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RESEARCH ARTICLE

THE EFFECT OF *GANODERMA LUCIDUM* POLYSACCHARIDE PEPTIDE (GLUCAN) ON CASPASE 8, CASPASE 9, AND APOPTOSIS, LEIDIQ CELL OF 18 MONTHS MALE RATTUS NORVEGICUS TESTIS

***,[1]Bambang Wasito Tjipto, [1]Aris Widodo, [1]Basuki Purnomo and [2]WIBI Riawan**

[1]Medical Science Doctoral Program of Biomedical Interest Postgraduate Program of Faculty of Medicine, University of Brawijaya, Malang
[2]Department of Biochemistry, Faculty of Medicine, University of Brawijaya, Malang

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ABSTRACT

Introduction: Ganoderma Lucidum was reported contained several active components biologically, such as polysaccharides, triterpenes, sterols, proteins, peptides, amino acids, adenosine, oleic acid, vitamins and minerals. Vitamins were contained among B1, B2, C, niacin, and biotin. While the mineral content included potassium, fofor, calcium, magnesium, copper, germanium and some other makroelemen which totaled more than 200 active elements. Late Onset Hypogonadism (LOH) was a syndrome of physical abilities decline, sexual or psychological abilities associated with decreased testosterone in the blood. By middle age of 45-59 years of age the decline of bodily functions was started, including the decline of reproduction function and hormones such as testosterone, which was known as the aging process. With increasing life expectancy then so did the number of elderly in the future, the result will be increasing problems of the elderly.

Objective: This study aimed to analyze the effect of β Glucan extract Ganoderma lucidum Polysaccharide Peptide (PSP) in decoction way, in 21 days compared with β Glucan of ethanol extract of G lucidum Glucan Polysaccharide Peptide (PSP), and control. On how was apoptotic of leidig cell of 18 months male rattus norvegicus testes happened, whether through the pathway of caspase 8 (extrinsic) or through the pathway of caspase 9 (intrinsic).

Methods: To determine how was apoptotic of leidig cell of 18 months male R norvegicus testes happened, it used CCTV incaged inside the cage made of acrylic for 21 days. caspase 8, caspase 9, Mn SOD and apoptotic was examined using immuno histochemistry.

Results: At the studies of caspase 9, there was a meaningful difference to the mean in the control with mean of $7,78 \pm 2,438^{a}$ on the water extract with mean of $4,89 \pm 2,472^{b}$, and the ethanol extract with mean $2,56 \pm 1,333^{c}$. with $p = 0.000$, ($\alpha < 0.001$) There was a decrease in the mean value of caspase 8 significantly in the ethanol extract is $3,56 \pm 2,455^{c}$, the water extract is $7,56 \pm 1,424^{b}$, meanwhile in control is $14,00 \pm 2,598^{a}$. It turned out that all the groups A, B, C each had an average of caspase 8 of rat testes Leidiq cells that different significantly with $p=0.000$, ($\alpha < 0.0001$). In apoptosis test on testicular Leidiq cells immunohistochemically, there was a decrease of apoptotic in the given of ethanol extract with a mean is $2,78 \pm 1,202^{c}$, compared to water extract / decoction with mean $5,22 \pm 1,302^{b}$, and control the mean apoptotic is $9,78 \pm 1,716^{a}$ in average. It turned out that all the groups A, B, C each had different rat testis Leidiq cell apoptotic average significantly with $p = 0.000$, ($\alpha < 0.0001$).

