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Evaluation of Antimutagenic and Antioxidant Properties in *Fomes fomentarius* L.: Potential Development as Functional Food

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Abstract: Numerous studies derived from medicinal herbs have been conducted to explore bioactive compounds as potential alternatives to synthetic drugs, aiming to mitigate harmful side effects and alleviate economic burdens. In this study, we assessed the safety and potential biological activities of extracts from *Fomes fomentarius* L. (FFL). The FFL extracts were obtained through various ethanol concentrations, as follows: 0%, 30%, 50%, 70%, and 100%, respectively. All extracts did not induce mutagenicity even up to 5 mg/plate concentration. In the assessment of antioxidant activity, only the hot water extract exhibited weaker antioxidant activity than the other ethanol extracts. Notably, all extracts exhibited significant antimutagenetic effects only with a metabolically active enzyme system (S9 mix). The condition of 70% ethanol extract displayed the most robust antimutagenic activity; thus, the extract was sequentially fractionated with solvents of varying polarities to isolate inhibitory components. After the fractionization, the diethyl ether and butanol fractions effectively suppressed the growth of mutated colonies, suggesting that those such as essential oils, vitamins, alkaloids, and flavonoids can be considered major active compounds. Overall, our study demonstrated that FFL extracts induce potent antioxidant and antimutagenic effects. Further investigations are warranted to verify specific active compounds which induce an antimutagenic effect. Our findings provide valuable insights into FFL as a promising source for potential functional food development.

Keywords: *Fomes fomentarius* L.; ethanol extraction; antimutagenicity; antioxidants; diethyl ether; butanol fractions

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1. Introduction

In recent years, there has been a surge in research focused on harnessing natural products for the development of novel materials. These substances offer the potential to alleviate the economic strain associated with synthetic drugs while frequently presenting fewer adverse effects [1]. Natural products consist of primary and secondary metabolites, which are biosynthesis products found in plants and animals. Primary metabolites are essential compounds produced by all types of living cells, crucial for basic cellular functions. On the other hand, secondary metabolites, although not essential for cell growth or basic functions, exhibit physiological activity in specific plants and do not participate in fundamental metabolic processes [1,2], thereby prompting endeavors to explore novel active agents derived from natural products [3]. Due to these unique properties, natural products are increasingly used as functional foods, promoting health benefits beyond basic nutrition. Functional foods are foods that have been formulated to strengthen specific health functions, offering more health benefits compared to conventional foods [4–6]. These foods contain components that not only provide nutrients but can also help improve specific health conditions or reduce the risk of diseases [4,6]. To be considered functional foods, they either enhance general and physical well-being or reduce the risk of developing diseases. In addition, they need to meet key criteria such as safety, scientific evidence, consumption, and

the presence of biologically active compounds [4,5]. Therefore, numerous efforts have been continuously undertaken to discover potential functional foods, and many natural products have demonstrated the capability to improve physiological functions and decrease the risk of chronic diseases [5,6]. This is why they have become a key focus in the development of health-promoting food products.

Mushrooms, classified as fungi, have garnered significant attention among natural products due to their distinctive nutritional profile, blending animal protein with plant nutrients. Widely valued for their taste and flavor, mushrooms are utilized extensively for both culinary and medicinal purposes, offering a rich content of carbohydrates, proteins, lipids, minerals, and vitamins [3]. Mushrooms also contain various bioactive compounds including flavonoids, folates, phenolics, enzymes, lectins, terpenoids, carotenoids, polysaccharides, minerals, proteins, glycosides, tocopherols, fats, organic acids, volatile oils, and alkaloids [7]. As numerous studies have highlighted [7,8], mushrooms exhibit diverse bioactivities, ranging from anticancer properties to the regulation of biological functions, and even ameliorative effects on conditions like stroke and cardiac disease. Consequently, the appealing health benefits of mushrooms have captured the interest of both the public and researchers [3,7,8].

Fomes fomentarius L. (FFL), belonging to the Polyporaceae family and the genus Fomes, typically thrives on aged or hardwood trees over extended periods. Its surface, characterized by a thick, gray exterior encased in a hard shell, displays distinctive yellowish-brown or blackish-brown wavy patterns or horizontal wrinkles [9]. FFL has served as a traditional natural remedy for numerous ailments worldwide for centuries. FFL, also known as the tinder fungus, has been utilized for various medicinal purposes since ancient times. FFL has traditionally been employed for wound healing, inflammation reduction, immune enhancement, and digestive improvement [10,11]. This fungus exhibits diverse physiological activities, including antimicrobial, anti-inflammatory, antioxidant, and antiviral effects [11–15]. However, studies on its pharmacological effects are still in the early stages and require other effects such as antimutagenicity. Furthermore, most of the studies on biological activity were focused on polysaccharides by hydrothermal extraction. The assessment of FFL has not yet been fully studied in terms of safety and antimutagenicity. Therefore, we aim to investigate antioxidant and antimutagenic effects in FFL extracts. We extracted FFL with different ethanol percentages and evaluated the biological activities. Our main goal is to identify FFL as a promising source for potential functional food development and show its appealing health benefits.

