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# Inhibitory effects of *Armillaria mellea* (Vahl) P. Kumm. on liver glutathione S-transferase activity

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# *Armillaria mellea* (Vahl) P. Kumm'nın karaciğer glutatyon S-transferaz aktivitesi üzerine baskılayıcı etkileri

**Abstract:** *Armillaria mellea* (Vahl) P. Kumm. commonly-known as the honey fungus is an edible mushroom and its antioxidant, antitumor, neuroprotective, and insulin resistance reductive effects have been well-characterized. Glutathione S-transferases (GST) are the group of detoxification enzymes has a function to conjugate glutathione to the variety of electrophile, making them more water-soluble for excretion. Their inhibition or activation could have profound toxicological or clinical implications. This study is conducted to investigate the inhibitory effects of *A. mellea* on GST enzymes. Total GST activities were measured using glutathione and 1 chloro-2,4-dinitrobenzene as substrates spectrophotometrically, and enzyme kinetic studies were conducted to determine $V_{max}$ and $K_m$ values. Additionally, aqueous and methanolic extracts of *A. mellea* were tested to see how they modulate the kinetic parameters. $V_{max}$ for liver GST enzyme was calculated as 443.90 ± 11.52 U/mg, $K_m$ value for GSH, and CDNB were determined as 4.88 ± 0.53 mM and 10.43 ± 1.07 mM, respectively. The decrease in $V_{max}$ and the increase in $K_m$ values with *A. mellea* extracts demonstrated a mixed-type inhibitory mechanism. Methanolic extract inhibits the GSH-dependent GST activity much more than the CDNB-dependent activity, but aqueous extract mainly affects CDNB-dependent GST activity. In conclusion, *A. mellea* could suppress the hepatic GST enzymes that might have toxicological consequences such as reduced cellular detoxification of electrophilic xenobiotics as well as alleviated drug resistance in the treatment of several diseases.

**Key words:** *Armillaria mellea*, glutathione S-transferase, enzyme inhibition, $V_{max}$, $K_m$

**Özet:** *Armillaria mellea* (Vahl) P. Kumm. yaygın olarak bal mantarı olarak bilinen yenilebilir bir mantardır ve antioksidan, antitümör, nöroprotektif ve insülin direncini azaltıcı etkileri iyi karakterize edilmiştir. Glutatyon S-transferazlar (GST), detoksifikasyon enzimlerinin bir grubudur ve glutatyonu çeşitli elektrofillere konjüge etme fonksiyonuna sahiptir. Bu işlem onları atılım için suda daha fazla çözünür hale getirir. GST enzimlerinin baskılanması veya aktive edilmesinin toksikolojik veya klinik etkileri olabilir. Bu çalışma *A. mellea*'nın GST'ler üzerindeki baskılayıcı etkilerini araştırmak için yapılmıştır. Toplam GST aktiviteleri, spektrofotometrik olarak glutatyon ve 1 kloro-2,4-dinitrobenzen substratları kullanılarak ölçülmüş, $V_{max}$ ve $K_m$ değerlerini belirlemek için enzim kinetiği çalışmaları yapılmıştır. Ek olarak, *A. mellea*'nın sulu ve metanolik özütleri, kinetik parametreleri nasıl modüle ettiklerini değerlendirmek için test edilmiştir. Karaciğer GST enzimi için $V_{max}$ 443,90±11,52 U/mg, GSH ve CDNB için $K_m$ değerleri sırasıyla 4,88±0,53 mM ve 10,43±1,07 mM olarak belirlenmiştir. *A. mellea* ekstreleri ile $V_{max}$'daki azalma ve $K_m$ değerlerindeki artış, GST enziminin karışık tipte inhibisyon mekanizmasıyla baskılandığını göstermiştir. Metanolik ekstraktın GSH-bağımlı GST aktivitesini CDNB-bağımlı aktiviteden çok daha fazla inhibe ettiği, fakat su ekstraktlarının esas olarak CDNB-bağımlı GST aktivitesini baskıladığı gösterilmiştir. Sonuç olarak *A. mellea*'nın, karaciğer GST enzimini baskılayarak ksenobiyotiklerin hücresel detoksifikasyonunu azaltabilecek ve çeşitli hastalıkların tedavisinde ilaç direncinin azaltılması gibi toksikolojik sonuçları olabilecek bir mantar türü olabileceği değerlendirilmiştir.