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INTRODUCTION

Ganoderma lucidum was a favorite medicine in oriental medication for centuries. Fruiting bodies was called "Lingzhi" in China and "Reishi" in Japan. It had been known as a traditional medicine, which was used in Chinese and Japanese traditional medicine for the treatment of several diseases, such as hepatitis, hypertension, chronic bronchitis, bronchial asthma, cancer and others (Habijani *et al*., 2001; Boh *et al*., 2007). A study demonstrated that antioxidants in plasma after consumption of G lucidum was increased for 10 days, and it was associated with a trend PJK biomarker profile trend. The

******Corresponding author: Bambang Wasito Tjipto,*
Medical Science Doctoral Program of Biomedical Interest Postgraduate Program of Faculty of Medicine, University of Brawijaya, Malang

long term toxicity of *G. lucidum* in a study conducted by Gao & Han (2008) have shown that it is safe to consume its capsule within dose rage of 0.47 g/kg to 1.87 g/kg body weight. G lucidum in the report contained several active biological components, such as polysaccharides, triterpenes, sterols, proteins, peptides, amino acids, adenosine, oleic acid, vitamins and minerals. Vitamins conceived including B1, B2, C, niacin, and biotin. Meanwhile the mineral content included potassium, fofor, calcium, magnesium, copper, germanium and some other makroelemen which had totaled more than 200 active elements (Boh *et al*., 2007).

Late Onset Hypogonadism (LOH) was a syndrome of decline in physical ability, sexual or psychological ability associated with decreased of testosterone in the blood. By middle age started from 45-59 years of age, there was a decline of bodily functions, including the decline in reproductive function and hormones such as testosterone. Decreased levels of testosterone at the age of 55 and above indicated significant difference compared with the last 12 years. In accordance with the increasing age, the testosterone production also decreased, which was known as the aging process. Testosterone is a anabolic hormone. With increasing life expectancy then so did the number of elderly in the future, this result in increasing the elderly problems (Dotson, 2003; Kaufman *et al*., 2005). Some men have experienced LOH syndrome in their thirties, but with relatively small amount of approximately 5% (Wibowo 2002). If deduced based on the facts and realities that many factors that contribute to LOH can be found in Indonesia including pollution, workplace burden, and life style, then it is possible that Late Onset Hypogonadism (LOH) more experienced by men in Indonesia compared to western countries (Wibowo 2002). Some preliminary studies had shown prevalence of LOH in some area. In Jakarta, around 70,94 % respondent experienced LOH (Taher 2005).

MATERIALS AND METHODS

Thirty (30) *R. norvegicus*, aged 18 month-old each, divided in to three groups. Group A (control) , group B (Hot water extract 50 mg/kg body weight in 2 ml *Ganoderma lucidum* polysaccharide peptide (ß-glucan)), and Group C (ethanol extract 50 mg/kg body weight in 2 ml *Ganoderma lucidum* polysaccharide peptide (ß-glucan)). Each group consists of ten rats . Rats in group A were subdivided into 2 cages; each cage consists of 5 male rats. The rats in group B were subdivided in 3 cages each cage consist of 3, 3 and 4 male rats respectively. The group C were also subdivided in 3 cages as previously described in group B. One female rat (12 month-old) was added in each cage. At the 21st day post treatment, the rats were sacrificed using ether. In each rat, one testicle was dissected and removed for histology preparation and the other for immunohistochemistry evaluation of caspase 9, caspase 8, and and apoptotic of cells leidiq testis with chemical imunohisto test done on Faculty of Medicine, in University of Brawijaya.

WORKING PROCESS of Immunohistochemistry
Prestaining.
Sample preparation (slide),
Ensuring the 370C incubator for 24 hours
Deparafinisasi
Xilol and alcohol use radiant.
Store in 40C to do immunostaining.
Blocking endogenous peroxide using a 3% H2O2 for 20 menit.
wash using PBS pH 7.4 three times, diving 5 minutes.
Wash using PBS pH 7.4 three times, diving 5 minutes.
Then incubation using conjugated anti-mouse biotin for 2 hours
Wash using PBS pH 7.4 three times, diving 5 minutes. And drops with DAB (Diamino Benzidine) and incubation for 10 min.
Wash using PBS pH 7.4 three times, diving 5 minutes. And wash using dH20, for 5 minutes.
Then counterstaining using Mayer Hematoxilen were incubated for 10 minutes and wash using tap water.
Blocking protein unspesifik using 5% FBS containing 0.25% Triton X-100. And washing using PBS pH 7.4 three times, for 5 minutes.
Incubation using monoclonal anti (primary antibody), for 24 hours at 40 ^{o}C
Wash using PBS pH 7.4 three times, diving 5 minutes.
Then incubation using conjugated anti-mouse biotin for 2 hours
Rinse and dry using dH2O aired.
Mounting using entelan and cover with a cover glass.
observation of cells under a light microscope with a magnification of 400 X, 1000 X .

