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PROXIMATE NUTRITIVE COMPOSITION AND TERATOGENIC EFFECT OF *Lentinus sajor-caju* COLLECTED FROM BANAUE, IFUGAO PROVINCE, PHILIPPINES

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ABSTRACT

Lentinus sajor-caju is a wild edible mushroom that is usually found growing on decaying logs in the forest. In the present study, the proximate composition and teratogenic activities of *L. sajor-caju* collected from Ifugao Province were investigated. Based on the proximate analysis, this mushroom contained crude protein (22.06%), crude fiber (21.48%), carbohydrates (59.51%), energy value (338.25 kcal), moisture content (12.21%), ash content (4.89%) and crude fat (1.33%). In zebrafish assay, 100% mortality of embryos was observed at 3% and 1% extract concentrations after 36 hours of exposure and at 0.5% extract concentration after 48 hours. Delayed growth was recorded after 12 hours at 0.5%, 1% and 3% concentrations, which resulted to 100% coagulation after 48 hours. Coagulation was the most lethal effects of the extract. Hatchability was noted after 48 hours at 0.1%, 0.05% and the control embryos. Malformations such as bent tail, hook like tail, scoliosis, yolk deformity, and light pigmentation were observed after 48 hours exposure. Thus, *L. sajor-caju* contained valuable nutrients but exhibited toxic and teratogenic effects to zebrafish embryos.

Keyword: *L. sajor-caju, D. rerio,* teratogen, nutritive composition, Ifugao community

INTRODUCTION

Lentinus is a multicultural genus capable in enduring a wide temperature range, dominant in tropics, and are often located in temperate regions **[1]**. This genus has been categorized under the family Polyporaceae which are group of fungi that becomes corky or woody with age and frequently forming shelflike growths on trees **[1, 2]**. One of the species of this family is the *Lentinus sajor-caju*. This mushroom is a practically usual fungus of the tropical rainforests after good rain and can frequently be seen in troops along fallen logs and up dead trees. Also, it is grayish to brown in relation to their color and appeared as leathery-textured wild edible mushroom with a well-developed central stipe. Moreover, *L. sajor-caju* was considered as edible mushroom **[3]** due to its ability to lessen the difficulties on hunger and malnutrition given that they are very healthful and significantly could impart an alternative remedy for various diseases. Aside from its typical role as being a source of food, *L. sajor-caju* can also enhanced physiological activities such as the antihypertensive effect in hypertensive rats **[4]**.

Nutritionally, wild edible mushrooms are low in calories, fat-free, cholesterol-free, gluten-free, and very low in sodium **[5]**, yet they provide powerful nutrients such as protein, carbohydrate, minerals and vitamins **[6]** that certainly play a role in improving food nutrition. In relation to its antimicrobial activity, edible mushrooms are used in medicine treatments to protect against free radicals. Also, they have been shown to possess antagonistic effects against bacteria, fungi, viruses and cancer **[7]**. Similarly, they are suitable in curing headache, cold and fever as well as in treatment for malnutrition in infants, obesity, and protein deficiency **[8]**. Mushrooms also exhibit toxic and teratogenic effects at certain concentration against *Danio rerio*. Teratogens are agents that cause malformation in the developing embryos. However, teratogenicity testing can be a desirable property because many anticancer drugs are teratogenic and teratogens can be used as anticancer drugs **[9]**.

Igorots of Ifugao Province also known as “people of the mountain” **[10]** culture usually revolves around rice, which is considered as their prestige crop. In rural communities worldwide like them, they considered that edible mushrooms are important food sources and income. Also, they collect mushrooms as food for their family, and sometimes sold in the market for extra income. However, many of the traditional knowledge of indigenous people remained undocumented, particularly on the species richness and relative nutritive composition and also the medicinal features of the mushrooms found in their community. In line with this, there is a need to establish the proximate compositions as well as medicinal properties of mushrooms particularly *L. sajor-caju* collected from Banaue, Ifugao Province.