**Anahtar Kelimeler:** *Armillaria mellea*, glutatyon S-transferaz, enzim baskılama, $V_{max}$, $K_m$

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## 1. Introduction

Fungi are eukaryotic and heterotrophic organisms that are free of chlorophylls and composed of tubular filamentous cells and spores. Commonly known as honey fungus, *Armillaria mellea* (Vahl) P. Kumm. is a plant pathogen and classified as a part of a cryptic complex of morphologically similar species that are closely related to each other. They also have green bioluminescent properties and produce mushrooms around the bases of the infected trees. Honey fungus is also classified as edible, even though some people might be intolerant (Pegler, 2000). As ingested, its metabolites reached the hepatic tissues and metabolized internally.

One of the main detoxification enzymes present in the liver tissues is Glutathione S-transferases (GSTs), which plays a crucial role in the detoxification of endobiotic metabolites and xenobiotics. They conjugate the reduced glutathione (GSH), an intracellular thiol-containing compounds to the electrophilic substances to make them more hydrophilic and less toxic. When food and/or its metabolites enter an organism, protective mechanisms against oxidative stress and other detrimental effects are activated, which also involve the activity of GST enzymes (Strange et al., 2001). There is increasing evidence that some foods and food additives might affect GSTs either by direct suppression of enzymatic activity or indirectly by declining the levels of the GST substrates. Therefore, this study is conducted to see if edible mushroom; *A. mellea* could interfere with the detoxification of organic xenobiotics or endobiotic metabolites, which would eventually lead to unpredictable toxicological consequences for organisms. Our study provides a detailed *in vitro* study on inhibitory effects of *A.mellea* on rat liver GST enzymes.

## 2. Materials and Method

### 2.1. Reagents and chemicals

All the chemicals were analytical grade having more than 99% purity if available. They were obtained from Sigma-Aldrich (Steinheim, Germany) or Merck (USA). Analytical grade methanol, potassium phosphate dibasic trihydrate, sodium carbonate, bovine serum albumin (BSA), ethylene diamine tetraacetic acid (EDTA), sodium chloride, trizma base, 1 chloro-2, 4 dinitrobenzene (CDNB), L-glutathione (reduced), sodium diphosphate, potassium sodium tartrate, Folin and Ciocalteu's phenol reagent, copper sulfate, potassium chloride, hydrochloric acid, disodium monophosphate were the used chemicals in this study.

### 2.2. Preparation of fungal extracts

*Armillaria mellea* that was used in this study was collected from the Western Black sea region of Turkey and kept at Karamanoglu Mehmetbey University, Kamil Özdağ Science Faculty, Department of Biology. Dr. Yasin UZUN kindly provided the specimen. Water and methanol extracts were prepared to scrutinize *in vitro* enzyme inhibitory effects over liver GST enzymes. Accordingly, dried entire mushrooms weighing ten grams were pulverized using liquid nitrogen, mortar, and pestle and extracted with the Soxhlet extraction apparatus throughout 24-h with methanol or water as solvents. Then, a rotary evaporator concentrated the extracts which were freeze-dried in a lyophilizer and stored at +4°C until further use.

### 2.3. Preparation of cytosolic fractions from rat liver tissues

After washing with the ice-cold homogenization medium (50 mM potassium phosphate, 5 mM EDTA, 0.5 mM PMSF, 1.15% KC, pH:7.0), rat liver tissues were minced and homogenized using the bladed homogenizer (Tissue Ruptor™, Qiagen, USA). Following homogenization, the samples were centrifuged at 1.500 *g* for 15 minutes for the separation of nuclear part and non-degradable cells. Supernatants were transferred to the Eppendorf tubes and kept at -85°C until further use. The total protein contents of the homogenates were measured with the Lowry method (Lowry et al., 1951).

### 2.4. *In vitro* enzyme assay for liver glutathione S-transferase

For measuring the total GST enzyme activity, 1 chloro-2, 4 dinitrobenzene (CDNB), and reduced glutathione (GSH) were used as substrates. According to our optimized protocol which is developed from a previously published procedure (Habig et al., 1974), 15 µl of 2 mg protein-containing homogenates were mixed with 250 µl of phosphate buffer (50 mM, pH:7.0), 20 µl of GSH (50 mM), and 15 µl of CDNB (50 mM dissolved in 2/3 ethanol) in a UV-transparent 96-well plate and mixed thoroughly. After that, the changes in absorbance was monitored at 340 nm for 2 min with a spectrophotometric microplate reader (MultiScan GO™, Thermo Scientific, USA). Besides, the rate of non-enzymatic CDNB-GSH conjugation was determined with an enzyme-free reaction. Total GST activity was determined as the amount of chromogenic product formed by the homogenate containing one mg of protein for one minute.

