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ROMINA ASHRAFI

EVALUATION OF GENOTOXIC AND ANTIGENOTOXIC PROPERTIES OF REISHI (*GANODERMA LINGZHI*) AND LION'S MANE (*HERICIUM ERINACEUS*) MUSHROOM EXTRACTS

Master Thesis

Genetics study programme

Academic Supervisor
PhD student
Milda Babonaite

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ABBREVIATION

ACE	Acidic extract
AKE	Alkaline extract
ALS	Alkaline-labile sites
AO/EtBr	Acridine Orange/ Ethidium Bromide
DSBs	Double-strand breaks
EAHE	Erinacine A-enriched Hericium erinaceus
HWE	Hot water extract
IA	Immune Assist
Ir	Irregular-shaped
LM	Lion's Mane
LMP	Low melting point
LPS	Lipopolysaccharide
MN	Micronucleus
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
MWE	Microwaved 50% ethanol extract
NGF	Nerve growth factor
NMP	Normal melting point
PBMC	Peripheral blood mononuclear cells
R	Reishi
Reg	Regular-shaped
SCGE	Single-cell gel electrophoresis
SSBs	Single-strand breaks
TCM	Traditional Chinese medicine

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SUMMARY

This study evaluated the cytotoxic, genotoxic, and antigenotoxic effects *of Ganoderma lucidum* (Reishi) and *Hericium erinaceus* (Lion's Mane) mushroom extracts on human peripheral blood mononuclear cells (PBMCs) from five healthy donors. Cytotoxicity was assessed using a dual acridine orange/ethidium bromide staining technique, showing no significant cell viability reduction. The genotoxic and antigenotoxic effects were measured using the alkaline comet assay.

Both mushroom extracts induced some DNA damage, though the changes were not statistically significant. Certain doses (10, 20, and 40 µL of Lion's Mane and 40, 50, and 80 µL of Reishi) resulted in less DNA damage and were selected for further antigenotoxic assessment.

Antigenotixic effects of mushroom extracts against H_2O_2-induced DNA damage were evaluated in the pre- and post-treatment groups. In pre-treatment, cells treated with 80 µL of Reishi extract showed slight DNA damage reduction. For Lion's Mane, 10 µL decreased damage, while 20 and 40 µL increased it. Post-treatment with Reishi extracts improved DNA repair, with 80 µL being the most effective. Lion's Mane extracts also reduced DNA damage at 10, 20, and 40 µL concentrations.

Overall, both mushroom extracts showed potential antigenotoxic properties, varying with concentration and treatment duration. Further research is needed to confirm these findings and their implications for DNA repair.

VILNIAUS UNIVERSITETAS
GYVYBĖS MOKSLŲ CENTRAS

Romina Ashrafi
Baigiamasis magistro darbas

Reishi (Ganoderma lingzhi) ir liūto karčių (Hericium erinaceus) grybų ekstraktų genotoksinių ir antigenotoksinių savybių įvertinimas

SANTRAUKA

Šiame tyrime buvo įvertintas *Ganoderma lucidum* (Reishi) ir *Hericium erinaceus* (Lion's Mane) grybų ekstraktų citotoksinis, genotoksinis ir antigenotoksinis poveikis žmogaus periferinio kraujo vienbranduolėse ląstelėse *in vitro*. Ekstraktų citotoksiškumas buvo įvertintas panaudojant akridino oranžo / etidžio bromido dažų mišinį. Pastebėta, kad tiriamieji ekstraktai nesumažino ląstelių gyvybingumo, priešingai, daugeliu atvejų jį padidino, lyginant su neigiama kontrole. Galima genotoksinis ir antigenotoksinis grybų ekstraktų poveikis buvo įvertintas šarminiu kometos metodu.

Abu grybų ekstraktai indukavo nežymų DNR pažaidų kiekį, tačiau pokyčiai nebuvo statistiškai reikšmingi. Tam tikros koncentracijos (10, 20 ir 40 µL Liūto karčių ir 40, 50 ir 80 µL Reishi) buvo atrinktos tolesniam antigenotoksiniam ekstraktų vertinimui.

Grybų ekstraktų antigenotoksinės savybės buvo tirtos dviem būdais - išankstinio poveikio (angl. *pre-treatment*) būdu, kai ląstelės buvo veikiamos grybų ekstraktais ir tik vėliau H_2O_2. Ir vėlyvojo poveikio (angl. *post-treatment*), kur ląstelės iš pradžių buvo veikiamos H_2O, o vėliau grybų ekstraktais. Išankstinio poveikio tyrimo metu, ląstelėse, paveiktose 80 µL Reishi ekstraktu, DNR pažaidų lygis ląstelėse sumažėjo. Taip pat DNR pažaidų lygis ląstelėse sumažėjo, jas paveikus Liūto karčių ekstrakto 10 µL doze, nors didesnės ekstrakto dozės (20 ir 40 µL) DNR pažaidų kiekį padidino. Vėlyvojo poveikio tyrimo metu, visos tirtos abiejų grybų ekstraktų dozės sumažino DNR pažaidų lygis ląstelėse, paveiktose H_2O_2.

Gauti rezultati leidžia manyti, kad abu grybų ekstraktai pasižymi potencialiomis antigenotoksinėmis savybėmis. Tačiau, šiom savybėm įrodyti, reikalingi papildomi tyrimai.

INTRODUCTION

Nowadays studying medicinal mushroom properties grabs a lot of attention. They have been used since ancient times as a valuable food source, moreover, they have therapeutic effects such as immunomodulatory, anticarcinogenic, antiviral, antioxidant, anticancer and antiobesity properties (Degreef et al., 1997; Assemie & Abaya., 2022; Thu et al., 2020).

Reishi mushroom is mostly known for immunomodulatory and antiaging properties (Lysakowska et al.,2023) and Lion's Mane mushroom is mostly known for modulating neurological disorders, particularly neurodegenerative diseases (Skulbel et al.,2020; He et al., 2017).

We hypothesize that consuming specific doses of Reishi or Lion's Mane mushrooms can repair the damage (antigenotoxic effect) induced by various endogenous and exogenous factors which could be utilized as primary or supplementary treatments for various diseases or induce more damage (genotoxic effect).

In this study, we investigated the genotoxic and antigenotoxic properties of Reishi (*Ganoderma lucidum*) and Lion's Mane (*Hericium erinaceus*) mushroom extracts, manufactured by a Lithuanian company "Mishkay", by utilizing the alkaline comet assay or single-cell gel electrophoresis (SCGE), which is a cheap and sensitive method for investigating primary DNA damage in single cells (Collins et al.,2012; Kucharova et al., 2019).

The aim of this thesis was to evaluate the probable genotoxic and antigenotoxic properties of Reishi and Lion's Mane mushroom extracts in human peripheral blood mononuclear cells (PBMCs) using *in vitro* comet assay.

Main objectives:

1. To evaluate the cytotoxicity of Reishi and Lion's Mane mushroom extracts in human PBMCs.
2. To evaluate the genotoxic potential of Reishi and Lion's Mane mushroom extracts in human PBMCs using an alkaline comet assay.
3. To estimate the genome protective effects of mushroom extracts (pre-treatment and post-treatment).
4. To evaluate the kinetics of H_2O_2-induced DNA damage attenuation in human PBMCs treated with Reishi and Lion's Mane mushroom extracts.

1. LITERATURE REVIEW

1.1 Phytochemicals present in mushrooms and their properties

Fungi, commonly known as mushrooms, are distinct from plants as they lack chlorophyll and obtain their nutrition heterotrophically from other organisms (Arabaain et al., 2019). Despite their non-plant status, fungi are rich in nutrients and are considered beneficial for health, often likened to non-toxic drugs (Mirfat et al., 2010).

Metabolic compounds in plants can be categorized into primary and secondary metabolites. Primary metabolites are essential for the growth and sustenance of organisms, whereas secondary metabolites, though not crucial for development, play a role in defence against stress, pests and diseases (Nofani., 2008; Triani et al., 2017).

Edible mushrooms, a type of fungus, are easily visible and can be hand-collected, often referred to as "functional foods" due to their nutritional benefits (Assemie & Abaya., 2022).

Of the estimated 1.5 million fungi species, at least 2000 are edible, and Edible mushrooms can be sourced from the wild or cultivated (Chang., 2006; Rahi., 2016; Thu et al., 2020).