RESULTS

During treatment

During treatment, the rats in the control group were only given food and water *ad libitum*. Rats in group B were given hot water extract 50 mg/kg body weight in 2 ml *G. lucidum* polysaccharide peptide (ß-glucan) daily using oral tube for 21 days. Rats in group C were given ethanol extract 50 mg/kg body weight in 2 ml *G. lucidum* polysaccharide peptide (ß-glucan) daily using oral tube for 21 days. After the 5th day, there was one dead rat in each group. So, there were nine rats remain in each group. The A group was divided into 2 cages (Cage 7 and Cage 8); each cage consists of 5 male rats. The B group was divided in 3 cages (Cage 4, Cage 5, and Cage 6); Cage 4 and Cage 5 consist of 3 male rats; Cage 6 consists of 4 male rats. The C group was also divided in 3 cages (Cage 1, Cage 2, and Cage 3); Cage 1 and Cage 2 consist of 3 male rats; Cage 3 consists of 3 male rats. Within each cage (Cage 1-8) added one female rat (12 months old).

From the statistical test one-way ANOVA for sexual behavior after the treatment between the control, water extract / decoction of polysaccharide peptide ganoderma lucidum (β glucan) in rattus novergicus rates aged 18 months (old), and the treatment of the ethanol extract of Ganoderma lucidum polysaccharides peptide (β glucan) in rattus novergicus rats aged 18 months (old). It showed the mean control of 0,50, water extract mean of 2.7 and ethanol extract of 2,1. Its significant value = 0.00 (p α,> 0,05). Post hoc test results table showed no difference between group B (water extract) with group C (ethanol), where its significant value = 0,210 (p α.> 0.05). While the groups A is differed significantly from

group B (0,000) p = 0,000 (p α <0,05). And group C (0,002), p = 0.002 (p α . <0,05).

Caspase 9 test results

Test results of Active-Caspase-9 in immuno histochemistry of a process leading to apoptotic in the mitochondrial pathway (intrinsic), after the treatment in the testes of rat in group A (control), group B (water extract / decoction), and group C (ethanol extract) shown in the [Figure 1]

treatment at the testes of rat in group A (control), group B (water extract / decoction), and group C (ethanol extract) shown in the image; see [Figure 2].

Different test results using the Caspase-8 variant analysis

Group	n	Mean ± standard deviation	p value
control	9	14,00 ± 2,598[a]	<0,0001
Exctraks Dekok G.lucidum	9	7,56 ± 1,424[b]	
Ethanol Extracts G.lucidum	9	3,56 ± 2,455[c]	

Description: Superscript different shows significant differences based on the results of LSD test