### 2.5. Determination of maximum velocity ($V_{max}$) and Michaelis-Menten constants ($K_m$) for liver glutathione S-transferase

Enzyme kinetic studies were conducted to determine $V_{max}$ and $K_m$ values for different substrates of GSTs. Abovementioned protocol was conducted with varied concentrations of CDNB (1- 2- 5- 10- 20- 25- 30- 40- 50- 60- 70 mM) and GSH (1- 2- 5- 10- 25- 50- 75- 100 mM), each time one substrate concentration was kept constant and the other changed. Velocity versus substrate concentration graphs (Michaelis-Menten curve) and double reciprocal plots (Liveviever-Burk plot) were developed, and the maximum enzymatic activity for liver GST and $K_m$ values for each substrate were calculated with the enzyme kinetics module of GraphPad Prism 6.0 software.

### 2.6. Effects of *A. mellea* extracts on liver glutathione S-transferase activity and its kinetic parameters

Substrate dependent enzymatic activity measurements were conducted to determine the mechanism by which *A. mellea* extracts suppress the GST enzyme. $K_m$ and $V_{max}$ values were calculated in the absence and presence of *A. mellea* extracts (0- 0.25- 0.5- 1- 2- 4- 6- 8- 10 mg/ml) in accordance with Micheals-Menten kinetics. First, the Michaelis-Menten plot that was obtained by changing CDNB concentrations were reconducted for the *A. mellea* water and methanol extracts, and then $V_{max}$ value for CDNB-dependent GST activity and $K_m$ values for CDNB substrate were estimated. Changes in $V_{max}$ values with different extracts were determined, and the rate of enzymatic suppression was calculated for aqueous and methanolic extracts. Another substrate of the GST enzyme is reduced glutathione (GSH). Similar enzyme kinetics studies again determined the effects of *A. mellea* on GSH-dependent GST activity. In these studies, the CDNB concentration was kept constant, and GSH-dependent total GST activity was determined at different doses of *A. mellea* extracts. $K_m$ and $V_{max}$ values were determined accordingly.

### 2.7. Statistical analyses

All the assays were carried out at least in triplicate measurements. The results are expressed as mean values and the standard error of the mean (SEM). $K_m$ and $V_{max}$ values were calculated from double reciprocal plots using the enzyme kinetics module of GraphPad 6.0 software.

## 3. Results

### 3.1. The kinetic parameters of liver glutathione S-transferases

One of the main substrates of the GST enzyme is the GSH molecule. All GST isozymes conjugate the GSH group from the thiol groups to the compounds to be detoxified, making them more water-soluble. Additionally, CDNB is another commonly used substrate of GST *in vitro* since it resembles the common xenobiotics that are the target of all GST isozymes. Enzymatic GSH conjugation to the CDNB produces a chromogen that can be followed at 340 nm spectrophotometrically.

At the first stage of our study, kinetic parameters of liver GST enzymes were determined. To do this, by keeping the concentration of CDNB constant, GSH-dependent enzymatic activity of GST were analyzed. Michaelis-Menten and Lineviewer-Burk plots for GSH-dependent

GST activity were given in Figures 1A and 1B, respectively. Additionally, CDNB-dependent GST activity was also studied at constant GSH levels, and the changes in GST activity has been monitored. According to the Michaelis-Menten (Figure 1C) and Lineviewer-Burk (Figure 1D) plots, the maximum velocity of GST ($V_{max}$) was calculated as 443.9±11.52 U/mg, $K_m$ value for GSH and CDNB were determined as 4.88±0.53 mM and 10.43±1.07 mM, respectively.

### 3.2. Effects of *A. mellea* extracts on CDNB and GSH-dependent GST enzyme activity and its kinetic parameters

The abovementioned studies were also conducted in the presence of different amounts of (0- 0.25- 0.5- 1- 2- 4- 6- 8- 10 mg/ml) aqueous and methanolic extracts of *A. mellea*. In the presence of *A. mellea* extracts, the changes in $V_{max}$ and $K_m$ values for both substrates reveal the enzymatic inhibitory mechanism, such as competitive, non-competitive, uncompetitive, or mixed-type. The effects of *A. mellea* aqueous extracts on kinetic parameters of the GST enzyme have been summarized in Figure 2. Using these graphs and their equations, changes in the $V_{max}$ and $K_m$ values revealed that aqueous extracts inhibited both GSH and CDNB-dependent GST activity. A decrease in $V_{max}$ and an increase in $K_m$ values (Figure 2A-2D) with the increasing doses of *A. mellea* extracts suggests the mixed-type inhibitory mechanism over the GST enzyme. According to Table 1, aqueous extracts inhibited the GSH-dependent GST activity at 19.71% with maximum doses. However, its effect on CDNB-dependent activity is much more pronounced. Since maximum inhibition takes place two-fold higher. That is, CDNB-dependent activity was inhibited by approximately 40% with 10 mg/ml of aqueous extract.

