelium and systematically.^{2,4–7} Acute and chronic exposure of cigarette smoke can result in tissue damage that can increase lipid peroxidation products and degradation products of extracellular matrix proteins, such as malondialdehyde (MDA), 8-isoprostane, hydrogen peroxide, and 4-Hydroxy-2-nonenal (HNE).^{2,8,9}

HNE is one of the cytotoxic lipid peroxidation end-product lipids from $\omega 6$ polyunsaturated fatty acids. Linoleic acid and arachidonic acid are potential precursors of HNE. 4-Hydroxy-2-nonenal can react with proteins and form HNE-adduct proteins with histidine, cysteine, and lysine. This complex can change the function of these proteins.^{6,8} Cigarette smoke can increase lipid peroxidation and HNE formation, which then activates mitogen-activated protein kinase (MAPK) and the release of TGF β 1 and induction of γ GCS. This eventually causes inflammation because there is a redox imbalance.^{6,8}

Oxidative stress can be inhibited by the body's defense system through antioxidants which can eliminate free radicals and minimize damage. Various enzymatic and non-enzymatic systems play a role in this process.^{10,11} Antioxidants can be defined as chemical molecules containing monohydroxy/polyhydroxy phenols and work by inhibiting lipid peroxidation.^{12–14} The appropriate antioxidants, both endogenous and exogenous antioxidants are important in the overall defense mechanism.^{9,15} Glutathione (GSH) is the primary antioxidant because it produces in the body and is important for the first defense mechanism to free radicals. It is also a secondary antioxidant with N-acetylcysteine (NAC) and flavonoids that act as radical scavenging to prevent the chain reaction of free radicals, play a role in various transcription factors, and detoxifications. Cigarette smoke can make ineffectively of endogenous antioxidants and the body needs to supply exogenous antioxidants to maintenance the defense.^{9,15,16} Several studies have shown that smokers have lower levels of vitamins E, C, β carotene, and GSH in plasma compared to nonsmokers.^{2,10,17} Glutathione is abundant in the body and are found in Epithelial lining fluid (ELF) in high concentrations and serve to protect various oxidants that enter inhalation. GSH concentrations in ELF 10–100x higher than the levels in plasma. Exposure to chronic cigarette smoke can reduce GSH levels in ELF, plasma, and lungs after the GSH adaptive response period is exceeded.^{18–20}

Previous studies have shown that supplementation with vitamins C and E is expected to reduce the harmful effects of cigarettes. The effectiveness of most antioxidants is generally seen in proportion to the number of hydroxyl (OH) groups present in the aromatic ring so that natural substances appear to have better antioxidant activity than synthetic antioxidants.^{10,16,21,22} One exogenous antioxidant that has been found in foods that have strong antioxidant effects is a white oyster mushroom (*Pleurotus ostreatus*)^{7,12,23}

P. ostreatus are nutritious, high in fiber, and contain many vitamins and minerals.^{10,24–26} This oyster mushroom has strong antioxidant activity due to secondary metabolite compounds contained in it. Components in white oyster mushrooms that are believed to have antioxidant effects include vitamin C, beta-carotene, selenium, ergothioneine, and phenolic components for the primary component. Phenolic antioxidant activity is mainly due to its ability as a hydrogen donor reducing agent and oxygen quencher singlet and has a potential metal chelation effect.^{7,12,27} Phenolic antioxidant derivatives will also induce GSH enzyme synthesis in human alveolar epithelial cells.^{12,21,24} *P. ostreatus* as a whole when compared with winter and shitake mushrooms have antioxidant activity, reducing power, scavenging abilities, and higher total phenol content.^{7,10,23,28} Previous studies have shown that *P. ostreatus* extract has a protective effect on the liver, kidneys, and brain.^{7,29} Other studies have shown that ethanol extract of white oyster mushrooms has the effect of preventing an increase in MDA¹¹ levels and a decrease in lung density in rats induced by cigarette smoke.^{16,24,28,30} In this study, we assess the antioxidant effect of *P. ostreatus*, their influence in preventing a decrease in GSH levels and preventing an increase in HNE levels, and their correlation in Wistar rat lung cells that are exposed to cigarette smoke.

