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Biochemical evaluation of commercially available Reishi Supplement Against The Enzymes On Oxidation And Phosphorylation of Cellular Proteins.

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ABSTRACT
Antioxidant enzyme activity protect body from free radicals and its effect in human life. However, it can be resist for the some drugs such as anti-tumor, chemotherapy drugs and etc. In this study, antioxidant effect of *Ganoderma Lucidum* which is known as Reishi and, its extract that is prepared by using 0.1 mg/mL, was analyzed by measuring its total phenolic and total flavonoid contents, protein content and lastly measuring the effect of mushroom extract on the activity of the antioxidant enzymes; glutathione-S-transferase, superoxide dismutase and protein tyrosine kinase. The mushroom extract was prepared in by using water and its total phenolic and flavonoid contents of extract was measured and they were used in the further analysis. The activated effect of reishi was observed on these three enzymes and due to these activities; reishi is not available to use with cancer drugs as a supplement or alternative medicine. Also, observed that reishi can be used as food supplement and etc. Finally, cytotoxicity test was performed with K-562 cells and observed that reishi extract was increased the viability of cells.

Keywords: Reishi mushroom, antioxidant enzymes, Superoxide dismutase, Glutathione-S-transferase, Protein tyrosine kinase, cytotoxicity

Hücresel Proteinlerin Oksidasyon ve Fosforilasyonundaki Enzimlere Karşı Reishi Desteğinin Biyokimyasal Değerlendirmesi

ÖZ
Antioksidan enzim aktivitesi vücudu serbest radikallerden ve insan yaşamındaki etkilerinden korur. Bununla birlikte, anti-tümör, kemoterapi ilaçları vb. gibi bazı ilaçlara karşı direnç gösterebilir. Bu çalışmada, Reishi olarak bilinen Ganoderma Lucidum'un antioksidan etkisi ve 0.1 mg/mL hazırlanan ekstraktı analiz edilmiştir. Toplam fenolik ve toplam flavonoid içeriği, protein içeriği ve son olarak mantar ekstraktının antioksidan enzimlerin aktivitesi (glutation-S-transferaz, süperoksit dismutaz ve protein tirozin kinaz) üzerindeki etkisi ölçülmüştür. Mantar özü, su kullanılarak hazırlandı ve toplam fenolik ve flavonoid kontent içerikleri ölçülmüştür ve daha sonraki analizlerde kullanılmıştır. Reishi'nin aktif etkisi bu üç enzim üzerinde ve bu aktivitelerden dolayı gözlendi. Reishi, kanser ilacı ile takviye edici veya alternatif bir ilaç olarak kullanılamayacağı yorumu yapıldı. Ayrıca, reishinin besin takviyesi ve benzeri olarak kullanılabileceği gözlendi. Son olarak, K-562 hücreleri ile sitotoksisite testi yapıldı ve reishi özütünün hücrelerin canlılığını arttırdığı gözlendi.

Anahtar Kelimeler: Reishi Mantarı, Mantar, antioksidanlar, Süperoksit dismutaz, Glutatyon – s-transferaz, Protein tirozin kinaz, sitotoksisite

1. Introduction

Nowadays herbal medicine solution has picked up ubiquity in medical services all over the world. They are especially used for food cosmetic and pharmaceutical industries all over the world. Most of the chemical compounds in pharmaceutical

industry are first isolated from medicinal plants and used in natural product derived drug formulations, then used as effective foods supplements. Among with ancient plant archive of human health and nutrition, mushrooms are also important in both, and also well known ingredients in folkloric medicine approaches.

Their benefits in strengthening the immune system and anti-cancer effects, in addition to their nutritional value, attracted many scientists over centuries [1]. Mushroom cultivation is common in China, India and some of developed countries because of cheap raw material and easily available. Medicinal mushrooms have been used as traditional treatment in Japan, China and Korea, over centuries. In some countries like Japan and China, there are scientific and medical studies in which mushroom extracts are used for the treatment of cancer. [1] Among thousands of mushrooms with medicinal value**,** Reishi (Ganoderma Lucidum) is one the well known herb for folkloric use throughout the world. It has been known in China for more than 2000 years. Since known to provide health improvement without any side effects, it is distinct from all medicinal mushrooms. Reishi was rare until the late twentieth century, when it was planted by the Japanese and made available to the public. The western world has been interested in Reishi mushrooms for the past 30 years, and there are only 6 varieties were evaluated for their health benefits: Red, black, blue, white, yellow, and purple. Among those, the red and black are the most known types having high therapeutic capacity [3].