The changes in the kinetic parameters of GST enzyme with *A. mellea* methanolic extracts are also studied, and the results are summarized in Figure 3. Similar to the water extracts, methanolic extracts also decreased the $V_{max}$ and increased the $K_m$ values for both substrates with rising concentrations. The decrease in $V_{max}$ and increase in $K_m$ values suggests the mixed-type inhibitory mechanism similar to the aqueous extracts. On the contrary to the results of water extracts, the methanolic extract inhibited the GSH-dependent GST activity much more than the CDNB-dependent activity. According to Table 2, methanolic extracts suppressed the GSH-dependent GST activity at 48% with maximum doses, but CDNB-dependent activity decreased only 4% with similar doses. How the GST activity is regulated with different doses of *A.mellea* has been schematized in Figure 4, and the differences between different extracts are visualized.

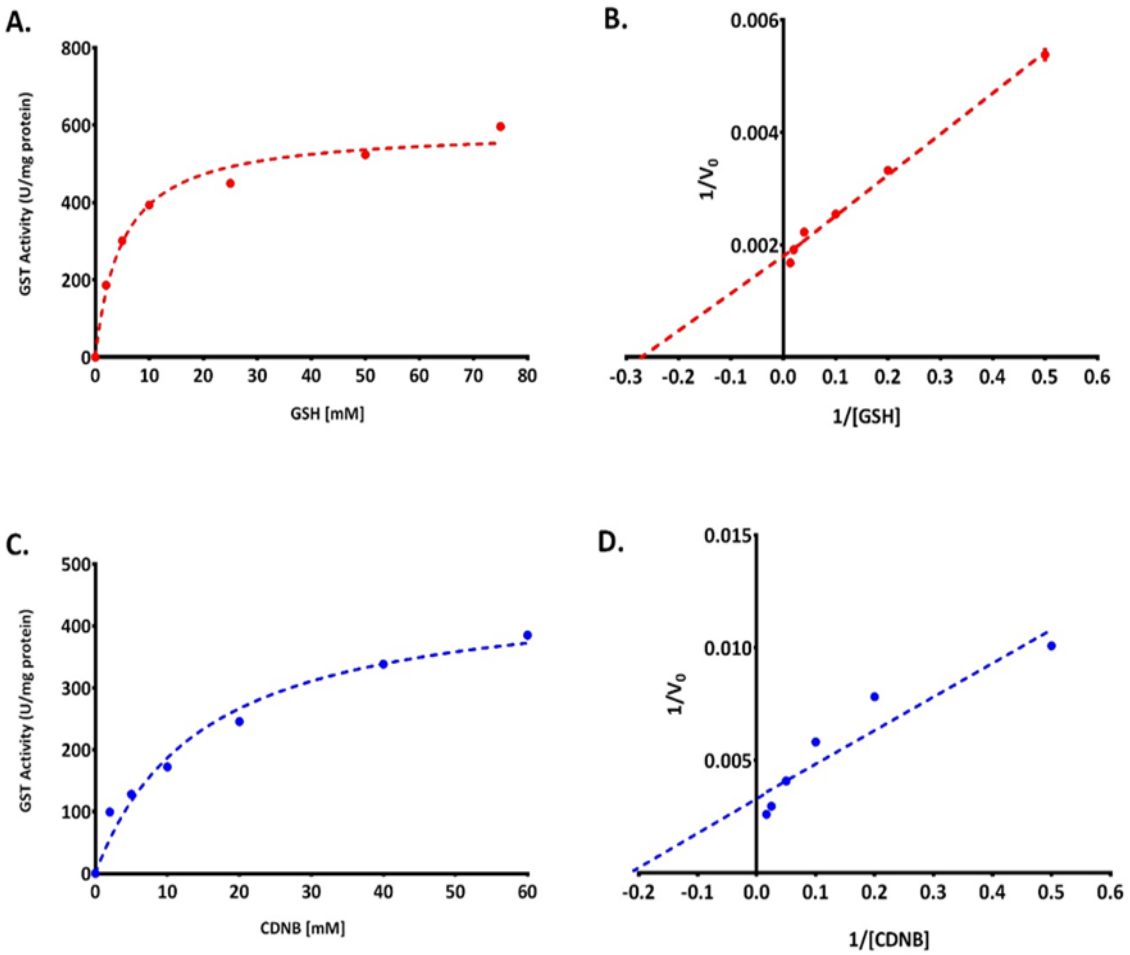


**Figure 1.** Michaelis-Menten (A) and Lineviewer-Burk plots (B) for GSH-dependent GST activities and Michaelis-Menten (C) and Lineviewer-Burk plots (D) for CDNB-dependent GST activities.