Various biochemical methods, including methanol, ethanol and water extraction, have been employed to assess the chemical composition, food value and efficacy of different parts of edible mushrooms (Assemie & Abaya., 2022).

Edible mushrooms typically contain 85-95% moisture, 35-70% carbohydrates, 10% fat, 6-10.9% minerals, and 3-8% nucleic acids (Rahi., 2016). Mushroom protein is notable for containing all nine essential amino acids required by humans, with leucine, aspartic acid, valine, glutamine, and glutamic acid being the most common (Kalac., 2009; Thu et al., 2009).

The nutritional value of some commercially available edible mushrooms is presented in Table 1.1.

Table 1.1 Nutritional value of some commercial edible mushrooms (on a dry wt. basis) (Ahlawat et al., 2016).

Nutritional parameters	Mushrooms			
	Agaricus bisporus	*Pleurotus spp.*	*Volvariella volvacea*	*Lentinula edodes*
Proteins %	29.14	19.59	38.10	18.85
Carbohydrates %	51.05	64.34	42.30	63.60
Fat %	1.56	1.05	0.97	1.22
Vitamins D (IU/g)	984	487	462.04	205
Sodium (mg/kg)	500.8	208.87	345.34	82.49
Iron (mg/kg)	85.86	183.07	72.51	37.55
K:Na	84:1	129:1	120:1	255:1

Edible fungi serve as a rich source of vitamins such as thiamine, riboflavin, niacin, biotin, ascorbic acid, pantothenic acid and folic acid (Hossain et al., 2007). They are also abundant in vitamins B1, B2, B12, C, D and E (Heleno et al., 2010; Mattila et al., 2001). Furthermore,

minerals including calcium, iron, manganese, magnesium, zinc and selenium have been identified in edible mushrooms (Alam et al., 2007).

These mushrooms boast unsaturated fatty acids, making them low in calories, fat-free, cholesterol-free, gluten-free and low in sodium (Sanchez., 2004). Among the fatty acids prevalent in edible mushrooms are linoleic acid, oleic acid, and palmitic acid, which contribute to reducing lipid levels in the blood (Podkowa et al., 2021).

While edible mushrooms lack unsaturated fatty acids, they contain ergosterol, which prevents cardiovascular diseases and facilitates vitamin D production (Javed et al., 2019).

With their high nutritional value, edible mushrooms are not only utilized as food but also exhibit therapeutic effects such as immunomodulatory, anticarcinogenic, antiviral, antioxidant, anti-inflammatory, antimicrobial, anticancer and antiobesity properties (Figure 1.1) (Degreef et al., 1997; Assemie & Abaya., 2022; Thu et al., 2020).

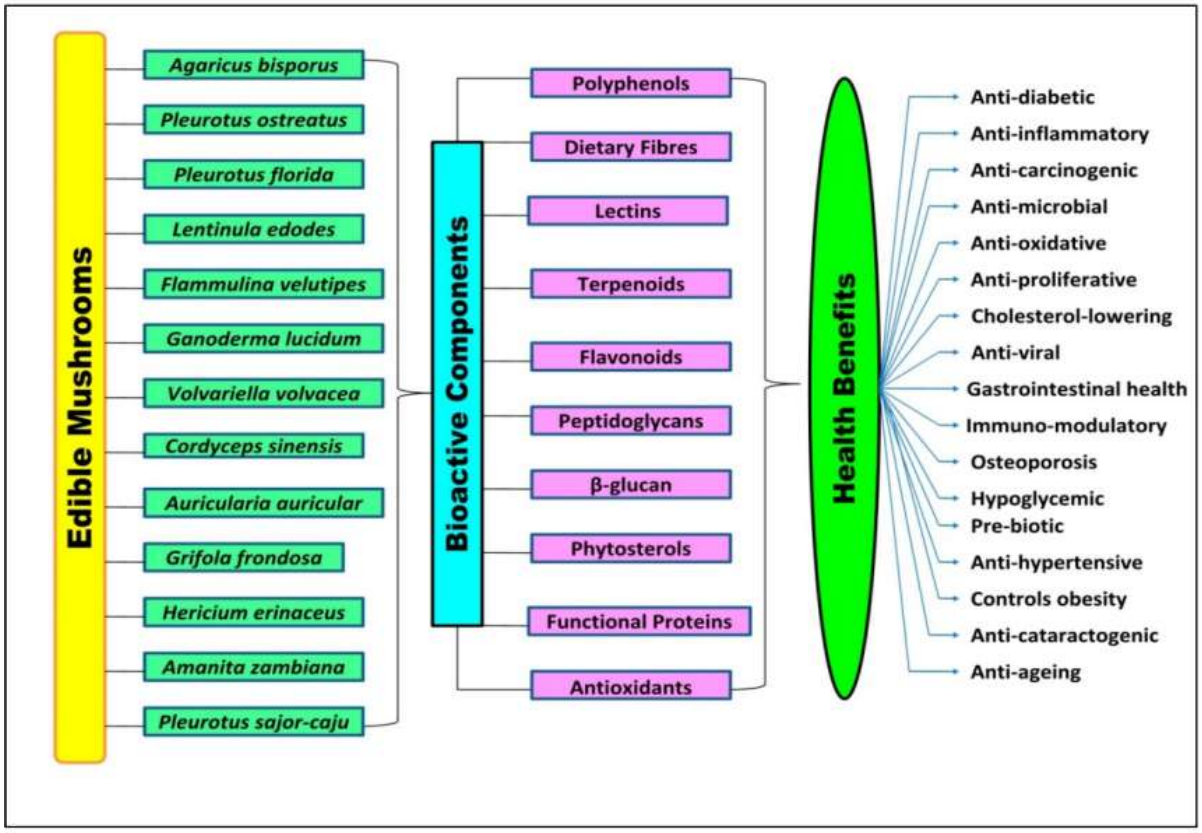

Figure 1.1 Edible mushrooms, bioactive components, and health benefits. (Kumar et al., 2021).

1.2 Lion's Maine mushrooms

1.2.1 Taxonomy

Hericium erinaceus, commonly known as Lion's Mane (LM), is a versatile mushroom with culinary and medicinal applications (Lee et al., 2020). It falls under the class

Bisidiomycete, subclass *Holobasidiomycetidae*, order *Hericiales* and Family *Hericiaceae*. This unique fungus is recognized by various names across different regions, including Yamabushitake in Japan, Houtou in China, Bear's Head, White Beard, Old Man's Beard, Pom Pom, Monkey's Head Mushroom, Bearded Tooth Mushroom and Hedgehog Mushroom and it is particularly popular in China, Taiwan and Japan (He et al., 2017; Huet al., 2020; Khan et al., 2013).

1.2.2 Properties and Applications

Lion's Mane mushroom boasts a rich nutritional profile, comprising polysaccharides (such as glucose, galactose, mannose, and rhamnose), proteins, 16 amino acids (including 7 essential ones), vitamins (notably B12) and minerals (Mn, P, K, Fe, Zn). Additionally, it contains unsaturated fatty acids like linoleic acid, alongside various bioactive compounds such as B-glucan polysaccharides, hericenones, erinacines, isoindoline, sterols, triterpenes, alkaloids, lactones and glycoproteins (Hobbs, C., 2020; Rogers & Robert., 2012; Friedman & Mendel., 2015; He et al., 2017; Spelman et al., 2017; Sheu et al., 2013; Khan et al., 2013).

Lion's Mane mushroom exhibits diverse therapeutic applications, including the treatment of metabolic disorders, gastrointestinal ailments and memory impairment. It is also recognized for its anti-obesity effects and enhancement of lipid metabolism (Lee et al., 2020; Hiraki & Eri et al., 2017). Furthermore, Lion's Mane demonstrates antioxidant and anti-inflammatory properties, along with benefits in mitigating hyperlipidemia, hyperglycemia, fatigue and ageing. Notably, it holds promise in the treatment of neurological disorders, particularly neurodegenerative diseases (Table 1.2) (Skulbel, et al., 2022; He, Xirui, et al., 2017).

Table.1.2 Some bioactive compounds and their action (David& Williams, (2023) indicates (as cites in Sokół S et al., 2016)).

