TOXICITY, ANTIMICROBIAL, AND ANTIMUTAGENCICITY POTENTIALS OF THE ETHYL ACETATE EXTRACT FROM THE MUSHROOM *Coriolus versicolor* (L. :Fries.) Quelet

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ABSTRACT: *This study evaluated the toxicity, antimicrobial, and antimutagenicity potentials of the crude extracts prepared from the fruiting bodies of the mushroom, Coriolus versicolor (L.:Fr.) Quel.. Crude ethanol extract (EE) obtained from the sample was solvent partitioned into hexane extract (HE), ethyl acetate extract (EAE), dichloromethane extract (DE), and aqueous extract (AE) by serial extraction. The extracts obtained were tested for antimicrobial activity using paper disc diffusion assay. The extracts possessed no antimicrobial activity against Bacillus subtilis, Escherichia coli, and Saccharomyces cerevisiae, except for EAE which is partially active against B. subtilis. Toxicity of the extracts was tested using brine shrimp lethality test (BSLT). BSLT results showed that the extracts were bioactive against Artemia salina. Among the extracts, EAE was chosen to be fractionated further and subjected to antimutagenic testing since it has an appreciable LC_{50} value of 86.44 ppm. Trace amounts of phenolic antioxidants were detected in EAE through chromatographic screening. EAE also showed antimutagenic activity against the mutagen, methylmethane sulfonate (MMS), through the peripheral blood micronucleus assay. It was also further observed that the EAE significantly reduced the frequencies of micronucleated reticulocytes (MNRETs) in a dose-dependent manner.*

Keywords: brine shrimp lethality test, paper disc diffusion assay, peripheral blood micronucleus test, *Coriolus versicolor*

1. INTRODUCTION

Fungi play an important role in the development of new compounds that could be used in the manufacture of new pharmaceutical products, agrochemicals and industrial enzymes [1]. Several natural products derived from fungi have been developed into important human medicines. Examples include penicillin and cephalosporin antibiotics; cyclosporin, an immunosuppressant used in organ transplantation surgery; and several of the "statin" drugs (i.e., lovastatin) used for lowering blood cholesterol levels [2]. There is a huge number of fungal species in the world but only a small number have been examined chemically. The potential to discover novel bioactive compounds from fungi remains enormous [3]. One of the medicinally important mushrooms is *Coriolus versicolor*. The multi-colored cap resembles a 'turkey tail' and occurs as overlapping clusters on dead logs and trees in most parts of the world [4]. This is not an edible fungus but hot water extracts have been used in traditional medicine for a wide range of ailments [5].

The focus of the modern clinical use and research (over 400 published studies) has been the immuno-modulating and anti-tumor properties of the hot water extracted polysaccharides from *C. versicolor* (L.:Fr.) Quel. [6]. This research exclusively deals with another scope of analysis using three more solvents: hexane, ethyl acetate and dichloromethane. Further fractionation of the ethyl acetate extract was also conducted. The antimutagenic potential of the ethyl acetate extract of the mushroom *C. versicolor* (L.:Fr.) Quel. was evaluated in this study using Peripheral Blood Micronucleus Assay with albino mice as test organism [7].

2. MATERIALS AND METHODS

Sampling and Sample Preparation

Fruiting bodies of the mushroom, of approximately the same size, were collected from Buru-un, Iligan City. Collection was done by separating the fruiting bodies from the dead logs or trees using a knife. The samples were packed in thick plastic bags, stored in an ice cooler box and transported to the laboratory.

Extraction and Solvent Partitioning

A 900 g of fresh samples were chopped into small pieces and macerated at room temperature for 48 hours using a sufficient amount of absolute ethanol to completely submerge the material. The resulting mixture was then filtered using ordinary filter paper and with the aid of a suction pump to hasten the process. The filtrate was concentrated *in vacuo* using a rotary evaporator at reduced pressure and at 40^0 C to give the crude aqueous ethanolic extract. This was then freeze dried yielding a brown residue. The resulting crude ethanol extract was then subjected to sequential solvent partitioning to give hexane, ethyl acetate, dichloromethane, and aqueous extracts.

Brine Shrimp Lethality Test

Toxicity of the extracts was evaluated through brine shrimp lethality test using *Artemia salina Leach* as the test organism [8]. Toxicity of the test extracts were tested at 10, 100, and 1000 ppm. Median Lethal Concentration (LC_{50}) was determined from the 24-hour death count using Probit Analysis [9, 10].