2. Materials and Methods

2.1. Fomes fomentarius L. Extraction

The FFL used in this study was procured from Kwangchi Agricultural Center in Namwon, Jeonbuk Province, South Korea. After removing some soil, the FFL was stored at room temperature. Before being employed in the experiment, the sample was ground with a grinder. Then, the sample was utilized for extraction.

Various ethanol percentages were employed to extract FFL. The ethanol solvent concentrations ranged from 0% to 100%. For the ethanol-free extract (0%), 20 volumes of distilled water were added to 50 g of dried FFL powder, boiled at 100 °C for 3 h, and repeated three times. Other extracts were prepared by adding 20 volumes of different ethanol concentrations to 50 g of dried FFL powder and conducting an extraction three times over 3 days. Each extract was concentrated via vacuum filtration using a rotary vacuum evaporator (8 mm adventech, USA). After cooling to −70 °C, crude extracts were lyophilized using a laboratory freeze dryer (IlShinBiobase Co., Ltd., Dongducheon, Republic of Korea) and the yields were determined.

2.2. Nutritional Composition Analysis

To assess the nutritional composition of FFL, the methods outlined by AOAC (2016) were followed [16]. The moisture content was determined using the atmospheric pressure method in a 105 °C dryer. The crude fat content was calculated by comparing the weights of normal FFL and FFL extracted with ether, followed by complete drying. Crude protein was assessed using the micro Kjeldahl nitrogen determination method, with the nitrogen content multiplied by 6.25. The saccharide content was quantified excluding moisture, protein, fat, and ash.

2.3. Anti- and Mutagenicity Tests

Anti- and mutagenicity tests were assessed in *S. typhimurium* strains using the Ames test [17]. We utilized the TA98 strain for detecting frame-shift mutations and the TA100 strain for detecting base-pair substitutions with and without metabolic activation (S9 mix) through direct plate incorporation. Positive controls included 4-nitroquinoline-1-oxide (4NQO; 1.0 μg/plate) and sodium azide (SA; 0.5 μg/plate) for direct mutagens (without S9 mix) and 2-aminoanthracene (2AA; 0.5 μg/plate) for indirect mutagenesis (with S9 mix). The S9 mix (protein content: 23.6 mg/mL) was purchased from Moltox Co. (11-05L.2; Boone, NC, USA); it was isolated from the liver of a Sprague Dawley rat (7-week-old, male) induced by phenobarbital and 5,6-benzo-flavone. DMSO was used as the negative control. The highest test sample concentration was 5000 μg/plate. We mixed 2 mL of top agar, 100 μL of DMSO-dissolved test sample at various concentrations, 100 μL of cultured strains (1–2 × 10^9 CFU/mL), and 500 μL of S9 mix or 0.2 M sodium phosphate buffer in a tube at 45 °C, vortexed it briefly, and spread it on minimal-glucose agar plates. After 48 h of incubation at 37 °C, the revertant colonies were counted. The antimutagenic activity was quantified as the percent reduction in the mutagenic effects, determined by the following equation:

$$\text{Inhibition (\%)} = (M - B)/(M - A) \times 100 \quad (1)$$

where M represents the count of revertant colonies induced by the mutagen, A denotes the count of revertant colonies occurring spontaneously, and B signifies the count of revertant colonies observed when both the test sample and mutagen were introduced to the test strains.

2.4. Antioxidant Activity

2.4.1. Total Phenolic Content

The total phenolic content in the FFL extracts was determined using the total phenol assay method [18]. The Folin–Ciocalteu reagent reacts with the polyphenolic compounds to form a measurable molybdenum V complex. To 100 μL of the ethanolic FFL extract (1.0 mg/mL), 2 mL of 2% Na_2CO_3 solution was added and allowed to sit at ambient temperature for 3 min; then, 0.1 mL of 50% Folin–Ciocalteu reagent was introduced. The mix was incubated at room temperature for 30 min, after which its absorbance at 750 nm was recorded using a spectrophotometer (DU-800, Beckman Coulter Inc., Brea, CA, USA). Calibration with a gallic acid standard curve allowed for the reporting of the total phenolic content as micrograms of the gallic acid equivalent per milligram of extract (μg/mg).

2.4.2. DPPH Radical Scavenging Activity

The radical scavenging activity of the FFL extracts was assessed using a DPPH (Sigma-Aldrich, Waltham, MA, USA) solution based on a modified Aderogba method [19]. DPPH was diluted in 99.9% ethanol to 0.005 g/100 mL and left in darkness for 2 h, achieving an initial absorbance of 1.5–1.7 at 520 nm. For the test, 0.2 mL of the FFL extract (1.0 mg/mL) was mixed with 0.8 mL of this DPPH solution, shaken, and incubated at room temperature for 30 min before measuring the absorbance at 520 nm. L-ascorbic acid was selected as a

positive control. The DPPH scavenging efficiency was calculated as a percentage reduction in absorbance at 520 nm compared to the blank, using the following equation:

$$AA\% = (Abs\ blank - Abs\ sample)/Abs\ blank \times 100 \quad (2)$$

where Abs blank is the control's absorbance and Abs sample is the extract's absorbance.