Methods

Study design

The laboratory experimental study was conducted *in vivo* using Wistar strain male rats. The study design used was a randomized block design. This study is reported in line with the Animal Research: Reporting of *in vivo* Experiments (ARRIVE) guidelines.⁴⁹

Subjects

This study uses male Wistar strain rats that were selected based on the inclusion criteria and exclusion criteria that have been set. Inclusion criteria were animals of 8-12 weeks of age and a weight of 200-250 grams at the beginning of the treatment. Exclusion criteria were rats that showed behavioral changes during the adaptation period and had weight loss >10% during the adaptation period. Some animals were dropouts if they died during the research treatment period.

Sample size

Rats were randomly selected for each group and the number of samples was determined based on the Power Analysis formula, using *G * Power* 3.1.9.2 software. The sample size required for this study is a minimum of 20 experimental animals and considering the possibility of dropout (DO), the total number of experimental animals in this study will be 24.³¹

Research variable

The variables used in this study consisted of three groups of variables, namely: independent variables (ethanol extract of white oyster mushrooms, exposure to cigarette smoke); dependent variables (HNE level, GSH level); and controlled variables (rat strain, sex, weight, age, food and drink).

Research tools and materials

The equipment used in this study were: a smoking pump/tobacco smoke discharger (designed for this research, more information below), HNE Elisa Kit Assay (Abbexa) 83, Reduced Glutathione (GSH) Assay Kit (Colorimetric) (K464-100) 84, tissue homogenizer, multichannel micropipette reservoirs; Multiscan GO Reader (Thermo Scientific 1510).

A tobacco smoke discharger (**Figure 1**) is a device that functions as a means of sucking cigarette smoke and flowing it into the test room (smoking room) with a certain flow speed. This tool utilizes electric power which is then converted into mechanical energy so as to produce a pressure difference sufficient for the suction and flow of cigarette smoke. The use of this tool is for research purposes because this tool helps to simulate a test room containing cigarette smoke with a certain intensity and its impact on test animals is investigated. The advantage of using this tool is the achievement of steady and measurable smoke flow stability so as to produce valid research data.

White oyster mushroom determination

Determination of white oyster mushrooms was carried out at the Sekolah Tinggi Ilmu Hayati (STIH) Institut Teknologi Bandung (ITB), aimed at ensuring macroscopic identification of white oyster mushrooms based on size, shape, color, surface characteristics, and texture of natural materials.

Preparation of ethanolic extract of *Pleurotus ostreatus* and dosage

Plant material was derived from fresh white oyster mushrooms taken from Panandaan oyster mushroom cultivation, Jambudipa Village, Cisarua District, West Bandung Regency. Fresh mushrooms were washed, drained, thinly torn with a thickness of 1-2 cm, then put in an oven with temperature of 50°C for 5-6 days. The dried mushroom was mashed and then put into the macerator and macerated by ethanol 96% in a ratio of 1:10. This mixture was macerated for 24 hours with occasional stirring and then filtered. Maceration was repeated 3 times. The aqueous extract was then concentrated using a rotary evaporator and heated in a water bath.³² The concentrated extract is in the form of a paste.

Based on empirical data and previous research on white oyster mushrooms, this study used a dose of 250 mg/kg BW.^{7,10} The comparison group was given the drug N-acetyl-cysteine (NAC) which is a precursor of GSH and acts as a free radical

Figure 1. Tobacco smoke discharger.

scavenger. The dose of NAC used in chronic obstructive pulmonary disease (COPD) cases is 600 mg once a day.³³ The dose was converted to the Paget & Barnes (1964) conversion table for rats.

Induction of cigarette smoke with smoking pump (tobacco smoke discharger)

A tobacco smoke discharger can smoke 2 cigarettes in an average time of 6 minutes 30 seconds and the time needed to refill cigarettes and restart combustion is about 30 seconds. In this study, each group received exposure to cigarette smoke for 60 minutes/day or the equivalent with eight cigarettes/group/day with a time lag of about 5 minutes for burning two cigarettes. The cigarettes given were clove cigarettes with a tar content of 38 mg.