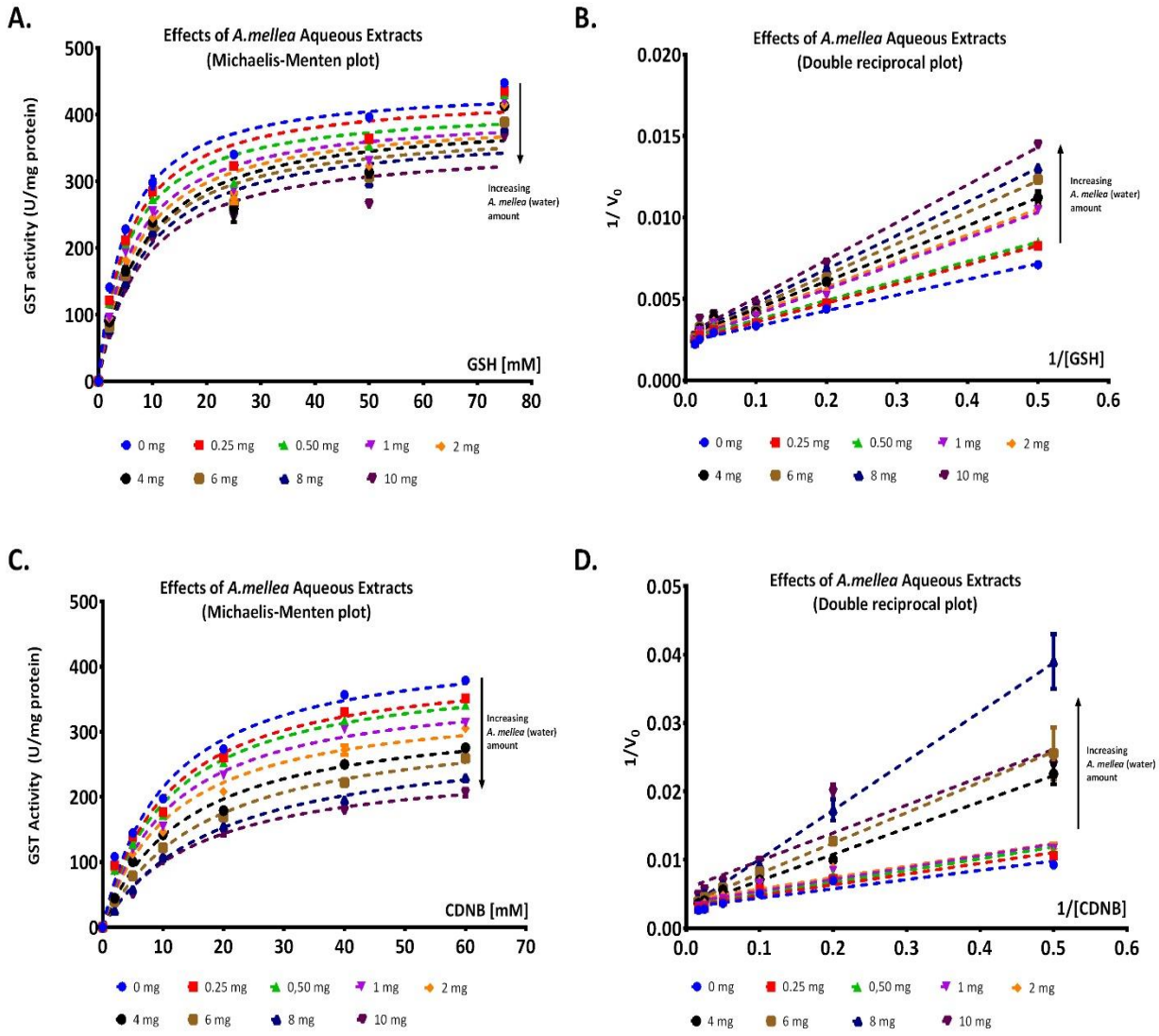


**Figure 2.** Effects of *A. mellea* water extracts on GSH and CDNB-dependent GST activities. A and C demonstrate the simple substrate vs. velocity curves, and B and D demonstrate the double reciprocal plots of A and C, respectively.