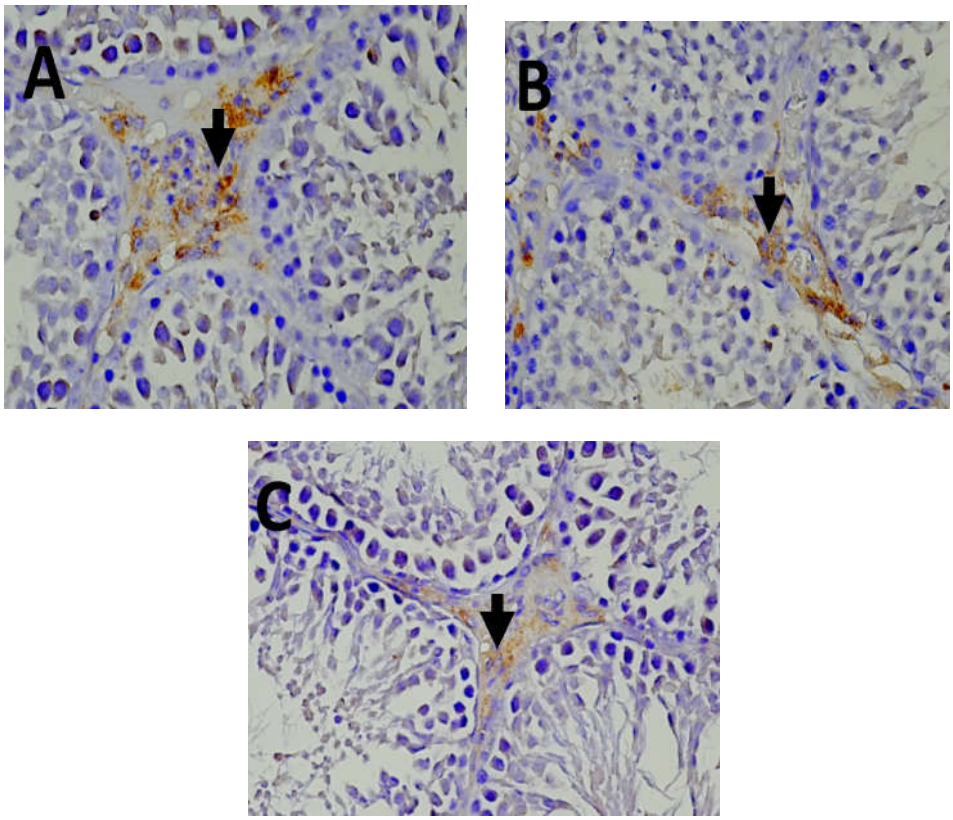

Figure 1. Figure of active test results of caspase 9 in immuno histochemical way of testicular Leidiq cells, in the control group (A) was clearly brown and it is a lot compared to the group of water extract / decoction (B) and ethanol extract group (C)

Different test results using the Caspase-9 variant analysis

Gruop	n	Mean ± standard deviation	p value
control	9	7,78 ± 2,438[a]	<0,0001
Extracts dekok G lucidum	9	4,89 ± 2,472[b]	
Ethanol Extracts G.lucidum	9	2,56 ± 1,333[c]	

Description: Superscript different shows significant differences based on the results of LSD test

Results of analysis of variance at Table 1. It appears that there is a significant difference (p <0.05) caspase-9 after treatment among the three groups. Therefore conducted a further test anava to see where the different groups using LSD (Least Significant Different). The LSD test results that the three groups differ significantly.

Caspase 8 test results

Test results of active-Caspase-8 in immuno histochemistry of a process leding to the extrinsic pathway of apoptotic, after the

Results of analysis of variance at Table 2. it appears that there is a significant difference (p <0.05) caspase-8 after treatment among the three groups.

Therefore conducted a further test anava to see where the different groups using LSD (Least Significant Different). The LSD test results that the three groups differ significantly.

Apoptotic test result (TUNEL)

Apoptotic test results in immuno histochemistry for the occurrence of Testicular Leidiq cell apoptotic, after treatment on rat testicular at group A (control), group B (water extract/decoction), and group C (ethanol extract) shown in the [Figure 3]. Results of analysis of variance at Table 3. It appears that there is a significant difference (p <0.05) apoptosis after treatment among the three groups.