Measurement of HNE levels

Measurement of HNE levels of protein adducts was taken using 4-HNE ELISA Assay Kit as per manufacturer's instructions, a competitive enzyme-linked immunosorbent assay technology developed for quantitative rapid examination. Samples were taken from rat lung tissue, washed with phosphate buffer then homogenized and centrifuged at 1,000 rpm for 20 minutes at 4 ° C. The supernatant from the centrifugation result was then used for 4-HNE examination, compared with standard of 4-HNE.³⁴

Measurement of GSH levels

Measurement of glutathione (GSH) levels in lung cells was done using BioVision's Reduced Glutathione Kit as per manufacturer's instructions. Samples were taken from tissue homogenates from lung cells. This tool uses the principle of colorimetric reaction, by measuring the quantification of GSH levels from the absorbance of chromophore reduction formed at a wavelength of 450 nm. This tool can detect GSH at a minimum level of 50 pmol/well GSH in a 100 µL reaction. In this study samples were taken from rat lung tissue and homogenized by centrifuged at 10,000 rpm for 20 minutes at 4° C. The centrifugation supernatant is used for glutathione examination.³⁵

Research flow

Rats were adapted to a laboratory atmosphere for 7 days. During the adaptation period, the experimental animals were kept in comfortable cages, one group/cage, with a large enough cage size (60x45x35 cm) so that the animals could move actively, with sufficient lighting in the room where the rat cage is stored, and low humidity. The animals in this study were given the type of food and food calories that were adjusted to their needs. Drinking water was provided in the cage and given *ad libitum*. The rats were randomized with a completely randomized design (CRD) into 4 groups each consisting of 6 rats and given different treatments: Group I: Negative control group (rats were given standard food and drink for 42 days, were not exposed to cigarette smoke and did not get ethanolic extract of *P. ostreatus*), Group II: positive control group (rats given cigarette smoke exposure for 60 minutes/day for 42 days using a smoking pump in the smoking chamber. Cigarettes were kretek brand. Without the administration of ethanolic extract of *Pleurotus ostreatus*, only given carrier fluid 3 cc/rat/day). Group III: treatment group (rats were given cigarette smoke exposure for 60 minutes/day in 42 days and were given ethanolic extract of *Pleurotus ostreatus* dose 250 mg/kg BW orally), group IV: comparison group rats were given cigarette smoke exposure for 60 minutes/day in 42 days and given N-acetyl-cistein (NAC) dose 10.8 mg/day orally. On the 43rd day, rats were taken for lung tissue to be examined for levels of HNE and glutathione in rat lung tissue.

The rats were previously anesthetized using ketamine and the rat lung tissue was surgically removed. The rat was supine, then the ventral part of the thoracic cavity was opened using surgical instruments and the lungs were removed using a scalpel. After that, the rats were euthanized by deep anesthesia or increasing the anesthetic dose until the animals died. The carcasses of the rats were exterminated using incinerator.

During the research, experts or laboratory assistants supervised the experimental animal research process from the adaptation process of the experimental animals to execution. Sampling and analysis of the results are carried out by the research team.

Data analysis

Data analysis with Shapiro-Wilk for normal data and Bartlett's test for equal variances was done. After that Kruskal Wallis test was used for HNE and the analysis of variance (ANOVA) for GSH, followed by post hoc Bonferroni test. A 95% confidence level ($P < 0.05$) was considered significant. A Spearman's rank test was also done to test the correlation between the GSH and HNE parameters. SPSS v 20.0 was used to analyze data of 4-HNE and GSH, and Stata MP 16 was used for testing the correlation among the two variables.

Ethical aspects

This research applies the ethical principles of research in general: Respect, Benefit, and Justice and the principle of using experimental animals, namely 3R: replacement, reduction, and refinement. This research got ethical approval from The

Health research ethics committee of Faculty medicine of Padjadjaran University No: 210/UN6.C10/PN/2017, in accordance with "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985). All efforts were made to ameliorate harm to the animals by following appropriate procedures for animal experiments.

Time and place researched

The experimental study was conducted at the Faculty of Pharmacy, Universitas Islam Bandung, and Aretha Medika Main Biomolecular Research Center from January 2018 to January 2019.

Results

On macroscopic examination of the lungs, it was seen that the rat lung tissue was pink and did not show any significant color differences in each group. With a smooth lung surface, there were no lumps or nodules visible or palpable.

Table 1 shows that the group with the highest average HNE value was group 2 with HNE levels of 47.70 ± 5.35 pg/mol, while the group with the lowest HNE was group 1 with HNE 40 values, 40 ± 1.93 pg/mol.⁴⁹ HNE levels in group 3 and group 4 were higher than group 1, but lower than group 2. Group 3 had a lower HNE value than group 4.

Table 2 shows the K-Wallis test outcome of HNE data with a value of $P=0.03$ which means that there is a significant difference in the mean HNE between the test groups. In this study, it may be concluded that cigarette smoke caused a significant increase in HNE levels compared to normal controls and ethanolic extract of *Pleurotus ostreatus* significantly reduced the level.