In China and Japan, Reishi was used for insomnia, in muscle diseases with mental and neurological pain, loss of appetite and debility after illnesses. It is also shown to exert positive effect against asthma, skin inflammation and used in the treatment of stiff neck and shoulders, bronchitis, rheumatism and chronic hepatitis, as well as in treating the patients with liver failure [4].

The chemical composition of Reishi has been solved to some extent, and high levels of organic germanium, polysaccharides and triterpenes were shown to exist among the six varieties analyzed. One of the polysaccharides is Beta D-glucan, known with anti-cancer activity, was shown to stimulate, or modify the immune system by activating the immune cells (macrophage) and helper T-cells, as well as increasing the immunoglobin levels to produce high immune responses [4] .

Human body was evolved with sophisticated and huge defense against many toxicants, known as antioxidant protection or defense system. The need for creation of human antioxidant system is due to the oxygen dependent cellular processes, and it involves many components, both endogenous and exogenous in origin, that function interactively to neutralize the reactive chemical species with toxic effects [20]. The vast majority of the oxidants , the reactive species, delivered by cells are the consequence of normal aerobic metabolism: approximately 90% of the oxygen utilized by the cell is consumed by the mitochondrial electron transport system.These species are either radical in nature, with unpaired electrons, and mostly involved the oxygen that is extremely reactive atom that can change into a portion of the potentially harmful elements known as reactive oxygen species (ROS).The other reactive components known as free radicals and reactive nitrogen species (RNS). The neutralization of such harmful species require the exogenous dietary antioxidants, including Vitamin C and E, Polyphenols (flavonoids, flavones, flavono's and proanthocyanidins), Beta-carotene and alternative carotenoids and oxy carotenoids. Under normal conditions, human body also employs the endogenous metabolites and biomolecules as antioxidants, such as enzymes that catalyze ROS removal (GST, SOD, NOX, and GPX), bilirubin, Thiols (glutathione), lipoic acid, N-Acetyl aminoalkanoic acid, nucleotide derived

koenzymes (NADPH and NADH), Ubiquinone, Uric acid. Also metal binding proteins such as Transferrin , Myoglobin and Ferritin for iron, Albumin, Ceruloplasmin and Metallothionein for copper.

The types of ROS targets in cells are generally biomolecules such as nucleic acids, proteins and unsaturated fatty acid, as well as carbon hydrates. The properties of the intrinsic membrane will be altered by these reactions which include liquidity, ion transport, protein synthesis, damage of DNA, enzyme activity loss, eventually leading to necrobiosis (Uday Bandyopudya et al., 1999).

The exogenous antioxidants are mainly extracted from food and medicinal plants, such as fruits, vegetables, cereals, mushrooms, beverages, flowers, spices and traditional medicinal herbs. [5]. Besides, the formulated and processed components of herbs are also important sources of natural antioxidants. These types of natural antioxidants are typically polyphenols (phenolic acids, flavonoid anthocyanins, lignans and stilbenes), carotenoids (xanthophylls and carotenes) and vitamins (vitamin E and C). Generally, these natural antioxidants, especially polyphenols and carotenoids, exhibit a variety of biological effects, such as anti- inflammatory, antibacterial, antiviral, anti-aging, and anticancer activities.

Phenolic compounds are the common ones and considerably present in the fungi kingdom. Phenolic constituents display several bioactivities such as antimicrobial, antioxidant, antiviral, antiinflammatory. Dietary phenolics that have been researched deeply in the last decades are divided into various subgroups and the leading categories of phenolic compounds are flavonoids, phenolic acids, and tannins [5]. Some of the other species of phenolics are coumarins, lignans, quinones, and stilbenes [6]. On the other hand, almost all flavonoids have antioxidant activity due to their 3-4 dihydroxy configurations. Flavonoids are the main and most studied phenolic phytochemicals that are widely distributed in plants [6]. More than 6,400 flavonoid structures were determined in the performed studies [8]. Flavonoids are consisting of many subclasses such as; flavones, flavonols, flavanones, flavanonols, chalcones, isoflavonoids, anthocyanins, bioflavonoids [6]. Flavonoids are essentially split into two groups; anthocyanins and anthoxanthins. Anthocyanins have some colour pigments such as red, blue, and purple. An-thoxanthins possess colourless or white to yellow molecules (flavonols, flavones, isoflavones) [7].