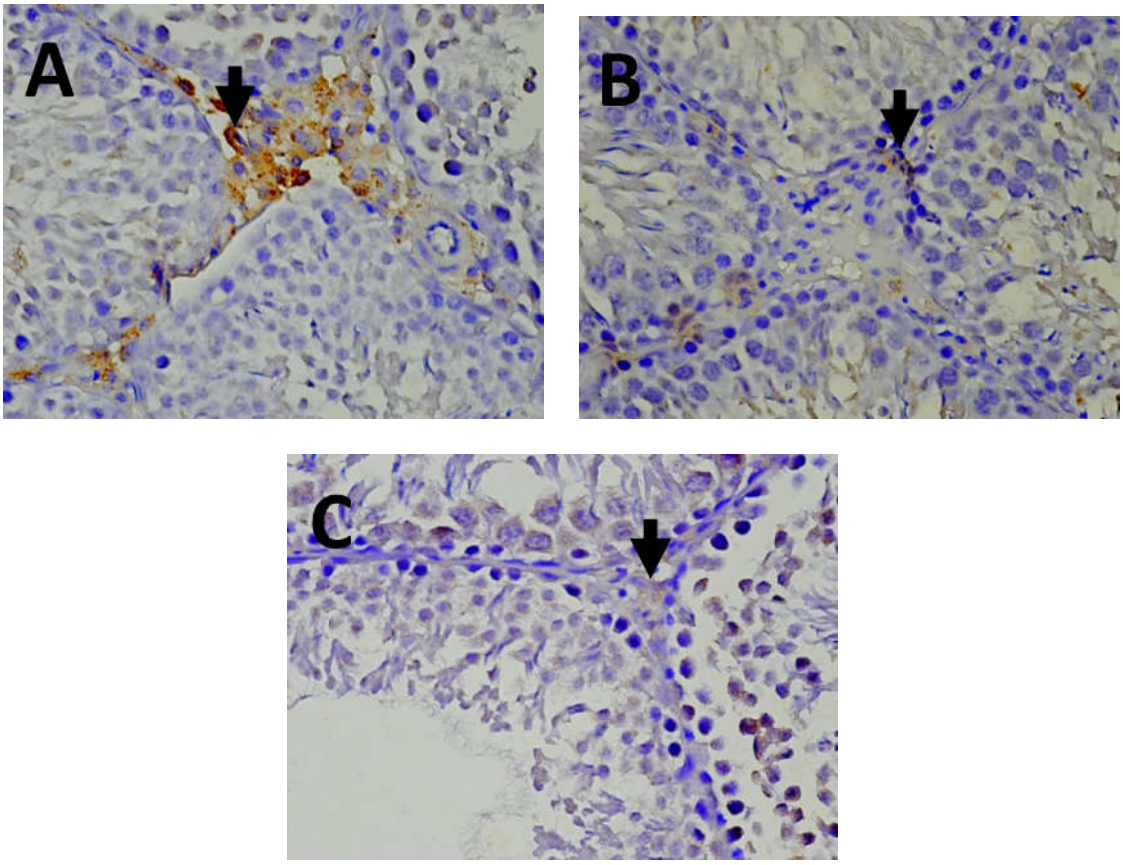

Figure 2. Figure of caspase 8 active test results in immuno histochemistry of testicular Leidiq cells, in the control group (A) is more obvious and many brown lot than the water extract group / decoction (B) and ethanol extract group (C)