2.4.3. ABTS Total Antioxidant Capacity

Total antioxidant activity was determined using the ABTS cation decolorization assay [20]. ABTS (Sigma-Aldrich) at 7.4 mM and potassium persulfate at 2.6 mM were mixed in darkness to generate the ABTS cation. This solution was then diluted with distilled water to achieve an absorbance of 1.4–1.5 at 735 nm based on a molar extinction coefficient of $3.6 \times 10^4\ M^{-1}\ cm^{-1}$. The antioxidant capacity was quantified by the percentage reduction in absorbance compared to the control using the following equation:

$$ABTS\ scavenging\ effect\ (\%) = (Abs\ blank - Abs\ sample)/Abs\ blank \times 100 \quad (3)$$

where Abs blank is the control's absorbance and Abs sample is the extract's absorbance.

2.4.4. DTT Antioxidant Activity

The redox cycling activity of the FFL extracts was assessed using the 1,4-Dithiothreitol (DTT) assay [21]. In brief, phenanthrenequinone (PQN) is a polyaromatic hydrocarbon species (PAH) with oxidative properties. The PQN was utilized to determine the consumption of the DTT reagent, which reacts with oxidative materials. Thus, the consumption rate of the DTT reagent was measured upon treatment with the PQN and FFL extracts. A total of 350 µL of FFL extracts and 500 µM of DTT were incubated in PBS (1 mL total volume) at 37 °C for 60 min (at 15 min intervals). Then, 1 mL of 10% trichloroacetic acid was added to the mixture and incubated for 5 min. Subsequently, 1 mL of 0.4 M Tris-HCl buffer at pH 8.9, containing 20 mM of EDTA and 25 µL of 10 mM DTNB, was added in the reaction mixture. A total of 100 µL of the reaction mixture was added into a 96-well plate, and then the absorbance was measured at 412 nm using a microplate reader (BioTek, Winooski, VT, USA). The rate of DTT consumption, indicating redox cycling activity, was calculated based on the linear regression of the time-response data, normalized by sample mass [22].

2.5. Solvent Fractionation

To fractionize different polarity, the 70% ethanol extract (34 g) extracted from FFL (500 g) was mixed with 200 mL of distilled water and an equal volume of diethyl ether. This mixture was placed at room temperature for 12–24 h, allowing it to separate into water and diethyl ether. The diethyl ether layer was collected, and the water layer was sequentially extracted with chloroform, dichloromethane, and butanol to produce different organic solvent fractions in the same manner. The left residue was considered to be the water fraction. All fractions were filtered, evaporated under reduced pressure, and then freeze-dried. The scheme and yield of the separation and purification processes are shown in Figure 1. The samples were dissolved in DMSO to a 60 mg/mL concentration, filtered (0.45 µm), and used for the tests.

2.6. Statistical Analysis

Experimental data are shown as means ± standard deviations (SDs) based on a minimum of three experimental runs. To analyze the differences in outcomes across various FFL extract tests, both a one-way ANOVA and Student's *t*-test were utilized. All graphs were created by GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA).

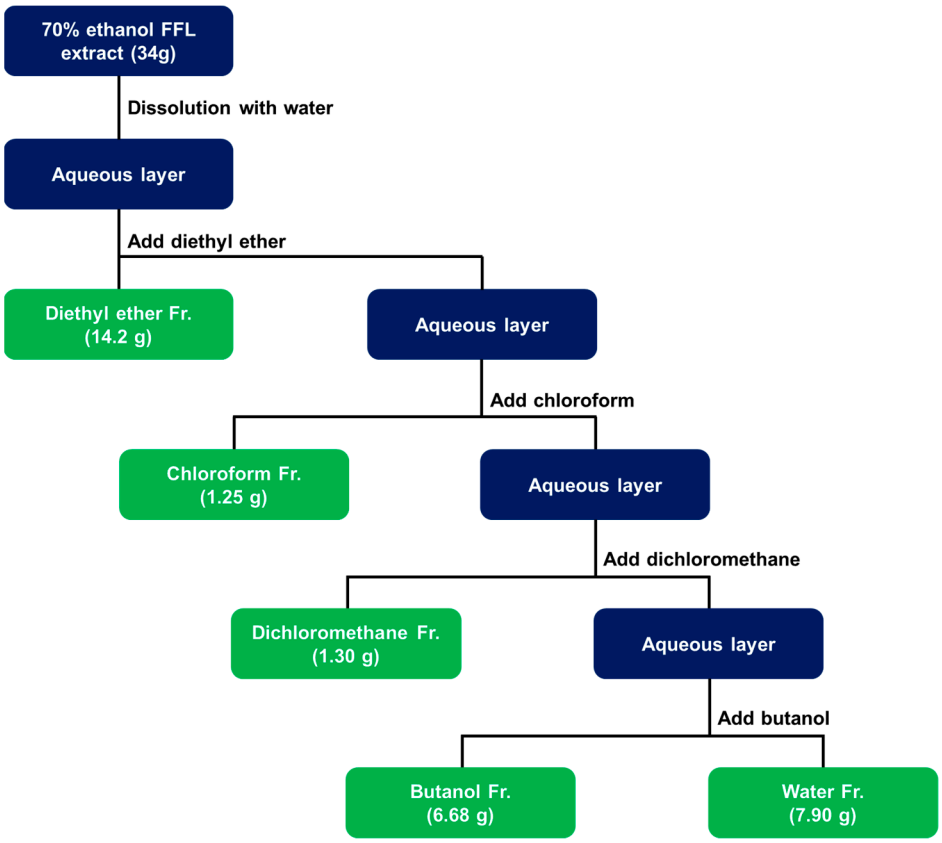

Figure 1. Procedure and yields for solvent fractionations.