MATERIALS AND METHODS

Source of the Mushrooms

Fruiting bodies of *L. sajor-caju* was collected from Banaue, Ifugao Province, Philippines. These were brought in the laboratory for tissue culture in a potato dextrose agar plates and incubated at 30°C for 7 days to allow fungal growth. After tissue culture, the fruiting bodies were air-dried for proximate composition analysis.

Proximate Composition Analyses

The nutritional content of *L. sajor-caju* was brought to Lipa Quality Control Center, Bocaue Bulacan, Philippines for proximate analyses. Analyses were based on the guidelines of the Association of Official Analytical Chemist **[11]**. The moisture, ash, crude protein, crude fiber, and crude fat were analysed. The total carbohydrates and energy value were calculated.

Mycelial Mat Production

Twenty ml of mature coconut water was dispensed in a sterile microwavable container. These were sterilized in an autoclave at 121°C, 15 psi for 30 minutes. After which, these were inoculated with mycelia disc from the pure culture of *L. sajor-caju* and incubated at 30°C for 10 days to allow fungal growth. The mycelial mats were harvested, air-dried and pulverized for extraction for teratogenic assessment.

Hot Water Extraction of Mycelia

The functional components of milled mycelia of *L. sajor-caju* were obtained through hot water extraction following the procedure of Eguchi et al. **[12]**. Six hundred ml of distilled water was added to 20 grams of powdered mycelia in a 1000 ml capacity flask. The mixture was placed into a double boiler water bath at 80 to 90°C for two hours. Extract was filtered using a Whatman filter paper. The filtrate extract was diluted using embryo water to prepare 10 ml of the different concentrations (3%, 1%, 0.5%, 0.1%, 0.05% and control) served as treatments.

Maintenance and Acclimatization of Zebrafish

An aquarium comprising of untreated and clean water with continuous aeration at 26±1°C was maintained for adult female and male adult zebrafish at a ratio1:2, respectively. Fish was then acclimatized for one week before using for spawning and fertilization. They were nourished daily with a high protein diet flakes. Excess food was taken out from the aquarium to maintain the high quality of water.

Zebrafish Spawning and Fertilization

Zebrafish was confined in a plastic mesh to prevent cannibalism. After which, the zebrafish was subjected in dark condition by wrapping the aquarium with black plastic bag to allow spawning. After 12 hours in the dark condition, the aquarium was exposed to light condition for another 12 hours. Fertilization occurs 30 min after the light was turned on. Twelve hour post fertilized embryos were siphoned out from the aquarium using a hose and transferred in a beaker. Embryos was rinsed three times with distilled water and placed in a petri plates to check the uniformity and normal conditions of embryos using a compound microscope. Unfertilized or coagulated eggs were discarded.

Zebrafish Teratogenicity and Toxicity Assay

Two ml of each treatment concentration of mushroom extract was dispensed into each well of the 12-well ELISA plate. Four embryos at segmentation phase were transferred into each well containing the different treatments. The plate was incubated at 26°C±1°C. Teratogenic activity was examined under 40X magnification using a compound microscope after 12, 24, 36 and 48 hours of incubation. Morphological endpoint evaluation of treated zebrafish was based on the parameters established by Schulte and Nagel **[13]** and Nagel **[14]**: lethal (coagulation, tail not detached, no somites, and no heart-beat), teratogenic (malformation of head and tail, scoliosis, growth retardation, stunted tail, and limited movement), and normal. Percentage of tail and head malformation was observed per treatment. Hatchability, malformation and mortality rates were recorded.

Statistical Analysis

Experiment was laid out in a completely randomized design (CRD). Data were analyzed using analysis of variance (ANOVA). Duncan's Multiple Range Test (DMRT) was carried out to compare the treatment effects at 5% level of significance. The SPSS program was used for analysis.