The ANOVA test P-value for GSH is 0.00 ($p \leq 0.05$). There is a significant difference in the mean GSH between groups, with Bartlett's P-value greater than 0.05 so that the post-hoc Bonferroni test can be used seeing the difference in mean GSH between groups. Significant mean differences were found in almost all groups, except for differences between groups 3 and 4 (**Table 3**).

In this study, cigarette smoke was shown to significantly reduce GSH compared to normal controls, and ethanol extract of white oyster mushroom significantly increased GSH levels in the lung of rats exposed to cigarette smoke. The effect of the ethanolic extract of *Pleurotus ostreatus* in experimental animals was as good as the effect of NAC. The GSH and HNE

Table 1. Results of Analysis of 4-hydroxy-2-nonenal (HNE) and glutathione (GSH) levels of Rat Lung Network on Day 43. Group 1: normal control group (normal feed, without treatment); Group 2: negative control group (normal feed, cigarette smoke 60 minutes/day); Group 3: treatment group (normal feed, ethanol extract of *Pleurotus ostreatus* 250 mg/kg BW, exposure to cigarette smoke 60 minutes/day); Group 4: comparison group (normal feed, NAC, cigarette smoke exposure 60 minutes/day); SD: standard deviation; 4-HNE: 4-hydroxy-2-nonenal; GSH: glutathione.

Groups	Average group concentration			
	4-HNE (pg/mL)		GSH (pg/mL protein)	
	Value	SD	Value	SD
1	40.40	1.93	0.02	0.00
2	47.70	5.35	0.01	0.00
3	44.18	2.09	0.04	0.00
4	46.65	5.43	0.03	0.00

Table 2. Results of Statistical Analysis and Correlation of 4-HNE and GSH Levels. 4-HNE: 4-hydroxy-2-nonenal; GSH: glutathione; P (F) *: significant <0.05; P (Bartlett) *: significant > 0.05; P (K-Wallis) *: significant <0.05; P (correlation) *: significant <0.05; rs (correlation coefficient): significant > 0.00.

Variable	P(F)	P value	P (K-Wallis)	correlation	
		(Bartlett)		P	r_s
4-HNE			0.03*		
GSH	$\leq 0.01^*$	0.68*			
GSH-4-HNE				0.78	0.06

Table 3. The post-hoc Bonferroni test for glutathione (GSH) intergroup.

Variable	Row-col mean	1	2	3
GSH	2	−0.01 (0.00)		
	3	0.02 (0.00)	0.02 (0.00)	
	4	0.01 (0.00)	0.02 (0.00)	−0.004 (0.13)

parameters were then tested for correlation using Spearman's rank analysis. The results show the value of $P = 0.78$ and $r_s = 0.06$ so it can be concluded that there is no correlation between GSH and HNE.

Discussion

In this study, exposure to kretek cigarette smoke in rats for six weeks was expected to cause significant oxidative stress in rats.^{4,36} Free radicals in cigarette smoke can cause membrane damage through lipid peroxidation, which will cause a cascade of further oxidative damage, increase cell membrane rigidity, osmotic fragility, decrease the life span of erythrocytes, and disrupt lipid fluidity.⁷

Lipid peroxidation will produce secondary metabolites from peroxidation products such as malondialdehyde (MDA) and HNE.^{7,8} Secondary product levels of lipid peroxidation are thought to have a correlation with the degree of obstructibility of the respiratory tract in COPD patients.³⁷ Membrane damage or lipid peroxidase also can cause changes in phospholipase to arachidonic acid which can further increase HNE and induce the release of inflammatory mediators such as prostaglandin and leukotrienes. This can cause the respiratory tract to become more vulnerable to inflammation and develop cell proliferation changes and cause airway obstruction.^{37,38}

4-Hydroxy nonenal is a specific metabolite which is very toxic, reactive and has high diffusion ability. It also has an important role as a signaling molecule for cell differentiation, proliferation, regulation of the cell cycle and regulation and expression of various stress-response genes. The concentration of HNE in cells can be used for the determination of signaling for apoptosis, differentiation, or proliferation.^{8,39} In this study the high HNE in rats exposed to cigarette smoke indicates that lipid membrane damage has occurred and poses a risk for further damage and can initiate a heavier process. Increased 4-HNE adducts have a good correlation with pulmonary function measurement through FEV1. This reinforces the suspicion that 4-HNE has an important role in the pathogenesis of COPD.^{40,41}

The ethanolic extract of *P. ostreatus* at a dose of 250 mg/kg body weight in this study showed a preventive ability or could inhibit the increase in HNE levels in rats exposed to cigarette smoke exposure, so it can be concluded that this extract can inhibit pulmonary lipid peroxidation due to free radicals from cigarette smoke.