3. Results and Discussion

3.1. Nutritional Analysis and Extraction

The nutritional composition of the dried fruit body of FFL is detailed in Table 1. The moisture content, crude carbohydrate, crude protein, crude fat, and crude ash were 11.04 ± 0.26%, 75.83 ± 1.44%, 5.82 ± 0.11%, 5.72 ± 0.17%, and 1.58 ± 0.18%, respectively, which aligns with a previous report [23]. Typically, edible dried mushrooms contain varying amounts of protein (ranging from 13.8 to 38.5 g/100 g), fat (0.4 to 5.9 g/100 g), carbohydrates (32 to 61.4 g/100 g), and ash (1.3 to 14.4 g/100 g) [24,25]. Despite FFL being a lignified, non-edible mushroom, its biological activities make its composition noteworthy. Therefore, this experiment confirmed that FFL shares constituents like those found in other common mushrooms, suggesting a potential practical application in food material development.

Table 1. Nutritional composition of FFL fruit body.

Properties	Content (%)
Moisture	11.04 ± 0.26
Crude carbohydrate	75.83 ± 1.44
Crude protein	5.82 ± 0.11
Crude fat	5.72 ± 0.17
Crude ash	1.58 ± 0.18

After nutritional analysis, FFL was extracted using various ethanol concentrations and their yields were assessed (Table 2). The hot water extraction (0%) yielded the highest at 10.4 ± 3.4%, followed by 70% ethanol (6.8 ± 1.0%) and 30% ethanol (5.1 ± 0.6%), with lower yields observed for 50% and 100% ethanol extracts. Pressurized hot water extraction typically involves pretreating biomass with hot water via liquid or steam, garnering significant

attention due to water's unique solubility properties, the potential for increased hydrolysate compared to organic solvents, and the ability to extract diverse compounds [26]. Elevating the pressure and temperature leads to a reduction in the dielectric constant, bringing it closer to that of ethanol. This suggests that water becomes capable of extracting a broader spectrum of both polar and non-polar constituents [27,28]. In contrast, organic solvent extraction using hexane, ethanol, and methanol is widely employed for extracting essential oil components, polyphenols, and steroids [29]. These differences are considered the main reason for a higher yield in the hot water extraction compared to ethanol extracts.

Table 2. Extraction yields of *Fomes fomentarius* L. (50 g).

Ethanol Solvent Percentage (%)	Yields (g)	Yields (%) *
0	5.20 ± 1.67	10.4 ± 3.4 [a]
30	2.55 ± 0.31	5.1 ± 0.6 [b]
50	2.51 ± 0.40	5.0 ± 0.8 [b]
70	3.40 ± 0.50	6.8 ± 1.0 [ab]
100	1.32 ± 0.28	2.6 ± 0.5 [c]

* Yield (%) = solid extract (g)/raw material (dry weight) × 100. [a–c] The same letters are not significantly different ($p < 0.05$).

3.2. Mutagenic Activity of FFL Extracts

The potential mutagenicity of the FFL extracts was assessed using TA98 and TA100 strains (Figure 2 and Supplementary Table S1). The test was carried out separately in the presence or absence of a metabolically active enzyme system (S9 mix). For the TA98 strain, which screens for frameshift mutagens, control groups with and without the S9 mix had 22 ± 4 and 25 ± 3 colonies, respectively. Positive controls showed significantly higher colonies, 735 ± 68 without S9 and 531 ± 31 with S9, respectively (Supplementary Table S1). All the FFL extracts showed no significant mutagenicity compared to the negative controls. Similarly, the TA100 strain, detecting base-pair mutagens, revealed control colonies of 121 ± 11 without S9 and 118 ± 16 with S9, and positive controls of 792 ± 28 and 837 ± 15, respectively (Supplementary Table S1). Colony counts from all the FFL extracts did not significantly change by a factor of two compared to the control without treatment, showing no increase or decrease in colony numbers according to the dose–response.

The Ames test is a method used for detecting potential genetic alterations in cellular DNA [17]. Thus, the Ames test plays a crucial role in the safety assessment of chemicals, contributing to public health protection and environmental safety [17]. This test targets substances such as radiation, UV light, chemicals, and internal factors, known for their high DNA reactivity and potential to cause unrepairable DNA damage, leading to cancer [30,31]. Considering this fact, FFL extracts showed no mutagenic activity in the presence or absence of the metabolic enzyme system, indicating that FFL has no mutation-inducing properties in both strains. Further studies are necessary to assess the extracts in various cell lines and animal models to confirm their potential mutagenic effects and safety.