Bioactive compounds	Some known health effects
Polysaccharides	Anti-inflammatory, immunomodulatory, anticancer, antimicrobial, gastroprotective, hepatoprotective, glucose-lowering, cholesterol-lowering
Hericenones A-B	Anticancer or cytotoxic, reduces platelet aggregation
Hericenones C-H, erinacines A-I	Neuroprotection, prevention of neurodegenerative disorders
Hericirine	Anti-inflammatory
Polyphenols	Antioxidants

The primary bioactive compounds derived from Lion's Mane fruiting bodies include polysaccharides (like B-glucan), hericenones, erinacines and ergosterols (Basko et al., 2023).

B-glucan stands out for its anti-tumour, immune-stimulating, anti-cancer, hypolipidemic, antioxidant and neuroprotective properties (Hu et al., 2020; Ren., 2012; Szydlowska-Tutaj., 2021; Basko et al., 2023).

Hericenones, aromatic compounds extracted from the mushroom's fruiting body, comprise 11 known types (A-K), with four of them (C, D, E, H) shown to promote NGF synthesis (Kawagishi et al., 1991; Ma et al., 2010).

Hericenones A and B play roles in neuronal growth and development and show potential in managing depression and anxiety disorders (Kumar, A., 2013).

Erinacines, predominantly isolated from mycelium but also present in the fruiting bodies, consist of 15 identified types (A-K, P, Q, S), with erinacines A-I demonstrated to stimulate neurogenesis and enhance nerve growth factor (NGF) activity, contributing to neurological health (Chong et al., 2020; Kawagishi et al., 1994; Shimbo et al., 2005).

Ergosterol, the predominant sterol in Lion's Mane cell membranes, exhibits notable anti-inflammatory effects by inhibiting the production of pro-inflammatory cytokines such as TNF-alpha, IL-6 and IL-1. It also demonstrates anti-cancer properties, aids in reducing high cholesterol levels and serves as the precursor to vitamin D (Huan et al., 2017; Kang et al.,

2015; Abe et al., 2009). The content of ergosterol is highest in fresh mushrooms and decreases with increasing temperature from 20°C to 70°C (Heleno et al., 2016). Additionally, irregular-shaped Lion's Mane (Ir-LM) by-products contain a higher ergosterol content compared to regular-shaped Lion's Mane (Reg-LM) (Jordan et al., 2022).

Furthermore, Lion's Mane is noted for macrophage activation, key cells within the innate immune system (Saitu et al., 2019).

1.3 Reishi mushrooms

1.3.1 Taxonomy

Ganoderma lucidum (G. lucidum) holds significant importance in traditional medicine, earning the title of the "king of mushrooms" within traditional Chinese medicine (TCM) (Ho et al., 1994; Li et al., 2019). With a history spanning over 4 thousand years in Asian countries, it boasts both economic and medicinal value (Wasser., 2005). Known by various names across different cultures, such as "Reishi" in Japan, "Lingzi" in China, and "Yeongji" in Korea, *G. lucidum* symbolizes longevity, spiritual potency and good health (Wasser., 1999; Kumar et al., 2021).

Its presence extends globally, including countries like France, England, Canada, North America, Taiwan, China, Korea, Japan and Iran (Hong & Jung., 2004; Fakoor et al., 2007). Typically found on trees like oak, Pyrus, Quercus, magnolia and plums. The fruiting body of *G. lucidum* is characterized by its thick, hard texture and bitter taste (Jong & Birmingham., 1992; Kumar et al., 2021). Notably, it's shiny appearance distinguishes easily, with "*lucidum*" translating to "shiny" in Latin (Figure 1.2) (Kumar et al., 2021).

Figure 1.2 Reishi mushroom(Dabhi., 2023).

Taxonomically, *G. lucidum* belongs to the class *Agaricomycetes,* the order *Polyporales,* and the Family *Ganodermataceae* (Dabhi., 2024).

Among the approximately 2000 known types of Reishi mushrooms, only six are recognized for their potential health benefits: black, red, purple, blue, white and yellow mushrooms (Table 1.3) (Ma et al., 2018).

Table 1.3 Categories of Reishi and potential uses (Dabhi., 2023).

Appearance(colour)	Taste	Use
Black	Brackish	Enhance lung function.
Red	Bitter	Refine memory and improves internal organs function.
Blue	Sour	Remediate liver function and eyesight.
Purple	Sweet	Aid complexion and intensify function of eyes joints.
White	Hot	Defend kidney
Yellow	Sweet	Strengthen spleen role

1.3.2 Properties and Applications

Reishi mushrooms are rich in various bioactive components, including polysaccharides (alpha/beta-D-glucan), alkaloids, triterpenoids (such as ganoderic acid, ganderenic acids, ganoderol, ganoderiol, lucidenic acids), sterols/ergosterol, proteins (LZ-8, LZ-9), nucleosides (adenosine, inosine, uridine), nucleotides (guanine, adenine), steroids, phenols and glycoproteins (Boh et al., 2007; Ahmad et al., 2021). Their biological composition is detailed in Figure 1.3.

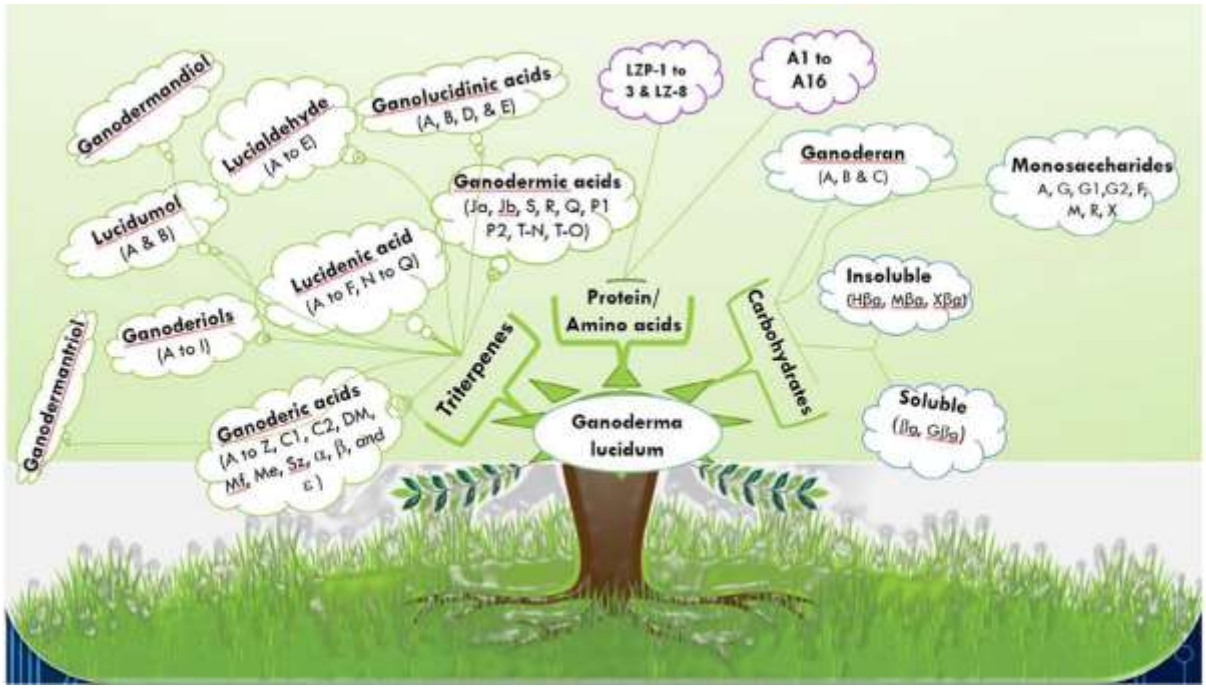

Figure 1.3 Biologically active isolated compounds of *G. lucidum*. (Ahmad et al., 2021).

Polysaccharides, particularly, exhibit anti-inflammatory, hypoglycemic, antitumor and immunostimulatory effects (Jong & Birmingham., 1992). Polysaccharides like beta-glucan, along with micronutrients and antioxidants such as glycoproteins, triterpenoids, flavonoids and ergosterols found in mushrooms, contribute to their ability to combat infections and toxins (Chugh et al., 2022). *Ganoderma* polysaccharides also demonstrate antioxidant activity, ameliorating oxidative stress (Zhang et al., 2012).