It is not surprising that the entire body depends on many endogenous defensive systems which assist in protecting the cell against radical injury by endogenous free radicals. Also this metabolic detoxification system turns xenobiotics such as drugs and environmental pollutants, or antibiotics like hormones from lipid soluble substances into water soluble, less poisonous substances that can be excreted from the entire body via kidneys. This system includes of phase I and phases II metabolizing enzymes in which their activities lead to biotransformation of xenobiotics.

Some of those are mostly cytosolic enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Also glutathione utilizing enzymes are also included in antioxidant defense system such as Glutathione Transferase (GST) enzyme family members. They participate in metabolism of oxidative, toxic and harmful intermediates, in the presence of certain cofactors. Some of the cofactors are metal elements such as zinc, copper, iron, whereas, some of them are vitamin and nucleotide derived organic molecules such as folate, lipoate, biotin and NADH for optimum catalytic action in detoxifying reactions. They may also require Glutathione, GSH an essential soluble antioxidant, which is synthesised from the amino acids cysteine, glycine and glutamate. Glutathione directly quenches ROS like lipid peroxides and

plays a substantial role in the metabolism of xenobiotics.

The glutathione S-transferases (GSTs) are primary phase II detoxifying enzymes that has also many other important roles in the cell [9]. Their main function in the cell is catalyze the attack of reduced glutathione to compounds that have electrophilic nitrogen, sulphur or carbon atom. They have a wide range of substrate specificities which include nitrobenzenes, epoxides, heterocyclic amines, quinones, arene oxides, α,β-unsaturated carbonyls [10]. GST enzymes are evolved from thioredoxin enzymes which are antioxidants and found in a lot of organisms. GSTs have structure and sequence similarity with other stress related proteins and it is suggested that a common stress related ancestor that is formed before thioredoxin is responsible for this relationship [11]. Besides having these activities, cytosolic GST enzymes can bind covalently and noncovalently to non-substrate ligands and have roles in intracellular transport and disposition of xenobiotics. These non-substrate hydrophobic ligands are some steroids, bilirubin, heme and lipophilic anticancer drugs [12-14]. Mitochondrial GST activity might be present to protect against genotoxic and cytotoxic electrophiles, which is produced within the mitochondria by the activity of mitochondrial cytochrome p-450 species or might result from the decomposition of lipid peroxides produced during respiration [15-18].

The main role of GST is to catalyze the conjunction of glutathione with electrophilic compounds and eliminate the foreign compounds including anti-cancer drugs [19]. There are evidences that the activity of GST leads to drug resistance, in addition to the elimination of foreign compounds, it has a role in removal of free radicals which are resulted from natural evolution [17]. The antioxidant enzyme SOD has more simple function when compared with GST, since it catalyses the dismutation of the superoxide radical (O2-) into hydrogen peroxide (H_20_2) and elemental oxygen (O_2) and as such provides an important defence against the toxicity of the superoxide radical [20].

Recent studies have shown the concerted action of different metabolic pathways to exert antioxidant defense. Cellular oxidation level and protein phosphorylation has been evaluated in many studies (21). It was also shown that oxidative stress may mediate signaling pathways independent of ligand-binding initiation. Such pathways are controlled by thiol oxidation and involved protein tyrosine kinase activation (22).

Protein tyrosine kinases (PTK) are members of a large multigene family responsible for regulating cell functions, including cell-to-cell signaling, growth, differentiation, motility, cytoskeletal rearrangement, and adhesion. About one-third of oncogenes involved in human malignancies are derived from tyrosine kinases. Targeting of specific tyrosine kinase signaling inhibits tumor growth and proliferation, and thus has emerged as a promising strategy for cancer treatment. To date over 90 human PTKs have been identified, which can be divided into two major groups based on their structure and localization. About two-thirds of the known PTKs are transmembrane receptors, while the other major group contains intracellular tyrosine kinases [23].

The aim of this study was to analyze the the antioxidant and anticancer potential of commercially available Reishi Mushroom supplement.