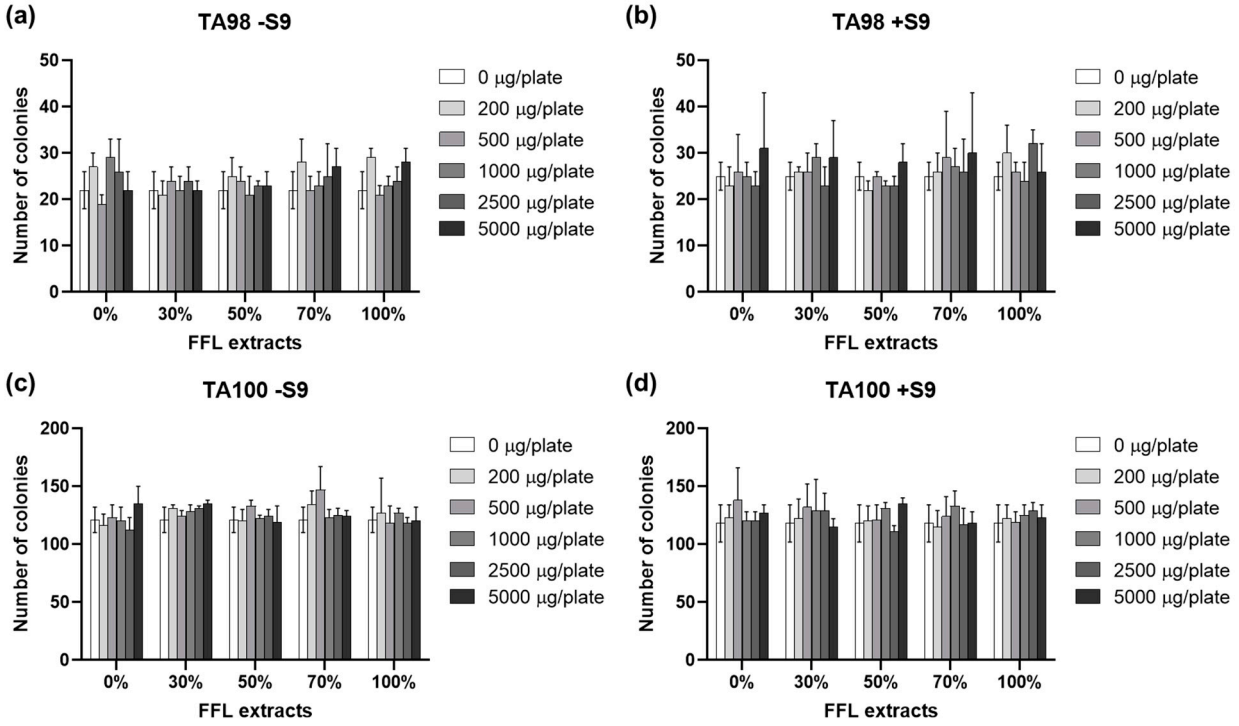

Figure 2. Mutagenicity activity of two strains by FFL extracts in the presence and absence of S9 mix; (**a**,**b**) TA98 strain; (**c**,**d**) TA100 strain. The number of spontaneous revertant colonies is shown as means ± SD.

3.3. Antioxidant Activities of FFL Extracts

In general, polyphenols contain numerous OH groups, and their contents are positively correlated with antioxidant capacity since the hydroxyl group attached to the free radical terminates the radical with an attached electron [32]. The total polyphenolic content of each FFL extract was quantified and compared using the Folin–Ciocalteu reagent. Table 3 illustrates that there were no significant differences in the polyphenol content among the ethanol extracts. Only the hot water extract showed lower polyphenol contents compared to the other ethanol extracts. This tendency was observed in the results of other plant extracts [29,33]. Abugri and McElhenney quantified the polyphenol and flavonoid contents in edible mushroom extracts. The results showed higher polyphenol contents in ethanol extracts compared to the hot water extract [33]. In addition, Hu et al. observed that an increased proportion of organic solvents facilitated the extraction of an increased range of components, including essential oils, organic acids, polyphenols, and steroids, compared to hot water extraction methods [29]. Collectively, organic solvents seem to provide a more favorable condition for the extraction of polyphenols in FFL.

Table 3. Total phenolic contents of the *Fomes fomentarius* L. extracts.

Ethanol Solvent Percentage (%)	Contents (μg/mg)
0	84.4 ± 13.5 [b]
30	111.3 ± 13.8 [a]
50	123.8 ± 16.2 [a]
70	120.7 ± 13.5 [a]
100	119.9 ± 15.3 [a]

[a,b] The same letters are not significantly different ($p < 0.05$).

The radical scavenging activity of the FFL extracts was assessed with DPPH reagent. As shown in Figure 3a, FFL extracts at concentrations of 100 μg/mL or lower exhibited no significant differences among all the extracts. The condition of the 50% FFL extract

displayed the highest activity at a concentration of 500 μg/mL, whereas the condition of the 0% FFL extract revealed the lowest activity among all the FFL extracts. This result aligns with the result of the total phenolic contents. The DPPH radical scavenging activity is known to be attributed to the antioxidant effects of phenolic acids, flavonoids, and other phenolic compounds [34].