RESULTS AND DISCUSSION

Nutritive Composition of *L. sajor-caju*

Edible mushrooms are believed to be a healthy food source due to their high and qualitatively good protein content, low fat and cholesterol content, minerals and vitamins **[15]**. Proximate composition was analysed using the air-dried fruiting bodies of *L. sajor-caju* and the amount of crude protein, crude fiber, crude fat, moisture and ash content as well as the energy value and total carbohydrates were determined per 100g of the sample. The proximate composition of air-dried fruiting bodies of *L.*

sajor-caju is presented in Table 1. Apparently, L. sajor-caju contained crude protein, fiber, carbohydrates, ash, moisture and can consider as source of energy.

Table 1. Proximate composition of air-dried fruiting bodies of *L. sajor-caju*	
Nutrients	**Amount (%)**
Crude Protein	**22.06**
Crude Fiber	**21.48**
Crude Fat	**1.33**
Ash Content	**4.89**
Moisture Content	**12.21**
Total Carbohydrates	**59.51**
Energy value (kcal)	**338.25**
Total carbohydrates and energy were calculated according to the following equation: Total carbohydrates (%) = 100 - (protein+ fat + moisture content +ash content); Total energy (kcal) = 4 x (protein + carbohydrates) + 9 x (fat)	

Proteins of mushroom are known in encompassing nearly all the essential amino acids **[16]**. The presence of proteins in mushrooms is a proof that they are highly nutritious and good for human consumption **[15]**. This crude protein content obtained in this study is closer to the values obtained in *Lentinus squarrosulus* with 22.82% **[17]**. This value is slightly lower than the *Lentinus conatus* (24.62%) but higher than *Auriculariaauricula-judae* (8.36%) **[18]** and *Volvariella volvaceae* (13.38%) **[19]**. Bernas et al. **[20]** disclosed that the differences in the protein contents of mushrooms might be due to the type of species or strain, development stages, the growth substrate, time of harvest and level of nitrogen available in the growth substrate. The result indicates that *L. sajor-caju* is a good protein source. As compared to the other essential foods, only 7.3% proteins are contained in rice, 12.7% in wheat, and 9.4% in maize, thus, *L. sajor-caju* contains much more proteins which are an ideal food source to human nutrition, and therefore, can be able to supplement low protein diets **[21]**.

Crude fiber is also part of the healthy diet. It has a significant role in weight management by functioning as a bulking agent in the digestive system. Mushrooms fibers can reduce appetite, making an individual feel fuller longer and can lower overall calorie intake **[22]**. In the present study, *L. sajor-caju* is also rich in crude fiber (21.48%), which is within the reported value of 3-35% fiber on a dry weight basis **[23]**. The value obtained is closed with the result of Afiukwa et al. (2015) who reported 21.00% protein is present on the same species. These values are much higher than the content of other edible mushrooms such as *Ganoderma* spp. with 3.5% **[24]**, *V. volvaceae* with 3.77% **[19]**, *Plerotus tuber-regium* with 5.3% **[25]** and *L. squarrosulus* **[17]**. The distinctions of the fiber content of mushrooms species was due to their growing location or maybe on nutritional intake during fruiting cycle **[19]**. In contrast with cereals and vegetables, *L. sajor-caju* was good as cereals and vegetables due to its high fiber value in which carrots has only 0.6% and lettuce has 0.2% fiber content. Given that *L. sajor-caju* contained significant amounts of crude fiber, this might be a good source of dietary fiber for supplementation of certain food products with fewer amount of fiber **[21]**.

Generally, mushrooms are a low calorie food which protects heart health and reduces blood pressure with small amount of fat and sugars and without starch and cholesterol **[26, 27]**. *L. sajor-caju* had 1.33% crude fat content, which is much lower than *Pleurotus tuber-regium* with 3.73% (Osuala et al., 2009) and higher than *V. volvaceae* (0.77%). This fat content of *L. sajor-caju* is closed to the fat content of *Lentinus edodes*, *Pleurotus* sp., and *Agaricus bisporus* having 1.70-1.92% fat content **[19]**. Moore and Chi **[28]** reported that this crude fat consists of representatives of all groups of lipid compounds which include free fatty acids, glycerides, sterols, and phospholipids. Among those fatty acids, linoleic acid constitutes higher proportion in which it is the only necessary fatty acid required in the human diet. Likewise, mushrooms are cholesterol-free food similar with vegetables and this is favorable in relation with good health since cholesterol is considered as a risk factor of coronary heart disease and related conditions.