The antioxidant activity of *Ganoderma lucidum* contributes to its anticancer, antioxidant, antidiabetic, immunomodulatory, metabolic acceleration, anti-obesity and anti-ageing properties (Lysakowska et al., 2023). Moreover, the antioxidant properties of *Ganoderma lucidum* vary among strains and even within different parts of the same strain (Rahman et al., 2020).

Among the 18 types of amino acids found in Reishi mushrooms, leucine exhibits antioxidant or hypoglycaemic effects, while terpenoids, particularly triterpenes, possess anti-inflammatory and anticancer properties (Jong & Birmingham., 1992).

The bioactive compounds of *G. lucidum* play a crucial role in the prevention and treatment of various diseases, including diabetes, skin diseases, autoimmune diseases, viral infections (influenza, HIV, hepatitis), heart diseases, hypertension, high cholesterol, sleep disorders,

insomnia, hepatopathy, bronchitis, asthma, arthritis, stomach ulcers, antitumor, hepatoprotective, hypocholesterolemic, antiviral, antibacterial, antidiabetic and anti-parasitic properties (Weng et al., 2009; Wasser., 2005; Dabhi., 2024).

Furthermore, the bioactive compounds in Reishi mushrooms enhance cellular activity and survival of immune cells, protect against oxidative damage, suppress reactive oxygen species, and exhibit hyperglycaemic properties by reducing blood glucose levels, promoting glycogen synthesis and DNA protection from strand breakage. They also possess neuroprotective properties (Ahmad et al., 2021; Kim et al., 1999; Xu et al., 2022).

1.4 Comet Assay

The comet assay, also known as single-cell gel electrophoresis (SCGE), is a straightforward, cost-effective and sensitive technique that provides semi-quantitative and semi-qualitative measurements of DNA damage (Collins et al., 2014; Collins et al., 2012; Kucharova et al., 2019). Originally described by Ostling and Johanson in 1984 and later termed the comet assay by Olive and colleagues, this method is also known as alkaline unwinding or alkaline elution (Ostling & Johanson., 1984; Olive et al., 1990).

Primarily used for genotoxicity testing and as a biomarker, the comet assay helps explore the fundamental mechanisms of DNA damage and repair (Collins et al., 2012). It can detect low levels of DNA damage and repair in single cells (Moller & Peter., 2022), specifically identifying single-strand breaks (SSBs), double-strand breaks (DSBs) and alkaline-labile sites (ALS) (Jiang et al., 2019).

1.4.1 Main steps, principles and applications

Applications of the comet assay include detecting oxidative DNA damage (Fikrova et al., 2011), studying the physicochemical behaviour of DNA, examining cellular responses to DNA damage and biomonitoring human populations. This method provides insights into how strand breaks relax supercoiled DNA loops, enabling them to migrate towards the anode during electrophoresis (Collins et al., 1997).

The comet assay is versatile, measuring primary DNA damage and repair in both eukaryotic and some prokaryotic cells. It involves the electrophoretic separation of DNA from nucleoids and applies to various non-animal and animal species, including invertebrates,

vertebrates, and humans (Dhawan et al., 2009; Brunborg et al., 2018; Gajski et al., 2019a; Gajski et al., 2019b).

There are different types of comet assays, with the standard or alkaline comet assay version being one of the most prevalent. This version detects single and double-strand breaks as well as alkali-labile sites and is widely used for detecting DNA damage in genotoxicology and as a biomarker in human studies (Table 1.4). The enzyme-modified comet assay, which employs various enzymes, enhances the detection of specific DNA lesions, increasing the assay's sensitivity and specificity (Muruzabal et al., 2022; Neri et al., 2015; Kucharova et al., 2019).

Table 1.4 Overview of the various modifications of the comet assay (Vodenkova et al., 2020).

Assay	Starting material to embed in the gel	Enzyme treatment during incubation step	Endpoint	Data interpretation
Standard comet assay	Cells from samples	No incubation step needed	Single- and double-strand breaks	More migration of DNA to the tail indicates higher level of DNA damage
Enzyme-modified comet assay	Cells from samples	DNA glycosylases/ endonucleases isolated from bacteria or human cells	Specific lesions; for example, oxidized, alkylated, methylated, or dimerized bases	More migration of DNA to the tail as compared with controls (not incubated with enzyme) indicates higher levels of specific DNA lesions
Cellular repair assay	Cells from samples	No incubation step needed, but cells are grown and harvested at various time points	Induction and removal of DNA lesions over time	Decreasing migration of DNA to the tail with time indicates repair of DNA lesions
Comet-based in vitro DNA repair assay	Substrate cells containing specific DNA lesions	DNA repair enzymes present in protein extracts from samples will incise at lesions in substrate DNA	DNA repair incision activity	More migration of DNA to the tail as compared with controls (not incubated with protein extract) indicates higher DNA repair activity

The alkaline comet assay consists of five main steps - preparation of cells from fresh or frozen samples, processing gels for the comet assay, lysis, comet formation during electrophoresis, and comet visualization and analysis (Figure 1.4) (Collins et al., 2023).

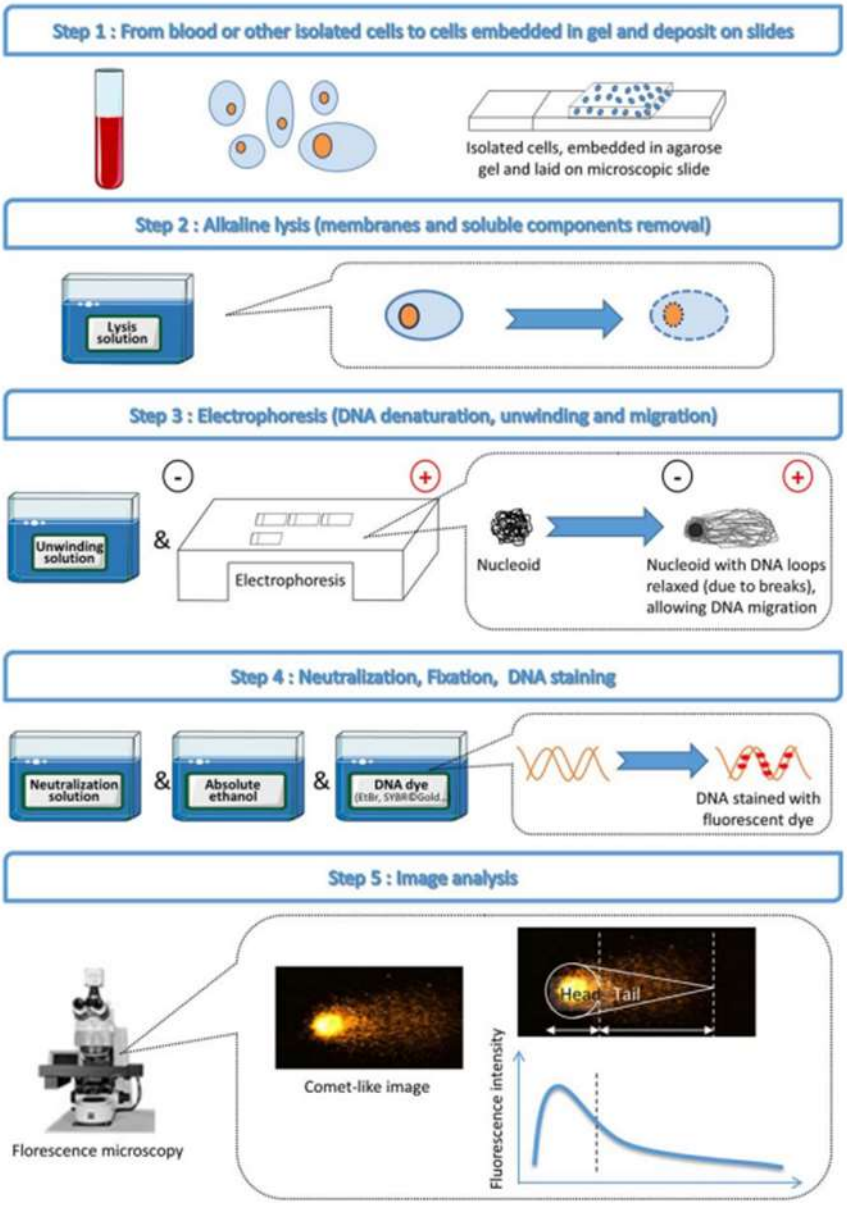

Figure 1.4 Scheme of the comet assay (Azqueta et al., 2019).