**Table 1.** Effects of *A. mellea* aqueous extracts on inhibition levels, $V_{max}$ and $K_m$ values of two different substrates. Data is represented as mean ± SEM.

| | ***A. mellea* aqueous extracts GSH-dependent GST Activity** | | | ***A. mellea* aqueous extracts CDNB-dependent GST Activity** | | |
|---|---|---|---|---|---|---|
| **Amount** | **$V_{max}$ (U/mg)** | **$K_m$ (mM)** | **% Inhibition** | **$V_{max}$ (U/mg)** | **$K_m$ (mM)** | **% Inhibition** |
| **0 mg** | 443.9 ± 11.5 | 4.88 ± 0.53 | 0 ± 0 | 438.5 ± 14.3 | 10.43 ± 1.06 | 0 ± 0 |
| **0.25 mg** | 433.4 ± 14.6 | 5.54 ± 0.71 | 2.37 ± 3.29 | 408.5 ± 12.6 | 10.54 ± 1.01 | 6.84 ± 2.88 |
| **0.50 mg** | 413.8 ± 16.8 | 5.49 ± 0.91 | 6.78 ± 3.80 | 400.1 ± 11.0 | 11.07 ± 0.93 | 8.76 ± 2.52 |
| **1 mg** | 405.3 ± 18.6 | 6.41 ± 1.14 | 8.70 ± 4.20 | 375.2 ± 13.3 | 11.40 ± 1.22 | 14.44 ± 3.04 |
| **2 mg** | 400.0 ± 19.8 | 6.88 ± 1.29 | 9.89 ± 4.46 | 351.2 ± 14.6 | 11.62 ± 1.45 | 19.91 ± 3.34 |
| **4 mg** | 398.4 ± 23.5 | 7.81 ± 1.69 | 10.25 ± 5.30 | 331.4 ± 8.7 | 13.67 ± 1.02 | 24.42 ± 2.00 |
| **6 mg** | 384.7 ± 17.0 | 7.54 ± 1.24 | 13.34 ± 3.84 | 323.4 ± 6.7 | 16.91 ± 0.94 | 26.25 ± 1.55 |
| **8 mg** | 379.9 ± 17.1 | 8.25 ± 1.35 | 14.42 ± 3.87 | 295.5 ± 6.3 | 18.54 ± 1.02 | 32.61 ± 1.45 |
| **10 mg** | 356.4 ± 20.4 | 8.07 ± 1.68 | 19.71 ± 4.60 | 257.3 ± 8.5 | 15.84 ± 1.42 | 41.32 ± 1.96 |