Paper Disc Diffusion Assay

Antimicrobial property of the crude extracts was determined via paper disc diffusion method against a panel of laboratory strains which includes the following test organisms: *Bacillus subtilis* (gram positive bacteria), *Escherichia coli* (gram negative bacteria), and *Saccharomyces cerevisiae* (fungi). Ampicillin and Nystatin were used as positive control for bacteria and fungi, respectively. Respective solvent of each extract was used as negative controls to verify that any zones of inhibition obtained were from the extracts and not from the solvents [8].

Gravity Column Chromatography

The fractionation of the ethyl acetate extract was done through a series of gradient elution normal phase gravity column chromatography (GCC) on a 60 – 230 mesh silica gel stationary phase [11]. Hexane, hexane-ethyl acetate mixtures, ethyl acetate, ethyl acetate-methanol mixtures, and methanol were used as mobile phases in the gradient elution. The isolation process was monitored by TLC on a silica-coated plate and was guided by BSLT [12]. Purification and characterization of the bioactive components of the sample were not covered in this study.

Antioxidant and Micronucleus Test

Presence of phenolic antioxidant in the ethyl acetate extract was determined qualitatively using chromatographic screening [8]. Ethyl acetate extract was subjected to antimutagenic testing through Peripheral Blood Micronucleus Assay using Swiss albino mice of different sexes aging 7 to 12 weeks and acridine orange as staining agent [13, 14]. The antimutagenic activity of the extract was evaluated through its effect towards the formation of micronuclei induced by methylmethanesulfonate (MMS), a mutagen. Microscopy based scoring was employed in counting the number of micronucleated reticulocytes [15].

Statistical Evaluation

Statistical analyses of the results were obtained using the one-way analysis of variance (ANOVA) with square root transformation of raw data and Duncan's Multiple Range Test (DMRT) [16, 17].

3. RESULTS AND DISCUSSION

The extracts obtained exhibited similar color and texture having a dark brown and sticky consistency. Solvent partitioning of the crude ethanol extract with hexane, ethyl acetate, dichloromethane, and water separated the nonpolar, slightly polar, and polar compounds.

Toxicity and Antimicrobial Tests

The test organisms are chosen because of their ability and their medical importance as common pathogens for known diseases. The activity of the extracts against the representative test organisms would determine their possible activity to other species belonging to the groups presented.

Results were obtained by measuring the diameter of zones of inhibition in millimeter scale using a ruler. The size of the paper disc used was 6 mm in diameter, which was incorporated in measuring the zones of inhibition. The results of the antimicrobial activity are presented in Table 1.

Table 1. Antimicrobial activity of the crude extracts of *C. versicolor* (L.:Fr.) Quel. by paper disc diffusion method

Crude Extracts	Mean zone of inhibition in mm ± SD		
	B. subtilis	***E. coli***	***S. cerevisiae***
EE	7.33 ± 0.50	6.0 ± 0.0	6.0 ± 0.0
HE	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0
EAE	10.0 ± 0.87	6.0 ± 0.0	6.0 ± 0.0
DE	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0
AE	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0
Ampicillin	35.93 ±1.83	32.00 ± 0.85	-
Nystatin	-	-	16 ± 1.77
Solvent	6.0 ± 0.0	6.0 ± 0.0	6.0 ± 0.0

Note: Size of filter paper disc is 6 mm in diameter

Results showed that all extracts did not inhibit the growth of *S. cerevisiae and E. coli*, thus all extracts did not possess antimicrobial activity against these microbial strains. However, the EAE and EE exhibited weak inhibition on *B. subtilis*. Moreover, it can be noted that the antibacterial activity of EAE was weak with mean diameter of zone of inhibition equal to 10 mm and thus, considered to be partially active. It can also be noted that the antibacterial activity for EE was very weak with mean diameter of zone of inhibition less than 10 mm and thus, considered to be inactive [8].

Brine shrimp lethality test (BSLT) is an *in vivo* test which is used as a convenient monitoring assay for screening and fractionation in the discovery of bioactive natural products. It was found out that this bioassay has positive correlation with the human solid tumor cell lines [18]. Table 2 tabulates the LC_{50} of the crude extracts. For a 24-h test, LC_{50} of 450 ppm warrant further evaluation and fractionation of crude extracts. In toxicity evaluation of plant extracts by brine shrimp lethality bioassay, LC_{50} values lower than 1000 ppm are considered bioactive. The lower the LC_{50}, the more bioactive is the extract. Based on the results, all values are lesser than 1000 ppm which means that the plant extracts contain bioactive compounds which needed to be investigated. Based on the LC_{50} values of the extracts, bioactive compounds are definitely present in these extracts. Moreover, it is possible that a broad range of structurally diverse compounds contribute to the overall pharmacological activity of the crude extracts [18].