The mechanism of *P. ostreatus* ethanol extract in inhibiting the increase in HNE can be caused by several mechanisms, mainly because the hydroxyl group in the ethanol extract of white oyster mushroom can function as an excellent hydrogen donor so as to prevent membrane damage due to free radicals. Another possibility is to increase the synthesis of endogenous antioxidants such as glutathione, so that further damage from oxidative stress can be prevented.

These results are reinforced by *in vitro* data in previous studies, which showed the potential antioxidant activity of ethanolic extracts of white oyster mushrooms. *In vitro*, the ethanolic extract of white oyster mushroom with a concentration of 10 mg/mL has an effectiveness of 56.20% in inhibiting lipid peroxidation (LPO) activity, while ascorbic acid as a standard can inhibit 67.15%. The 50% inhibitory concentration for lipid peroxidation activity for extracts and ascorbic acid was 8 and 6 mg/mL.^{7,28} The water-soluble *P. ostreatus* polysaccharide extract on Caco-2 cells showed inhibition of lipid peroxidation with MDA markers with fluctuating results for three days, the first day showed a decrease in MDA, but the third day the effect of the inhibition disappeared. This is thought to be correlated with GSH levels whose results are the opposite of MDA levels, on the same day.⁴²

In vitro test of ethanol extract *P. ostreatus* has a potent reducing power and this effect increases with increasing extract concentration. The reduction ability at 10 mg/mL is 1.367, which is better than BHT (1,192).²³ In previous studies, ethanol extract of *P. ostreatus* *in vivo* was proven to be able to prevent increased levels of malondialdehyde (MDA) in the liver, kidney and brain in mice given CCl₄ as hepatotoxic.²³ The above studies confirm the hypothesis that ethanol extract of *P. ostreatus* can prevent lipid peroxidation.

Antioxidant agents can inhibit the process of lipid peroxidation.¹¹ One of the most abundant endogenous antioxidant in the body is GSH.^{21,36} In this study rats exposed to cigarette smoke showed a significant reduction in GSH compared to normal rat. The imbalance of oxidants and antioxidants in the body will cause oxidative stress.^{43,44}

GSH levels in the body depend on a balance between the synthesis process and the metabolic process of GSH which causes GSH to be inactive. Synthesis of *de novo* GSH is influenced by γ GLCL and GLS and is influenced by GPx and glutathione reductase (GR) in the process of reducing GSH to GSSG. The highest GSH synthesis is in the liver, which is the place of primary synthesis, so that if there is a decrease in other organs there should also be a fairly extreme decrease before the liver. Oxidative stress that occurs in cells will cause GSH to reduce H₂O₂ and change into the reduced form of GSSG that accumulates in cells or can also form sulfhydryl form proteins that form a mixed disulfide or released from the cell. So far the GSH:GSSG ratio is one of the best indicators for assessing oxidative stress conditions in cells.^{18,45,46} The body's defense system or endogenous antioxidants are not fully able to fight free radicals in the body, so exogenous antioxidant supplements are needed.¹⁰

Exogenous antioxidants can be synthetic antioxidants or antioxidants derived from natural ingredients such as medicinal plants. The most commonly used synthetic antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate and tert-butylhydroquinone. However, the use of BHA and BHT is very limited because it is suspected to be carcinogenic and cause liver damage. So now it is still very necessary to develop antioxidants that are sourced from natural sources.⁷ *P. ostreatus* are medicinal plants that have high antioxidant potential and, in this study, increased GSH levels in male rats exposed to cigarette smoke. The effect given is significant, and the result is the same as the effect arising from the administration of GSH precursors, namely NAC.

The mechanism by which ethanol extract of *P. ostreatus* enhances the synthesis of GSH is suspected to be through several mechanisms as follows: 1) increasing the expression of enzymes γ GLCL and GLS which play an important role in the synthesis of glutathione in the body; 2) increasing cysteine residues in the body, which is one of the glutathione amino acids, this mechanism is almost the same as the mechanism of action of NAC; 3) *P. ostreatus* contain selenium which is considered as one of the precursors of glutathione with a mechanism that cannot be clearly explained.^{7,24,26,45} All three of the above mechanisms as a whole cause ethanol extracts of *P. ostreatus* to increase levels of glutathione in the body.

