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Toxicity Assessment of *Lactococcus lactis* IO-1 Used in Coconut Beverages against *Artemia salina* using Brine Shrimp Lethality Test

Olaide Olawunmi Ajibola[1*], Samuel Lihan[2], Ahmad Hussaini[1], Rosmawati Saat[1], Idris Adewale Ahmed[3], Wahab Abideen[4], Fazia Mohamad Sinang[1], Ngieng Ngui sing[1], Gbadebo Clement Adeyinka[5]

1- Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, Kota Samarahan, 94300, Malaysia.

2- Institute of Biodiversity and Environmental Conservation, Universiti Malaysia Sarawak, Kota Samarahan, 94300, Malaysia.

3- Center for Natural Products Research and Drug Discovery, University of Malaysia, 50603, Kuala Lumpur, Malaysia.

4- Department of Microbiology, Faculty of Basic and Applied Science, Osun State University, Osogbo, Nigeria.

5- Department of Chemical Engineering, Mangosuthu University of Technology, 51, Umlazi, Durban, South Africa.

Abstract

Background and objective: Plant-based fermented foods containing favorable micro-organisms have been used to improve diets. Starter microorganisms may produce toxic compounds that are hazardous to consumers. Brine shrimp lethality test is a convenient and appropriate assay to check toxicity of samples. The aim of this study was to investigate toxicity of pasteurized coconut beverages at 70°C, 80°C and 90°C for 25, 15 and 5 min, respectively, and unpasteurized coconut beverages fermented by *Lactococcus lactis* against *Artemia salina* nauplii.

Material and methods: After extraction of coconut beverages fermented by *Lactococcus lactis* using methanol, cytotoxicity was assessed using (lethality concentration). Newly 10 hatched *Artemia salina* nauplii were transferred into various concentrations (in replicates) of the fermented sample extracts. After 24 h, survived *Artemia salina* nauplii were counted and lethality concentration was assessed. The brine shrimp lethality test was used to investigate sample toxicity at various doses from 1 to 500 µg ml^{-1} at various time intervals.

Results and conclusion: The fermented extracts included low larvicidal potential against *Artemia salina* nauplii. Correlations were reported between the extract doses and percentage mortality of nauplli brine shrimp. The pasteurized fermented extracts were less toxic and cheaper. Interestingly, starter culture, fermentation, thermal treatment and time contributed to breaking down of hydrolysable tannins and larger polyphenolic compounds, producing smaller compounds with lower toxicity responses in brine shrimp lethality test. The four probiotics beverage extracts included non-cytotoxic activity as presented by low mortalities in brine shrimp lethality test. In conclusion, these extracts can be used to justify probiotic production of beverages.

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***Corresponding author:**
Olaide Olawunmi Ajibola,
Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, Kota Samarahan, 94300, Malaysia.

Tel: +60166513450
Fax: +6082583160
E-mail: 16020010@siswa.unimas.my

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1. Introduction

Coconut (*Cocos nucifera* Linn.) is one of the perennial plants cultivated for their pharmacological benefits. It belongs to family of Arecaceae [1,2]. Scientific reports have revealed that coconut beverages include a wide range of pharmacological effects e.g. antithrombotic, anti-bacterial, antioxidant, immunostimulatory, hypolipidemic, cardioprotective, antidiabetic and antiviral effects [3,4]. Fermented coconut beverages are famous in Asian countries, especially South-East Asian countries. People in these regions commonly believe that consumption of traditionally prepared fermented plant beverages includes potentials to cure most illnesses [5].

Fermented foods are rich in bioactive microorganisms, which offer health benefits to their consumers and enhance

the overall food quality [6]. Production of the plant-based fermented foods using specific microbial cultures such as lactic acid bacteria (LAB) (Lactobacillus, Bifidobacterium) and yeast strains (Saccharomyces) for the enhancement of food nutritional and pharmaceutical values has widely been reported [7]. *Lactococcus* (*L.*) *lactis* subsp. *lactis* IO-1 has successfully been used as a starting culture to develop lactic acid from lactose and hydrolysis of citric acid and casein by fermentative pathways [8].