Table 2. Cytotoxicity of the crude extracts of *C. versicolor* (L.:Fr.) Quel. against brine shrimp nauplii (*Artemia salina*)

Crude Extract	dose, ppm	Mean % mortality	LC_{50} , ppm
EE	10	26.7	125.42
	100	43.3	
	1000	73.3	
HE	10	40	16.56
	100	83.3	
	1000	100	
EAE	10	20	86.44
	100	60	
	1000	76.7	
DE	10	23.3	53.01
	100	56.7	
	1000	93.3	
AE	10	6.7	874.77
	100	26.7	
	1000	50	

Figure 1 shows a graphical comparison of LC_{50} values of the crude extracts from *C. versicolor* (L.:Fr.) Quel. Among the extracts obtained after partitioning EE, AE showed very little activity at LC_{50} value of 874.77 and the most promising extract came from HE with an LC_{50} of 16.56 µg/mL. This shows that HE has the greatest bioactivity followed by DE, EAE, and AE, respectively. Since EAE has an appreciable LC_{50} value and a considerable amount of weight, EAE was

chosen to be fractionated further and subjected to antimutagenic testing instead of the more active HE.

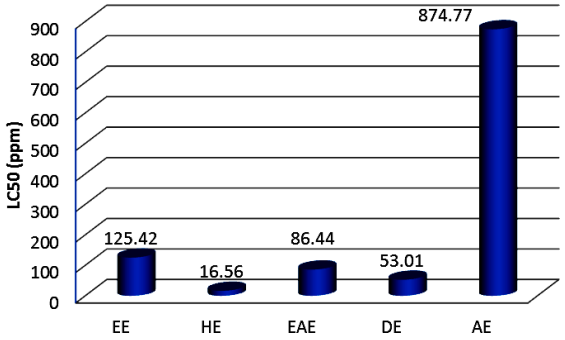

Figure 1. Graphical comparison of LC_{50} values of the crude extracts.

Gravity Column Chromatography

A total of 9 pooled fractions (EAE1 – EAE9) were obtained when EAE was fractionated using gradient elution normal phase gravity column chromatography (GCC). Table 3 lists the color and percent mortality of the nine fractions towards BSLT. Among the 9 pooled fractions, EAE 1 showed the highest percentage mortality against the brine shrimps at 100 ppm concentration. It is possible that compounds responsible for the bioactivity of EAE might be found in EAE1. EAE1 was then selected to be fractionated further.

Table 3. Percent mortality of *A. salina* nauplii in EAE pooled fractions at 100 ppm

EAE pooled fraction	Color	% mortality
EAE 1	brownish yellow	100
EAE 2	dark brown	73.33
EAE 3	dark brown	90.00
EAE 4	light brown	80.00
EAE 5	Brown	53.33
EAE 6	dark brown	43.33
EAE 7	dark brown	30.00
EAE 8	dark brown	36.67
EAE 9	Brown	26.67

Fractionation of EAE revealed 5 pooled fractions. Percent mortality of the collected fractions is shown in Table 4. Based on the results, it can be noted that somehow fractionation and purification process can enhance the concentration of the active components, thus bioactivity is also enhanced [11, 12].

Table 4. Percent mortality of *A. salina* nauplii in EAE1 pooled fractions at 100 ppm

EAE1 pooled fraction	Weights (mg)	% mortality at 100 ppm
EAE 1.1	15.3 mg	86.67
EAE 1.2	27.0 mg	100.00
EAE 1.3	48.2 mg	83.33
EAE 1.4	10.7 mg	73.33
EAE 1.5	6.3 mg	76.67

Chromatographic Screening of Phenolic Antioxidants

These 5 pooled fractions were subjected to chromatographic screening of antioxidants. A blue color was developed lightly in the spot which correspond to EAE 1.2 and the positive standard, BHT. This revealed the presence of phenolic antioxidants in EAE 1.2. This result also suggested that the ethyl acetate extract from *C. versicolor* is a potential source for antioxidant compounds [8].

