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MYCOCHEMICAL, ANTIOXIDANT, ANTIBACTERIAL AND TERATOGENIC ACTIVITY OF *Tyromyces chioneus* COLLECTED FROM BAMBANG, NUEVA VIZCAYA, PHILIPPINES

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ABSTRACT

Tyromyces chioneus, commonly known as the white cheese polypore is a species of polypore fungus. It is widely distributed macrofungus and has a circumpolar distribution in temperate boreal pine forests, of Asia, Europe, and North America, causes white rot in dead hardwood trees, especially birch. In order to establish its nutraceutical mycochemical composition, antioxidant, antibacterial activity and teratogenic property were assessed. The mycochemical screening revealed the presence of phenols, triterpenes, steroids, fatty acids, sugars, anthraquinones, coumarins, anthrones, tannins, flavonoids, and alkaloids. *T. chioneus* acetate-methanol extract showed 26.53% DPPH radical scavenging activity and 31.13 mg GAE/g total phenolics. Hot water extract of *T. chioneus* also showed bacterial inhibitory activity against *Escherichia coli* ATCC 25992 and *Staphylococcus aureus* ATCC 29213 with diameter zone of inhibitions of 16.58 mm and 15.67 mm, respectively. The teratogenic assay revealed that the hot water extract of *T. chioneus* have teratogenic activities against zebrafish model and that it has a potential to be used in the development of anticancer drugs. Thus, fruiting bodies of *T. chioneus* contain bioactive compounds with functional activities such as antibacterial and antioxidant and it could be harnessed as an anti-cancer drug.

Keywords: *Tyromyces chioneus*, mycochemical, antioxidant, antibacterial, teratogenicity

INTRODUCTION

Nowadays, there are many diseases that are damaging the human health such as cancer, diabetes and heart disease that can lead to death when not treated immediately. These diseases affect the lives of the people not only physically but also emotionally. Nevertheless, there are also many things the world could offer especially nature that can help us prevent or even cure those diseases and most of them can be seen everywhere. One of them are mushrooms that may grow on trees, decomposing leaves, dung, mulch, soil or compost, feeding off the dead or decaying matter in those substances. Furthermore, mushrooms are considered as an abundant source of wide range of useful compounds such as tannins and flavonoids, and steroids with documented bioactivities against diabetes, hypercholesterolemia, and even cancer **[1, 2]**. Aside from this, mushroom has been found to have antioxidant activity which gives the mushroom the ability to neutralize free radicals that are unstable **[3][4]**. Researchers have also showed both fruiting body and the mycelium contain compounds with wide-ranging antimicrobial activity **[5]**. Lastly, it also contains teratogens that cause malformation in the developing embryos. Embryos of zebara fish *(D. rerio)* are now used as standard model in toxicity testing due to their similarity to higher forms of vertebrates **[6]**. But despite of its negative effect, teratogenicity testing can be desirable property because many anticancer drugs are teratogenic and teratogens can be used as anticancer drugs **[7]**. Since, scientific or clinical studies about mushrooms are not sufficient especially here in the Philippines where there are lot of undiscovered species of mushrooms, it is proposed then to have a close attention to carry out further research of functional mushrooms that can add to the pool of knowledge and could give awareness to the people about helpful benefits and properties of mushroom that could be used as an alternative medicine. Thus, this research was done to determine the functional components and bio-assay activities of *T. chioneus* collected from Bambang, Nueva Vizcaya.

MATERIAL AND METHODS

Source of Mushroom

The fruiting body of *T. chioneus* that was collected from the area inhabited by the Isinai tribe in Bambang, Nueva Vizcaya, Philippines was used for the study.