The strain is regarded as generally recognized as safe by scientists because of its probiotic properties, including production of bacteriocins [8]. Special properties of the strain from other LABs include its ability to ferment xylose to produce L-lactic acid [8]. Bioactive compounds from fermented crop beverages can be good alternative beverage supplements in regular medications to control metabolic disorders [9]. However, consumer safety includes great concerns since control of specific microorganisms is difficult and contamination may result in intoxication by pathogenic microorganisms [10].

Although coconut beverages are used in traditional medicines to treat several diseases, the current knowledge on fermented coconut beverages is rather scarce, especially fermentation with probiotic bacteria such as Lactobacillus and Bifidobacterium [10]. However, scientific evidence is necessary to prove that lactic acid fermentation systems can enhance medicinal and pharmacological activities of coconut beverages using probiotic *L. lactis*. However, probiotic coconut beverages are consumed as functional beverages in human medicine and nutrition [11].

These effects have not been proven scientifically. This can be due to production of micronutrients, vitamins, amino acids and glucuronic acid during lactic acid fermentation. Mixed cultures of yeasts and LAB have been used to produce inexpensive probiotic coconut beverages [12]. However, some of these probiotic bacteria can produce toxins during metabolic processes, which can decrease their uses and create hazards for the consumers. Various assays have been developed to investigate potential toxicity of these bacteria on species of *Thamno cephalus platyurus*, *Artemia* (*A.*) *urmiana*, *A. salina* and *A. franciscana*. The brine shrimp lethality test (BSLT) was investigated as a suitable probe for the preliminary assessment of toxicity [13].

Over the last decade, toxicity screening of wider varieties of food and plant products have been carried out using BSLT as a method of choice by many researchers [13]. Of various *Artemia* species, *A. saliana* is the most commonly studied species, appearing in more than 90% of all studies on *Artemia* species [14]. As an alternative bioassay for the toxicity investigation of plant extracts [13], BSLT is widely used in toxicity assessment of cyanobacteria, dental materials, metal ions, heavy metals, algae, nanoparticles as well as screening of food products [14]. The procedure is simple, affordable, non-aseptic and high potential for bioactive chemicals [14]. Furthermore, this popular In vivo assay has shown a successful assay for bioassay-guide fractionation of active cytotoxins [14]. More importantly, good correlations have been reported for the lethal concentrations that kill 50% of the exposed population lethality concentration (LC_{50}) [14]. In this study, toxicity of various coconut beverages fermented by *L. lactis* were assessed against *A. salina*. This study was carried out based on the mortality of *A. salina* brine shrimps at six different concentrations (1, 10, 50, 100, 250, and 500 µg ml^{-1}) of fermented coconut beverage (UPW= unpasteurized coconut beverages, pasteurized coconut beverages= PCW90, PCW80 and PCW70) extracts at 0, 24 and 48 h.

Several researchers previously focused on beneficial effects of probiotic bacteria on the general health of humans and animals, including improvement of immune system and enhanced protection against intestinal pathogens [5-8]. However, the bacterial safety is less reported. Therefore, the present study assessed toxicity of the selected coconut beverages fermented by *L. lactis*.

2. Materials and methods

2.1 Sample preparation

Fresh crops of *Cocos nucifera* Linn were harvested in Kota Samarahan, Sarawak, Malaysian Borneo. The coconut crops were transferred to the laboratory for assessment with no microbial or physical damages. The *Cocos nucifera* Linn juices were prepared by perforating crops using stainless steel knife after main epicarps were cleaned with sterile water.