In this study the antioxidant ability of ethanolic extracts of *P. ostreatus* compared was with N-acetylcysteine (NAC). N-acetylcysteine is also an antioxidant that contains thiol which is also a precursor of GSH. N-acetylcysteine increases GSH synthesis and maintains a balance of GSH levels in cells. This antioxidant can prevent liver damage due to high doses of paracetamol in humans and prevent liver damage and GSH depletion in mice. NAC antioxidants are widely used in COPD cases. The thiol group in NAC can reduce free radicals and has functioned as a chelating agent which is very useful in the case of heavy metal poisoning. N-acetyl cysteine can also penetrate cell membranes, so that its level are high intracellular.^{34,47}

In this study, the potency of the ethanolic extract of *P. ostreatus* at a dose of 250 mg/kg BW in preventing the decrease in GSH levels in rats exposed to cigarette smoke was as good as the potency of NAC.

Previous studies have shown that intraperitoneal administration of *P. ostreatus* extract at a dose of 200 mg/kg body weight in old rats can increase levels of enzymatic antioxidants such as CAT, SOD, Gpx, GST and GR in liver, kidney and brain organs.⁷ In other studies, the administration of ethanolic extracts of *P. ostreatus* with the same dosage and administration method has been found to inhibit lipid peroxidation and increase enzymatic antioxidants such as CAT, SOD, Gpx, GST. The ethanolic extract also can increase non-enzymatic antioxidants (GSH, vitamins C and E) in CCl₄-induced rat liver, kidney and brain tissue.^{7,23}

The antioxidant potential of *P. ostreatus* is supported by the content of secondary metabolites contained therein. The results of phytochemical tests previously showed that white oyster mushrooms contain many alkaloids, steroids, flavonoids, tannins, saponins and phenolic components. Previous research also showed that *P. ostreatus* can contain about 100 different bioactive components. This fungal cell wall contains many non-starch polysaccharides, where β -glucans are the most attractive components, and contain phenolic components such as protocatechuic acid, gallic acid, homogentisic acid, rutin, mirisetin, chrysin, naringin, tocopherol like α -tocopherol, γ -tocopherol, ascorbic acid and β -carotenoids.^{12,30}

Other research also states that the ethanol extract of *P. ostreatus* contains many polyphenols or phenolic components in which there are many flavonoids such as routine, also contain β -carotenoids, vitamin A and C, and selenium. In this study,

the results of testing of compound markers using HPLC showed that the ethanol extract of *P. ostreatus* used in the study contained rutin (flavonoids) and cinnamic acid (phenolic acid).^{12,47}

The ethanolic extract of *P. ostreatus* *in vitro* has antioxidant power that can trap hydroxyl radicals and superoxide, inhibit lipid peroxidation, reduce ferric ions, chelating ferrous ions and quenching 2,3-diazabicyclo[2,2,2]oct-2-ene (ene DBO). This extract also shows good antioxidant activity *in vivo* by reducing lipid peroxidation and increasing enzymatic and non-enzymatic antioxidant activity.^{24,28}

Other studies mention that water soluble crude polysaccharide from *P. ostreatus* shows the superior ability of free radical scavenging and NOS, due to the alleged role of the *P. ostreatus* β -glucan antioxidant potential. This means that *P. ostreatus* can be a herb that can be developed into a good natural antioxidant.⁴⁸ Some of the above studies are strongly related to the mechanism of aging, whereas studies to see the effect of ethanol extracts of white oyster mushrooms on endogenous antioxidant levels in rat models with COPD have not been examined. This is why this research can pave the way to develop the use of natural antioxidants in COPD cases. Provision of antioxidants and anti-inflammatory drugs alone in COPD patients can be used based on several conditions that need to be considered. Currently exogenous antioxidants are often used in COPD patients among mucolytic antioxidants such as erdostein, carbocystein and NAC.^{34,46}