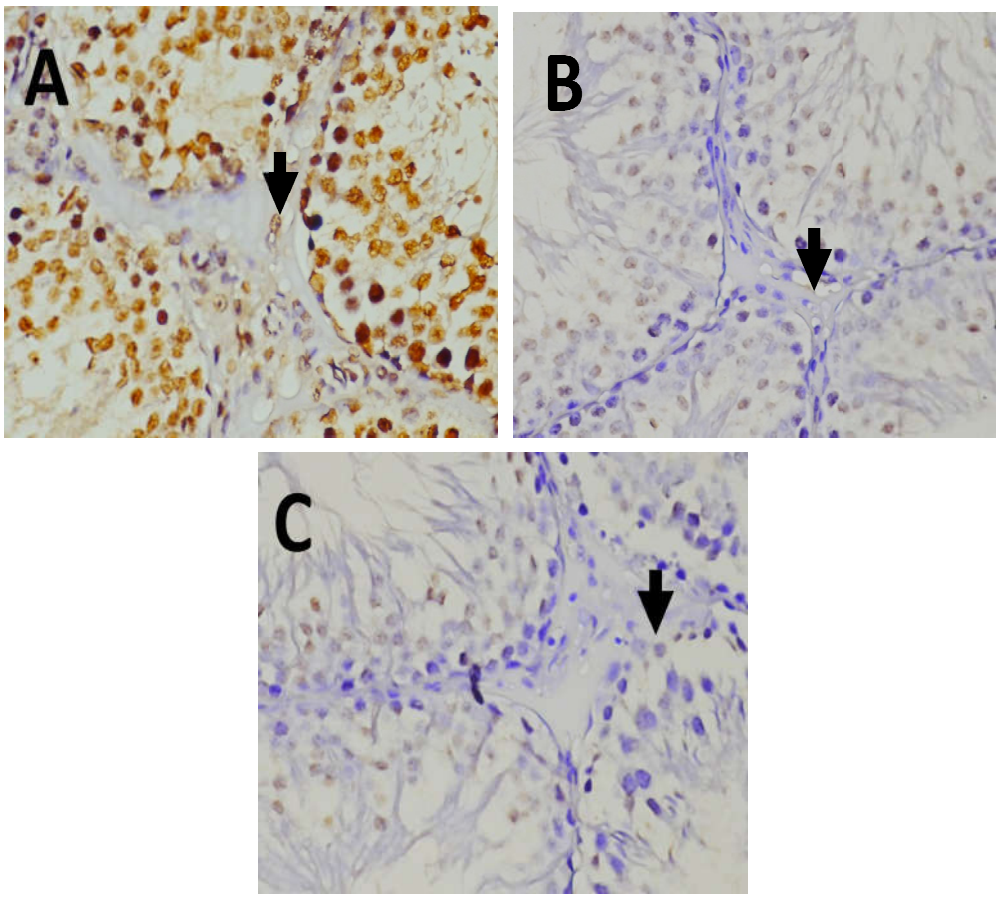

Figure 3. Figure of Immunohistochemistry apoptotic test results of testicular Leidiq cell, in the control group (A) is more brown (apoptotic) compared with the water extract group / decoction (B) and ethanol extract group (C)

Apoptosis different test results using Varian analysis

Group	n	Mean ± standard deviation	p value
control	9	$9,78 \pm 1,716^{a}$	<0,0001
Ectraks Dekok G.lucidum	9	$5,22 \pm 1,302^{b}$	
Ethanol Extracts G.lucidum	9	$2,78 \pm 1,202^{c}$	

Description: Superscript different shows significant differences based on the results of LSD test.

Therefore conducted a further test anava to see where the different groups using LSD (Least Significant Different). The LSD test results that the three groups differ significantly.

DISCUSSION

Ability of lifestyle and environmental changes to affect reproductive health was an interesting and relevant field of research. Leydig cells responsible for the production of testosterone in the testes of mammals. Testosterone production depends on stimulation of these cells by LH secreted in pulses into the peripheral circulation by the pituitary gland (pituitary) below the brain in response to GnRH from the hypothalamus. Testosterone and its aromatase products, estradiol, and then provide input back to the hypothalamus and pituitary to suppress the production, on a temporary basis, LH and thus testosterone. In response to reduced testosterone, GnRH and LH produced again. This negative feedback cycle (negative feed back) produces LH pulsatile secretion followed by testosterone pulsatile production (Bremner *et al*., 1993; Ellis *et al*., 1983). During human life of men, decreased serum testosterone usually begins in the fifth decade (Belanger *et al*., 1994), In humans, the decline was accompanied by an increase in FSH serum levels and an increase or no change in the level of LH (Zwart *et al*., 1996). This observation, although they do not rule out age-related deficits from hypothalamic-pituitary axis during human aging, showed a deficit of primary testicular. For this purpose, we chose to study aging in Leydig cells at Rattus norvegicus rats aged 18 months as a model for humans. In this strain, as in humans and other rat, serum levels of testosterone declines with age.