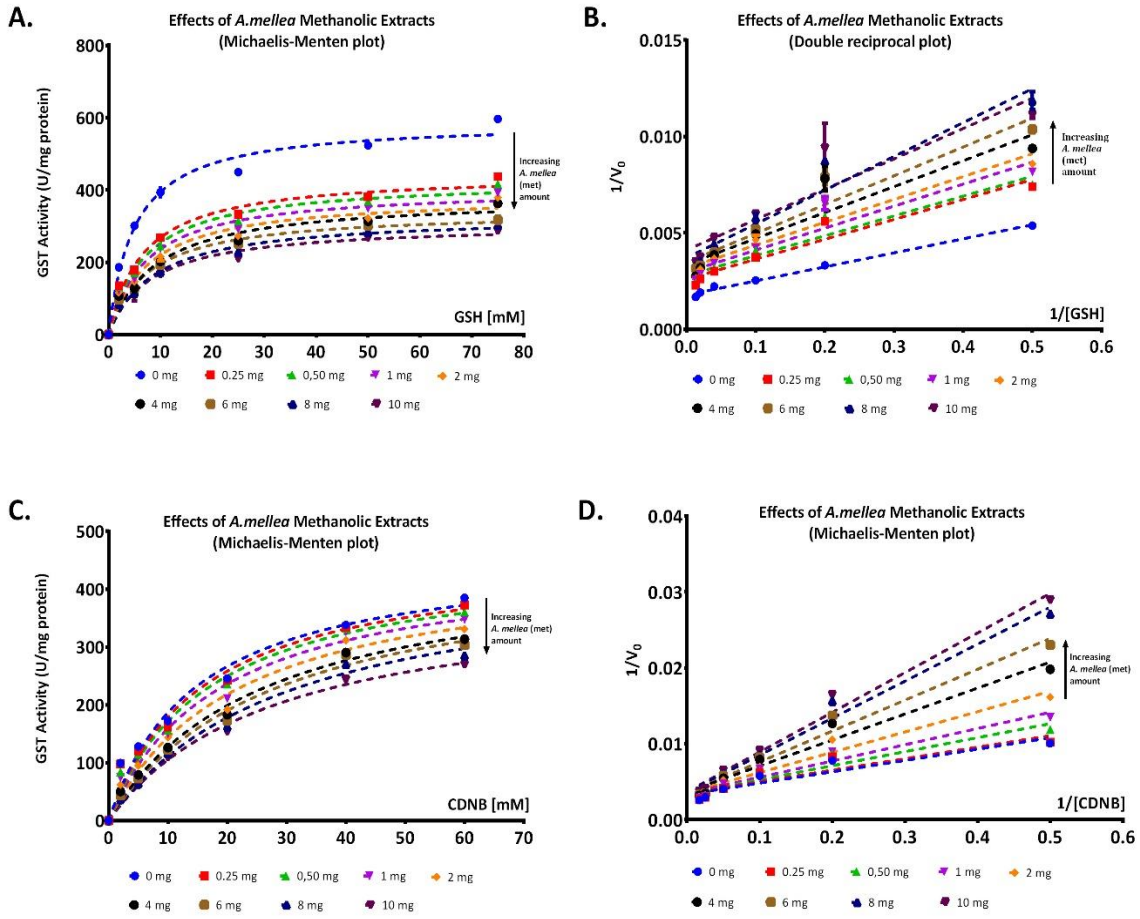


**Figure 3.** Effects of *A. mellea* methanolic extracts on GSH and CDNB-dependent GST activities. A and C demonstrate the simple substrate vs. velocity curves, and B and D demonstrate the double reciprocal plots of A and C, respectively.

## 3. Conclusion and Discussion

The world population is getting faced with several health and nutritional problems due to irregular growth patterns. Today, the use of natural resources in the diet becomes a necessity due to the economic difficulties, and the use of macrofungi arises due to their high nutritional properties. Additionally, several types of fungus exhibit pharmacological activities with few side effects and are used medically. *A. mellea*, an edible and medicinal fungus, has been reported to exhibit antioxidant and antitumor activities (Wu et al., 2012). Besides, some data reported its neuroprotective roles against degenerative disorders such as Alzheimer's diseases (An et al., 2017), and it's insulin resistance reductive effects have recently been proposed (Yang et al., 2019).

**Table 2.** Effects of *A. mellea* methanol extracts on inhibition levels, $V_{max}$ and $K_m$ values of two different substrates. Data is represented as mean ± SEM.

| | ***A. mellea* methanol extracts GSH-dependent GST Activity** | | | ***A. mellea* methanol extracts CDNB-dependent GST Activity** | | |
|---|---|---|---|---|---|---|
| **Amount** | **$V_{max}$ (U/mg)** | **$K_m$ (mM)** | **% Inhibition** | **$V_{max}$ (U/mg)** | **$K_m$ (mM)** | **% Inhibition** |
| **0 mg** | 589.3 ± 16.1 | 4.95 ± 0.56 | 0 ± 0 | 464.3 ± 27.1 | 14.86 ± 2.39 | 0 ± 0 |
| **0.25 mg** | 445.9 ± 13.8 | 6.64 ± 0.79 | 24.33±2.35 | 460.6 ± 27.9 | 15.67 ± 2.57 | 0.80 ± 6.01 |
| **0.50 mg** | 428.0 ± 14.4 | 6.93 ± 0.88 | 27.37±2.45 | 458.8 ± 23.8 | 16.74 ± 2.30 | 1.18 ± 5.13 |
| **1 mg** | 404.6 ± 14.1 | 7.13 ± 0.93 | 31.34±2.39 | 456.2 ± 27.7 | 18.67 ± 2.89 | 1.74 ± 5.96 |
| **2 mg** | 384.2 ± 15.9 | 7.46 ± 1.15 | 34.80±2.70 | 454.0 ± 28.1 | 21.62 ± 3.24 | 2.22 ± 6.06 |
| **4 mg** | 378.9 ± 15.7 | 8.71 ± 1.29 | 35.70±2.68 | 453.4 ± 23.2 | 25.56 ± 2.97 | 2.35 ± 4.99 |
| **6 mg** | 342.2 ± 9.2 | 7.54 ± 0.75 | 41.93±1.57 | 453.9 ± 26.1 | 27.72 ± 3.52 | 2.24 ± 5.62 |
| **8 mg** | 328.5 ± 9.5 | 8.71 ± 0.90 | 44.26±1.62 | 447.7 ± 30.9 | 30.27 ± 4.19 | 3.58 ± 6.65 |
| **10 mg** | 306.5 ± 12.1 | 8.16 ± 1.16 | 47.99±2.05 | 440.0 ± 16.2 | 28.04 ± 2.50 | 5.17 ± 3.49 |

