

Anti-inflammatory and immunosuppressant activity of *Coprinus comatus* ethanol extract in carrageenan-induced rats of *Rattus norvegicus*

Nuniek Ina Ratnaningtyas ^{1*}, Fajar Husen ², Hernayanti ¹, Nuraeni Ekowati ¹, Bambang Heru Budianto ¹

¹Department of Biology, Faculty of Biology, Jenderal Soedirman University. Jl. Dr. Soeparno 63 Karangwangkal 53122, North Purwokerto, Banyumas, Central Java, Indonesia

²Department of Medical Laboratory of Technology, Bina Cipta Husada College of Health Science, Pahlawan Road V/6, Tanjung, South Purwokerto, 53144, Banyumas, Central Java, Indonesia

*Corresponding author email: nuniek165@yahoo.com

Received May 13, 2022; Accepted October 02, 2022; Available online November 20, 2022

ABSTRACT. *Coprinus comatus* (O.F. Mull.) is an edible mushroom that is used as an anti-inflammatory agent. Therefore, this study aims to determine the effect of inflammation treatment on symptoms alleviating, function maintenance, and inhibiting the process of tissue damage due to an increase in free radicals using drug formulations with high antioxidant compounds. This is a true experiment conducted using a Completely Randomized Design (CRD) with a post-test and a control group. The rats were divided into 6 categories, which include 1 healthy and 5 groups induced with 1% carrageenan. Out of the treatment groups, 3 were treated with ethanol extract of *C. comatus* fruiting body at doses of 250 (T1), 500 (T2), and 750 mg/kg BW (T3), 1 received diclofenac sodium (PC) and the other as a negative control (NC), were given extract for 14 days and induced with 0.5 mL carrageenan in paw of rats at day 15. The qualitative identification showed the extracts contains flavonoid, polyphenol, alkaloid, triterpenoid, steroids and saponins, and GC-MS analysis showed 10 putative metabolites compound. T2 group significantly decreased the levels of IL-1 β (70.63%), IgE (59.04%), total leukocyte count (31.24%), plantar thickness (12.5%), edema volume (33.3%), and increased endothelial NO levels (48.2%).

Keywords: Antioxidant, *Coprinus comatus*, Cytokine, Inflammation, Phytochemicals compounds.

INTRODUCTION

Inflammation in vascular disease involves circulating inflammatory/immune cells such as neutrophils, lymphocytes, monocytes, macrophages, and constitutive cells of vascular tissue, namely smooth muscle, endothelial cells, and fibroblasts. This cellular interaction produces inflammatory mediators including cytokines, interleukins (IL, IL-1 β , IL-6), and tumor necrosis factor (TNF- α) that activate the production of prostaglandin (PG) E2 (Gomez et al., 2013). In an inflammatory state, the increase in IL-1 β , TNF- α , the number and migration of leukocytes and neutrophils, as well as edema occur after 3 hours of being induced by carrageenan (Silva et al., 2013). The reactive oxygen species (ROS), which are highly reactive radicals interact molecularly with the results of protein oxidation in form of aminoacyl residues, leading to mutations in DNA. They also react with endothelial cell lipids which cause peroxidation chain reactions and produce more free radicals reactive. Excess ROS production in cells causes oxidative stress, leading to inflammation and cell or tissue damage (Arief & Widodo, 2018). Meanwhile, the ROS is formed in large quantities more than the endogenous antioxidant capacity and causes necrosis (Husen et al.,

2021) or damages the endothelial cells, leading to impaired vasodilation (Rodríguez et al., 2020).

Mushrooms are among the ingredients that are developed as herbal medicine because they contain therapeutic compounds with anti-inflammatory and minimum toxic effects compared to synthetic drugs (Yuan et al., 2006). One of the potential bioactive compounds, which is usually used as anti-inflammatory agents is derived from the fungus *Coprinus comatus* (OF Mull.) Pers. (Agaricaceae, Agaricomycetes) (Husen et al., 2021). The *C. comatus* grows in tropical forests using decomposed forest organic waste as substrate and available nutrients (Gupta et al., 2005). These bioactive metabolites include phenolic, terpenoids, polysaccharides, lectins, steroids, glycoproteins, and some lipid components as well as special compounds possessed by fungi such as *C. comatus* in suppressing the inflammatory process (Reis et al., 2012). Although the extract of *C. comatus* has been investigated, the identification of bioactive compounds as anti-inflammatory and antioxidant was only limited to qualitative and quantitative characterization using high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) (Ratnaningtyas et al., 2021). It

was also discovered that ethanol and ethyl acetate extracts of *C. comatus* contained rutin, vitamins C and E, as well as flavonoids and saponins. (Husen et al., 2021; Ratnaningtyas et al., 2022).

In this study, the identification and characterization of *C. comatus* compounds were carried out using qualitative identification and quantitative analysis using gas chromatography–mass spectrometry (GC-MS) to obtain putative metabolites compound that may role as an anti-inflammatory effect. Meanwhile, anti-inflammatory with 1% carrageenan induction and evaluation of nitric oxide (NO[·]) levels after the administration of *C. comatus* extract have not been widely investigated, where only the levels of enzymatic antioxidants and malondialdehyde (MDA) were evaluated (Zhao et al., 2018). The novelty is to use the *C. comatus* mushroom as an anti-inflammatory drug in rats induced by carrageenan in form of ethanol extract, tested on rats with edema/ inflammation, and also evaluate their plantar thickness.

EXPERIMENTAL SECTION

Mushroom and Chemical Materials

The materials used were the *C. comatus* mushroom fruiting body from CV. Asa Agro Corporation, Cianjur, 30 male Wistar rats, 1% carrageenan, 0.9% physiological NaCl, 0.012 g/200 g BW sodium diclofenac, ethanol (PA), Alcohol 70%, carboxymethylcellulose (CMC) 5%, Rat ELISA Kit BT-Laboratory (IgE, IL-1 β) nitric oxide (Kit of BioVision), ether, distilled water, feed pellets, and rice husks. The tools used were a 50 x 30 x 20 cm rearing cage, analytical balance, vacuum rotary evaporator, ELISA reader and washer, water bath, plethysmometer, microscope, hemocytometer, and caliper.

Animal Treatment Design

Before the treatment, the rats were acclimatized for 10 days. The rats were given food and drink *ad libitum*, food was given twice in the morning (07.00) and afternoon (16.30). A number of 24 Male Wistar rats were used and divided into six groups: healthy control/HC (without any treatment), negative control/NC (with 0.5 mL carrageenan induction), positive control/PC (given diclofenac sodium 0.012 g/200 g), treatment group 1/T1 (given 250 mg of ethanol extract), treatment group 2/ T2 (given 500 mg of ethanol extract), and treatment group 3/ T3 (given 750 mg of ethanol extract). *C. comatus* ethanol extract and metformin were given orally for 14 days, and the induction of carrageenan conducted in day 15, after 6 hours the paw edema and blood of the rats were taken as a sample. All animal after the treatment was done were eliminated with diethyl ether and neck dislocation.