2. MATERIALS AND METHODS

2.1. Materials

Reishi mushroom extracts, Gallic Acid (Merk), Dimethyl Sulfoxide (DMSO) (Carloerba), Folin Ciocalteus Phenol Reagent (Sigma-Aldrich), Sodium Carbonate (Fisher Scientific), Quercetin (Sigma), Aluminium Chloride (Merk), Methanol, 1-Chloro-2,4-Dinitrobenzene (CDNB) (Fluka), Xanthine (Sigma-Aldrich), Nitro Blue Tetrazolium Chloride (NBT) (Thermo).

2.2. Methods

2.2.1. Preparation of the Reishi Mushroom Extracts:

mushroom supplement capsules used to prepare 0,1 mg/ml standardized mushroom content is water. The solution was stir mixed and heated at 40°C overnight. Following day, extract was centrifuged at 14000rpm for 15 minutes, then the supernatant filtered through with 0.2 µ filter (Fig.1). the same extraction procedure was repeated with use of methanol instead of water solvent.

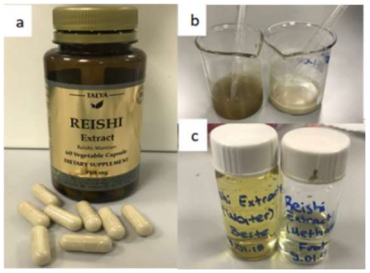

Figure 1: Commercially available and standardized formula of Reishi Extract (a), using water (left) and methanol (right) solvents for extraction (b), after final centrifugation of water (left) and methanol (right) extracts.

2.2.2. Determination of Total Phenolic Contents (TPC) of Reishi Mushroom:

The total phenolic contents of the samples were determined using Folin reagent with 1 mg/mL gallic acid (GA) as standard reagents. To each 100 µL Mushroom samples, 1 mL Folin reagent was added after 5 minutes in dark 1 mL Na_2CO_3 was added to the mixture and incubated 1 hour in the dark at room temperature. Here, the assay samples containing 100 µL DMSO without mushroom was used as blank. The presence of phenolics were observed with green color formation (Fig 2), and the absorbance at 750 nm was measured for both samples and standard (GA) dilutions. The total phenolic concentration was calculated from Gallic acid (GA) calibration curve.

2.2.3. Determination of Total Flavonoid Content (TFC) of Reishi Mushroom:

The total flavonoid contents in the samples were measured by using the aluminium chloride colorimetric method (2).

Figure 2: Phenolics were determined with the green color formation where the intensity is concentration dependent.

The assay was started with 250 µl of extract or standard diluted with 750 µl ethanol. To this diluents, then 50 µl of 10% (w/v) Aluminum Chloride solution, 50 µl of 1 M Sodium acetate and 1 µl DMSO was added. The samples and standard dilutions, then, incubated at room temperature for 30 min at dark. The presence of TFCs were observed with yellow color formation (Fig 3), and the absorbance at 415 nm was measured for both samples and standard (quercetin) dilutions. The standard curve was also employed by measuring the quercetin diultions (50-200 µg/mL) following the same assay protocol. The blank samples were prepared with water.

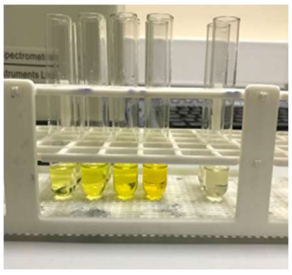

Figure 3: TFCs were determined with the yellow color formation where the intensity is concentration dependent.

2.2.4. The effect of Reishi on model cell line : Cytotoxicity analysis by XTT Assay

The possible cytotoxic effect of reishi extracts were analyzed on K562 cell line via colorimetric method with use of a formazan dye XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide). Simply, cells were seeded and harvested using conventional culturing methods, then reseeded at a density of 1×10^5 cells/ml into 96 well plates. Each plate was incubated at 37°C for 48 hours in a humidified, 5% CO2 chamber, that is the laminar flow hood for culture. Then 50µl of the Dye Solution (XTT and electron coupling agent phenazine methosulfate in 50:1 ratio) was added to to each well, and plates were incubated again at 37°C for up to 4 hours in laminar flow hood. Then plates were mixed well to obtain to obtain uniformly colored solution. The plates then transferred to 96-well plate reader and the absorbance was recorded at 450nm (Fig 4). The analysis was performed in triplicate wells in independent duplicate experiments, in each media control (no cell), cell control (no extract but buffer) was used.