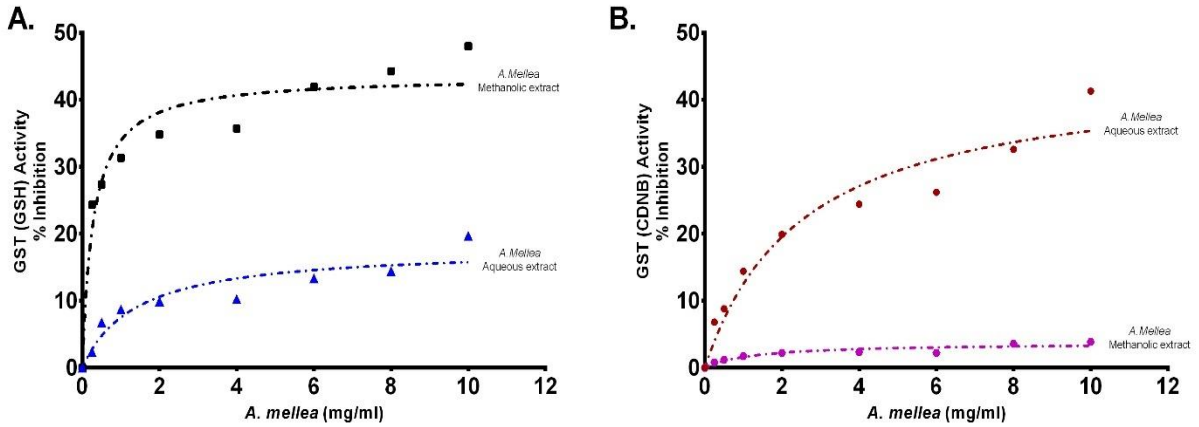


**Figure 4.** The suppressive effects of *A.mellea* aqueous and methanol extracts on GSH-dependent (A) and CDNB-dependent (B) total GST enzyme activity.

Glutathione S-transferases assure efficient enzymatic conjugation of the main antioxidant molecule, GSH, to the reactive electrophiles making them more water-soluble and excetable. They take part in the detoxification of xenobiotics and endobiotic metabolites, mainly in the liver tissues. However, several compounds can also be converted to highly reactive or toxic products through the GSH conjugation (Appiah-Opong et al., 2009). The crucial roles of GSTs in drug metabolism and cellular physiology might be regulated as a result of active compounds that are present in our diet. Therefore, their inhibition or activation could have profound toxicological or clinical implications. For instance, the induction of GSTs would diminish the carcinogenic properties of some compounds and provides chemoprevention to the organisms. Their inhibition might have toxicological consequences such as reduced cellular detoxification of electrophilic xenobiotics as well as alleviated drug resistance in the treatment of several diseases (Appiah-Opong et al., 2009). Therefore, interferences with the functionality of GSTs caused by the nutrients may have unpredictable toxicological consequences for the organisms. Nevertheless, it is hard to make an overall assessment of the level at which dietary supplementation, over-nutritional dietary supplementation, natural exposure, or poisoning will show effects (Gweshelo et al., 2016). Thus, this study is conducted to investigate the effects of *A. mellea* on liver glutathione S-transferases.

The results of this study confirm the general inhibitory potential of both aqueous and methanolic extracts of *A. mellea* on liver glutathione S-transferase enzyme. They decreased $V_{max}$ values for GST activity at all concentrations while relatively increased $K_m$ values of its substrates, namely GSH and CDNB. This type of suppression is one of the general characteristics of mixed-type inhibition. Although similar to the effects of non-competitive inhibitors, mixed-type inhibitors reduce the affinity of the substrate to the enzyme without competing with the substrate. The decrease in $V_{max}$ and the increase in $K_m$ demonstrate *A.mellea* suppresses GST activity with a mixed-type suppression mechanism. Form the experimental results, it is essential to note that the inhibitory mechanism of aqueous and methanolic extracts was slightly different from each other in such a way that methanolic extract mainly inhibits the GSH-dependent GST activity much more than the CDNB-dependent activity. However, aqueous extracts mainly affect GSH-dependent GST activities.

The inhibition of GSTs has been studied *in vitro*, and several plant-derived compounds such as hematin, thonningianin, cibacron blue, tannic acid, ellagic acid, ethacrynic acid, ferulic acid, stilbene, caffeic acid, quercetin, and curcumin have been reported to decrease GST activities (Hayeshi et al., 2007; Gweshelo et al., 2016). Therefore, *A.mellea* extracts might have high levels of these compounds, since elevated doses caused increased enzyme suppression. The inhibition of GSTs by *A.mellea* may support the uses of some drugs which are detoxified with the GST enzyme system and would increase the bioavailability of the drugs in the body. However, further studies should focus on separating the bioactive compounds responsible for inhibiting GSTs and identify their structures.

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