Coprinus comatus Extraction

A total of 1.5 kg *C. comatus* mushroom fruiting body was prepared and cut into thin slices in preparation of the creating the extract. The samples then oven-dried at a temperature of 40-45°C until the

dried fruit bodies of the mushrooms were obtained. The dried mushrooms obtained were crushed using a blender and weighed 200 g with an analytical balance. Subsequently, Pro-Analyst (PA) ethanol solvent was added in a ratio of 1:5, 1:3, and 1:2 and macerated (Ratnaningtyas et al., 2019).

C. comatus Phytochemicals Screening and Identification

Qualitative identification; identification of flavonoids was carried out using amyl alcohol reagent, powdered magnesium (Mg) or zinc (Zn), and hydrochloric acid (HCl) (positive sign is orange, reddish-orange, or reddish yellow). Alkaloids test was carried out using Mayer's solution, and Dragendorff-bouchardat reagent (positive sign is dark brown or blackish brown). Triterpenoids and steroids were tested using a solution of acetic anhydride (CH₃COOH) and concentrated sulfuric acid (H₂SO₄) (the positive sign is purple-black). Testing of polyphenols using 1 mL of 10% Fe(III) chloride (FeCl₃) solution (positive sign is dark blue, blackish blue, or blackish green). The saponin test was carried out by boiling the sample extract with distilled water and shaking and adding methanol (CH₃OH), a positive sign for the formation of a stable foam for 30 seconds.

GC-MS quantitative identification; The *C. comatus* mushrooms fruiting body were analyzed using GCMS Agilent 6980N Network GC System with Agilent 5973 inert MSD detector (70eV direct inlet). Subsequently, approximately 2 μ L of *C. comatus* mushroom ethanol extract solution was injected into GC-MS with J&W Scientific, HP-5MS capillary column (Mohan et al., 2016).

Carrageenan Induction

The rats were anesthetized with diethyl ether (DE), placed on a surgical board in a supine position, and induced sub-plantarly with 0.5 mL of 1% carrageenan in 0.9% physiological NaCl. The inflammatory state can be detected immediately on the first day (6-7 hours after induction) to obtain the blood sample.

Measurement of Edema Volume and Plantar Thickness

The volume of edema was measured 6 hours after carrageenan induction using a plethysmometer (Chakraborty & Yolmo, 2019), while plantar thickness measurements were carried out with a caliper (Nigrovic & Shin, 2015).

Measurement of Immunoglobulin E (IgE)

Approximately 50 μ L of the solution was added to the standard well, 40 μ L of the sample and 10 μ L of anti-IgE antibody to the sample well, followed by 50 μ L of streptavidin-HRP respectively. 50 μ L of substrate solution A was added to each well, followed by 50 μ L of solution B. Optical density value was determined at 450 nm for 10 minutes (Waner et al., 2003).

Measurement of Nitric Oxide (NO[·]) Levels (Griess Method)

Griess solution was made by preparing solution I, where 0.5 g of sulfanilic acid was dissolved in 150 mL

of 30% v/v acetic acid. Meanwhile, solution II was prepared by boiling 0.1 g of naphthyl ethylenediamine in 20 mL of aqua-bidest until dissolved and poured hot into the 150 mL of 30% v/v acetic acid and mixed in a brown bottle. The absorbance was read using a UV-Vis spectrophotometer.

Measurement of Interleukin 1 β (IL-1 β)

A total of 50 μ L antibody was added to each well. The well plate was washed with 3x350 μ L 1X Wash Buffer by aspirating or decanting from wells and dispensing 350 μ L 1X Wash Buffer to each well. The optical density value was measured at a wavelength of 450 nm (AbCAM, 2016).

Research Ethics

Experiments and treatment of experimental animals have received ethical approval from the health research ethics committee of the regional general hospital (RSUD) Dr. Moerwadi, Solo with number 515/IV/HREC/2021. The experimental animals were terminated using diethyl ether, put in a glass beaker with ether, closed, and allowed to stand. Subsequently, neck decapitation was carried out a few moments after the rat appeared weak and the dead ones were buried in the ground. The termination process was carried out by minimizing or eliminating the suffering of the animals based on the institutional animal care and use committee (IACUC).

Statistical Analysis

All the data parameters were expressed as mean \pm standard error (SE) and independent sample groups. One-way analysis of variance (ANOVA), Duncan's multiple-range, and correlation test were carried out using SPSS statistical package (v.25.0) to compare the main parameters. Moreover, the P values were indicative of statistical significance.

RESULTS AND DISCUSSION

Phytochemicals Screening and Identification of Ethanol Extract of *C. comatus*

The screening and identification compounds of *C. comatus* mushroom ethanol extract with their retention

time, the molecular formula and weight, as well as groups are shown in **Table 2**. Meanwhile, the putative metabolites compounds were identified using GC-MS to know the types of compounds in the extracts. This process provided an overview of variations and types of the compounds that are more diverse (Roy et al., 2018). The qualitative results identification of extracts was presented in **Table 1**.

Phytochemical screening in the extract showed the content of flavonoids, polyphenols, alkaloids, saponins, triterpenoids and steroids. Qualitative identification shows that the content of flavonoids and polyphenols is the highest with a very strong indication (+++). Previous research also showed that qualitative identification of *C. comatus* ethyl acetate extract contained strong flavonoids (++), and medium (+) of alkaloids (Husen et al., 2021). Meanwhile, the compounds were identified using GC-MS to obtain more diverse types with the potential as anti-inflammatory agents, antioxidants, and immunomodulatory effects. This process provided an overview of variations and types of the compounds that are more diverse (Roy et al., 2018).

Furthermore, GC-MS is an ideal technique for qualitative and quantitative analysis of volatile and semi-volatile compounds due to the combination of the separation technique (GC) and the best identification technique (MS) (Adeoye-Isijola et al., 2018). Due to the polarity of ethanol, which can capture compounds with high polarity and/or those with semi-polar properties, it is used as a solvent in extraction to obtain a variety of compounds with a high polarity level (Hussain et al., 2020). Based on the results in Table 1, the compound with the highest molecular weight was cholestan-3-ol, trifluoroacetate, (3-beta, 5-alpha)-(CAS) with 484.70 g.mol⁻¹, retention time 20.645, and belongs to the group of alkaloids. Meanwhile, previous reports identified the ethyl acetate extract of *C. comatus* fruiting body using a UV-Vis spectrophotometer showed that the alkaloid content was 2.97 mg.L⁻¹ or 5.94% (Husen et al., 2021).