Carbohydrates are major class of biological macromolecules that are essential in the human diet and provide energy to the body. The carbohydrates contents of some wild edible mushrooms from nitrogen free extracts were found to be between 41.00% and 65.00% **[29]**. In this study, *L. sajor-caju* had 59.51% total carbohydrates. This value is higher than the *Ganoderma* spp. *Omphalotus olearius*, and *Hebeloma mesophaeum* having 50.3-50.9% **[24]** and in *P. tuber-regium* and *V. volvaceae* with 31.31- 36.25% **[25]** and lower than *Rusulla vesca*, *Lactarius trivilaris* and *L. tigrinus* **[30]**. This result showed that *L. sajor-caju* is a good source of carbohydrates and may perhaps be able to stimulate the immune system of the human body. In addition, carbohydrates can be used in pharmaceutical purposes, for instance, in type 1 diabetic who use up high fiber diets produce lower blood glucose levels while type 2 diabetics may perhaps improve blood sugar, lipids and insulin levels **[22]**. Also, the beta glucan fibers located in the cell walls of mushrooms accelerate the immune system to fight cancer cells and inhibit tumor from forming **[22]**. Furthermore, ash content is one of the leading nutrients of mushrooms. The result of this study showed that *L. sajor-caju* contains 4.89% ash content which is higher than *L. conatus, L. squarrosulus, L. torulosus*, and *L. cladopus* ranging from 1.52-2.21% **[31]** and inferior to *Pleurotus* sp., and *A. bisporus* with 5.50-6.70% **[19]**. The differences in the amount of nutrients attained might be due to some factors, for instance, the substrate used can influence the chemical composition of mushroom **[32]**. The cultivated mushrooms may perhaps have higher ash content due to organic fertilizers, chemicals or even growth hormone for rapid cultivation of mushrooms used **[19]**. Ash contents can be able to determine the amount and type of minerals in food which is valuable because this reveals the physiochemical properties of foods and growth of microorganisms. Thus, it is vital component in a food nutrition, quality and microbial activity. Also, this ash content indicates that it is relatively contains high mineral content **[15]**.

The moisture content of *L. sajor-caju* was 12.21%. Moisture content of mushrooms indicates that they are highly perishable and promotes susceptibility to microbial growth and enzyme activity which accelerates spoilage **[24]**. Also, Shewfelt **[33]** reported that the removal of moisture in the mushrooms will eventually lessen the risk of microbial spoilage or harmful effects triggered by enzyme. Nutritional composition of mushrooms are greatly affected by its moisture contents which may vary from their harvesting time, maturation period and environmental conditions such as humidity, temperature, growing period, storage condition etc **[34]**.

The energy value of *L. sajor-caju* was calculated as 338.25 kcal. This is higher than the values of wild edible mushrooms collected from Uganda including *Volvariella speciosa*, *Polyporus tenuiculus*, *Termitomyces microcarpus*, *T. tyleramus* and *T. clypeatus* ranging from 220.62-250.46 kcal energy value **[21]**. This result indicates that *L. sajor-caju* was ideal food source and can also be a good source of energy.

Toxic Effects of *L. sajor-caju* Extract in Zebrafish Embryos

Zebrafish has been used as a model in several studies in the field of molecular genetics, vertebrate biology, neurobiology and transgenic research (Nagel, 2002), hence, their importance on toxicology along with drug discovery has been recognized. In this study, the toxic effect of the mushroom extract in developing embryos of zebrafish was investigated. Mortality was determined after 12, 24, 36, and 48 hours of treatment exposure and the results are presented in Table 2. Apparently, after 12 hours of exposure, 8.33% mortality was observed at 1% and 3% concentrations while no mortality was observed at the lower concentrations. However, after 36 hours of exposure, 100% mortality was significantly recorded at 3% and 1% concentrations whereas those at 0.5% concentration had 83.33% mortality. This rate of mortality increased to 100% after 48 hours of exposure. Although some embryos died at 0.05% concentration, no significant difference was observed when compared with the control embryos. These results strongly indicate that the effects of the extract are concentration and time of exposure dependent.