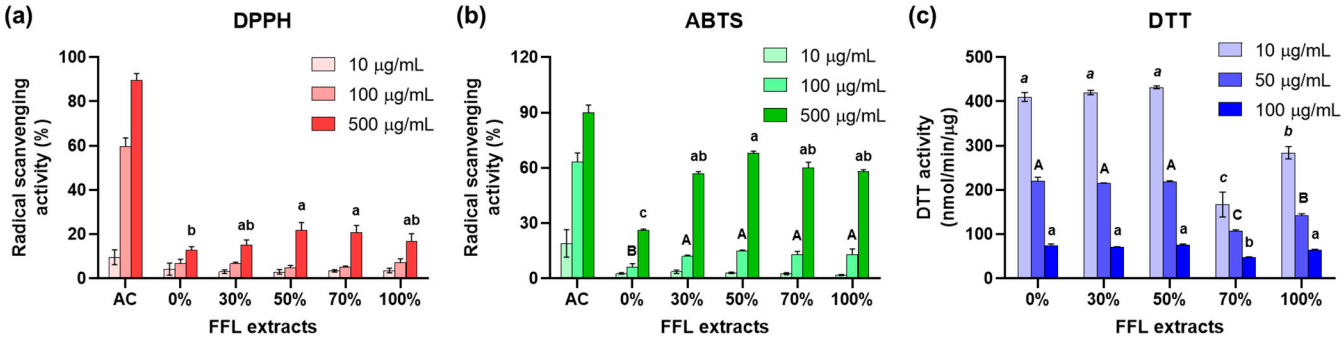

Figure 3. The antioxidant activities of the *Fomes fomentarius* L. (FFL) extracts. The activities were assessed through (**a**) DPPH free radical scavenging, (**b**) ABTS total antioxidant capacity, and (**c**) DTT activity. Different letters (a–c, A–C, and *a–c*) in the same concentrations are significantly different at $p < 0.05$ by Duncan's test. The data are shown as means ± SD. AC: ascorbic acid.

The total antioxidant capacity was also measured by the ABTS cation decolorization assay (Figure 3b). At a concentration of 10 μg/mL, there were no significant differences among the FFL extracts. However, the higher antioxidant activity in the ethanol solvent extracts was observed over 100 μg/mL concentrations than in the hot water extraction. Lastly, the antioxidant capacity was assessed by measuring the reaction between an oxidant and the FFL extracts using the DTT assay method. PQN is in the PAHs group with oxidative properties and was utilized to determine the consumption of the DTT reagent, which reacts with oxidative materials. Thus, the consumption rate of the DTT reagent was measured upon treatment with PQN and FFL extracts (Figure 3c). The condition of PQN exhibited a high DDT consumption of 652.5 ± 32 nmol/min/ng. However, when PQN was combined with FFL extracts, the consumption of the DTT reagent decreased in a concentration-dependent manner starting from 10 μg/mL. Particularly, the 70% ethanol extract displayed minimal DTT consumption of 167 ± 28 nmol/min/μg at 10 μg/mL, indicating robust reactivity with PQN even at low concentrations.

Through the antioxidant tests, we confirmed that ethanol extractions displayed higher polyphenol contents and potent antioxidant abilities compared to the hot water extract. Consistent with our results, Koludizic et al. reported that the methanol extract of FFL exhibited a higher content of polyphenols and DPPH scavenging activity compared to the aqueous extract [23]. In a prior study assessing the antioxidant capacity of FFL, Lee reported that FFL increased antioxidant activity by enhancing superoxide dismutase (SOD) and catalase (CAT) levels and decreasing thiobarbituric-acid-reactive substances (TBARS) in rats induced with diabetes [35]. Taken together, the ethanol solvent extraction of FFL appears to exhibit more potent antioxidant activities than the hot water extraction of FFL.

3.4. Antimutagenic Effect of FFL Extracts

The antimutagenic effect of the FFL extracts was evaluated using the mutagens (4NQO, SA, and 2-AA), as shown in Figure 4. Based on the results of the mutagenicity test, the highest concentration of FFL extracts was selected, exhibiting no toxicity or mutagenicity on both strains. The positive controls were 4NQO (TA98 −S9 mix), SA (TA100 −S9 mix), and 2-AA (TA98 and TA100 +S9 mix), respectively. In the TA98 strain, all the FFL extracts did not induce any inhibitory effect in the condition without the S9 mix; however, they exhibited a dose–response inhibition with the S9 mix (Figure 4a,b). The half maximal inhibitory concentration (IC_{50}) values for each FFL extract were 1984 (0%), 1926 (30%),

1020 (50%), 473.2 (70%), and 585.3 (100%) μg/plate, respectively. They were ranked in the following order: 70% > 100% > 50% > 30% > 0%. A similar tendency was also observed in the TA100 strain (Figure 4c,d). Only the inhibitory effect was observed with the S9 mix. The IC_{50} values were 1603 (0%), 2191 (30%), 1544 (50%), 1163 (70%), and 1579 (100%) μg/plate, respectively. The were ranked in the following order: 70% > 50% ≥ 100% ≥ 0% > 30%.