Table 1. Qualitative screening results of phytochemical compounds of *C. comatus* ethanol extract

Phytochemicals	Reagent Test	Results
Flavonoids	Zn/Mg + amyl alcohol + HCl	+++ (reddish yellow)
Polyphenols	FeCl ₃ 10%	+++ (dark blue)
Saponins	Boiled aquades + methanol (CH ₃ OH)	++ (formed stable foam)
Alkaloids	Mayer's, Dragendorff-Bouchardat's reagents	++ (reddish orange)
Triterpenoids + Steroids	Acetic acid (CH ₃ COOH) + sulfuric acid (H ₂ SO ₄)	++ (brownish purple)

Note: The positive sign indicates the presence of the target compound in the extract; + (medium level), ++ (strong), +++ (very strong).

Table 2. Putative metabolites compound profiling and identification using GC-MS analysis

Compound Name	Retention Time	Molecule Formula	Molecule Wight (g.mol ⁻¹)	%Area
(4,6-Diphenyl-pyrimidin-2-ylsulfanyl) acetic acid benzylcarbamoyl-methyl ester	2.805	C ₁₃ H ₁₂ N ₂ O ₄	260.24	9.6
Stannane	7.305	H ₄ Sn	203.81	8.9
Piperazine, 2,5-dimethyl-3-(2-methylpropyl)- (CAS)	11.225	C ₉ H ₂₀ N ₂	156.27	11.44
3-Diethylamino-4-methyl-5-methylthio-2-(trifluoroacetyl)furan	11.549	C ₆ H ₈ OS ₂	128.19	12.53
Bis(ethylmercury)-cyanamide	11.935	C ₁₁ H ₁₇ NO	179.26	9.37
Methyl 6-deoxy-3,4-O-isopropylidene-2-O-(dimethyl- <i>t</i> -butylsilyl)-beta-D-galactopyranoside	12.76	C ₁₂ H ₂₀ O ₆	260.28	8.41
N-(4-Cyanomethyl-phenyl)-2-(3,5-dimethyl-phenoxy)-acetamide	4.315	C ₁₈ H ₁₈ N ₂ O ₂	294.34	17.64
2-Hydroxy-3,5,5-trimethyl-2-cyclohexenone	19.145	C ₉ H ₁₄ O ₂	154.21	9.5
Cholestan-3-ol, trifluoroacetate, (3-beta,5-alpha.)- (CAS)	20.645	C ₂₉ H ₄₇ F ₃ O ₂	484.70	9.76
Trisulfide, dipropyl (CAS)	21.28	C ₆ H ₁₄ S ₃	182.37	16.91

The results also showed that there were 10 kinds of compounds identified with the lowest retention time (4,6-diphenyl-pyrimidine-2-ylsulfanyl)-acetic acid with 2.805 and molecular weight of 260.24 g.mol⁻¹, which belongs to the acetic acid group. Although 2-oxanone has the highest retention time, it has the lowest molecular weight of 142.20 g.mol⁻¹. The retention time is the duration of sample analysis and in the reverse phase, the more polar substances will elute first and faster than non-polar substances (Jandera & Hájek, 2018). Several compounds in the ethanol extract of *C. comatus* (Table 1 and Table 2) have various biological and biochemical activities. In addition to phenolic compounds that can act as antioxidants, others also have anti-inflammatory and immunomodulatory roles. Previous studies on clinical applications reported that the compound 2-oxonanone (CAS) plays a role in inhibiting cancer development by suppressing HIF-1 α (Cheng et al., 2019). The qualitative identification of *C. comatus* contains alkaloids, saponins, flavonoids, polyphenol, and phenolic. The phenolic compounds act as antioxidant agents (Gomathi et al., 2015), while monoterpenoids and triterpenoids function as an anti-inflammatory (Wang et al., 2016). Methyl 6-deoxy-3,4-O-isopropylidene-2-O-(dimethyl-*t*-butylsilyl)-beta-D-galactopyranoside compounds which are included in isopropylidene derivatives exhibit immunomodulatory function (Catelani et al., 2003).

Inflammatory Effects of Carrageenan

This study used the principle of a 1% carrageenan-induced rat paw edema model to evaluate the effectiveness of drugs as well as the anti-inflammatory

extracts and assess their anti-edema effect (Amdekar et al., 2012). The inflammatory conditions can be exacerbated by the increase of free radicals. The induction of carrageenan increases the formation of nitric oxide free radicals (NO[•]), whose formation is mediated by inducible nitric oxide synthase (iNOS), and NO modulates the immune response and the process of inflammation (Sareila et al., 2008).

Immunoglobulin E (IgE) Levels

The effectiveness of the ethanol extract *C. comatus* fruiting body on decreasing levels of Immunoglobulin-E (IgE) showed significant results ($p < 0.05$) after 14 days of treatment (Figure 1). The highest IgE level was in the NC group at 37.65 \pm 3.50 g/mL, while the HC (healthy rats) was 18.63 \pm 3.26 g/mL. Furthermore, the IgE levels in the T1, T2, and T3 groups showed a significant decrease ($p < 0.05$) with an average of 19.03 g/mL, where T1 has 22.87 \pm 2.52 g/mL, T2 which was the lowest has 15.42 \pm 5.33 g/mL, and while the T3 was 18.81 \pm 3.27 g/mL.

Generally, the IgE levels of the rats that received the ethanol extract of *C. comatus* were close to the HC group. The levels in the PC group were also below the NC, with 25.69 \pm 4.12 g/mL, which indicated that the effect of the administration of diclofenac sodium is the same as the ethanol extract of *C. comatus* fruiting body in reducing IgE levels in inflammatory rats. The highest percentage decrease in IgE levels compared to the NC group was the T2 group with a value of 59.04%, while the T1 group experienced a decrease of 39.25% and the T3 group 50.09%. The induction of 1% carrageenan in the previous study increased the formation of free radicals, which were evaluated from

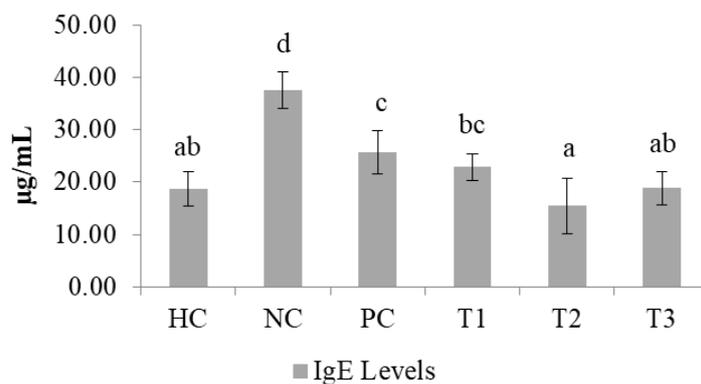


Figure 1. Immunoglobulin-E (Ig-E) levels after treatment. Note: The different letters on the bars represent the significant differences ($p < 0.05$)

the level of malondialdehyde (MDA), where the carrageenan-induced rats were 233.3 mmol/L while the healthy group was 207.5 mmol/L (Dinanti & Handajani, 2020). It was also discovered that the subcutaneous induction of 1% carrageenan in 190-200 g Wistar rats showed that the paw edema of those in the negative control group was very high, with 1.36 mL 3 hours after induction. Meanwhile, the administration of 0.9 mL of diclofenac sodium inhibited the inflammatory reaction by 13.79% (Yusuf et al., 2014).