Mycochemical Screening Test

Mycochemical screening was carried out on the acetate-methanol extract of the fruiting body of *T. chioneus* to detect the secondary metabolites present. The acetate-methanol extract was spotted on marked and labeled TLC (thin layer chromatography) 7 X 4 cm, and was developed in the acetate-methanol

(7:3) mixture in the developing chamber. The spots for a certain metabolite were visualized on the TLC plates and were exposed under UV light and hot plate to check the separation on the different compounds. For typical visualization of secondary metabolites, vanillin-sulfuric acid reagents was utilized. This solution can determine the presence of phenols, sterols, triterpenes and essential oils. Methanolic potassium hydroxide was used to test anthraquinones, coumarins and anthrones while phenolics compounds and tannins were detected through the use of potassium ferricyanide-ferric chloride reagent. Dragendorff's reagent was used to spot alkaloids and Antimony (III) chloride was used to detect the presence of flavonoids **[8]**.

Radical Scavenging Activity Assay

For the determination of DPPH radical scavenging activity, the procedure described by Kolak *et al.* **[9]** was use. The aqueous extract was dissolved in methanol to a final concentration of 500 ppm. A 0.1 mM DPPH in methanol was freshly prepared by diluting 1 mL DPPH stock solution (3.49 mg DPPH in 10 mL methanol) to 100 mL methanol. Then, 1 mL of each extract and 4 mL of DPPH solution were mixed and incubated in the dark at 37ºC for 30 minutes. Triplicate test was done in each extract. The absorbance reading was monitored at 517 nm using UV-Vis spectrophotometer (APEL-100). Lastly, the ability to scavenge the DPPH radical was calculated using the formula:

% DPPH scavenging effect = [($A_{control}$– A_{sample})/ $A_{control}$] x 100

Wherein $A_{control}$ is the absorbance of the control and the A_{sample} is the absorbance of the test sample containing the mixture of DPPH and the aqueous extract. The synthetic antioxidant cathechin was used as positive control.

Estimation of Total Phenolic

The acetate-methanol extract of *T. chioneus*, Folin – Ciocalteu method as described by Hodzic *et al.* **[10]** was use. Firstly, a calibration curve was made at different concentrations (0.25, 0.5, 1.0, 2.0 and 4.0 mg/ml) of ascorbic acid, using APEL-100 UV-Vis spectrophotometer (PD-303UV). These ascorbic acid solutions (volume of 20 mcL) were placed in vials. To each vial, 200 mcL of Folin – Ciocalteu reagent was added and incubated at room temperature for 5 minutes. Next, 20 ml of sodium carbonate (1 mg/ml concentration) was added to the mixture of ascorbic acid and FC reagent. This was then transferred to cuvettes and read using UV-Vis spectrophotometer at 680 nm wavelength. After standardization of the calibration curve, 3 mg of the aqueous extract was dissolved in a 3 ml distilled water. From each of the extracts, 200 mcl was transferred to vials (in triplicates). Then, 200 mcL of FC reagent was added and incubated at room temperature for 5 minutes. Then, 1 ml sodium carbonate was added to the mixture and read using the APEL-100 UV-Vis

spectrophotometer (PD-303UV) at 680 nm wavelength. Absorbance values of the extracts was compared with the calibration curve using the ascorbic acid. The phenolic compound was calculated based on the standard curve of the ascorbic acid and its linear regression as shown in the formula: y = mx+b. Where y represents the OD, m represents the slope of ascorbic acid, and b represents the y-intercept.

Preparation of Hot Water Extract

For the preparation of hot water extract that was used for antibacterial testing, the fruiting body of *T. chioneus* was air-dried and cut into pieces. The shredded pieces were powdered using a blender. In 600 ml of distilled water, 20 g of powdered fruiting body was dissolved in a 1000 ml capacity flask. It was then boiled in a double broiler water in a temperature of 80ºC to 90ºC for two hours as described by Eguchi *et al*. **[11]**. The aqueous extract was filtered using Whatman filter paper no. 1 and stored in a refrigerator prior to the assay.