2.2 Laboratory Preparation of Coconut juice

The *Cocos nucifera* Linn juice was prefiltered through 0.8-µm polysulfone filters (TISH Scientific, USA) before being filtered through 0.65-µm filter papers (TISH Scientific, USA). The filtrate was dispensed into sterile transparent containers sealed for UPW Then, UPW was transferred into disinfected sealed bottles for pasteurization. Bottles were immersed in water bath at temperatures of 90, 80 and 70°C for 5, 15 and 25 min (PCW90, PCW80 and PCW70) respectively, prior to storage at 4°C until further analyses. In this study, thermal processing of the coconut water was carried out at various temperatures and times to recreate the processing scenario that coconut juice products undergo after fermentation.

2.3 Cell collection and cultivation environment

The *L. lactis* IO-1 strain was previously isolated by Chinachoti et al. [15] and its In vitro potential probiotic properties were reported by Carvajal-Zarrabal et al. [16]. The probiotic IO-1 (provided by the Biochemistry Labor-

atory, Universiti Malaysia Sarawak) was sub-cultured thrice to achieve an active cell culture for the propagation of inoculums by inoculating the strain into artificial broth media (containing 20 g of glucose and 5 g of yeast extract per L) and incubating them at 37°C for 18 h. To prepare starting cultures, cells were harvested by centrifugation at 7500 ×g for 12 min and washed once with 0.85% sodium chloride. Then, cultures were diluted with sterile CW to achieve an optical density of OD_{575} = 0.401 using UV-1601 Spectrophotometer [Shimadzu, Kyoto, Japan] of approx. 10^8 CFU ml^{-1} using standard plate count method.

2.4 Sample preparation

The four media (400 ml) were inoculated using 0.4% (v v^{-1}) pure *L. lactis* IO-1 (10^8 CFU ml^{-1}). Batch processing was carried out statically at 30°C for 2 days using conical flasks (500 ml). Flasks were then agitated at 120 rpm with initial pH of 6.8. Fermented samples were collected at 24-h intervals.

2.5 Fermented sample extraction procedure

In this study, method of Mahayothee et al. [17] was adopted with mild modifications. Briefly, extraction was carried out using methanol as solvent with a ratio of 1:5 v v^{-1} of fermented sample to methanol. Sample was agitated at 120 rpm for 6 h at 25°C using orbital shaker (New Brunswick Scientific, Edison, New Jersey, USA). Sample was filtered through Whatman no. 4 papers. The fermented filter sample extract was heated at 40°C using rotary evaporator (R-114, Buchi, Switzerland). For toxicity analysis, a concentrated extract of 10 mg was volumetrically adjusted using 10 ml of dimethyl sulfoxide.

2.6 Toxicity testing against brine shrimps

2.6.1 Hatching brine shrimp cysts

Hatching of the brine shrimp cysts (Figure 1a) was carried out with little modifications using method of Omeke et al. [14]. Active nauplii (Figure 1b) were prepared after hatching with plastic pipettes.

2.7 Brine shrimp bioassay

The lethality bioassay of the extracted fermented samples was carried out using BSLT based on a method by Omeke et al. [14]. Each well was filled with ten freshly hatched *A. salina* nauplii. The positive control group included combinations of 5 mg of thymol in 5.0 ml of sea water with ten nauplii. The negative control group included 0.5 ml of dimethyl sulfoxide and 4.50 ml of sea water with ten nauplii. Each experiment was carried out in triplicate. Plates were incubated at 25°C for 24 h under direct light. Every 6 h, mortality rate of the nauplii was investigated using light microscope. During the study, no feed or air was needed since feed of brine shrimps with dry yeast suspension during the toxicity assessment was considered insignificant [18]. Populations of dead and survived nauplii were counted in each well and LC_{50} was calculated. Based on Thangapandi Veni calculations [19], toxicity characteristics of the extracts were estimated as follows [20]:

$$\text{Mortality (\%)} = \frac{D\ test - D\ control}{A\ control} \times 100 \qquad \text{(Equation 1)}$$

Where, D_{test} was the population of dead larvae in each test plate, $A_{control}$ was the population of live larvae in control plates, and $D_{control}$ was the population of dead larvae in each control plate.