Interleukin-1 β (IL-1 β) Levels

The results of the measurement of Interleukin-1 β (IL-1 β) levels were significant between the rats that received the ethanol extract of the *C. comatus* and the NC group ($p < 0.05$). The highest level of IL-1 β was in the NC group with 24.79 ± 5.41 g/mL, which was three times higher compared to the HC (healthy control) with 8.91 ± 2.42 g/mL (**Figure 2**).

The IL-1 β levels in the experimental group treated with the ethanol extract of the fruiting body of *C. comatus* and diclofenac sodium showed a very significant decrease compared to the NC after 14 days of the experiment. The levels of IL-1 β in the PC were 8.86 ± 3.65 g/mL, which is almost the same as the HC group, while the extract T1 had 9.51 ± 2.09 g/mL, the T2 was 7.40 ± 2.20 g/mL, and T3 had 7.28 ± 1.07 g/mL which was the group with the lowest levels

(**Figure 2**). The highest percentage decrease compared to the NC group was the T3 group with 70.63%, while the T2 was 70.1% and the T1 was 61.63%.

Induction of carrageenan led to the formation of inflammation which consisted of two phases, namely 1-2 hours after injection, which caused trauma. In the first phase, there is a release of serotonin and histamine to the site of inflammation and an increase in prostaglandin synthesis in the damaged tissue. The second phase is the release of prostaglandins, which is mediated by bradykinins and leukotrienes (Ramadhani & Sumiwi, 2016). These prostaglandins are inflammatory mediators that can cause tissue swelling (tumor) and pain (dolor), causing an increase in TNF- α , IL-1 β , and nitric oxide (NO) from iNOS (Andriyono, 2019).

Previous studies showed that sub-plantar induction of carrageenan caused an increase in IL-6 levels of 70.45 pg/mL, TNF- α of 669.23 pg/mL, and a decrease in IL-10 of 14.15 pg/mL compared the healthy rats with IL-6 levels. Moreover, 54.42 pg/mL, TNF- α 530.75 pg/mL and IL-10 25.075 pg/mL were measured 24 hours after induction (Amdekar et al., 2012). Acute rat paw inflammation occurs after carrageenan induction which is characterized by the migration of inflammatory cells into the microvascular system and fluid that enters the interstitial tissue.

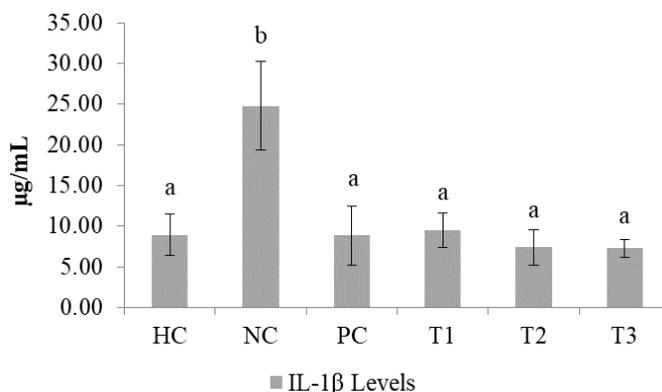


Figure 2. Interleukin-1 β (IL-1 β) levels after treatment. Note: The different letters on the bars represent the significant differences ($p < 0.05$)

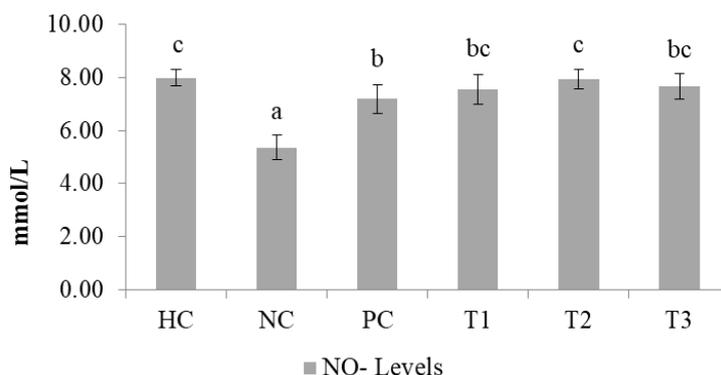


Figure 3. Nitric oxide levels of eNOS after treatment. Note: The different letters on the bars represent the significant differences ($p < 0.05$)

These events are induced by mediators that bind to specific receptors on inflammatory and endothelial cells (Albertini et al., 2007). *C. comatus* mushroom contains various compounds that play a role in counteracting and reducing free radicals by stimulating the formation of endogenous antioxidants, which act as an immunomodulator (Husen et al., 2021). The steroid compounds have anti-inflammatory activity by activating glucocorticoid receptors by increasing or decreasing the transcription process of genes involved in the inflammatory process and inhibiting the production of cytokines such as IL-6, IL-12, and TNF- α (Luliana et al., 2017).

Nitric Oxide of eNOS Levels

The measurement of nitric oxide (NO) levels from eNOS showed significant results at the level ($p < 0.05$). The highest NO level after 14 days of treatment and 6 hours was in the T2 group, while the lowest level was in the NC with 5.35 ± 0.47 mmol/L (Figure 3).

The NO levels in the PC, T1, T2, and T3 groups which were given anti-inflammatory drugs and ethanol extract of *C. comatus* mushroom fruiting body significantly increased after 6 hours of carrageenan induction. The values of NO levels in the PC, T1, T2, and T3 were 7.18 ± 0.55 mmol/L, 7.55 ± 0.55 mmol/L, 7.93 ± 0.35 mmol/L, and 7.66 ± 0.49 mmol/L, respectively. The average levels in the three groups treated with the ethanol extract of *C. comatus* were 7.71 mmol/L, while the healthy or HC group had 7.98 ± 0.32 mmol/L, which is almost the same value as the T2 group. The highest increase in NO eNOS levels was in the T2 group with 48.2% compared to the NC, while T1 and T3 had an increase of 41.1% and 43.2%, respectively.

The decrease in the levels of IgE, IL-1 β , and increased levels of NO expressed in eNOS occurs due to the role of bioactive compounds including flavonoids contained in the ethanol extract of the *C. comatus* fruiting body (Ratnaningtyas et al., 2019). The content of flavonoids (quercetin) in *C. comatus* is 30.1 μ g P/g DW and 1.46 μ g P/g DW rutin (Tešanović et al., 2017), while ethyl acetate extract contains 132.342 mg/L of vitamin C, 102.32 g/L of vitamin E, and 351.133 ppm of rutin (Husen et al., 2021). The

flavonoid content in the fruiting body *C. comatus* in a previous study was 16.40 mg/L (Husen et al., 2021). This significant value can prevent the activity of COX and lipoxygenase enzymes directly, inhibiting the biosynthesis of prostaglandins and leukotrienes which are the end products of the COX and lipoxygenase pathways. Flavonoids can also reduce the number of leukocytes and complement activation, thereby decreasing leukocyte adhesion to the endothelium and the inflammatory response (Wardani, 2020). Furthermore, the ethanol extract of the fruiting body of *C. comatus* also contains terpenoids (Ratnaningtyas et al., 2019), which provide anti-inflammatory activity. This is because the compounds can inhibit the production of TNF- α which is a pro-inflammatory cytokine. Flavonoids act as an anti-inflammatory by decreasing the main cytokines that are important in the process, namely TNF- α and IL-1 β (Lee et al., 2013).