First of all, cells are first embedded in agarose and placed on a microscope slide. They then undergo lysis to remove membranes and soluble components, leaving nucleoids that are subjected to alkaline treatment and electrophoresis.

During electrophoresis, damaged DNA fragments migrate more rapidly than undamaged genomic DNA. The greater the number of DNA breaks, the more chromosomal fragments are generated, resulting in longer tails during electrophoresis. The intensity and length of the comet tail relative to the head indicate the number of double-strand breaks (DSBs) in the cell (Jiang et al., 2019). A common explanation for comet formation is that when histones are removed (a process called lysis), the DNA remains connected to the nuclear matrix but in a

tightly wound state. If there are breaks in the DNA, it loosens and stretches towards the positive electrode during electrophoresis, forming the characteristic "comet" shape (Figure 1.5) (Collins et al., 2004).

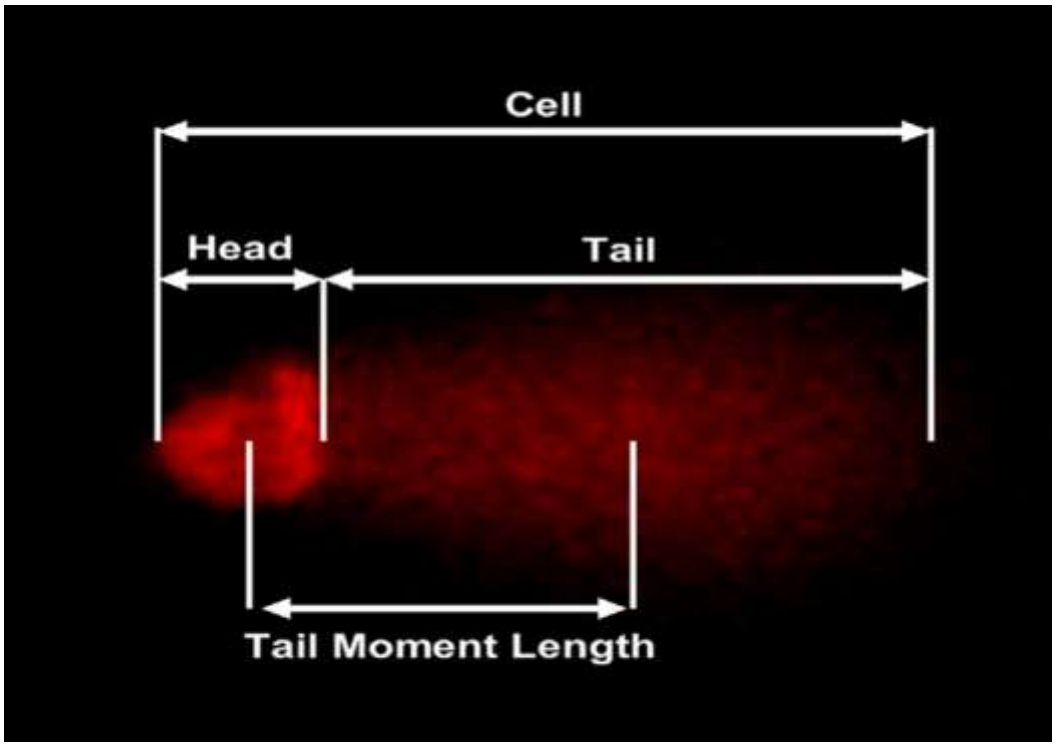

Figure 1.5 An example of damaged DNA in Comet. Representative comet tails of HEK293 cells exposed to γ-irradiation (10Gy) (Jiang et al., 2019).

Then samples are stained and visualized using fluorescence microscopy. A higher frequency of breaks results in increased DNA migration. The percentage of DNA in the comet tail reflects the frequency of DNA damage and is quantified through image analysis. Typically, 50-150 cells are counted per sample, with the mean or median used as the sample descriptor. Due to variations in DNA migration among comets within a gel, it is advised to analyze at least 50 comets per gel across two gels (Collins et al., 1997; Koppen et al., 2017).

The main advantages of comet assay is that it requires relatively low cell numbers, are applicable to all eukaryotic cells both in vivo and in vitro, and enables the detection of DNA damage at the individual cell level (Moneef et al., 2003). However, comet assay has limitations such as requiring viable single-cell suspensions and significant intra- and inter-laboratory variations (Moneef et al., 2003; Karbaschi et al., 2019).

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Reagents used:

1. Agarose (NMP, normal melting point) (Carl Roth GmbH+Co. KG, Germany)
2. Agarose (LMP, low melting point) (MBI Fermentas, USA)
3. Nutrient Medium RPMI 1640 (Sigma-Aldrich Co., USA)
4. Na_2EDTA (Carl Roth GmbH+Co. KG, Germany)
5. NaOH (Carl Roth GmbH+Co. KG, Germany)
6. Tris HCl (Carl Roth GmbH+Co. KG, Germany)
7. NaCl (B. Braun Melsungen AG, Germany)
8. DMSO (Sigma-Aldrich Co.,USA)
19. Triton X 100 (Carl Roth GmbH+Co. KG, Germany)
10. Ethidium Bromide (0,2 % Aqueous Solution) (Carl Roth GmbH+Co. KG, Germany)
11. Distilled Water
12. Acridine Orange (Sigma-Aldrich Co., USA)
14. Ficoll (Cytiva Co., Sweden)
15. H_2O_2(Valentis Co., Lithuania)

Solutions, used for comet assay and their preparation are written in Table 2.1

Table 2.1 Used solutions.

Solution	**Preparation**
AO/EtBr Dye Mix	1 μL Acridine Orange (5 mg/mL) /1 μL Ethidium Bromide (3 mg/mL) /1 mL PBS
Lysis Buffer NaCl Na2EDTA Tris HCl DMSO* Triton X–100*	 145 g/L 37,2 g/L 1,58 g/L 111,11 ml/L 11,11 ml/L
Alkaline Buffer Na2EDTA NaOH	 0,367 g/L 12 g/L
Neutralization Buffer Tris HCl	 63,03 g Tris HCl/L
Agarose (1%) NMP (Normal Melting Point) Agarose	 1 g Agarose/100 mL H2O

LMP (Low Melting Point) Agarose	0,5 g Agarose/50 mL PBS

* Added before use

Mushroom tinctures

The objective of this study was Reishi mushroom(R) and Lion's Mane mushroom (LM)extracts -tinctures, manufactured in Lithuania by the company Mishkay Co (Figure 2.1).

Figure 2.1 Lion's Mane (A) and Reishi (B) mushroom extracts (Mishkay Co., Lithuania)

A daily dose (3 mL) of one of the extracts contains 300 mg of Lion's Mane double (15:1) extract or 300 mg of Reishi (20:1) extract, respectively.

2.2. Isolation of human PBMCs from whole blood

Human blood samples were collected from 4 healthy donors (age range from 24 to 30). The extraction of the peripheral blood mononuclear cells was performed in the sterile centrifuge tubes, where equal parts of whole blood, cell culture media RPMI1640, and Ficoll were added. Samples were centrifuged for 20 minutes at 0.8 RCF, to separate blood to its components (Figure 2.2).

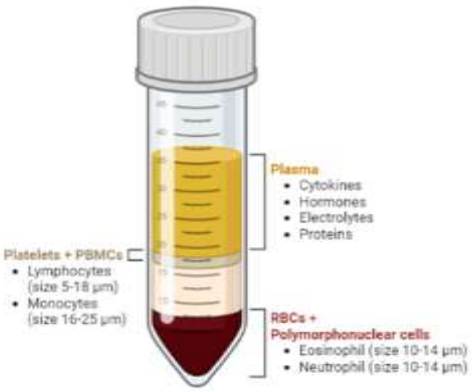

Figure 2.2 Extraction of peripheral blood mononuclear cells (PBMC) (created with Biorender.com)

The mononuclear cell layer was carefully collected, using a Pasteur pipette and transferred to a new, sterile centrifuge tube. Cells were washed with fresh cell culture medium and centrifuged again for 10 minutes at 0.8 RCF. After centrifugation, the supernatant was discarded, cells were resuspended with fresh medium, and cell number was evaluated using Countess 3 automated cell counter (Thermofisher Scientific, USA). Optimal cell number – 1×105 - 1×106 cells/mL. If the cell density was too high, cells were diluted up to the right density.