Table 2: Mortality of embryos treated with the varying concentrations of *L. sajor-caju* extract at different observation periods

Extract (%)	Mortality (%)			
	12 hour	24 hour	36 hour	48 hour
Control	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
0.05	0.00^{a}	0.00^{a}	8.33^{a}	8.33^{a}
0.1	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
0.5	0.00^{a}	0.00^{a}	83.33^{b}	100.00^{b}
1	8.33^{a}	8.33^{ab}	100.00^{b}	100.00^{b}
3	8.33^{a}	16.66^{b}	100.00^{b}	100.00^{b}

Values are means of three replicates per treatment. Treatment means with the same letter of superscript are not significantly different at 5% level of significance

This result was similar with the outcome of the recent study of Dulay et al. **[35]** showing that on 0.1% concentration of the ethanolic extract of *L. sajor-caju*, there were no mortality observed from 24 hours treatment exposure up to the last observation period. However, embryos exposed in 2.5% concentration, 16.67% mortality was recorded after 12 hours to 36 hours and significantly increased to 33.33% after 48 hours. Thus, the ethanolic extract of *L. sajor-caju* was considerably toxic to zebrafish embryos and this mushroom might be remarkable sources of toxic elements in pharmaceutical development. These toxic effects of mushrooms may possibly be accounted to their bioactive components with efficient inhibitory activity counter to the propagation of disease-causing agents like cancer cells **[35]**. Likewise, other species of *Lentinus* particularly *Lentinus tigrinus* hot water extract, embryos exposed to 0.05%, 0.1%, and 0.5% concentration survived at 24 hours exposure up to the preceding observation. But, embryos exposed at 0.1% and 0.5% died at 48 hours exposure. On the other hand, embryos treated with 1%, 5%, 10%, and 20%concentration the percentage mortality rate was significantly increased as the exposure to extract prolonged **[9]**.

Heartbeat of Zebrafish Embryos

Heartbeat rate is another notable sub-lethal effect on the parameter established by Shulte and Nagel **[13].** This was observed at the pharyngula stage of each embryo when the tail was prominently pigmented **[9]**. Heartbeat of the embryos was evaluated after 36 hours post treatment application. Table 3 shows the heartbeat rate of the embryos exposed to varying concentrations of *L. sajor-caju* extract. Absence of heartbeat was observed in higher concentrations such as 0.05%, 1% and 3% due to coagulation at earlier phase. The highest heartbeat was statistically recorded at the control group of embryos with a mean percentage of 106.67%. However, a 105.90 beat per minute was recorded at 0.1% concentration which shows no significant difference with the heartbeat rate observed in the control group of embryos. On the other hand, lowest heartbeat rate was recorded on 0.05% concentration with a mean percentage of 93.33%. The normal embryonic heart rate of zebrafish was reported closer to human heart rate at 120-180 beats per minute **[36]**. Thus, heartbeat rate observed in zebrafish embryos is closer but slightly lower than normal.

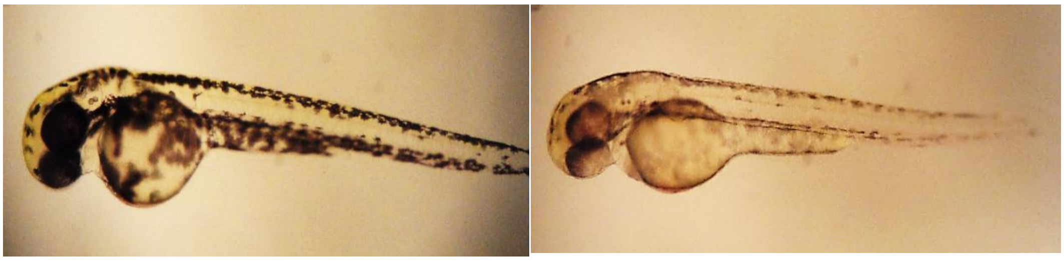
Table 3: Heartbeat rate and hatchability of embryos treated with varying concentrations of *L. sajor-caju* extract