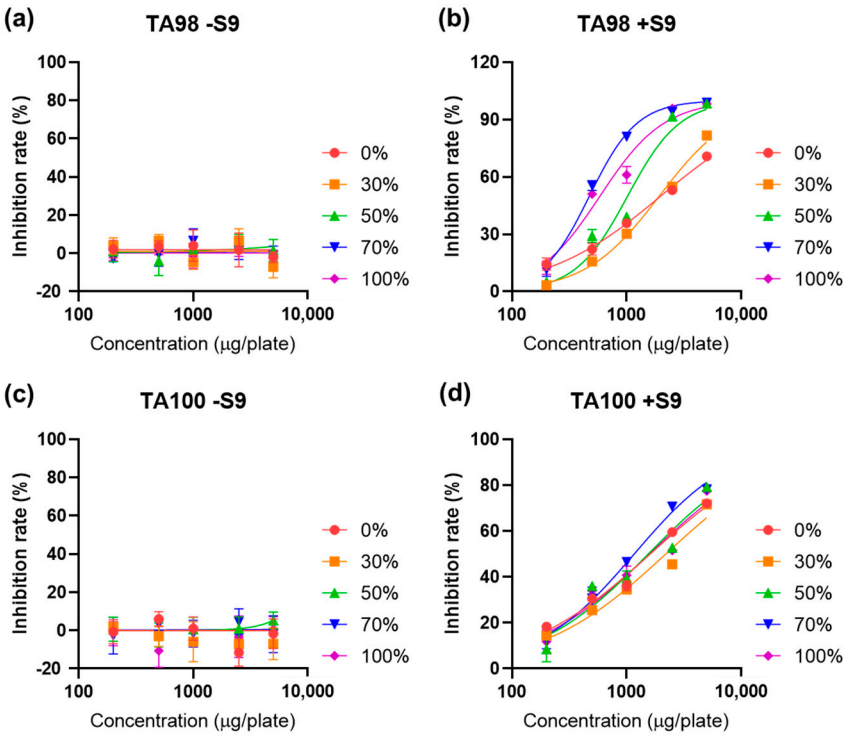

Figure 4. Antimutagenicity effects of two strains by FFL extracts in the presence and absence of S9 mix; (**a**,**b**) TA98 strain; (**c**,**d**) TA100 strain. The inhibitory effect was tested using the mutagens (4-nitroquinoline-1-oxide (4NQO), sodium azide (SA), and 2-aminoanthracene (2-AA)). For the indirect mutation (+S9 mix), 2-AA was applied at 0.5 μg/plate. For the direct mutation (−S9 mix), 4NQO (TA98) and SA (TA100) were applied at 1.0 and 0.5 μg/plate, respectively. The inhibition rate is shown as means ± SD.

Of note, a clear divergence was observed depending on whether metabolic activation occurred. This indicates that the metabolized compounds of the FFL extract significantly contribute to the antimutagenic effect in both strains. In general, the main reason for including the S9 mix in antimutagenic or mutagenic tests is to mimic the metabolic activation that occurs in a living organism, allowing for the assessment of a compound's mutagenic potential after metabolic conversion [36]. Larger antimutagenic effects were observed in other plant extracts under the S9 mix [37–39]. Notably, this was also observed in mushrooms. Alkan et al. tested the antimutagenic effects of eight mushroom species [40]. All extracts exhibited larger antimutagenic effects in the presence of the S9 mix compared to the absence of the S9 mix. This implies that the metabolized substances mainly contribute to the antimutagenic effects by preventing the activation of mutagens in or out of body cells [41]. Meanwhile, the extracts with higher ethanol percentages (over 70%) induced more robust antimutagenic effects compared to the extracts with lower percentages. This difference appears to be closely related to the increased ethanol proportion. An increased proportion of organic solvents enhances the extraction of components such as essential oils, organic acids, polyphenols, and steroids compared to hot water extraction methods [26]. Therefore, the enriched concentration of these components in the extracts likely contributes to their enhanced antimutagenic properties. Through this test, we could confirm the an-

timutagenicity of the FFL extracts under the metabolic activation and performed solvent fractionation of the crude extract (70% FFL extract).

3.5. Solvent Fractionation for Antimutagenic Active Compound Group

The fractionation was carried out with the 70% ethanol FFL extract to identify major active compound groups. The scheme and yield of the separation and purification processes are shown in Figure 1. The dried FFL powder (500 g) was extracted in 70% ethanol, and the extraction yield was 34 g. Thereafter, solvents with five different polarities were used to generate five fractions. The 70% ethanol extract was fractionated into diethyl ether (14.2 g), chloroform (1.25 g), dichloromethane (1.3 g), butanol (6.68 g), and water (7.9 g) fractions, respectively.

Then, each fraction was employed for the antimutagenicity test (Figure 5). As occurred with the result of the previous section (Figure 4), no antimutagenicity was observed without the S9 mix in both strains. In the TA98 strain, all extracts exhibited a dose–response inhibition in the presence of the S9 mix, except for dichloromethane (Figure 5a). The IC_{50} values for each FFL extract were 505.3, 3223, 525.3, and 2811 μg/plate for diethyl ether, chloroform, butanol, and water, respectively. They were ranked in the following order: diethyl ether ≥ butanol > water ≥ chloroform. In the TA100 strain, diethyl ether, chloroform, and butanol exhibited a dose–response inhibition with the S9 mix (Figure 5b). The IC_{50} values were 756.4 and 906.3 μg/plate for diethyl ether and butanol, respectively. They were ranked in the following order: diethyl ether ≥ butanol > chloroform.