2.3 Evaluation of genotoxic and antigenotoxic effects of Mushroom extracts

To evaluate the genotoxic and antigenotoxic properties of mushroom extracts, the alkaline comet assay was assessed. The study was divided into three stages (Figure 2.2).

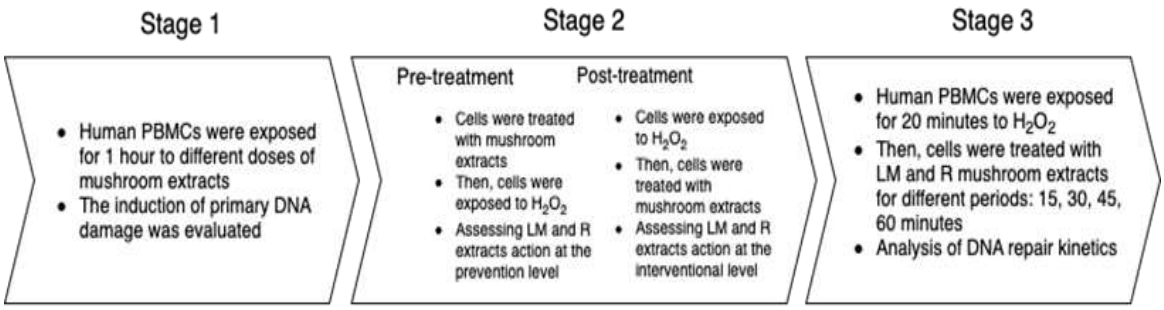

Figure 2.2 Scheme of the study

The first stage was devoted to evaluating the genotoxic potential of mushroom extracts. Then, the second stage was intended to investigate the antigenotoxic properties of Lion's

Mane and Reishi mushroom extracts. During the third stage, DNA repair kinetics were evaluated.

2.2.1 Stage1- Evaluation of genotoxic properties of mushroom extracts

To evaluate the cytotoxic and genotoxic potential of mushroom extracts, isolated human PBMCs were exposed to different doses of LM and R mushroom extracts.

In the initial stage, cells were exposed to different doses (10, 20, 30, 40, 50, 80 and 100 µl)of Reishi and Lion's Mane mushroom extracts for 1 hour at 37℃. Subsequently, the cells were centrifuged for 10 minutes at 0.8 RCF. Following centrifugation, the supernatants were carefully removed and replaced with fresh media.

After the treatment with different doses of mushroom extracts, cell viability was evaluated, using a dual AO/EB staining technique. 2 µL of staining solution and 20 µL of cell suspension were placed on a microscope slide, covered with a coverslip, and analysed with a fluorescent microscope (Nikon Eclipse 80i) at 200× magnification.

The colour green is commonly associated with living cells, whereas nuclei stained orange or red are indicative of apoptotic or deceased cells, respectively (García-Rodríguez et al.,2013). A total of one hundred cells were collected for each sample and the percentage of live and dead cells was evaluated.

Induction of primary DNA damage was evaluated by an alkaline comet assay, according to Kopjar et al.,2002 work. 40 µL of cells were immersed in 40 µL of LMP agarose on the microscope slide and subsequently covered with a 24x24 mm size coverslip for solidification in a refrigerator at a 4℃. Then, coverslips were removed, and the slides were immersed in a freshly produced cold lysis solution (pH = 10) for 1.5 h at a 4℃. After the lysis procedure, the slides were transferred into a horizontal gel electrophoresis chamber that was filled with an alkaline buffer (pH > 13) and left for 20 minutes to unwind. Then, electrophoresis was conducted with a power of 20W for 25 min. After electrophoresis, the slides were put into neutralization buffer (pH = 7.5) for 30 minutes at a temperature of 4℃. The slides were stained with 80 µL of ethidium bromide (0.2% aqueous solution) and imaged the next day using a 400-fold magnification fluorescence microscope (Nikon Eclipse 80i).

2.2.2 Stage 2 - Evaluation of genoprotective properties of mushroom extracts

The second stage consisted of two groups: pre-treatment and post-treatment.

Pre-treatment group samples were treated with different doses of Reishi (40, 50, 80 µl) and Lion's Mane (10, 20, 40 µl) mushroom extracts for 30 minutes, following a 20-minute exposure to H_2O_2.

While post-treatment group samples first were exposed to H_2O_2 for 20 minutes, following a 30-minute treatment with mushroom extracts.

Subsequently, the cells were centrifuged for 10 minutes at 0.8 RCF, the supernatants were then carefully removed and replaced with fresh media, and the alkaline comet assay procedure was performed as described in the 2.2.1 chapter.

2.2.3 Stage 3 - Evaluation of the DNA damage kinetics

During the third stage, the kinetics of DNA repair were evaluated.

Firstly, isolated human PBMCs cells were exposed to H_2O_2 for 20 minutes. Afterwards, the cells were centrifuged for 10 minutes at 0.8 RCF and the supernatants were replaced with fresh media. Then the cells were treated with different doses of Reishi (40, 80 µl) and Lion's Mane(10, 20 µl) mushroom extracts for four time periods: 15, 30, 45 and 60 minutes and the alkaline comet assay was performed as described in the 2.2.1 chapter. Simultaneously, for the negative control, the cells were not treated with anything and were examined at the same time intervals.

Subsequently, the cells were centrifuged for 10 minutes at 0.8 RCF, the supernatants were then carefully removed and replaced with fresh media, and the alkaline comet assay procedure was performed as described in the 2.2.1 chapter.

2.3 Statistical Analysis

Statistical analysis was done using t-tests. Data were presented as Mean ± SEM. Results were considered significant with $p \leq 0.05$.

3. RESULTS

3.1 Stage 1 - Cytotoxic and Genotoxic Potential of mushroom extracts

The viability of human PBMCs following 1-hour exposure to different doses of Reishi and Lion's Mane mushroom extracts was evaluated by a dual AO/EB (Acridine Orange/ Ethidium Bromide) staining technique (Figure 3.1, line charts). No relevant cytotoxicity was observed, regardless of the mushroom extract used, compared to the negative control (0 µL). On the contrary, Reishi mushroom extract tested at doses 10-80 µL, and Lion's Mane mushroom extract tested at doses 30-60 µL, significantly increased cell viability, compared to the untreated control (0 µL).