Cigarette smoke is known to reduce levels of total glutathione (GSH + GSSG) in the respiratory tract. Glutathione is widely known to be present in ELFs and airway epithelium. Glutathione plays an important role in cellular redox balance and is considered to be the most important antioxidant against inhalable reactive components. The mechanism underlying the total glutathione depletion is presumably because there is a part of the cigarette smoke phase which will react irreversibly with GSH to form a non-reduced GSH derivative (GSX), thereby causing GSH depletion. Under normal conditions, intracellular GSH will generally be stored in a reduced form, but the imbalance between GSH and oxidized glutathione (GSSG) will change under conditions of oxidative stress, and the ratio between GSH and GSSG will illustrate how intracellular cells condition. *In vitro* and *in vivo* studies state that under oxidative stress conditions the free sulfhydryl (-SH) group of GSH will become oxidized, and exposure to the cigarette smoke phase *in vitro* and *in vivo* will generally reduce GSH but the amount of GSSG does not increase significantly. Another study concluded that cigarette smoke does not cause GSH oxidation to GSSG, but rather causes a reaction so that it forms glutathione-aldehyde derivatives or non-reduced GSH derivatives (GSX), which in turn will reduce total GSH. This will cause chronic antioxidant deficiency. Chronic smokers will continue to be exposed to free radicals and with the decrease in GSH the negative effects of these free radicals will be even higher. This condition causes GSH synthesis which is essential for cell defense and lung protection. Depletion of GSH Intracellular depletion will significantly trigger oxidative stress and stimulate signal transduction pathways, cell proliferation, apoptosis and inflammation.⁴¹

4-Hydroxy nonenal is a toxic molecule and can further increase the level of ROS in cells through the mechanism of mitochondrial dysfunction 4-Hydroxy-2-nonenal, because mitochondria are organelles that are susceptible to HNE. This condition will further increase the inflammatory response that will occur.⁸ One of the mechanisms for detoxifying HNE from cells is by forming conjugations with GSH (glutathione-4-hydroxynonenal/GSHNE) to then be taken out of the cell. This conjugation process is catalyzed by glutathione-S-transferases (GSTs), which play an important role in regulating HNE levels in cells. There are two groups of proteins involved in transporting foreign species from within cells: namely ATP-binding cassette (ABC) transporters and non-ABC transporters. ABC transporters, including P-glycoprotein (Pgp) and multidrug resistance-associated protein (e.g., MRP1). Various xenobiotics and natural metabolites such as GSHNE will be transported past MRP1. Non-ABC transporters involved in transporting toxins, some drugs and antineoplastic agents and various glutathione conjugations are Ral-binding protein 1 (RalBP1), also known as RLIP76. This protein is abundant in various body tissues and is bound to cell membranes.³⁹

This reinforces the hypothesis that GSH will affect HNE levels in the network, because high GSH will cause HNE to decrease because it forms GSHNE. The data in this study also show that the levels of GSH and HNE for each group are contradictory, if the GSH rises then the HNE will decrease, but the statistical analysis states that the data obtained is not significant enough to reinforce that there is a correlation between GSH levels and HNE levels in the lung of rats exposed to cigarette smoke. Insignificant results between GSH and HNE correlations can also be due to the limited number of samples, so the results cannot be applied to the population.

One important factor that can break the cascade of cigarette smoke inflammatory processes early is antioxidants. Glutathione is an endogenous antioxidant found in many cells including lung cells and ELF. During the synthesis and use of glutathione in a balanced body, no further damage is expected, but high consumption of glutathione can cause levels to decrease in the body. In this condition exogenous antioxidant supplements are needed which can help the work

of endogenous antioxidants such as GSH. *P. ostreatus* are expected to optimize the body's endogenous antioxidants. Ethanol extract of *P. ostreatus* is expected to increase the synthesis of GSH in the body and help the body's defense of antioxidants through the mechanism of hydrogen donors from its hydroxyl groups. This plant can be developed into a potential source of antioxidants as well as a potential anti-inflammatory.

Conclusions

Ethanol extract of *P. ostreatus* inhibits the increase in levels of HNE and increases level of GSH in the lungs of male Wistar strain rats exposed to cigarette smoke exposure. There is no correlation between GSH levels and HNE in male Wistar strain rats exposed to cigarette smoke.

Data availability

Underlying data

Figshare: 4-HNE And GSH Lung rats exposed to cigarette smoke.

<https://doi.org/10.6084/m9.figshare.16550964>.⁴⁹

This project contains the following underlying data:

- Report of Data Analysis of GSH level at lung rats that exposed cigarette smoke.xls
- Report of Data Analysis of 4-HNE level at the lung rat that exposed cigarette smoke.xls

Reporting guidelines

Figshare: ARRIVE guidelines for 'Antioxidant effect of ethanolic extract of *Pleurotus Ostreatus* on 4-hydroxy-2-nonenal (HNE) and glutathione (GSH) level in lung rats exposed to cigarette smoke'. <https://doi.org/10.6084/m9.figshare.16550964>.⁴⁹

Data are available under the terms of the [Creative Commons Zero "No rights reserved" data waiver](#) (CC0 1.0 Public domain dedication).

