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Toxicity evaluation of coconut oil extracted with switchable hydrophilicity solvent (SHS) as a potential solvent contamination indicator

Jeleen Joyce F. Neri, Janus B. Pansacala*

Chemistry Department, College of Science and Mathematics, Western Mindanao State University, Normal Road, Baliwasan, Zamboanga City, Philippines

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Abstract

Toxicity screening of Coconut Oil Extracted with Switchable Hydrophilicity Solvent (COCOSHS) and consideration for presence of trace Switchable Hydrophilicity Solvent (SHS) from the extracted oil were assessed by brine shrimp lethality assay (BSLA). Evaluation of the toxicity were also compared to N,N-Dimethylcyclohexylamine (DMCHA) as a pure SHS solvent and pure Crude Coconut Oil (CCO). The total number of dead larvae was counted for each dosage at designated period exposures. For the percent mortalities, COCOSHS and DMCHA were found out to have similar trend of increasing percent mortality with respect to different concentrations at 6-hour and 24-hour period exposures but not with CCO sample. On the other hand, in terms of their LC_{50} values at 6-hours, all samples didn't exhibit acute toxicity since their LC_{50} values were above 1000 ppm. However, at 24-hour exposure, COCOSHS was classified as low toxic whereas DMCHA was classified as moderately toxic and CCO was classified as non-toxic indicating indirectly that SHS solvent might still remained in COCOSHS sample. In addition, it was found out that the difference between COCOSHS and CCO, COCOSHS and DMCHA were significant at the 95% confidence level for 6-hour and 24-hour exposure times which suggested that the null hypothesis was rejected. Based on the data and identified toxicity level of COCOSHS sample, it is highly possible that some DMCHA solvent used from the extraction process were still present and not sufficiently removed thus indicating that BSLA could serve as simple and cheap alternative in qualitatively assessing solvent contamination in SHS extraction.

* **Corresponding Author:** Janus B. Pansacala ✉ janus.pansacala@wmsu.edu.ph

Introduction

The utilization of vegetable oil in various industries is extensive, including refining for soap production and the manufacturing of biodiesel using acid oil and fatty acid distillates (Piloto-Rodríguez *et al.*, 2014). Additionally, vegetable oil finds applications in the production of plastics, paints, resins, lubricants, surfactants, pharmaceuticals, and fuels. The extraction of triglycerides from plants has been a longstanding practice in human culture, dating back 5000 to 6000 years, where oils were derived from sesame seeds, olives, almonds, walnuts, and flax seeds using crude presses (Gunstone, 1952). Vegetable oils are characterized by their high content of unsaturated fatty acids, which classifies them as unsaturated fats. As a result, these oils are considered essential nutrients in both human and animal diets, with many natural foods containing oils. Olive oil, palm oil, canola oil, sunflower oil, and coconut oil are among the various types of vegetable oils. With the exception of olive oil, these oils have been utilized for other purposes such as lubrication, medicine, and illumination. Notably, the Philippines remains one of the major global producer and leading supplier of coconut, while Brazil and Argentina are recognized as the primary suppliers of groundnut oil (Abbas & Baeten, 2016).

The process of producing vegetable oils involves the removal of oil from plant, typically from seeds. Two common methods usually employed are the mechanical extraction and the solvent extraction. Mechanical extraction by far is the oldest method used for extracting oils from the seeds where the oil from seeds can be recovered by means of mechanical pressing. In this process, a hydraulic filter press or a screw press used to force the oil from the pressed seed (Savoire *et al.*, 2012). On the other hand, a solvent extraction is a process for transporting materials from one phase to another for the purpose of separating one or more compounds from the mixtures (Kessler, 1985). Solvent extraction recovers almost all the oil (Nde & Foncha, 2020) than mechanical extraction. The desirable properties of a solvent suitable for extracting such vegetable oils from oilseeds are numerous.