Antibacterial Assay

Nutrient agar was aseptically poured in sterilized petri plates and were allowed to solidify. Once solidified, the bacterial cultures with approximately 1.5 x 10^8 cfu/mL were evenly spread over the media using sterile cotton swabs. Afterwards, treatment discs containing the extract were seeded in the center of the agar plates using flame sterilized forceps. The plates were then incubated for 18 to 24 hours at room temperature in inverted position. Three replications were prepared for each treatment.

Measurement of inhibition

After 24 hours of inoculation, zone of inhibition was measured using Vernier caliper. The zone of inhibition of bacterial growth caused by the mushroom extract was compared to the zone of inhibition of streptomycin sulphate which served as the positive control and sterile water as the negative control. Formation of zone of inhibition means effectivity of extract against the test pathogen and absence means resistance of the organisms to the extracts. The rating scale that was used are as follows: resistant if the zone of inhibition is less than or equal to 14 mm; intermediate if the zone of inhibition is from 15 to 16 mm; and susceptible, if the zone of inhibition is greater than or equal to 17 mm **[12]**.

Zebrafish (*Danio rerio*) teratogenicity and toxicity assay

The extract was diluted using embryo water (Hank's solution). Different concentrations (5%, 3%, 1%, 0.5% and control) was prepared and 3 mL of each solution was used.

Two milliliters (2 mL) of each treatment concentration was placed in each well of the 32-well ELISA plate together with three embryos at segmentation phase. The ELISA

plates were incubated at 26ºC ± 1ºC. Teratogenic activity was examined under 40× magnification using a compound microscope after 12, 24, 36, and 48 hours of incubation.

Morphological endpoint evaluation of zebrafish was based on the parameters established by Schulte and Nagel **[13]**: lethal (coagulation, tail not detached, no somites, and no heartbeat), teratogenic (malformation of head and tail, scoliosis, growth retardation, stunted tail, and limited movement) and normal. Percentage tail and head malformation was observed per treatment. Hatchability, malformation and mortality rate was recorded and pictures were taken out and death was defined as coagulated embryos.

Statistical Analysis

Experiment was laid out in a completely randomized design (CRD). Data was gathered and analyzed using Analysis of Variance (ANOVA). Duncan's Multiple Range Test (DMRT) was carried out in teratogenic and antibacterial activity to compare the treatment effects at 0.5 level of significance.

RESULT AND DISCUSSION

Mycochemical are chemical compounds derived from or naturally produced by a fungus that perform metabolic functions and serve as protection of the organisms. In this study, it was found out that there were eleven mycochemical compounds present on the mushroom acetate-methanol extract namely: phenols, triterpenes, steroids, fatty acids, sugars, anthraquinones, coumarins, anthrones, tannins, flavonoids, and alkaloids. In this study, the presence of phenols suggest that mushroom have a strong antioxidant and might prevent oxidative damage to biomolecules such as DNA, lipids and proteins which play a vital role in chronic diseases such as cancer and cardiovascular disease. Also, phenols may interfere with all stages of the cancer process, potentially resulting in a reduction of cancer risk **[14]**. Triterpenes was also present which play various important roles for the well-being of human body. Triterpenes frequently demonstrate bioactivity such as antifungal, antibacterial, and antiviral. It can also fight cancer cells and prevent the spread of tumors. Steroids are used as the main treatment for certain inflammatory conditions. They may also be used selectively to treat inflammatory conditions such as rheumatoid arthritis, lupus, and gout. While, the presence of fatty acids suggests that the increasing intake of essential fatty acids could enhance mental and physical performance, help treat some diseases, promote mental health, and improve body composition **[15]**. Sugar has health benefits, it is the source of instant energy, raises blood pressure immediately and helps proper functioning of the brain. Furthermore, some medical practitioners believe that sugar can also heal wounds much faster than medicines. This