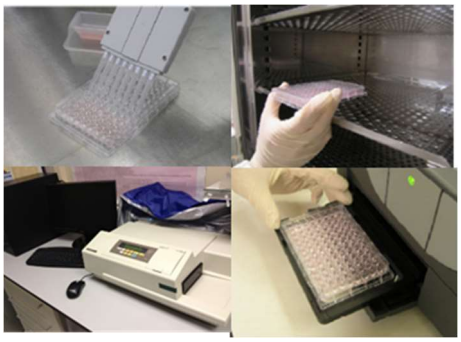

Figure 4: Simple cytotoxicity analysis with add, incubate, mix and read steps.

2.2.5. The Effect of Reishi Extract on Glutathione S-Transferase (GST) Enzyme Activity:

The bovine liver cytosol is used for the measurement of total GST activity, which is measured by conjugation of the thiol group of glutathione to the CDNB (1-chloro-2,4-dinitro benzene) substrate by using the miniaturized method of Habig (24). The change in total GST activities was measured against the substrate, 1-chloro-2,4-dinitro-benzene (CDNB), by monitoring the thioether (GSH-CDNB conjugate) formation at 340 nm. The measurements were performed with cytosol (0.1712 mg/mL) in a 100 mM potassium phosphate buffer at pH 6.5 with 2.4 mM CDNB and 3.2 mM GSH, using modified protocol. GST activity was measured by using multi-mode plate reader (Spectra maxM2) at 340 nm (18).

2.2.6. The Effect of Reishi Extract on Superoxide Dismutase (SOD) Enzyme Activity:

The principle of SOD activity is based on the inhibition of nitroblue tetrazolium (NBT) reduction by using the modified method [18]. Since the reduction of NBT results from the action of superoxide radicals to blue coloured formazan. The assay was performed using commercially available SOD source with the presence of 0.2 mM xanthine, 0.05U/mL XOD, 0.3 mM nitro blue tetrazolium (NBT), in 200 mM sodium carbonate buffer (pH 10.1) containing 0.5 mM EDTA. The absorbance was carried outafter addition of XOD at 550 nm.

2.2.7. Protein Tyrosine Kinase Assay:

Assay was performed with commercially available Universal Tyrosine Kinase Assay Kit (Takara) that monitors the transfer of γ-phosphate residue from ATP to peptide substrates immobilized on plate. where the phosphorylation level of substrate peptide immobilized on plates was explored with Horse Radish Peroxidase (HRP) conjugated anti-phosphotyrosine (pY20) antibody. The substrate is synthetic peptide with poly (Glu, Tyr) sequence with wide spectrum of tyrosine kinase specificity.. (Fig 5). The assay was initiated with the addition of ATP, in a final assay volume of 50 µL at 37°C. The change in absorbance at 450 nm was measured to determine the end-point of the kinase reaction. The effect of extracts on tyrosine kinase activity was monitored by the diminished activity of kinase at 450 nm.

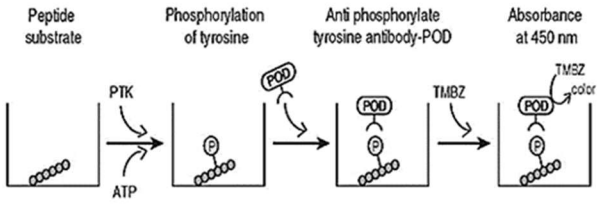

Figure 5: The basic principle of ELISA based PTK assay

3. Results And Discussion

The total phenolic and total flovanoid content of reishi mushroom was determined as 71,66 ± 8,33 gallic acid equivalent/mL of solution and 86,68 ± 17,08 quercetin equivalent/mL of solution(Fig 6, 7).