Mechanical extraction is a simple and cost-effective method commonly used for oil extraction from oilseeds. However, its drawback is a lower product yield compared to solvent extraction. Solvent extraction, on the other hand, involves the use of solvents to separate oil from oilseeds. Despite its effectiveness, one major disadvantage is that the solvents used are often corrosive, flammable, and contribute to smog formation (Durelle *et al.*, 2015). Additionally, solvent extraction is more economically viable for large-scale industrial operations due to the high cost of solvents. To address these challenges, researchers have explored alternative solvents with a focus on environmental friendliness. One approach involves utilizing non-volatile solvents with polarity switching characteristics, such as switchable hydrophilicity solvents (SHS), for oilseed extraction (Phan *et al.*, 2009). These solvents offer promising alternatives to conventional solvents, as they are less to non-toxic, non-flammable, and environmentally friendly. Furthermore, they possess the ability to switch between hydrophilic and hydrophobic states, enabling efficient extraction of oil from oilseeds. The development and application of such alternative solvents contribute to the advancement of sustainable and greener practices in the oil extraction industry.

Switchable hydrophilicity solvents (SHS) have shown effectiveness in the extraction of non-polar substances, including oil seeds. These solvents exist in two forms: a hydrophilic form with high miscibility in water and a hydrophobic form with low miscibility in water. The ability of SHS to switch between these forms allows for more efficient process design and reduces the volume of solvent required (Vanderveen *et al.*, 2014). One extensively studied and utilized SHS is N,N-dimethylcyclohexylamine (DMCHA), which was employed in the extraction of crude coconut oil from copra samples (Maguigad, 2018). The study demonstrated that the extracted coconut oil using DMCHA exhibited a satisfactory percent oil yield. However, a significant concern raised was the potential retention of DMCHA in the extracted coconut oil, which could pose inherent toxicity risks associated with amine compounds such as DMCHA.

This finding highlights the importance of carefully evaluating and addressing the potential adverse effects or residues of SHS in the extracted products, particularly when considering their applicability in food and pharmaceutical industries. Further investigations and risk assessments are necessary to ensure the safety and quality of the extracted oils when utilizing SHS as extraction solvents.

The presence of N,N-dimethylcyclohexylamine (DMCHA) contamination in samples can be evaluated using instrumental techniques such as proton nuclear magnetic resonance (HNMR), Ultra-Performance Liquid Chromatography-Mass Spectrophotometry (UPLC-MS), and Fourier transform infrared spectroscopy (FTIR) (Boyd *et al*., 2012; Holland *et al*., 2012; Phan *et al*., 2009). While these techniques provide highly precise and rapid detection of DMCHA, they may not be readily available and can be costly. However, an alternative approach for assessing DMCHA retention indirectly is through the evaluation of sample toxicity using the brine shrimp lethality assay (BSLA). The BSLA is an effective method for determining sample toxicity and can be utilized for various types of assessments, including plant extracts, heavy metals, metal ions, marine natural products, algae, pesticides, medicine, and nanoparticles (Sorgeloos *et al*., 1978; Wu, 2014). This technique offers several advantages, as it is easily mastered, cost-effective, and requires only a small amount of test material. By employing the BSLA, researchers can indirectly assess the potential toxicity associated with DMCHA contamination, providing a practical and accessible option for evaluating the safety of samples.

The toxicity risk of DMCHA retention in extracted coconut oil can be evaluated through the brine shrimp lethality assay (BSLA), which determines the concentration mortality relationship using the median lethal concentration, LC_{50}. The LC50 represents the concentration of DMCHA or the chemical under study that results in the death of half of the test subjects after a specific exposure time. The determination of LC_{50} values involves the use of the linear regression method, where % mortality is plotted against the corresponding logarithm of the concentration (Arambasic & Randhawa, 2014). By conducting the BSLA, this study aims to provide valuable information regarding the toxicity risk associated with the presence of DMCHA in the extracted coconut oil. The data abets in assessing the potential adverse effects and safety considerations related to DMCHA retention, contributing to a comprehensive understanding of the quality and suitability of the coconut oil for various applications.