Leukocyte Counts

The results of the number of blood leukocytes measured 24 hours after carrageenan induction showed that the highest count was in the NC group with 14362.5 ± 2192.2 mm³, while the lowest was in the T3 group (Figure 4). Based on the results of Duncan's further test analysis, it was discovered that each treatment group, except NC, was not significantly different at the level ($p < 0.05$).

In this study, the number of leukocytes showed that the group treated with the extract and diclofenac sodium decreased compared to the negative control ($p < 0.05$). Although it was not significantly different from the treatment group compared to the NC group. The administration of ethanol extract of *C. comatus* can reduce the number of leukocytes compared to the NC group without treatment. The PC group treated with diclofenac sodium had a leukocyte count of $11,250 \pm 973.8$ mm³, which is slightly higher than the T1, T2, and T3 with $10,956.25 \pm 1,665.13$ mm³, $10,050 \pm 1,206.92$ mm³, and $9,875 \pm 1,097.4$ mm³, respectively. The mean leukocyte counts in the three groups treated with the ethanol extract of *C. comatus* was 10,293.75 mm³, slightly below the PC and above the HC groups. The decrease in the number of

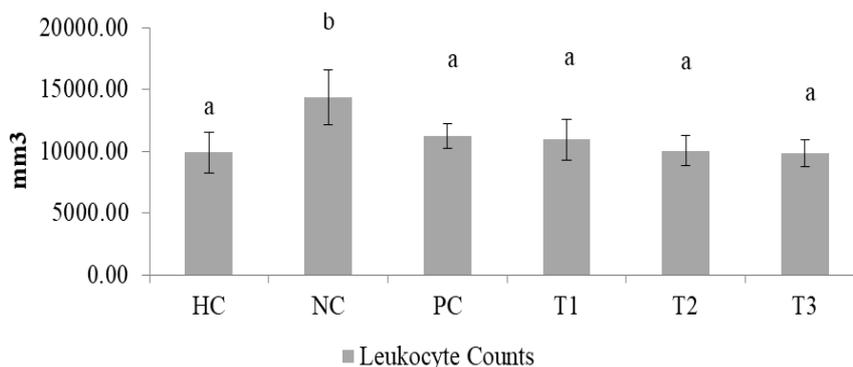


Figure 4. Leukocyte counts of the rats after treatment. Note: The different letters on the bars represent the significant differences ($p < 0.05$)

leukocytes in the treatment group with the highest extract was the T3 group with a decrease of 31.24%, while PC had 21.67% compared to the NC group.

Leukocytes in the group treated with extract significantly decreased compared to the NC group. This is in line with previous investigation, where 180-200 g Wistar rats induced with 1% of 0.1 mL carrageenan gave an inflammatory response with total leukocyte count of $4.18 \times 10^5/\text{mL}$ in the negative control group. Meanwhile, the group given indomethacin 10 mg decreased by 48.1% to $2.7 \times 10^5/\text{mL}$ and inhibited peritoneal leukocyte migration by 60.81% ($p < 0.05$) (Gupta et al., 2005). In this study, the results of the calculation of the number of leukocytes were also directly proportional to the increased levels of IL-1 β and IgE, especially in the NC group, and the decrease in leukocytes was directly related to the decrease in the levels of IL-1 β and IgE.

Plantar Thickness

Moreover, 6 hours after induction of carrageenan 1%, the plantar thickness was measured using a caliper. The results of the analysis showed that the treatment group induced and treated with the ethanol extract of *C. comatus* fruiting body was not significantly different in Duncan's follow-up test with a level ($p < 0.05$). The plantar thickness which represents the

effect of carrageenan induction, and the process of inflammation gave different responses between test groups. The NC group had the highest mean plantar thickness with 0.72 ± 0.07 cm (Figure 5). The group of rats treated with the extract showed a decrease in plantar thickness, where the T1 was the smallest compared to other groups.

The decrease in plantar thickness within 6 hours before inflammation showed that ethanol extract of *C. comatus* fruiting body can suppress the inflammatory response. Based on the results, the plantar thickness of the PC, T3, T2, and T1 groups were 0.68 ± 0.06 cm, 0.70 ± 0.05 cm, 0.65 ± 0.10 cm, and 0.63 ± 0.05 cm, respectively. Although the follow-up test analysis showed no significant difference for each treatment group, it was discovered that the group without extract and diclofenac sodium gave the highest plantar thickness response (Figure 5). The highest percentage decrease in plantar thickness in the group treated with the extract was the T1 group at 12.5% compared to the NC group. A high ROS in inflammatory conditions can cause a decrease in NO bioavailability due to the redox reaction (O^{2-}) that catalyzes ONOO \cdot . This is because NO plays an important role in several vascular functions, including the regulation of vasomotor tone and maintenance of vascular health (Laurson et al., 2001).

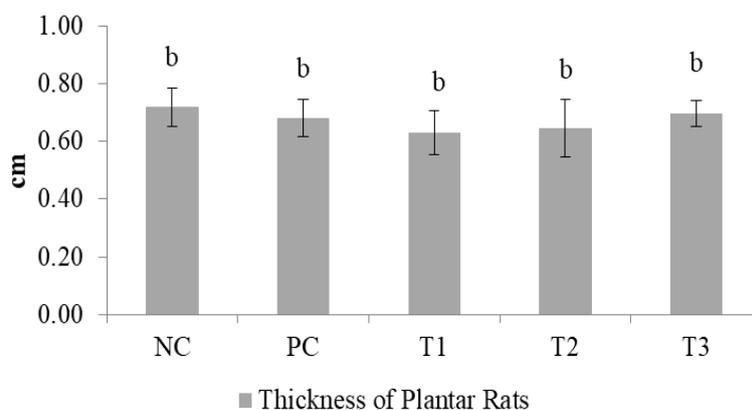


Figure 5. Plantar thickness of the rats' paws after treatment. Note: The different letters on the bars represent the significant differences ($p < 0.05$)

The fruiting body of *C. comatus* mushroom shows antioxidant activity that can reduce ROS due to the content of vitamins C and E. They synergize together in breaking lipid peroxidation reactions (Husen et al., 2021; Ratnaningtyas et al., 2022), where vitamin E breaks the lipid peroxidation chain propagation efficiently. Furthermore, their synergistic interaction is effective in increasing the antioxidant capacity of vitamin E and recovered by vitamin C which previously reacted with superoxide anions to form tocopherol radicals (Niki, 2014).