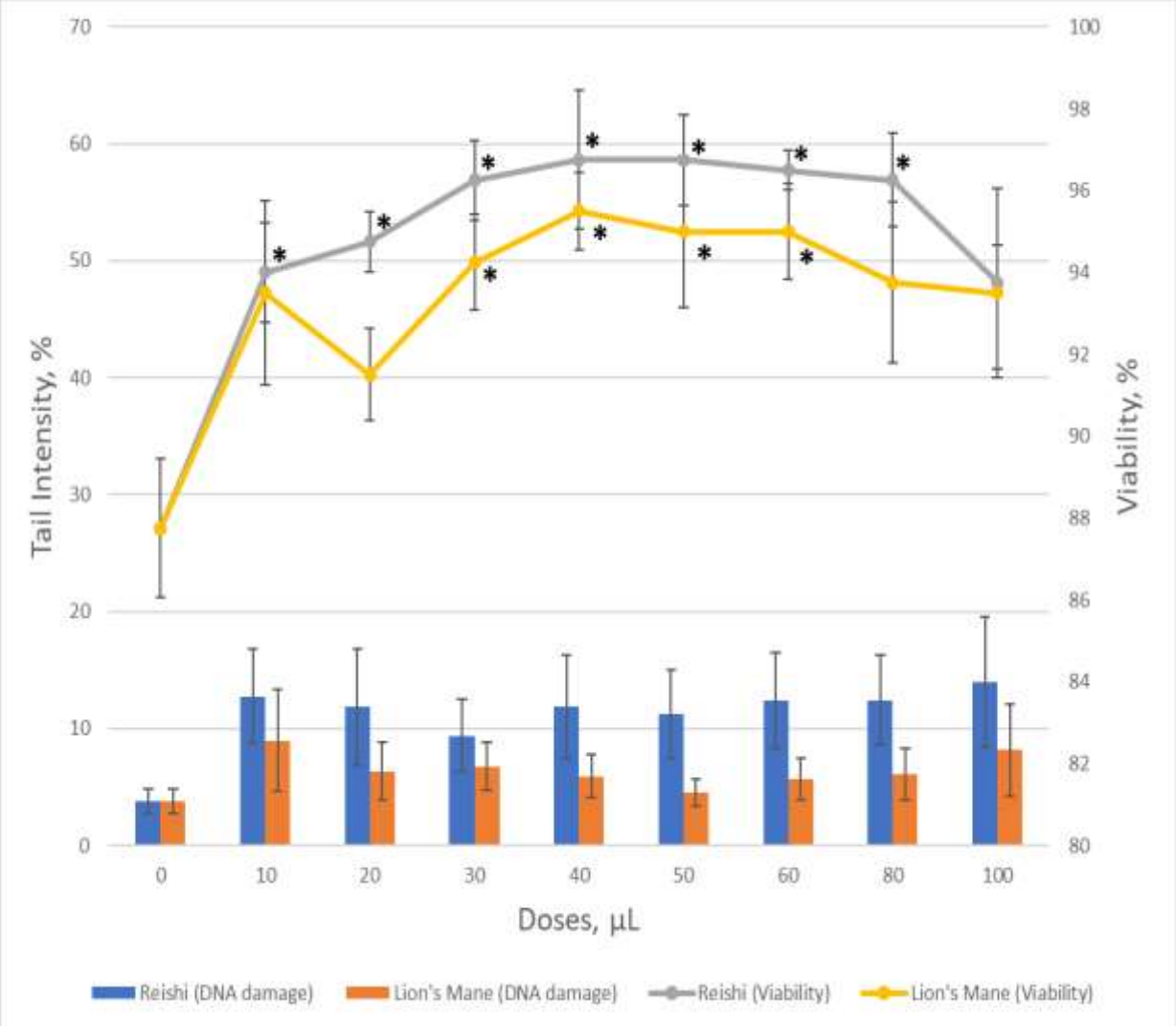

Figure 3.1 Percentage of live cells and percentage of DNA in a comet tail (Tail Intensity, %) after a 1-hour exposure of human PBMCs with different Reishi and Lion's Mane mushroom extract doses.

The genotoxic potential of Reishi and Lion's Mane mushroom extracts was evaluated by an alkaline comet assay in human PBMCs, following a 1-hour treatment (Figure 3.1.1, bar charts). Reishi and Lion's Mane mushroom extracts induced more DNA damage than the untreated control. Interestingly, Reishi mushroom extract induced more DNA damage than Lion's Mane extract, however, none of the tested doses induced significant amounts of DNA damage. Therefore, doses, that caused less damage (40, 50, and 80 µL) were selected for the second stage experiments.

3.2 Stage 2 - Genoprotective properties of mushroom extracts

To evaluate the genoprotective effects of mushroom extracts, two experimental approaches were conducted: pre-treatment, to evaluate the extract's action at the DNA damage prevention level, and post-treatment, to estimate the extract's action at the interventional level.

In the pre-treatment group, human PBMCs were exposed to mushroom extracts for 30 minutes and then treated to H_2O_2 for 20 minutes. Positive control (20-minute exposure to H_2O_2) induced significant amounts of DNA damage (14.28%), compared to the untreated control (Figure 3.2-4). As demonstrated in Figure 3.2A, pre-treatment application with Reishi mushroom extract, slightly reduced the H_2O_2-induced DNA damage (to14,28 %), while a dose of 80 µL exhibited the most prominent attenuation of DNA damage (to 11,08 %).

In the post-treatment group, human PBMCs were exposed to H_2O_2 for 20 minutes and then treated with different doses of mushroom extracts. In the post-treatment case with Reishi extract, all tested doses (40, 50, 80 µL) reduced the DNA damage induced by H_2O_2 to 8,11, 8,72, and 7,5 %, respectively, compared to the positive control (14,28 %) (Figure 3.2B). However, the results were not statistically significant.

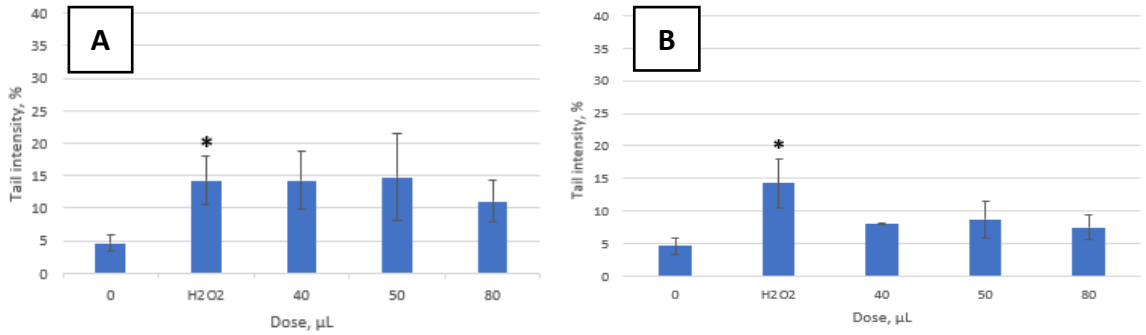

Figure 3.2 Percentage of DNA damage after (A) pre-treatment and (B) post-treatment with Reishi Mushroom.

For the pre-treatment and post-treatment with Lion's Mane mushroom extract, the same doses were used (40, 50, and 80 µL). However, an almost 2-fold increase in DNA damage, compared to positive control was observed, when cells were treated with 50 and 80 µL of Lion's Mane extract, therefore, lower doses (10 and 20 µL) were selected instead (Figure 3.3 A-B).

In the pre-treatment group with Lion's Mane extract, only a 10 µL dose was able to slightly reduce DNA damage (to 13,05 %), compared to the positive control (14,28 %), while doses of 20 and 40 µL increased more damage than the positive control (17, 13 and 17,7 %, respectively) (Figure 3.3A).

In the post-treatment group, cells treated with Lion's Mane extract were able to reduce DNA damage induced by H_2O_2 (Figure 3.3B). A 2-fold decrease in DNA damage was observed, when the cells were exposed to 10 and 20 µL of extract, compared to the positive control.

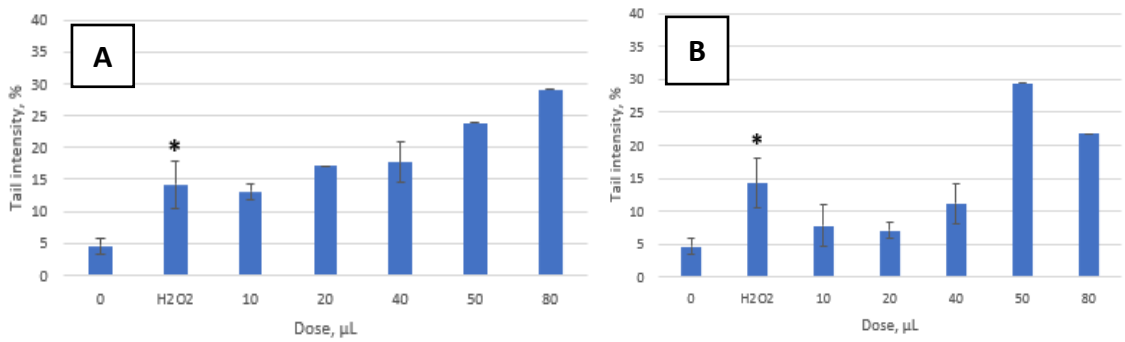

Figure 3.3 Percentage of DNA damage after (A) pre-treatment and (B) post-treatment with Lion's Mane Mushroom.

Overall, in the post-treatment groups, cells, treated with mushroom extracts after being exposed to H_2O_2, were able to attenuate DNA damage. However, the reduction of DNA damage may be due to natural cell properties to repair induced DNA damage. Therefore, to evaluate the kinetics of attenuation of H_2O_2-induced DNA damage in human PBMCs naturally and treated with Reishi and Lion's Mane mushroom extracts, stage 3 was performed, using the most successful Reishi (40 and 50 µL) and Lion's Mane (10 and 20 µL) mushroom extracts doses.