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A. Vijaya Anand

Department of Human Genetics and Molecular Biology, Bharathiar University, Coimbatore, Tamil Nadu, India

The authors have put effort to evaluate the "Antioxidant effect of ethanolic extract of *Pleurotus Ostreatus* on 4-hydroxy-2-nonenal (HNE) and glutathione (GSH) level in lung rats exposed to cigarette smoke". The authors describe the research undertaken with this in an organized manner, emphasizing the results obtained by them. The article needs modification for better cohesion of information to achieve the goal and the minor shortcomings which need to be considered. Hence, the paper can be accepted after MINOR REVISIONS are carried out:

1. There are some grammatical, alignment and typographical errors are noted in the manuscript and it should be thoroughly checked and corrected throughout the manuscript. For example, the words "and systematically" maybe as "systematically"; "to maintenance the" as "to maintain the"; "are found" as "is found"; "ELF 10-100x" as "ELF are 10-100x"; "with temperature" as "with a temperature"; "this study" as "this study,"; "incinerator" as "an incinerator"; "significantly" as "significantly"; "increasedGSH" as "increased GSH"; "prostaglanding" as "prostaglandins"; "but the third" as "but on the third"; "malondiadehyde" as "malondialdehyde"; "normal rat." as "normal rats"; "level are" as "level is"; "vitaminAand" as "vitamin A and"; "water soluble" as "water-soluble"; "Currently" as "Currently,"; "andHNElevels" as "and HNE levels"; "condition exogenous" as "condition, exogenous"; "increases level" as "increases the level".
2. Check the abbreviations throughout the manuscript and introduce the abbreviation when the full word appears the first time in the text and then use only the abbreviation (For example, malondialdehyde (MDA), TGFβ1, γGCS, glutathione (GSH), etc.,). And it should be in both abstract as well as in the remaining part of the manuscript. Make a word abbreviated in the article that is repeated at least three times in the text, not all words need to be abbreviated.
3. The full form of the species should be given when the first time appears and followed by only the first letter of the genus in both the abstract and the remaining part of the

manuscript (For example, *Pleurotus ostreatus* when the first time appears and followed by *P. ostreatus*). And the name should be italic all over the manuscript and the species name should start with lower case (small letter).

4. When referring to SPSS versions beginning from 19, authors should cite 'IBM SPSS Statistics for Windows, version 20 (IBM Corp., Armonk, N.Y., USA)'. Similarly should be for Stata MP 16 also.
5. The authors should properly mention the subscript and superscript (For example, in "CCl₄", 4 should be subscripted) and it should be checked throughout the manuscript, wherever applicable.
6. The authors may justify how the dose is optimized for the present investigation either in the materials and methods or any part of the results or discussion (either based on available literature or previous study). If from the others study should be supported with reference.
7. The conclusion seems simple, hence, it is highly recommended to include weaknesses or limitations of the study and potential future research goals.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Phytotherapeutics, Clinical Biochemistry, Medical Genetics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 14 January 2022

<https://doi.org/10.5256/f1000research.77366.r119854>

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Ali Akbar Nekooeian

Cardiovascular Pharmacology Research Lab, Department of Pharmacology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

The manuscript has examined the effects of ethanolic extract of *Pleurotus Ostreatus* on 4-hydroxy-2-nonenal (HNE) and glutathione (GSH) levels in lung rats exposed to cigarette smoke. Although the manuscript has an interesting message for the readers, however, it is not appropriately written and is in need of revision.

Materials and methods

The components of this section are usually written based on chronology. This needs to be followed in this manuscript.

What test was used after Kruskal Wallis for multiple comparisons?

The data presentation form has not been mentioned in the statistical analysis section.

Results

Tables 2 and 3 are outputs of statistical software used to analyze the data. They have to be deleted. Presenting correlation coefficient and P values in the text does the job.

Discussion

The discussion is too long. The manuscript does have results on two items. This indicates a short discussion. The discussion should include part of the changes in HNE and GSH by smoke and the effects of the extract on these variables. In the present form, the discussion is somehow chaotic and needs to be organized.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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