Edema Volume

The results of the volume of inflammation/edema measurement in rat paws after intraplantar injection of 1% carrageenan within 6 hours showed that each group gave a different response and was statistically significantly different in Duncan's follow-up test ($p < 0.05$) (Figure 6).

The measurement of the volume of edema in rat paws indicated the occurrence of an inflammatory process after carrageenan induction with reddish swelling characteristics. The group treated with diclofenac sodium (PC) showed a decrease with edema volume of 1.80 ± 0.46 mL, while the NC was 1.92 ± 0.38 mL. Furthermore, the effectiveness of the fruiting body of *C. comatus* ethanol extract was also indicated by the decrease in edema volume in the T1, T2, and T3 groups. The highest decrease was in the T3 group with extract 750 mg/kg BW with 33.3% and

edema volume. The edema volume of the T1 group was 1.56 ± 0.54 mL with a decrease of 18.8%, while T2 was 1.58 ± 0.33 mL with a decrease of 17.7% compared to NC (Figure 6).

The results also showed that the volume of edema and plantar thickness of the rats' feet in the NC group had the highest value compared to others. This is in line with a previous study that induced 100 μ L of 1% carrageenan sub-plantar in Wistar rats, where 4 hours after injection the negative control group had plantar thickness > 1.2 mm, while the group treated with indomethacin 10 mg was < 0.6 mm (Sadeghi et al., 2014). It was also stated that 100 μ L induction of 1% carrageenan in Wistar rats caused edema with an increase in plantar foot thickness, where the healthy group was 3.023 cm, the sick was 4.01 cm, and the group with diclofenac sodium was 3.17 cm for 24 hours after induction (Amdekar et al., 2012).

Body Weight of The Rats

The measurements of body weight before and after treatment showed mixed results, where the analysis showed that the extract was not significantly different in Duncan's follow-up test ($p > 0.05$). The highest initial body weight was in the HC group with 278.8 ± 11.28 g, while the lowest was in the T1 with a value of 209.6 ± 10.88 g. The highest final body weight was in the HC with 300.8 ± 6.50 g, and the lowest was 217.4 ± 11.44 g in the T1 group (Figure 7).

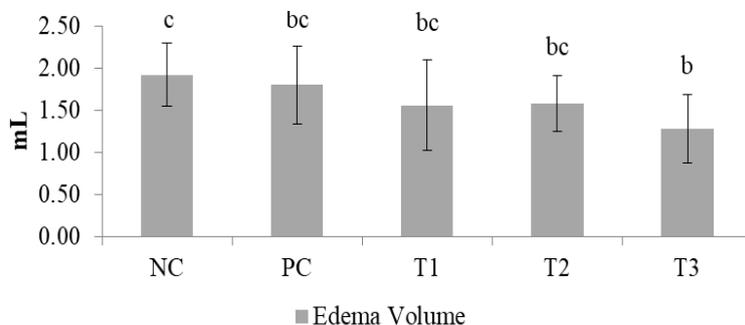


Figure 6. Edema volume of the rats after treatment. Note: The different letters on the bars represent the significant differences ($p < 0.05$)

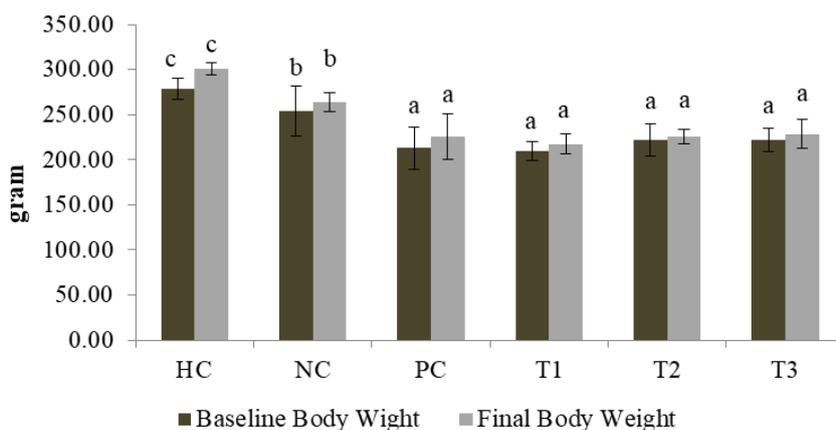


Figure 7. Body weight of the rats before and after treatment. Note: The different letters on the bars represent the significant differences ($p < 0.05$)

The initial and final body weight as supportive data was measured to determine whether inflammation in rats induced by 1% carrageenan affected weight loss due to inflammatory reactions and decreased appetite. However, there was no significant effect on weight loss, especially in the NC group. Based on the results, the HC, NC, PC, T1, T2, and T3 increased by 7.3%, 3.6%, 5.5%, 3.6%, 1.42%, and 3.1%, respectively (Figure 7). Furthermore, 0.5 mL of 1% carrageenan induction after 24 hours did not affect weight loss, while changes in body weight in the group treated with the extract showed an increase between 1 and 3.6%. The relatively fast inflammatory time of carrageenan which is less than 24 hours to cause an inflammatory reaction did not affect the appetite of experimental animals, therefore, changes (weight loss) that can occur because inflammation were not proven in this study.

CONCLUSIONS

The ethanol extract of the fruiting body of *C. comatus* has the potential as an herbal medicine with its activity in increasing NO eNOS levels, reducing IL-1 β and IgE levels as well as total leukocyte count, as shown by a decrease in edema and plantar thickness of the rats' feet. The administration of the extract to the inflammatory model rats did not have a significant effect on changes in body weight. A dose of 250 g of extract was effective in reducing plantar thickness, 500 mg reduced IgE levels and increased NO eNOS, while 750 mg decreased the volume of rat paw edema

ACKNOWLEDGMENTS

This study was carried out at the Faculty of Biology, Jenderal Soedirman University. It was supported and financed by the Institute for Research and Community Service (LPPM) through the Applied Leading Research Grant (RUT) under the supervision of the Service and Business Agency (BLU), Jenderal Soedirman University (UNSOED), with grant number T/215/UN23.18/PT.01.01/2020.