Extract (%)	Heartbeat (per min)	Hatchability (%)
Control	106.67^{c}	100.00^{c}
0.05	93.33^{b}	50.00^{b}
0.1	105.90^{c}	41.67^{ab}
0.5	0.00^{a}	0.00^{a}
1	0.00^{a}	0.00^{a}
3	0.00^{a}	0.00^{a}

Values are means of three replicates per treatment. Treatment means with the same letter of superscript are not significantly different at 5% level of significance.

Hatchability of Zebrafish Embryos

Hatching of the embryos was completed after 48 hours of treatment application (Figure 1). Control embryos showed the highest hatchability of 100%, while 50% hatchability was recorded in 0.05% treated embryos and 41.67% hatchability was observed in 0.1% treated embryos. However, no hatchability was observed in higher concentrations (0.5%, 1% and 3%) due to early arrested embryos (Table 3). The hatchability of embryos was affected by varying concentrations and time of exposure in *L. sajor-caju* hot water extract. In the previous studies, the varying concentrations of *L. tigrinus* extract reduced the hatchability of the embryos. When the concentration increases, the percentage of the hatchability decreases. Delayed development also might be the cause of the low hatchability of the embryos which is one of the main sub-lethal effects of the extract. Likewise, distinctive developmental abnormalities could interrupt the hatching process.



A **B**

C

Figure 1: Hatched zebrafish embryos after 48 hpta.(A) control embryo, (B) embryo at 0.05% concentration, (C) embryo at 0.1% concentration.

Teratogenic Effects of *L. sajor-caju* Extract in Zebrafish Embryos

The different embryos exposed to different treatment concentrations of *L. sajor-caju* hot water extract were evaluated based on the parameters established by Nagel **[14]**. Growth retardation or delayed growth is significant parameter in determining the teratogenic effects of the extracts tested **[35]**. In this study, delayed growth was the most manifested teratogenic effect of *L. sajor-caju* hot water extract that caused mortality on the zebrafish embryos at higher concentrations. On the other hand, coagulation was the highly observed lethal effects on the embryos. Figure 2 shows the delayed growth after 12 hours at higher concentrations (0.5%, 1% and 3%) and later resulted to 100% coagulation after 48 hours. Moreover, lower concentrations of the extract (0.05%, and 0.1%) were considerably not significantly different with each other. No growth retardation was noticed in the control embryos. This result is consistent with the findings of Dulay et al. **[35]** delayed growth of the embryos is due to the increasing concentration. *L. sajor-caju* ethanolic extract at 2.5% concentration revealed high percentage of delayed growth as well as in 1% of *Pleurotus ostreatus* ethanolic extract. In addition, *L. tigrinus* hot water extract at higher concentration (0.5% to 10%) causes delayed growth in the zebrafish embryos prior to severe malformations **[9]**.

One of the most usual morphological deformities in teratogenicity assessment is tail malformation **[35]**. In this study, *L. sajor-caju* extract exhibit a number of tail malformations such as; hook-like tail and bent tail on different concentrations after 48 hours. Also, scoliosis or flexure, yolk deformity and light pigmentation were also observed. However, malformations were not observed at 0.5% or higher concentrations after 48 hours due to early arrested growth of embryos.

Teratogenic effects of *L. sajor-caju* on zebrafish embryos were illustrated in Figure 3. Hook-like tail was observed in concentrations of 0.1% and 0.05% after 48 hours of exposure, whereas, bent tail was observed at 0.1% concentrations of the extract. In addition, scoliosis or flexure were significantly recorded at 0.05% concentration as well as light pigmentation at 0.05% and 0.1% and yolk deformity at 0.05% concentrations after 48 hours post treatment application. These tail malformations and pericardial edema are common endpoints of zebrafish embryos once exposed to the known embryo-toxic and teratogen compounds such as retinoic acid, valproic acid, methoxyacetic acid, boric acid and hydroxyurea **[37]**.