indicates that mushroom shows medicinal activity **[16]**. Anthraquinones which can also be found on the mushroom have promising anti-cancer and anti-inflammatory properties that make them useful for pain relief and tissue repair, including in the treatment of arthritis. Some other benefits are constipation relief, colon cleansing and other digestive benefits **[17]**. Coumarins which was also present in the mushroom are a group of important natural compounds that have been found to have multi-biological activities such as anti-HIV, anti-tumor, anti-hypertension, anti-arrhythmia, anti-osteoporosis, pain relief, and preventing asthma and antisepsis **[18]**. The anthrones are used in pharmacy as laxative. They stimulate the motion of the colon and reduce water reabsorption **[19]**. Tannins on the other hand, have also been reported to exert other physiological effects such as to accelerate blood clotting, reduce blood pressure, decrease the serum lipid level, produce liver necrosis, and modulate immune-responses. It can also demonstrate anti-cancer properties, being able under certain conditions to inhibit the growth of cancer cells. Tannins are used in cosmetics for anti- aging skin care and to combat hair loss **[20]**. It is shown that flavonoid was also present on *T. chioneus*. Flavonoids are known to have anti-oxidative activity, free-radical scavenging capacity, coronary heart disease prevention, and anticancer activity **[21]**. The presence of alkaloids suggest that mushroom is a potential anticancer agent since alkaloids are known for their potential use in the elimination and reduction of human cancer cell lines and toxic against cells of foreign organisms **[22]**. Moreover, alkaloid has been reported to have antimicrobial property **[23]**.

Antioxidants have been widely used as food additives to provide protection against oxidative degradation of foods by free radicals. Moreover, phenolic compounds are essential part of human diet and are of considerable interest due to its action as a strong antioxidants and free radical scavengers, to chelate metals, and to interact with enzymes, adenosine receptors, and biomembranes **[24]**. As shown in the Table 1, the mushroom has 31.13 mg GAE/g. This indicates high phenolic content of the mushroom which means it has antioxidant activity since antioxidant activity of plant materials was well correlated with the content of their phenolic compounds. On the other hand, *T. chioneus* exhibited quite low DPPH scavenging activity (26.53%) compared to the standard Cathechin (68.66%). The result from the mycochemical analysis of *T. chioneus* supports that it has anti-oxidant property because of the phenols, coumarins and flavonoids it contains.

Antibacterial Activity

Bioassay disc diffusion method was used to determine the antibacterial activities of the

mushroom hot water extract against *S. aureus* (ATCC 29213) and *E. coli* (ATCC 25992). As shown in the Table 2, the zone of inhibition of hot water extract of the *T. chioneus* was 16.57 mm and 15.66 mm (intermediate) against the gram negative bacteria *E. coli* and gram positive bacteria *S. aureus,* respectively. However, this result were not comparable with the positive control streptomycin sulphate with the mean value of 40.53 and 40.21 (susceptible), respectively. Thus, the anti-bacterial property of hot water extract of *T. chioneus* was more effective in *E. coli* compared to *S. aureus*. Based from these results, the hot water extract of *T. chioneus* can be used as a broad spectrum against the two bacterial species. This is supported with the presence of mycochemicals in *T. chioneus* extract such as triterpenes and alkaloids which are responsible for its antibacterial property. This is also the same with the study of Suffredini *et al.* **[25]** who studied about the antibacterial activity of *T. angulanta* which was considered active against *S. aureus*. Likewise, alkaloids and triterpenes were also detected and denoted that presence of alkaloids and triterpenes maybe the responsible for the antibacterial activity observed in the crude organic extract from *T. angulanta*. The smaller zone of inhibition of the hot water extract than the positive control streptomycin sulfate could possibly be due to the sensitivity of *T. chioneus* to different extraction protocols.