2.8 Statistical analysis

Data were analyzed using SPSS Software v.21.0 (IBM Analytics, USA) to calculate LC_{50} values of the *A. salina* nauplii for each extract concentration ($P<0.05$) [21]. All calculations were carried out in triplicate.

3. Results and discussion

Fermented crude extracts (UPW, PCW90, PCW80 and PCW70) samples at 24-h intervals showed various results for the mortality of brine shrimps (Table 1). For each 24-h interval sample, the four fermented extracts were tested at six concentrations (1, 10, 50, 100, 250, and 500 µg ml^{-1}). The LC_{50} was recorded at the highlighted intervals using BSLT (Table 1). Results showed that concentrations at 500 µg ml^{-1} killed shrimps. *Artemia* species was shown to help various toxicity assessments to identify bioactive compound in crude plant extracts [14]. The BSLT is one of the reliable routine assessments. This assay is appropriate to screen certain food additives, heavy metals, plant extracts, pesticide and pharmaceutical components for toxicity [14]. The BSLT has been popular due to its easy use, high sensitivity and low cost [14].

Natural deaths, which occurred in blank seawater and wells with negative and positive controls, usually did not exceed 24%. This was possibly due to the lack of oxygen because most of the shrimps did not survive 48 h after the assay [18].

Factors such as salinity temperature and composition of the media and larvae maturation affect the natural death. This study showed the maximum and the minimum lethal concentrations as 500 and 1 µg ml^{-1}, respectively. Direct correlations were demonstrated between the number of mortalities and the fermented samples. Order of the extract toxicity was as follows:

UPW > PCW90 > PCW80 > PCW70

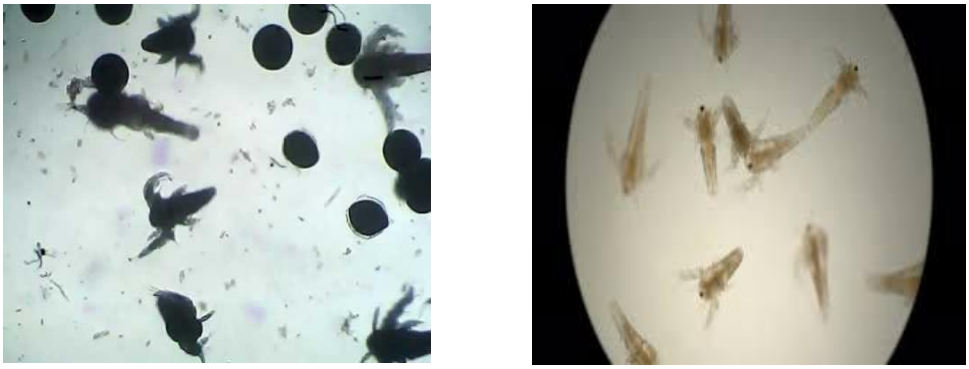

Figure 1. Microscopic observations of a) hatching *Artemia salina* cysts and b) nauplii of *Artemia salina*

Table 1. Average death rate of *Artemia salina* nauplii at various concentrations of coconut beverages containing *Lactococcus lactis* (mean ±SD, *n* = 3)