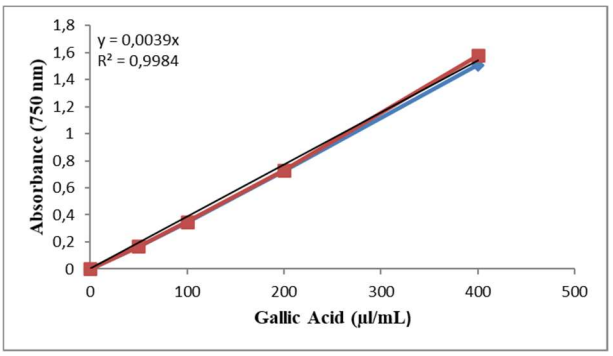

Figure 6: Phenolic Content Determination by using Gallic Acid as standard

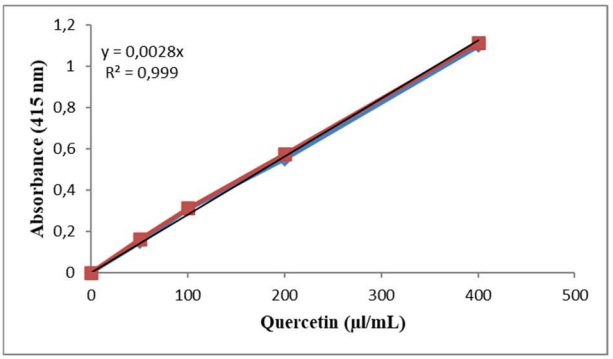

Figure 7: Flavonoid Content Determination by using Quercetin as standard

In the GST activity, the results showed that, reishi extracts increase the enzymatic activity of GST by decreasing concentrations. Also, total phenolic and flavonoid contents were measured to observe the GST activity with reishi however their content did not affect the measured GST activity (Fig 8. and Fig 9).

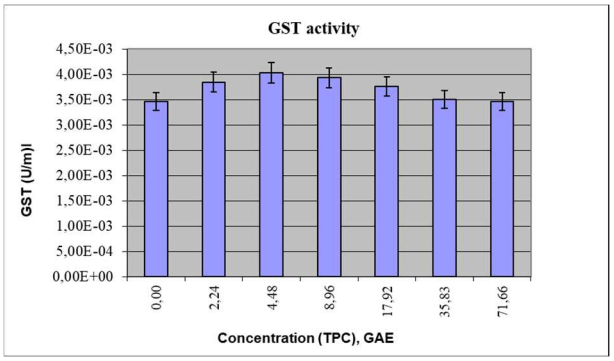

Figure 8: Dose Response Curve of GST Activity for varying GAE content of Reishi extract.

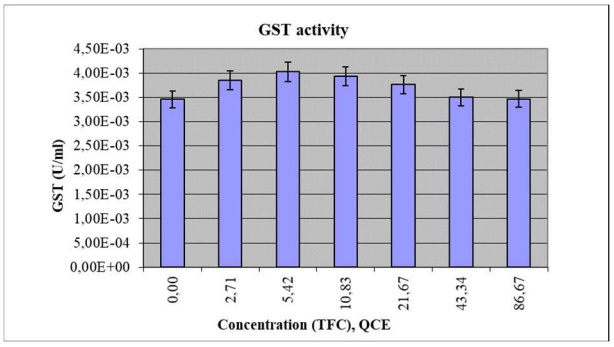

Figure 9: Dose Response Curve of GST Activity for varying QCE content of Reishi extract

The bovine liver cytosol and also commercially available enzyme was used as a source of SOD in assays. Without any difference between the enzyme source, the reishi increased the enzymatic activity by increasing concentration (Fig 10). The results presented here is with the commercially available SOD enzyme source.

Protein tyrosine kinase assay revealed that the reishi increased the enzyme activity only slightly in a dose dependent manner (Fig 11).

The possible cytotoxic effect of extracts on K562 model cells were determined via XTT analysis. In this assay, as reported with several other in vitro studies, reishi extract increased the viability of

model leukemia cells. In a dose dependent manner (Fig 12).

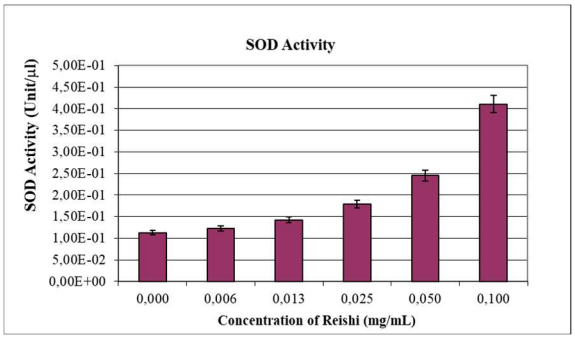

Figure 10: SOD Activity observed for standard quercetin concentration of extracts.