REFERENCES

- AbCAM. (2016). Sandwich ELISA protocol. *Discover*, 1–2.
- Adeoye-Isijola, M. O., Olajuyigbe, O. O., Jonathan, S. G., & Cooposamy, R. M. (2018). Bioactive Compounds in Ethanol Extract of *Lentinus squarrosulus* Mont - a Nigerian Medicinal Macrofungus. *African Journal of Traditional, Complementary and Alternative Medicines*, 15(2), 42–50. <https://doi.org/10.21010/ajtcamv15i2.6>
- Albertini, R., Villaverde, A. B., Aimbire, F., Salgado, M. A. C., Bjordal, J. M., Alves, L. P., Munin, E., & Costa, M. S. (2007). Anti-inflammatory effects of low-level laser therapy (LLLT) with two different red wavelengths (660 nm and 684 nm) in carrageenan-induced rat paw edema. *Journal of Photochemistry and Photobiology B: Biology*, 89(1), 50–55. <https://doi.org/10.1016/j.jphotobiol.2007.08.005>
- Amdekar, S., Roy, P., Singh, V., Kumar, A., Singh, R., & Sharma, P. (2012). Anti-inflammatory activity of lactobacillus on carrageenan-induced paw edema in male wistar rats. *International Journal of Inflammation*, (2012), 1–6. <https://doi.org/10.1155/2012/752015>
- Andriyono, R. I. (2019). *Kaempferia galanga* L. sebagai anti-inflamasi dan analgetik (*Kaempferia galanga* L. as anti-inflammatory and analgesic). *Jurnal Kesehatan*, 10(3), 495–502. <https://doi.org/10.26630/jk.v10i3.1458>
- Arief, H., & Widodo, M. A. (2018). Peranan stres oksidatif pada proses penyembuhan luka (The role of stress oxidative in the wound healing process). *Jurnal Ilmiah Kedokteran Wijaya Kusuma*, 5(2), 22–29. <https://doi.org/10.30742/jikw.v5i2.338>
- Catelani, G., Corsaro, A., D'Andrea, F., Mariani, M., Pistarà, V., & Vittorino, E. (2003). Convenient preparation of L-arabino-hexos-5-ulose derivatives from lactose. *Carbohydrate Research*, 338(22), 2349–2358. <https://doi.org/10.1016/j.carres.2003.08.001>
- Chakraborty, D., & Yolmo, N. L. (2019). *Costus speciosus* and its antipsoriatic arthritis activity: a review. *Asian Journal of Pharmaceutical and Clinical Research*, 12(1), 7–10.
- Cheng, J., Hu, L., Yang, Z., Suo, C., Wang, Y. J., Gao, P., Cui, C., & Sun, L. (2019). 2-Oxonanonoid antibiotic actinolactomycin inhibits cancer progression by suppressing HIF-1 α . *Cells*, 8(5), 439. <https://doi.org/10.3390/cells8050439>
- Dinanti, B., & Handajani, F. (2020). Pengaruh profilaksis ekstrak ubi jalar ungu (*Ipomoea batatas* L.) terhadap kadar malondialdehida hepar tikus putih (*Rattus norvegicus*) jantan galur wistar yang diinduksi karagenan (Effect of prophylactic extract of purple sweet potato (*Ipomoea batatas* L.) on liver malondialdehyde levels of male white rats (*Rattus norvegicus*) wistar strain induced by carrageenan). *Hang Tuah Medical Journal*, 15(2), 146–164.
- Gomathi, D., Kalaiselvi, M., Ravikumar, G., Devaki, K., & Uma, C. (2015). GC-MS analysis of bioactive compounds from the whole plant ethanolic extract of *Evolvulus alsinoides* (L.) L. *Journal of Food Science and Technology*, 52(2), 1212–1217. <https://doi.org/10.1007/s13197-013-1105-9>
- Gomez, I., Foudi, N., Longrois, D., & Norel, X. (2013). The role of prostaglandin E2 in human vascular inflammation. *Prostaglandins, Leucotrienes and Essential Fatty Acids*, 89, 55–63. <https://doi.org/10.1016/j.plefa.2013.04.004>
- Gupta, M., Mazumder, U. K., Kumar, R. S., Gomathi, P., Rajeshwar, Y., Kakoti, B. B., & Selven, V. T.

- (2005). Anti-inflammatory, analgesic and antipyretic effects of methanol extract from *Bauhinia racemosa* stem bark in animal models. *Journal of Ethnopharmacology*, 98(3), 267–273. <https://doi.org/10.1016/j.jep.2005.01.018>
- Husen, F., Hernayanti, H., Ekowati, N., Sukmawati, D., & Ratnaningtyas, N. I. (2021). Antidiabetic effects and antioxidant properties of the saggy ink cap medicinal mushroom, *Coprinus comatus* (Agaricomycetes) on streptozotocin-induced hyperglycemic rats. *International Journal of Medicinal Mushrooms*, 23(10), 9–21. <https://doi.org/10.1615/intjmedmushrooms.2021040020>
- Hussain, M. H., Fitrah, N., Bakar, A., Mustapa, A. N., Low, K., Othman, N. H., & Adam, F. (2020). Synthesis of Various Size Gold Nanoparticles by Chemical Reduction Method with Different Solvent Polarity. *Nanoscale Research Letters*, 15, 140–150.
- Jandera, P., & Hájek, T. (2018). Mobile phase effects on the retention on polar columns with special attention to the dual hydrophilic interaction-reversed-phase liquid chromatography mechanism, a review. *Journal of Separation Science*, 41(1), 145–162. <https://doi.org/10.1002/jssc.201701010>
- Laursen, J. B., Somers, M., Kurz, S., Mccann, L., Warnholtz, A., Freeman, B. A., Tarpey, M., Fukai, T., & Harrison, D. G. (2001). Endothelial regulation of vasomotion in ApoE-deficient mice implications for interactions between peroxynitrite and tetrahydrobiopterin. *Circulation*, 103(9), 1282–1288. <https://doi.org/10.1161/01.CIR.103.9.1282>
- Lee, C. W., Ko, H. H., Lin, C. C., Chai, C. Y., Chen, W. T., & Yen, F. L. (2013). Artocarpin attenuates ultraviolet B-induced skin damage in hairless mice by antioxidant and anti-inflammatory effect. *Food and Chemical Toxicology*, 60, 123–129. <https://doi.org/10.1016/j.fct.2013.07.029>
- Luliana, S., Susanti, R., & Agustina, E. (2017). Uji aktivitas antiinflamasi ekstrak air herba ciplukan (*Physalis angulata* L.) terhadap tikus putih (*Rattus norvegicus* L.) jantan galur wistar yang diinduksi karagenan (Anti-inflammatory activity test of ciplukan herb (*Physalis angulata* L.) water extract against white rats (*Rattus norvegicus* L.) of carrageenan-induced wistar). *Traditional Medicine Journal*, 22(3), 199–205.
- Mohan, K., Padmanaban, M., & Uthayakumar, V. (2016). Quantification of bioactive components from medicinal herb *Ganoderma lucidum* using HPTLC and GC-MS techniques. *Research Journal of Biotechnology*, 464, 42–49.
- Nigrovic, P. A., & Shin, K. (2015). Evaluation of synovial mast cell functions in autoimmune arthritis. *Mast Cells*, 423–442. https://doi.org/10.1007/978-1-4939-1568-2_26
- Niki, E. (2014). Role of vitamin E as a lipid-soluble peroxy radical scavenger: in vitro and in vivo evidence. *Free Radical Biology and Medicine*, 66, 3–12. <https://doi.org/10.1016/j.freeradbiomed.2013.03.022>
- Ramadhani, N., & Sumiwi, S. A. (2016). Aktivitas antiinflamasi berbagai tanaman diduga berasal dari flavonoid (The anti-inflammatory activity of various plants is thought to be derived from flavonoids). *Farmaka, Supp.* 14(2), 111–123.
- Ratnaningtyas, N. I., Hernanyanti, Ekowati, N., Husen, F., Maulida, I., Kustianingrum, R., & Vidiyanti, V. (2022). Antioxidant activities and properties of *Coprinus comatus* mushroom both mycelium and fruiting body extracts in streptozotocin-induced hyperglycemic rats model. *Biosaintifika: Journal of Biology & Biology Education*, 14(1), 9–21.
- Ratnaningtyas, N. I., Hernayanti, Ekowati, N., & Husen, F. (2021). Nephroprotective and antioxidant effects of ethanol extract of *Coprinus comatus* mushroom fruit-bodies on streptozotocin-induced diabetic rat models. *The 4th International Conference on Biosciences (ICoBio 2021)*, 948 (1-13). <https://doi.org/10.1088/1755-1315/948/1/012078>
- Ratnaningtyas, N. I., Hernayanti, Ekowati, N., Sukmawati, D., & Widiyanti, H. (2019). Chicken drumstick mushroom (*Coprinus comatus*) ethanol extract exerts a hypoglycaemic effect in the *Rattus norvegicus* model of diabetes. *Biocatalysis and Agricultural Biotechnology*, 19, 1–4. <https://doi.org/10.1016/j.bcab.2019.101050>
- Ratnaningtyas, N. I., Hernayanti, H., Ekowati, N., & Husen, F. (2022). Ethanol extract of the mushroom *Coprinus comatus* exhibits antidiabetic and antioxidant activities in streptozotocin-induced diabetic rats Nuniek. *Pharmaceutical Biology*, 60(1), 1126–1136. <https://doi.org/10.1080/13880209.2022.2074054>
- Reis, F. S., Barros, L., Martins, A., & Ferreira, I. C. F. R. (2012). Chemical composition and nutritional value of the most widely appreciated cultivated mushrooms: an inter-species comparative study. *Food and Chemical Toxicology*, 50(2), 191–197. <https://doi.org/10.1016/j.fct.2011.10.056>
- Rodríguez, C., Contreras, C., Sáenz-medina, J., Muñoz, M., López, M., Rivera, L., & Prieto, D. (2020). Redox biology activation of the AMP-related kinase (AMPK) induces renal vasodilatation and downregulates Nox-derived reactive oxygen species (ROS) generation. *Redox Biology*, 34, 1–15. <https://doi.org/>