Materials and methods

Materials and Sample Preparation

Three different samples of coconut oil were used in this study: coconut oil extracted with switchable hydrophilicity solvent (COCOSHS), N,N-Dimethylcyclohexylamine (DMCHA), and crude coconut oil (CCO). The CCO samples were sourced from a local coconut oil processing plant. The COCOSHS samples and DMCHA solvent were obtained from a previous study conducted (Maguigad, 2018). All necessary reagents were commercially obtained for the experiment. Additionally, a total of twenty grams of brine shrimp eggs were purchased in a transparent container from a local pet shop and used as received in the experiments.

Brine Shrimp Lethality Assay for COCOSHS, DMCHA, and CCO Samples

The toxicity of samples, namely COCOSHS, DMCHA, and CCO, was assessed using the brine shrimp lethality assay, a simple, cost-effective, and highly efficient method. The bioassay screening involved several steps, including the preparation of sterilized seawater for brine shrimp hatching, dosage preparation of COCOSHS, DMCHA, and CCO, bioassay testing, and the calculation of LC_{50} values.

Preparation of Seawater for Brine Shrimp Hatching

The natural seawater was collected using a cleaned bottle and subsequently filtered with Whatman filter paper (No.1) to eliminate any unwanted particles. The collected seawater was then subjected to sterilization by autoclaving at a temperature of 121 degrees Celsius for a duration of 15 minutes. Once sterilized, the

seawater was carefully transferred to a clean and sterilized container. For the hatching of brine shrimp, a round container and a colored plastic cup with ten holes in the lower body were utilized as a hatching chamber. Sterilized natural seawater was added to the container, and brine shrimp eggs were evenly sprinkled into the colored plastic cup. The cup was then covered and left undisturbed for hatching to occur. After a period of 24 hours, the active Artemia salina nauplii (larvae) that had hatched and separated from their eggshells were observed in the round container, exhibiting a natural attraction to light sources. To facilitate easy collection, the active larvae were carefully transferred using a clean 150-mL beaker, employing a Pasteur pipette for the transfer process.

Dosage Preparation for COCOSHS, DMCHA, and CCO

Solutions of different dosages for COCOSHS, DMCHA, CCO, thymol as the positive control, and sterilized natural seawater as blank control were prepared. The stock solutions for each sample (COCOSHS, DMCHA and CCO) with 50,000 ppm concentration were prepared. For COCOSHS, the 50,000 ppm concentration stock solution was done by mixing 2500 mg coconut oil extracted with switchable hydrophilicity solvent into 50mL sterilized natural seawater and tween 80 solution (47.5mL seawater + 2.5mL tween 80).

The 50,000 ppm stock solution of DMCHA was done by also mixing 2500 mg N,N-Dimethylcyclohexylamine into 50mL sterilized natural seawater and tween 80 solution (49.3mL seawater + 0.7mL tween 80). For CCO, 50,000 ppm stock solution of CCO was done by also mixing 2500 mg Crude Coconut Oil into 50mL sterilized natural seawater and tween 80 solution (48.3mL seawater + 1.7mL tween 80). Furthermore, all the prepared 50,000 ppm stock solution of each sample were utilized in preparing dosages with four different concentrations 10 ppm, 100 ppm, 500 ppm and 1000 ppm, respectively. The preparation of 50,000 ppm stock solution for positive control was done by dissolving 2500 mg thymol into 50mL sterilized natural seawater. The blank control was just sterilized natural seawater.

Bioassay Testing of COCOSHS, DMCHA, and CCO

After two (2) days when the brine shrimp are ready, two (2) milliliters of sterilized seawater was added to different vials of the prepared doses of COCOSHS, CCO and DMCHA samples. Ten (10) brine shrimps were introduced into each vial of different dosages. The volume of sterilized seawater was adjusted up to five (5) milliliters per vials. The vials were left uncovered under the lamp. After six (6), and twenty-four (24) hours, the number of surviving shrimps was counted and recorded. Also, the blank control and positive control were run parallel to test the sample. Three (3) replicates were done in each sample test. In each different dosages, the number of dead and alive nauplii was be determined after six (6) hour and twenty-four (24) hours of exposure (Sarah *et al.*, 2017). The corrected percent mortality of different concentrations (ppm) was calculated as shown in the equation 1 below.