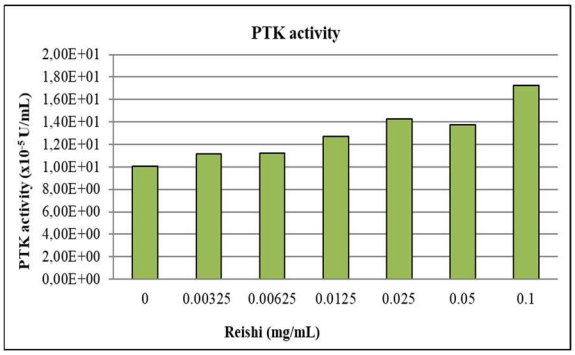

Figure 11: PTK Activity observed for standard quercetin concentration of extracts.

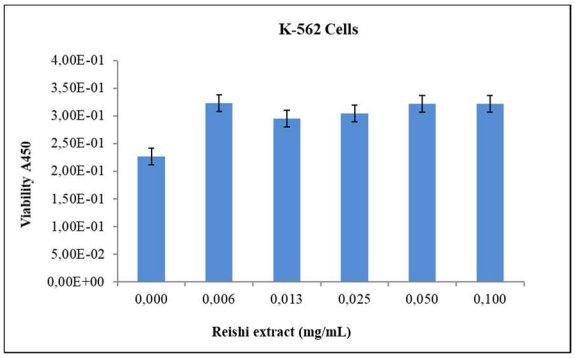

Figure 12: Cytotoxicity analysis of Resihi extract on K562 cells.

Discussion and Conclusion

In recent years, there are vast amounty of studies on phenolics and their biological effects, and this turned into a gold standard to measure the possible antioxidant capacity of natural products. Phenolic compounds obtained from plant extracts show great variety, with at least 8000 different structures. It was shown that differences in the profiles of phenolic compounds are dependent of the flora predominance (25). Also high flavonoid content and antioxidant activity is related and became another gold standard. Reishi supplements are claimed to be beneficial for the cancer treatment, as an antioxidant, anti-inflammatory and cell metabolic enhancements by its nuritent value. However there is no clear information in the literate whether the extracts, as standard formulations or not, exert any effect on glutathione utilizing cell defense, phase II detoxification enzymes, cellular antioxidant defense on enzymatic level or cell viability upon normalized flavonoid or phenolic doses of extracts were used. According to the manufacturer of the reishi supplement (Talya dietary supplement, 750 mg, 60 Vegetable Capsule) the daily dosage is one capsule, as suggested with other manufacturers in the market without exceeding 1000 mg of standardized formulations of extracts. Here, with 0.1 mg/ml standard solution of reishi extracts showed that total phenolic and flavonoid contents are high enough to exert antioxidant effect, that is 71,66 mg/g for TPC and 86,68 mg/g TFC. The extracts also exerted activity enhancement of PTK, an enzyme responsible for cellular signaling, cell viability, and SOD activity in a dose dependent manner. These results reveal the strong antioxidant and immune boosting effect of extracts as reported with animal models. However, PTK activity increase should be considered cautiously, especially for kinase related cancer treatments. It may not enhance but interfere with chemotherapies where kinase inhibitor drugs are used. On the other hand, GST is known in drug resistance mechanism, especially for cancer chemotherapeutics. For best therapeutic benefit of supplements, it is expected to induce GST inhibition in a dose dependent manner, however Reishi extracts shown to have no effects in terms of inhibition, but mild activity enhancements without the change in flavonoid or phenolics standardizations of the extracts. Therefore, the extracts or formulized supplements may not be used during or after the therapy with drugs where the drug metabolism is negatively affected with GST activity. Similarly SOD results is a warning sign of using the reishi extracts during

and after cancer chemotherapy, since enhanced SOD activity may seems beneficial for any during exposure to oxidative agents, however not beneficial for those receiving chemotherapy. On the other hand, the reishi supplements can be beneficial upon chemical poisoning, since GST mediated mechanisms are significant paths to detoxify agents.

The cytotoxicity analysis of reishi, here, revealed the viability of model cells improved in a dose dependent manner, without any toxicity observed even at highest doses under study. Such result also provide invaluable information together with enzyme assays, such that reishi supplemets should be used with caution,especially with other supplement and therapeutics. It is not clearly available in the literature that whether Reishi is safe to use during any therapy, especially for those involving synthetic drugs affecting the signaling pathways. As a supplement, as observed with several other food and health supplements of natural product origin, it should be considered as nutritional support, not therapeutic improving supplement, due to its interference with drug metabolism mechanisms and cytosolic cellular defence

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The authors declare no conflicts of interests.

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