3.3 Stage 3 - Kinetics of attenuation of H_2O_2-induced DNA damage in human PBMCs

Figure 3.4 represents the results of the attenuation of DNA damage induced by H_2O_2, in four time periods: 15, 30, 45, and 60 minutes, both in cells treated and not treated with mushroom extracts. The attenuation of H_2O_2-induced DNA damage was observed at all time points, in untreated cells, and as expected, in a time-dependent manner. The greatest reduction in DNA damage was observed at 45 and 60 minutes (40 µl of Reishi) and at 30 and 45 minutes (80 µl of Reishi) (Figure 3.4A). While in the case of Lion's Mane mushroom extract, the greatest reduction in DNA damage, induced by H2O2 was observed at 15 and 30 min (10 µl of Lion's Mane), while dose of 20 µl did not reduce DNA damage, compared to the untreated controls at the same time points (Figure 3.4B).

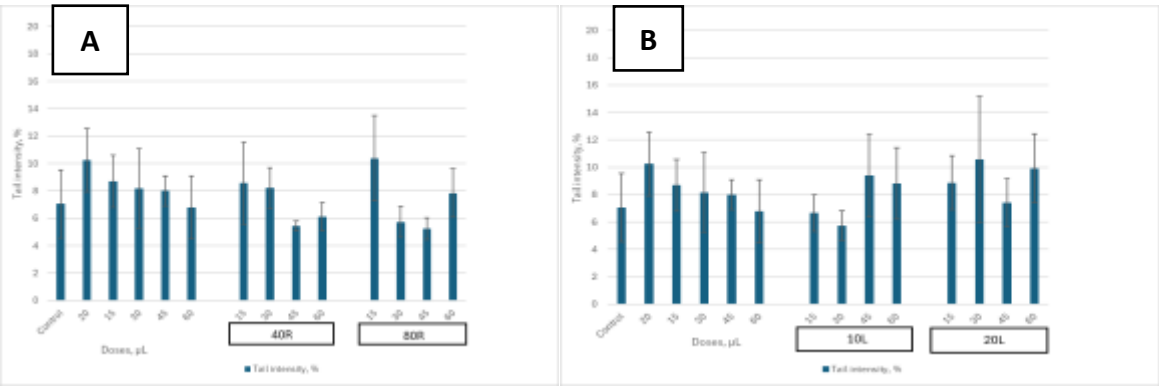

Figure 3.4 Evaluation of the kinetics of attenuation of H_2O_2-induced DNA damage in human PBMCs treated with Reishi (A) and Lion's Mane (B) mushroom extracts.

4. DISCUSSION

The primary aim of this study was to assess the genotoxic and anti-genotoxic properties of two medicinal mushrooms: Lion's Mane and Reishi mushroom extracts. Our findings indicate that both Reishi and Lion's Mane mushroom extracts do not exhibit genotoxic properties, as they induce non-relevant and insignificant amounts of DNA damage, compared to the negative control. However, it was observed, that Lion's Mane and Reishi mushroom extracts demonstrate slight antigenotoxic properties.

In our study, no relevant cytotoxicity was observed. 10-80 µL doses of Reishi and 30-60 µL doses of Lion's Mane mushroom extracts significantly increased the viability of human PBMCs, compared to the negative control (0 µL). In comparison, Savin and colleagues (2020) evaluated the cytotoxicity of Reishi mushroom chitosan extracts in L929 fibroblast cells, using MTT(3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay and detected a concentration-related decrease in cell viability. However, only the highest tested concentration (1000 µg/mL) reduced the viability significantly. Similarly, Ruan and colleagues (2015) worked specifically on the cytotoxic effects of 6 isolated triterpenoids from *G.Lucidum* on Caco-2, HepG2, and Hela cells using an MTT assay. A concentration-dependent decrease in cell viability was identified. Another study has been done on erinacine an enriched *Hericium erinaceus* (EAHE) and the study has found that no cytotoxicity was seen after long-time usage of Lion's Mane mushroom. (Lee et al., 2019). Kim and colleagues (2011) evaluated hot water (HWE), microwaved 50% ethanol (MWE), acidic (ACE), and alkaline (AKE) extracts of the fruitbody of Hericium erinaceus, it showed cell viability decreased treatment with HWE or MWE, while ACE or AKE were not cytotoxic. Our results in particularly similar to other scientists' work because mushroom extracts were not cytotoxic.

Reishi and Lion's Mane mushroom extracts induced more DNA damage than control using comet assay in human PBMCs, following 1h treatment. However, none of the tested doses induced a relevant and significant amount of DNA damage. Similarly, Žitkovič et al. (2019) tested the genotoxic effects of IA (Immune Assist), comprising six medicinal mushrooms, including *Ganoderma lucidum.* It was observed that none of the tested concentrations of IA induced significant amounts of DNA damage. As the work of Celik and collegues(2019) showed *G. lucidum* has no genotoxic effects; however, it did reduce spontaneous micronuclei (MN) formation and nuclear abnormalities. These results completely agree with ours.

While on the second stage, during post-treatment with H_2O_2, they induced less damage compared to the positive control.

A study has been done on Reishi mushroom and they tested on three doses of *G.Lucidum* they exhibited an antigenotoxic effect using MN(Micronucleus) technique, although the aqueous extract of *G. lucidum* exhibited no genotoxic effect, it did demonstrate an antigenotoxic effect (Celik et al., 2019).

In another study, they intend to explore the antigenotoxic effect of *Ganoderma Lucidum* against the damage caused by lipopolysaccharide (LPS) in rat bone marrow cells by micronucleus assay and comet assay and they conclude that *G.Lucidum* cannot be classified as a genotoxic substance (Baran et al., 2022).

A relevant study by Živković et al. (2019) focused on the genotoxic and antigenotoxic properties of IA (Immune Assist), comprising six medicinal mushrooms, including *Ganoderma lucidum.* Our experiment shares similarities with Živković's work, as both employ human peripheral blood mononuclear cells and utilize H_2O_2 to induce damage, with similar treatment durations. However, differences exist in the concentrations of mushroom extracts used. In Živković's study, the optimal time is 60 minutes at a concentration of 250 μg/ml but in our study no significant relation has been found between the time and mushroom extract doses.

Based on our findings across different mushroom types, it is clear that there are varying opinions regarding the genotoxicity, antigenotoxicity, viability, and cytotoxicity of Reishi and Lion’s Mane mushrooms. These differing views could be attributed to factors such as the concentration of mushroom extracts, treatment duration, experimental mishandling, bioactive components, and the mechanisms of repair or damage. Notably, the genotoxic and antigenotoxic properties of Lion’s Mane have been investigated in only a limited number of studies. Therefore, more comprehensive experiments are necessary to reach a definitive conclusion.

CONCLUSIONS

1. None of the tested doses (0-100 µL) of Reishi and Lion's Mane extracts reduced cell viability by more than 20 %.
2. Compared to the negative control (0 µL), none of the tested doses (0-100 µL) of mushroom extracts induced a statistically significant increase in DNA breaks.
3. Post-treatment of human PBMCs with Reishi (40, 50, 80 µL) and Lion's Mane (10, 20, 40 µL) mushroom extracts after exposure to H_2O_2 reduce DNA damage.
4. 45-minute treatment with 40 µl and 80 µl of Reishi mushroom extract reduced DNA damage, compared to the untreated control.
5. 15- and 30-minute cell treatment with 10 µl of Lion's Mane mushroom extract reduced more DNA damage than in the untreated control.

PARTICIPATION IN CONFERENCES

- Romina Ashrafi (2024, April) Evaluation of Genotoxic and Antigenotoxic Properties of Reishi (*Ganoderma lingzhi*) and Lion's Mane (*Hericium erinaceus*) Mushroom Extracts. Poster Presentation. Presented at Coin's Conference, Lithuania.

AUTHOR'S CONTRIBUTION

In this project, I have personally contributed to gathering, analysing, and interpreting the data.

I performed the following experiments: Cell viability using AO/EtBr staining and comet assay and also with the help of my supervisor carry out all individual experiments regarding Comet assay.

For every experiment, I prepared my dilutions, buffer solutions, microscope slides, etc.

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