Extract sample	Concentration (µg ml^{-1})							LC_{50} (µg ml^{-1})
	Time (h)	1	10	25	50	100	500	
UPW	0	0.33±0.58	1.00±1.73	1.33±0.58	1.67±1.15	2.33±1.53	3.00±0.00	4800.49
	24	0.00±0.00	0.67±0.58	1.00±0.00	1.30±1.15	2.33±0.58	2.33±0.58	6567.46
	48	0.00±0.00	7.33±0.47	1.33±1.10	1.67±1.15	2.33±0.58	2.33±0.58	7158.15
PCW90	0	0.33±0.58	0.67±1.15	1.33±1.53	2.00±1.00	2.33±0.58	2.67±1.15	5712.20
	24	0.30±0.58	0.67±0.58	0.33±0.58	1.33±1.15	2.00±1.00	2.33±2.08	6879.87
	48	0.00±0.00	0.33±0.58	0.67±1.15	1.33±1.52	1.33±0.58	2.00±1.00	8070.42
PCW80	0	0.00±0.58	0.33±1.53	1.00±1.53	1.00±1.00	1.33±0.58	2.33±1.15	5680.04
	24	0.33±0.58	0.67±0.58	0.33±0.58	1.33±1.15	1.67±1.00	2.33±2.08	7297.50
	48	0.00±0.00	0.33±0.58	0.67±1.15	0.67±0.58	1.33±1.15	2.00±1.00	8272.28
PCW70	0	0.00±0.00	0.67±0.58	1.00±1.73	1.33±0.58	1.67±1.15	2.33±0.58	6531.00
	24	0.00±0.58	0.33±0.58	0.67±1.15	1.33±1.52	1.33±0.58	2.00±1.00	8070.42
	48	0.00±0.00	0.33±0.58	1.00±0.00	1.00±1.00	1.33±0.58	2.33±0.58	9261.30
+C		5.00±0.82	7.33±0.47	10.0±0.00	10.0±0.00	10.0±0.00	10.0±0.00	10.58
–C		0	0	0	0	0	0	0

+C, positive control; -C, negative control
PCW= pasteurized coconut beverages, UPW= unpasteurized coconut beverages

Fermented coconut beverage extracts showed various mortality results for each sample as presented in Figures 2-5. Based on the results from this study, all fermented samples showed the least effect on mortality. Some lactic acid strains have been shown to produce antioxidant, GABA and essential amino acids, vitamin B_{12} as well as elements and minerals [12]. Furthermore, LAB are useful in improving animal health [22], preserving nutritional quality [23], preventing gastrointestinal infection [24], improving production in animals [25], improving digestibility of crude proteins [26], decreasing deleterious effects of mycotoxins [27] and enhancing bee health [28]. Results of this study are similar to those of Quiao-Won et al. [13], who reported kombucha fermentation cytotoxicity from various substrates. When comparing BSLT (LC_{50}) values of the fermented samples at 24-h intervals during the lactic acid fermentation, BSLT (LC_{50}) values of PCW70 were significantly the lowest ($P<0.05$), followed by those of PCW80, PCW90 and UPW. It was suggested that severity of the thermal treatment (pasteurization) in PCW90, PCW80 and PCW70 samples before lactic acid fermentation contributed to decreases in cytotoxicity through the oxidation and subsequent polymerization and degradation of the toxic hydrolysable compounds [29]. Moreover, heat treatment (pasteurization) duration of the three samples (PCW90, PCW80 and PCW70) before fermentation contributed to decreases in toxicity values, contrary to metabolic processing, processing duration and pasteurization [30]. Therefore, this decreased toxicity is one of the important technical characteristics used to select LAB starters. Furthermore, LC_{50} of these four extracts were 1-500 µg ml^{-1}. Thus, they showed low cytotoxicity and mortality (Table 1).

The lowest effect was from fermented PCW70 followed by PCW80 and PCW90. It can be suggested that fermented PCW70, PCW80 and PCW90 were more appropriate for the brine shrimps, compared to that the fermented UPW was. This could be due to the absorption and metabolism of the phenolic compounds, affected by 1) degrees of glycosylation/acylation, 2) fundamental structu-re, 3) conjugation with other phenolics, 4) molecular sizes, 5) degrees of polymerization, and 6) solubility [31].