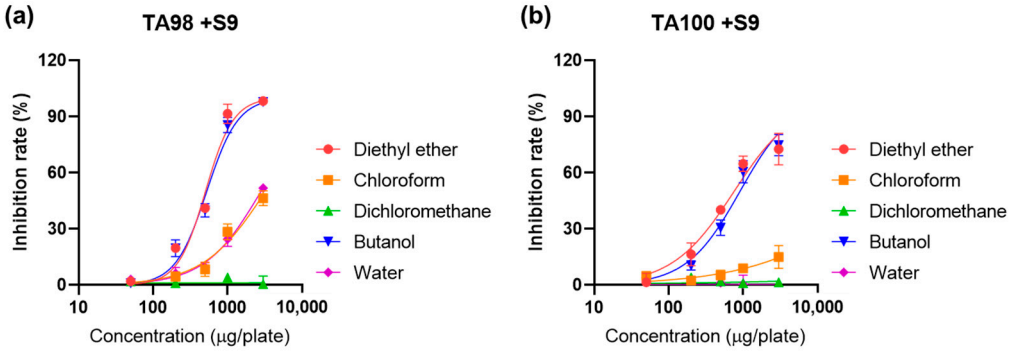

Figure 5. Antimutagenicity effects of two strains by the fractionized FFL extracts in the presence of the S9 mix; (**a**) TA98 strain; (**b**) TA100 strain. The inhibitory effect was tested using 2-aminoanthracene (2-AA; 0.5 μg/plate). The inhibition rate is shown as means ± SD.

Robust antimutagenic effects were observed in the diethyl ether and butanol fractions, which implies that they possess bioactive compounds. In general, solvent fractionation is applied in natural product research primarily to separate and characterize specific compounds or groups of compounds from complex mixtures [42]. This method is particularly useful for identifying active ingredients from natural sources, especially when natural products with diethyl ether, such as essential oils, terpenes, alkaloids, steroids, and lipid-soluble vitamins, are primarily extracted [43]. Some compounds of these groups revealed antimutagenic effects [44–46]. The essential oils extracted from *Ferula orientalis* L. exhibited antimutagenic effects in *S. typhimurium* [44]. The essential oils and monoterpenes extracted from *Salvia officinalis* L. also showed antimutagenic effects [45]. The isolated one triterpenoid and four steroids from the thorns of *Gleditsia sinensis* L. caused an inhibitory effect against mutagens using the *E. coli* PQ37 strain [46]. Lipid-soluble vitamins such as vitamins A and E were also reported to possess antimutagenic effects [47,48]. In the case of butanol fraction, butanol solvent is typically used to extract compounds with mid-to-high polarity [42]. The extract of butanol includes flavonoids, glycosides, and alkaloids [42]. It is particularly useful for separating compounds that are more polar than those soluble in water. Flavonoids were also reported to induce potent antimutagenic effects. The flavonoid structure contains the free 5-,7-hydroxyl group considered to be a major active

compound [49,50]. Two chalcone glycosides isolated from *Mentha longifolia* L. exhibited antimutagenic effects in the TA1537 strain [51]. In addition, Valentová et al. reported antimutagenic effects in quercetin glycosides [52]. Alkaloids such as quinolizidine alkaloids also exhibited antimutagenic effects [53–55]. Like this, many substances extracted from natural products present antimutagenic effects by preventing the activation of mutagens in or out of body cells, and the mentioned compound groups are commonly detected in mushrooms, including FFL [7,23]. Taken together, this result demonstrates the robust antimutagenicity of the diethyl ether and butanol FFL fractions, and further investigations focused on the isolation of compounds are required to identify major active compounds.

4. Conclusions

In this study, we extracted FFL through various ethanol concentrations and tested a safety profile and potential bioactive properties without inducing mutagenicity up to a concentration of 5 mg/plate. Among the different extraction methods, the ethanol extracts of FFL exhibited greater antioxidant activities than the hot water extraction. The 70% ethanol extract exhibited the most potent antimutagenic activity, particularly when tested with a metabolically active enzyme system. The fractionation process identified the diethyl ether and butanol fractions as being particularly effective in suppressing the growth of mutated colonies, highlighting those such as essential oils, vitamins, alkaloids, and flavonoids as possible key active components contributing to these effects. The findings underscore the significant antioxidant and antimutagenic capabilities of FFL extracts, suggesting their promise for functional food development. Our future study will focus on the further isolation of diethyl ether and butanol fractions to verify the specific compounds responsible for the antimutagenic effects and to explore their applications associated with healthcare. Overall, our study demonstrates for the first time that FFL ethanol extracts possess potent antimutagenic activity, and these can be safely used in medicinal applications after further investigations.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app14093927/s1, Table S1: The mutagenicity activity of the FFL extracts in TA98 and TA100 strains.

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