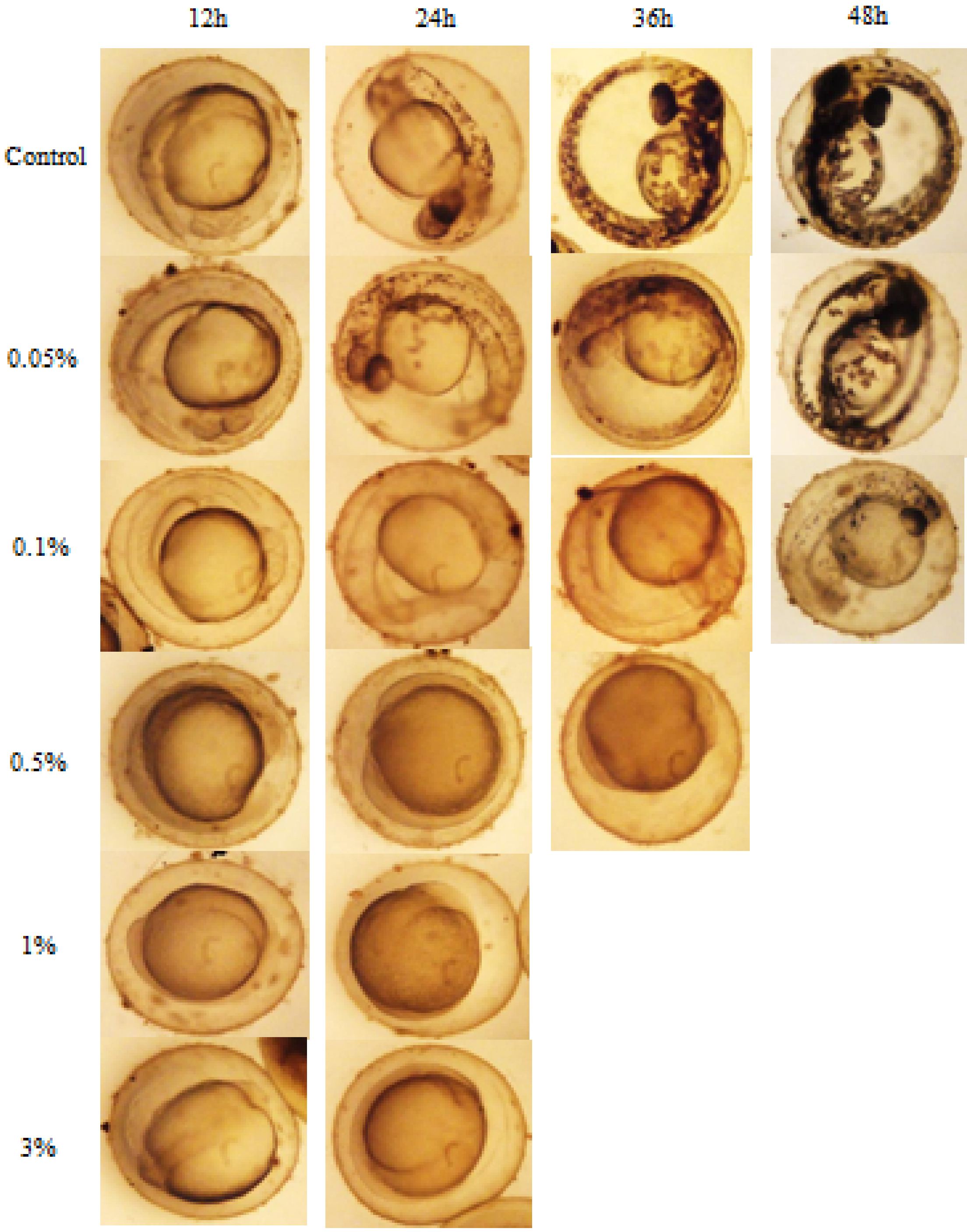


Figure 2: Morphology of embryos exposed to the different concentrations of *L. sajor-caju* extract after 12, 24, 36, and 48 hours