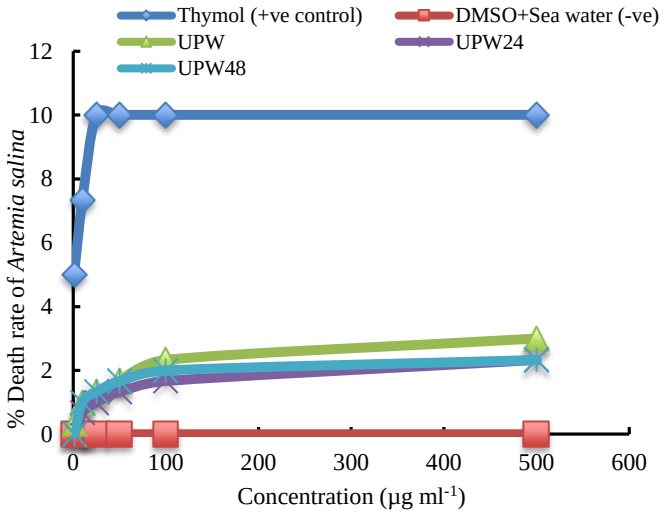

Figure 2. The mortality proportion of *Artemia salina*, 24 h after exposure to various concentrations of the fermented UPW extract at 0, 24 and 48 h

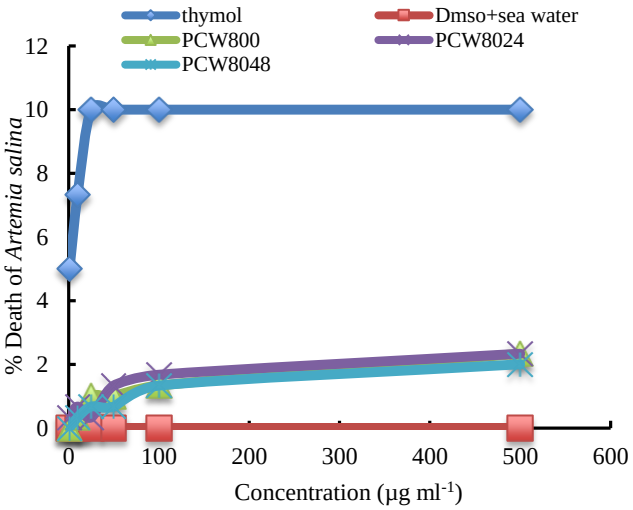

Figure 4. Mortality rate (%) of the brine shrimps, 24 h after exposure to various concentrations of the fermented PCW80 extract at 0, 24 and 48 h; pasteurized coconut beverages= PCW

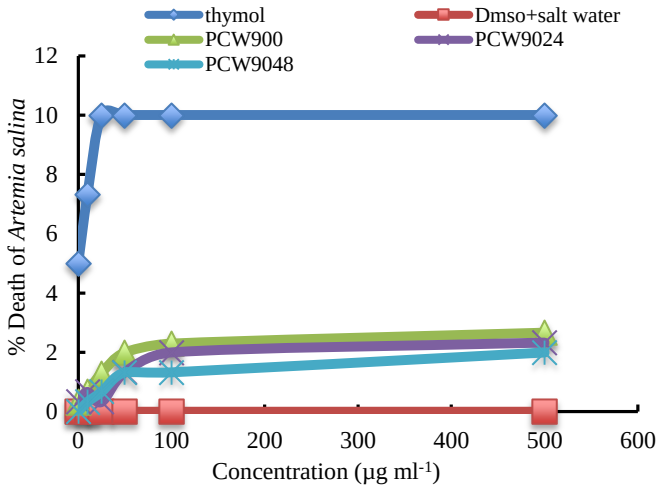

Figure 3. Death rate (%) of the brine shrimps, 24 h after exposure to various concentrations of the fermented PCW90 extract at 0, 24 and 48 h; pasteurized coconut beverages= PCW