Teratogenic Activity

The harvested fertilized embryos were exposed to different concentrations of *T. chioneus* hot water extract. Mortality was defined as coagulation and no visual heartbeat of embyros. The mortality rates of the *D. rerio* embryos were evaluated after 12, 24, 36, and 48 hours of treatment exposure. Figure 1 shows high mortality rate observed at 5% and 3% concentration at 12 hours observation. The result indicates that hot water extract of the mushroom at 5% and 3% concentration are toxic to *Danio rerio* embryos. These toxic effects of mushroom may possibly be accounted to their bioactive components with efficient inhibitory activity. The result revealed that there was significant difference between the higher concentration (5% and 3%) to lower concentration (1%, 0.5% and control) for 12, 24,36 and 48 hours of exposure (Table 3). This result reveals that mortality of the embryos was manifested based on the quantity of the extract and time of exposure. According to Hill *et al.* **[26]**, the heart is the first organ to develop and function in zebrafish and it is another notable sub lethal effect on the parameter established by Bachman **[27]**. Heartbeat of the embryos was evaluated after 36 hours post treatment application as shown in the Table 4. The highest heartbeat was recorded at the control group of embryos with a mean percentage of 123.00 beats per minute while 106.22 beats per minute for 0.5% concentration. On the other hand, lowest heartbeat rate was recorded on 1% concentration with a mean percentage of 105.33 beats per minute. While absence of heartbeat was observed in higher concentration such as 5%, and

3% due to coagulation at earlier phase. The normal embryonic heart rate of zebrafish was reported closer to human heart rate at 120-180 beats per minute **[28]**. Thus, heartbeat rate observed on zebrafish after 36 hours is closer but slightly lower than normal. As shown in the Table 5, 100% of hatchability was recorded in embryos treated with 0.5% concentration and control while 44.44% hatchability rate was observed at 1% concentration of the extract which is much lower than the first two concentrations. Meanwhile, no hatchability was observed in higher concentration due to early arrested embryos. Additionally, delayed growth of the embryos was observed after 12 hours exposure in 3% and 5%, which are at higher concentrations, later resulted in 100% coagulation after 48 hours. While, the embryos exposed to 1% concentration, hatched late after 36 hours of exposure. And no growth retardation was manifested on the embryos exposed in the control group. For the Sublethal effect of the mushroom extract on the development of embryos, only yolk deformity and malformation of tail (Figure 2) were observed on the hot water extract of the mushroom at 0.5% concentration after 48 hours of treatment and coagulation was the most evident lethal effect of the mushroom that was collected.

Table 1: Radical scavenging activity and total phenolic content of the mushroom

	%RSA	TPC (mg GAE/g sample)
T. chioneus	26.53	31.13
Cathechin	68.66	

Table 2: Zone of inhibition of different treatments

Treatments	Organisms	
	S. aureus	*E. coli*
Hot water extract	15.67 ± 2.26^{b}	16.58 ± 1.66^{b}
Streptomycin sulphate	40.21 ± 2.64^{a}	40.53 ± 2.46^{a}
Control, Distilled water	6.00 ± 0.00^{c}	6.00 ± 0.00^{c}

Means that do not share a letter in a column are significantly different at 0.05 level of significance

Table 3: Mortality rate of zebrafish embryo after 12, 24, 36 and 48 hours of treatment exposure

	Observation time (h)			
Concentrations (%)	12	24	36	48
Control	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	17.00 ± 0.24^{b}
0.5	22.22 ± 0.19^{b}	22.22 ± 0.19^{b}	22.22 ± 0.19^{b}	22.22 ± 0.19^{b}
1	22.22 ± 0.19^{b}	44.44 ± 0.19^{b}	55.56 ± 0.19^{b}	55.56 ± 0.19^{b}
3	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
5	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}

Means that do not share a letter in a column are significantly different at 0.05 level of significance

Table 4: Number of heartbeat rate of zebrafish embryos after 36 hours treatment

Concentrations (%)	Heartbeat (%)
Control	123.00^{a}
0.5	106.22^{a}
1	105.33^{a}
3	0.00^{b}
5	0.00^{b}

Means that do not share a letter in a column are significantly different at 0.05 level of significance