Anti-mutagenicity Test

The anti-mutagenic potential of EAE in three different concentrations, 30,000 ppm, 15,000 ppm, and 7,500 ppm, was compared to the chromosome-breaking activity induced by a mutagen, MMS (the positive control) and DMSO and water (the solvent control). Table 5 summarizes the mean number of micronucleated reticulocytes (MNRETs) found per one thousand cells from the peripheral blood of the test animals administered with the three different concentrations of EAE, DMSO, MMS and water as scored under the microscope.

Table 5. Mean number of micronucleated reticulocytes (MNRETs) found per one thousand cells from the peripheral blood of mice treated with different concentrations of EAE, DMSO, MMS and water

Treatment Groups	Mean MNRETS	
	raw data mean	transformed data mean ± SD
MMS	17.0333	4.4958 ± 0.3644^{a}
DMSO	0.7000	0.8313 ± 0.2256^{b}
Water	0.0000	0.7071 ± 0.0000^{b}
30,000 ppm EAE	0.7667	0.8534 ± 0.3091^{b}
15,000 ppm EAE	0.9000	0.9298 ± 0.3469^{b}
7,500 ppm EAE	1.0667	1.0333 ± 0.3420^{b}

Means having the same letter are not significantly different at $\alpha = 0.01$ DMRT.

The results in Table 5 show that the mean number of MNRETs was lower in animals that were administered with EAE extracts, DMSO, and water compared to the mean number of MNRETs of test animals administered with MMS. This increase in the MNRETS of test animals administered with MMS is due to the effect of the mutagenic MMS [19, 20]. This is clearly depicted in Figure 2.

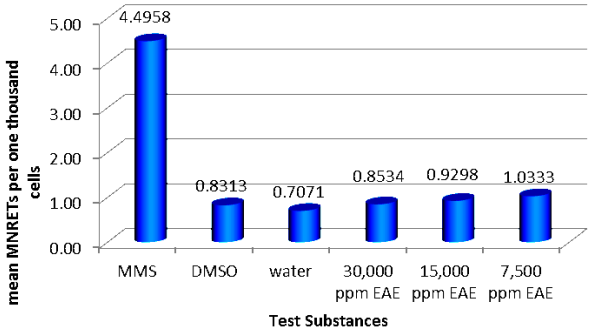

Figure 2. Chromosome-breaking potential of EAE.

The results were statistically evaluated using the one way analysis of variance (ANOVA). As shown in Table 6, the value of computed F was highly significant at 1% level of significance; this suggests that the treatment means are significant. Furthermore, the mean number of MNRETs produced by the test samples is not the same as for the five treatments. The treatment means were then evaluated further using Duncan's Multiplicity Range Test (DMRT) to determine which treatment means have significant or insignificant difference [16, 17].

Table 6. ANOVA-CRD calculations for mice treated with EAE

Source of variance	Degrees of Freedom	Sum of Squares	Mean Square	F_{cal}	F_{tab}
Treatment	5	55.0408	11.0082	561.4	4.43
Error	24	0.4706	0.0196		
Total	29	55.5114			

Results of the ANOVA revealed that the mean MNRETs of the different concentrations of EAE, DMSO, water, and MMS are significantly different as evidenced by the higher calculated value of the distribution ratio, F, which is equal to 561.4 compared to the tabulated F value that is 4.43 [16]. As depicted in Table 5, DMRT results show that there is a significant difference in the mean MNRETs for the EAE extracts, water, and DMSO with that of the mean MNRETs for MMS. However, there is no significant difference in the responses of all the three concentrations of EAE. Furthermore, MNRETs detected in the EAE extracts were comparable to the solvent [17]. This implies that all three doses of EAE do not have the capacity of inducing chromosome-breaking. Thus, the EAE of C. *versicolor* (L.:Fr.) Quel. does not have a comparable mutagenic activity like that of MMS [21].

The effect of EAE on the chromosome-breaking activity induced by MMS was evaluated by treating another set of mice with MMS prior to the administration of the same concentrations of EAE. Table 7 summarizes the mean number of MNRETs found per one thousand cells from the peripheral blood of the test animals as scored under the microscope.