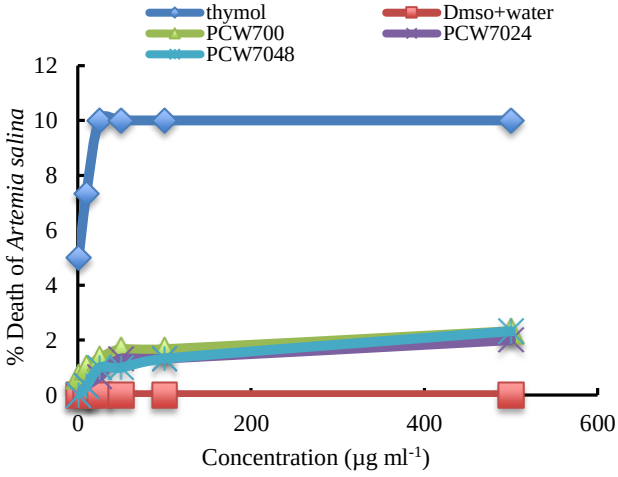

Figure 5. Mortality rate (%) of the brine shrimps, 24 h after exposure to various concentrations of the fermented PCW70 extract at 0, 24 and 48 h; pasteurized coconut beverages= PCW

Therefore, presence of tannin, coumarins, triterpenoids, flavonoids and another toxic elements were verified. Heating during the pasteurization distinguished toxic elements from these matrices and broke down larger hydrolyzable polyphenolics compounds, producing smaller compounds that displayed decreased toxicity before fermentation [32]. Furthermore, coconut water might contain phenolic compounds in stable forms of glycosides, which could be deglycosylated into aglycones by heat and hence increased their non-cytotoxicity. However, further investigations are necessary to support these suggestions. Based on the LC_{50} values ($P<0.05$), all fermented extracts showed a significantly lower mortality, compared to positive control (Table 1).

Cytotoxicities of the beverage extracts at LC_{50} were compared to the toxicity index of Meyer or Clarkson [33,34]. Extracts with LC_{50} less than 1000 µg ml^{-1} were toxic, while extracts with LC_{50} higher than 1000 µg ml^{-1} were referred to as non-toxic [33,34]. In addition, sample extracts were classified in the following order according to Clarkson's toxicity requirements: extracts with LC_{50} exceeding 1000 µg ml^{-1} were classified as non-toxic, LC_{50} exceeding 500-1000 µg ml^{-1} as low cytotoxicity, LC_{50} exceeding 100-500 µg ml^{-1} as medium toxic and LC_{50} exceeding 0-100 µg ml^{-1} as extreme toxic [32], Moreover, the reported high mortality in fermented UPW (> 1000 µg ml^{-1}) might not include significant toxicity concerns to wider uses in dietary supplements and foods. Nevertheless, thermal processing is necessary to decrease toxic bioactive compounds. Therefore, development of non-thermal processes that lead to better antioxidant and color retention may be beneficial, compared to the three samples [35]. This

significant lethality of *L. lactis* in the four coconut beverage extracts on brine shrimps can be used to establish an extremely low existence of potent cytotoxic compounds [19].

4. Conclusion

It can be concluded that all four probiotics beverage extracts used in this study include non-cytotoxic activities, as shown by low mortality rates in BSLT. These extracts can be used to justify the wide acceptability of *L. lactis* for the probiotic production of beverages, which are safe for consumption with no further health risks to humans.

5. Acknowledgements

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6. Conflict of interest

The authors declare no conflicts of interests.

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ارزیابی سمیت *لاکتوباسیلوس لاکتیس* IO-1 استفاده شده در شیره نارگیل در مقابل *آرتمیا سالینا* با آزمون مرگ و میر میگوی آب شور

اولاید اولاومی آجیبولا[1*]، ساموئل لیهان[2]، احمد صالحین حسینی[1]، روسماواتی سات[1]، ادریس آدوال احمد[3]، وهاب عابدین[4]، فاضیا محمد سیناگ[1]، نجینگ نجی[1]، گبادبو کلمنت آدینکا[5]

۱- دانشکده علوم و تکنولوژی منابع، دانشگاه ساراواک مالزی، کوتا ساماراهام، ۹۴۳۰۰، مالزی.