- 10.1016/j.redox.2020.101575
- Roy, R. D., Reena Roy, D., & Krishnappa, M. (2018). Mycochemical Profiling of *Lentinus squarrosulus* Mont., a Wild Edible Macrofungi Using Gc-Ms. *International Journal of Pharmaceutical Sciences and Research*, 9(10), 4349. [https://doi.org/10.13040/IJPSR.0975-8232.9\(10\).4349-54](https://doi.org/10.13040/IJPSR.0975-8232.9(10).4349-54)
- Sadeghi, H., Mostafazadeh, M., Sadeghi, H., Barmak, M. J., Talebianpoor, M. S., & Mehraban, F. (2014). In vivo anti-inflammatory properties of aerial parts of *Nasturtium officinale*. *Pharmaceutical Biology*, 52(2), 169–174. <https://doi.org/10.3109/13880209.2013.821138>
- Sareila, O., Korhonen, R., Auvinen, H., & Ha, M. (2008). Effects of levo- and dextrosimendan on NF- κ B-mediated transcription, iNOS expression and NO production in response to inflammatory stimuli. *British Journal of Pharmacology*, 155, 884–895. <https://doi.org/10.1038/bjp.2008.328>
- Silva, V. G., Silva, R. O., Damasceno, S. R. B., Carvalho, N. S., Prudeêncio, R. S., Aragão, K. S., Guimarães, M. A., Campos, S. A., Vêras, L. M. C., Godejohann, M., Leite, J. R. S. A., Barbosa, A. L. R., & Medeiros, J. V. R. (2013). Anti-inflammatory and antinociceptive activity of epiisopiloturine, an imidazole alkaloid isolated from *Pilocarpus microphyllus*. *Journal of Natural Products*, 76(6), 1071–1077. <https://doi.org/10.1021/np400099m>
- Tešanović, K., Pejin, B., Šibul, F., Matavulj, M., Rašeta, M., Janjušević, L., & Karaman, M. (2017). A comparative overview of antioxidative properties and phenolic profiles of different fungal origins: fruiting bodies and submerged cultures of *Coprinus comatus* and *Coprinellus truncorum*. *Journal of Food Science and Technology*, 54(2), 430–438. <https://doi.org/10.1007/s13197-016-2479-2>
- Waner, T., Mazar, S., Nachmias, E., Keren-Kornblatt, E., & Harrus, S. (2003). Evaluation of a dot ELISA kit for measuring immunoglobulin M antibodies to canine parvovirus and distemper virus. *Veterinary Record*, 152(19), 588–591. <https://doi.org/10.1136/vr.152.19.588>
- Wang, S., Wang, H., Liu, Y., Wang, Y., Fan, X., & Cheng, Y. (2016). Rapid discovery and identification of anti-inflammatory constituents from traditional Chinese medicine formula by activity index, LC-MS, and NMR. *Scientific Reports*, 6, 1–13. <https://doi.org/10.1038/srep31000>
- Wardani, I. G. A. A. K. (2020). Efektivitas gel ekstrak bunga kecombrang (*Etlingera elatior*) sebagai antiinflamasi terhadap mencit yang diinduksi karagenan (The effectiveness of kecombrang flower extract gel (*Etlingera elatior*) as an anti-inflammatory against carrageenan-induced mice). *Jurnal Ilmiah Medicamento*, 6(1), 66–71.
- Yuan, G., Wahlqvist, M. L., He, G., Yang, M., & Li, D. (2006). Natural products and anti-inflammatory activity. *Asia Pacific Journal of Clinical Nutrition*, 15(2), 143–152. <https://doi.org/10.11.495.8655>
- Yusuf, S. M., Rohmawaty, E., & Nusjirwan, R. (2014). Bixa orellana L. leaf infusion as an anti-inflammatory agent in carrageenan-induced wistar rats. *Althea Medical Journal*, 1(2), 60–64.
- Zhao, H., Zhang, J., Liu, X., Yang, Q., Dong, Y., & Jia, L. (2018). The antioxidant activities of alkaline-extractable polysaccharides from *Coprinus comatus* on alcohol-induced liver injury in mice. *Scientific Reports*, 8(1), 1–12. <https://doi.org/10.1038/s41598-018-30104-6>