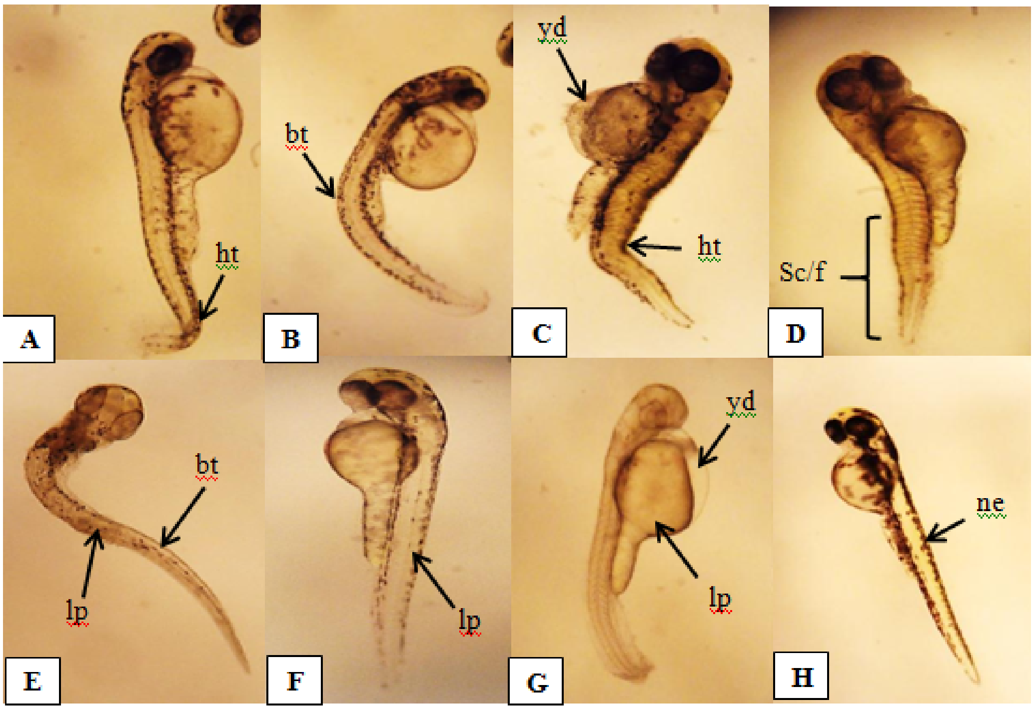


Figure 3: Teratogenic effects of *L. sajor-caju* on zebrafish embryos. (A and C) embryo with hook-like tail, (B and E) embryo with bent tail, (C and G) embryo with yolk deformity, (D) embryo with scoliosis or flexure, (E, F, and G) embryo showing light pigmentation, (F) normal hatched control embryo. ht – hook like tail; bt – bent tail; yd – yolk deformity; Sc/f – scoliosis or flexure; lp – light pigmentation; ne – normal embryo.

CONCLUSION

Based on the result of the study, it is concluded that *L. sajor-caju* consists of nutritive components which are considered as essentials to the human body. It was rich in crude protein which has 22.06%, 21.48% crude fiber, 59.51% carbohydrates, 338.25 kcal energy value, as well as, 12.21% moisture content, 4.89% ash content and low fat content which has only 1.33%. Therefore, *L. sajor-caju* is considered to be a healthy food and probable source of nutrients in which they contribute to the food value and highly nutritious in the diet of an individual. In the teratogenic activity, toxic effect was recorded particularly coagulation at higher concentrations. Sub-lethal effects including malformations of tail such as hook like tail, bent tail, scoliosis or flexure, yolk deformity, and light pigmentation were also observed at lower concentrations after 48 hours exposure. Thus, *L. sajor-caju* extract exhibit toxic effects at higher concentrations while malformations at lower concentrations as the exposure prolonged.

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