۲- انستیتو حفاظت از محیط زیست و تنوع زیستی، دانشگاه ساراواک مالزی، کوتا ساماراهام، ۹۴۳۰۰، مالزی.

۳- مرکز تحقیقات فرآورده های طبیعی و کشف مواد مخدر، دانشگاه مالزی، ۵۰۶۰۳، کوالا لامپور، مالزی.

۴- گروه میکروب شناسی، دانشکده علوم پایه و کاربردی، دانشگاه ایالت اوسان، اوزوگبو ، نیجریه.

۵- گروه مهندسی شیمی، دانشگاه فنی مانگوسوتو، ۵۱، اولمازی، دوربان، افریقای جنوبی.

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***نویسنده مسئول**

اولاید اولاومی آجیبولا،
دانشکده علوم و تکنولوژی منابع، دانشگاه ساراواک مالزی، کوتا ساماراهام، ۹۴۳۰۰، مالزی.

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پست الکترونیک:
olaideajibola@gmail.com

چکیده

سابقه و هدف: مواد غذایی تخمیری گیاهی حاوی ریزاندامگان‌های[1] مطلوبند و برای بهبود رژیم غذایی مورد استفاده قرار گرفته اند. ریزاندامگان‌های آغازگر ممکن است ترکیباتی سمی تولید کنند که برای مصرف کننده مضر می‌باشند. آزمون مرگ و میر میگوی آب شور روشی مناسب و راحت برای بررسی سمیت برخی نمونه‌ها می‌باشد. هدف این مطالعه بررسی سمیت شیره نارگیل پاستوریزه شده در درجه حرارت‌های ۷۰ ، ۸۰ و ۹۰ درجه سلسیوس به ترتیب به مدت ۲۵، ۱۵، ۵ دقیقه و شیره نارگیل پاستوریزه نشده و تخمیر شده توسط *لاکتوباسیلوس لاکتیس* علیه *آرتمیا سالینا ناپولی* بوده است.

مواد و روش‌ها: پس از استخراج شیره نارگیل تخمیر شده توسط *لاکتوباسیلوس لاکتیس* با متانول، سمیت سلولی نمونه‌ها (غلظت کشندگی) بررسی شد. *آرتمیا سالینا ناپولی* نسل دهم در غلظت‌های مختلف (به دفعات) به عصاره‌های نمونه تخمیری انتقال داده شدند. پس از ۲۴ ساعت، زنده‌مانی *آرتمیا سالینا ناپولی* شمارش و غلظت کشندگی بررسی شد. در آزمون مرگ و میر میگوی آب شور از محدوده غلظتی ۱ تا ۵۰۰ میکروگرم در میلی لیتر برای بررسی سمیت نمونه‌ها در فواصل زمانی گوناگون استفاده شد.

یافته‌ها و نتیجه‌گیری: عصاره‌های تخمیر شده پتانسیل کمی برای کشتن لارو *آرتمیا سالینا* ناپولی داشتند. بین مقدارهای عصاره و درصد مرگ و میر میگوی آب شور ناپولی همبستگی وجود داشت. عصاره‌های تخمیر شده پاستوریزه سمیت کمتر داشتند و ارزانتر بودند. جالب اینکه کشت آغازگر، تخمیر، تیمار حرارتی و مدت زمان لازم برای تجزیه تانین‌های قابل آبکافت و ترکیبات پلی فنولی بزرگتر، منجر به تولید ترکیبات کوچکتر با سمیت کمتر در آزمون مرگ و میر میگوی آب شور شدند. چهار عصاره زیست‌یار[2] شیره سمیتی برای سلول نداشتند که با کشندگی اندک در آزمون مرگ و میر میگوی آب شور نشان داده شد. در نتیجه، این عصاره‌ها می‌توانند برای تنظیم تولید نوشیدنی‌های زیست‌یار مورد استفاده قرار گیرند.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

[1] Microorganisms
[2] Probiotics