Since thousands of years man had been hunting wild mushrooms. Mushrooms had long been used as a food source (Mattila *et al*., 2001) because of its attractive chemical composition from source of nutrition. At the beginning of civilization, mushrooms were consumed mainly for palatability and unique flavors (Rai, 1994, 1997). The use of mushrooms completely different from the the traditional one, because many studies had been done on the mushroom chemical composition, which revealed that mushrooms can be used as a diet to treat the disease.

The results of Active-Caspase-9 in immuno histochemistry of a process leading to apoptotic in the mitochondrial pathway (intrinsic), after the treatment in the testes of rat in group A (control), group B (water extract / decoction), and group C (ethanol extract) are $7,78 \pm 2,438^{a}$, compare with $4,89 \pm 2,472^{b}$, and $2,56 \pm 1,333^{c}$. The results of active-Caspase-8 in immuno histochemistry of a process leding to the extrinsic pathway of apoptotic, after the treatment at the testes of rat in group A (control), group B (water extract / decoction), and group C (ethanol extract) are $14,00 \pm 2,598^{a}$, compare with $7,56 \pm 1,424^{b}$, and $3,56 \pm 2,455^{c}$. The results in immuno histochemistry for the occurrence of Testicular Leidiq cell apoptotic, after treatment on rat testicular at group A (control), group B (water extract/decoction), and group C (ethanol extract) are $9,78 \pm 1,716^{a}$, compare with $5,22 \pm 1,302^{b}$, and $2,78 \pm 1,202^{c}$.

Antioxidants were chemical compounds that protect cells from damage caused by unstable molecules known as free radicals. Free radicals were powerful oxidants and chemical entities that contain unpaired electrons. They were able to randomly destroy all body components, ie. lipids, proteins, DNA, sugars and were involved in mutations and cancer (Przybytniak *et al*., 1999). Oxygen was trapped by enzymes such as superoxide dismutase, catalase, and glutathione peroxidase. During the production of free radicals, it created oxidative stress. Antioxidants were an important defense of the body against free radicals and fungi which were a rich source of antioxidants (Mau *et al*., 2004; Puttaraju *et al*., 2006; Ferreira *et al*., 2007; Oyetayo *et al*., 2007). Antioxidant properties were a compounds correlated with phenolic compounds (Velioglu *et al*., 1998). Kim and Kim (1999) reported that mushroom extracts had properties to protect DNA. G. lucidum extract functioned as free radical trap (Jones and Janardhanan, 2000). Mau *et al*. (2004) found the antioxidant properties of some 'kuping' mushroom. Many species of mushrooms had been found to raise a strong immune, potentiation of animals and humans immune against cancer (Wasser and Weis, 1999; Borchers *et al*., 1999; Kidd, 2000; Feng *et al*., 2001). Tyrosinase and fractions of A. bisporus was an antioxidant (Shi *et al*., 2002). Triterpenoids were the major chemical compounds in G. lucidium. Camptothecin was responsible as an antioxidant in G. lucidum (Zhou *et al*., 2007). From the results of our study, male Rattus norvegicus rats aged 18 months (aging) had undergone oxidative stress that results in apoptotic through intrinsic pathways (mitochondria/ paftway) which activates caspase 9, and extrinsic pathways with the activation of caspase 8, when given polysaccharide peptide (β -glucan) Ganoderma lucidum of ethanol extract or water extract (decoction), the activation of caspase 8, caspase 9 and apoptotic can be inhibited, but the administration of inhibition ethanol extracts was better because it contained β glucan on the ethanol extract (55.25%) of Ganoderma lucidum polysaccharides peptide which is higher than the water extract (11%).

Conclusion

Ganoderma lucidum is a mushroom that contains ß-glucan with a concentration of 55.25% in the ethanol extract, and 11% in decoction extract, an antioxidant that may reduce the occurrence of caspase 9, caspase 8, and cell apoptosis in testes leidiq rattus norvegicus

Competing Interests

No competing financial interests exist.

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