Table 5: Hatchability rate of zebrafish after 48 hours	
Concentrations (%)	Hatchability (%)
Control	100.00[a]
0.5	100.00[a]
1	44.44[b]
3	0.00[c]
5	0.00[c]
Means that do not share a letter in a column are significantly different at 0.05 level of significance	

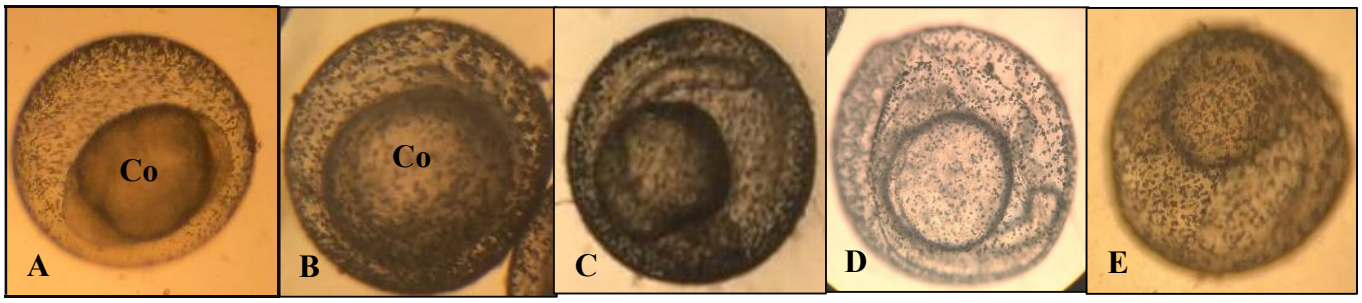

Figure 1: Morphological development of zebrafish embryos exposed on mushroom extract at 12 hours; (A) 5% concentrations; (B) 3% concentrations; (C)1% concentration; (D) 0.5% concentration; (E) control of mushroom extract; Co- coagulated

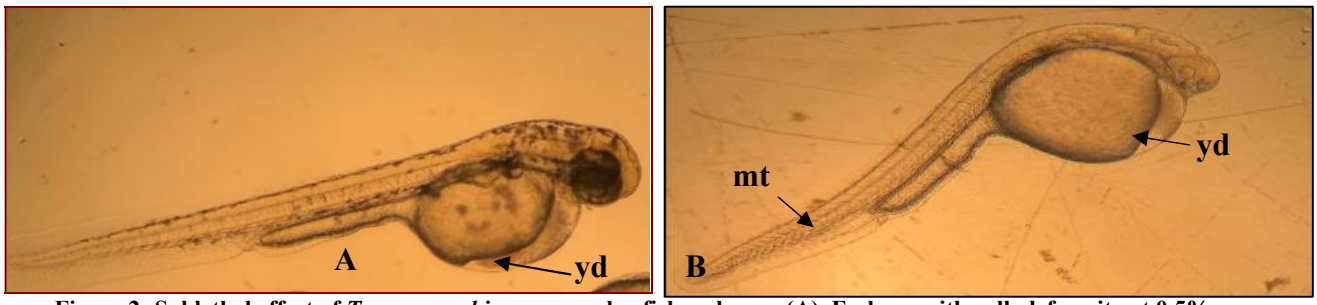

Figure 2: Sublethal effect of *Tyromyces chioneus* on zebrafish embryos. (A). Embryo with yolk deformity at 0.5% concentration after 48 hours post treatment application (hpta).(B) Embryo with malformation of tail at 0.5% concentration and yolk deformity after 36 hpta; mt- malformation of tail; yd- yolk deformity

CONCLUSION

T. chioneus has eleven mycochemical compounds present on the mushroom acetate-methanol extract. It also has an antioxidant although it is quite low as compared to Cathechin. While for the antibacterial of *T. chioneus* hot water extract, shows presence of zone of inhibition in both *S. aureus* and *E. coli* after 24 hours of incubation. Finally, *T. chioneus* also exhibit teratogenic activities against zefrafish embryo and therefore has a potential to be used in the development of anticancer drugs.

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