Table 7. Mean number of micronucleated reticulocytes (MNRETs) found per one thousand cells from the peripheral blood of mice treated with MMS prior to EAE

Treatment Groups	Mean MNRETS	
	Raw data mean	Transformed data mean
MMS	17.0333	4.4958 ± 0.3644^{a}
DMSO	0.7000	0.8313 ± 0.2256^{b}
Water	0.0000	0.7071 ± 0.0000^{b}
MMS + 30,000 ppm EAE	0.7667	1.4315 ± 0.6216^{c}
MMS + 15,000 ppm EAE	0.9000	2.2023 ± 0.3062^{d}
MMS + 7,500 ppm EAE	1.0667	2.8345 ± 0.2615^{e}

Means having the same letter are not significantly different at α = 0.01 DMRT.

Table 7 shows that the mice treated with MMS alone showed mean MNRETs which are significantly higher than the solvent control. This increase is due to the effect of the mutagenic MMS [19, 20]. However, the mean frequencies of the MNRETs of animals treated with MMS were reduced significantly upon treatment of the different concentrations of the EAE. This is clearly depicted in Figure 3.

Based on the results of ANOVA shown in Table 8, the responses of the test animals treated with MMS and EAE are significantly different with F value of 455.3 compared to the tabulated F value, 4.43. The calculated F value suggested that the mean MNRETs for MMS is significantly different to the other treatment groups, including the solvent control [16].

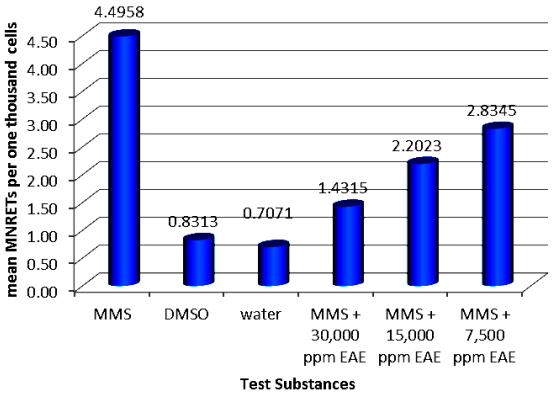

Figure 3. Effects of EAE on the chromosome-breaking induced by MMS.

DMRT results show that there is a significant difference in the responses of DMSO and the three concentrations of EAE against MMS at α=0.01. This is shown in Table 7, where the different concentrations of EAE have different letter superscripts, thus their means are significantly different [17]. These signified that the chromosome-breaking activity induced by MMS was significantly reduced by the treatment of EAE and that EAE binds with MMS prior to its reaction or EAE was promoted to the cell repair system resulting to the reduction of MNRETs count [19, 20]. Furthermore, a concentration-dependent response can be observed based on the DMRT calculations and as shown in Figure 3. Thus, increasing the concentration of EAE has increasingly inhibited the chromosome-breaking activity induced by MMS.

Table 8. ANOVA-CRD calculations for mice treated with MMS prior to EAE

Source of variance	**Degrees of Freedom**	**Sum of Squares**	**Mean Square**	**F_{cal}**	**F_{tab}**
Treatment	5	51.4249	10.2850	455.3	4.43
Error	24	0.5422	0.0226		
Total	29	51.9671			

In general, ethyl acetate extract of C. *versicolor* (L.:Fr.) Quel. does not have the capability of inducing chromosome-breaking in the peripheral blood cells of the test animals. Furthermore, this extract has the capacity to reduce chromosome-breaking effects of the mutagenic agent, MMS [21]. Thus, it has an antimutagenic activity against MMS.

5. CONCLUSIONS

All extracts obtained from *C. versicolor* were inactive against *B. subtilis*, *E. coli*, and *S. cerevisiae*, except for **EAE** which is partially active against *B. subtilis*. Furthermore, BSLT results showed that the extracts were bioactive against *A. salina*. Among the extracts obtained after partitioning **EE**, **EAE** was chosen to be fractionated further and subjected to anti-mutagenic testing since it has an appreciable LC_{50} value of 86.44 ppm. Further fractionation enhances the concentration of the active components, thus bioactivity is also enhanced. In addition, **EAE** contained trace amounts of phenolic

antioxidants as detected through chromatographic screening. **EAE** showed antimutagenic activity against the mutagen, methylmethane sulfonate (MMS), through the peripheral blood micronucleus assay. **EAE** exhibited a significant reduction of the frequencies of the micronucleated reticulocytes (MNRETs) in the peripheral blood cells of the test animals treated with MMS plus the different concentrations of **EAE**. Furthermore, the **EAE** significantly reduced the frequencies of MNRETs in a dose-dependent manner.

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