$$\text{Percent (\%)Mortality} = \frac{\text{number of dead larvae}}{\text{total number of test organisms}} \text{ x } 100 \quad \text{(eq. 1)}$$

LC_{50} Calculation for COCOSHS, DMCHA, and CCO

The median lethal concentration (LC_{50}) values was determined using Miller-Tainter method, a modified probit analysis (Arambasic & Randhawa, 2014; Randhawa, 2009). Each corrected percent mortality was converted into probit. The median lethal concentration (LC_{50}) of each different dosage was determined as log ppm was plotted versus the probit values of the percent (%) mortality. Also, calculation of LC_{50} was obtained through the equation of the line in the graph, y = mx+b, where y is the probit value, m is the slope, x is Log^{10} concentration, and b is the y-intercept. The LC_{50} of each sample was determined by computing the antilog of x (10x). In probit analysis, zero (0) and one hundred (100) percent mortality does not correspond to any probit values. Using the equations (eq. 2 and 3), corrected probit equivalents for zero (0) and one hundred (100) were calculated.

For 0% dead: $100 \left(\frac{0.25}{n}\right)$ (eq. 2)

For 100% dead: $100 \left(\frac{n-0.25}{n}\right)$ (eq. 3)

Where: n = total no. of organisms per trial

Use of statistical analysis were performed on the computed LC_{50} of COCOSHS, DMCHA and CCO samples. F-test and t-test were employed wherein F-test was used to compare the two variances of the said sample and t-test was used to compare the two means.

Results and discussion

Physical Properties of COCOSHS, DMCHA and CCO Samples

In terms of its physical properties, COCOSHS sample was observed to have a light-yellow color of solution (Fig. 1) and it was observed that the odor is somewhat a strong odor like a musky ammonia which is not normal for a coconut oil since it is normally associated with copra odor, its raw material. Likewise, DMCHA sample also have a musky ammonia odor although its color is colorless. Moreover, as what have noticed, COCOSHS sample has the odor like of DMCHA. The observed odor of COCOSHS might be due to the presence of unremoved SHS solvent from the sample. Also, the color of COCOSHS sample is not golden yellow like the CCO sample. Lastly, for CCO sample, its color is golden yellow, and its odor is like copra.

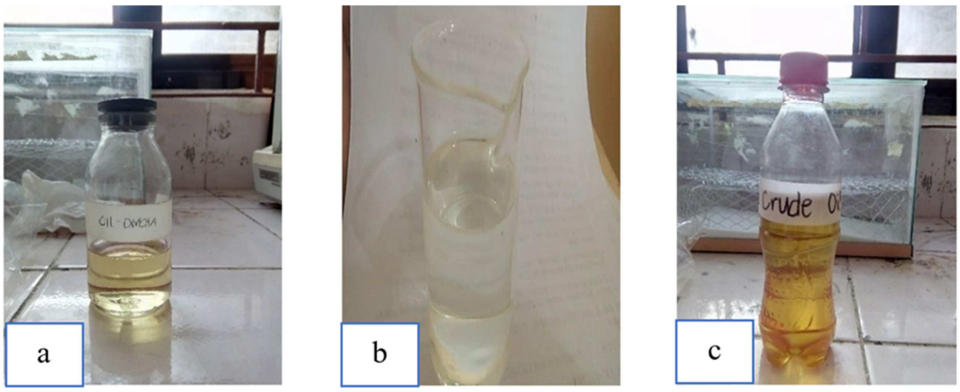

Fig. 1. Samples used in this study (a) COCOSHS, (b) DMCHA and (c) CCO.

Percent Mortality of COCOSHS, DMCHA, and CCO at 6-hour and 24-hour Exposure

In this study, the bioassay screening of COCOSHS, DMCHA, and CCO samples was conducted using the brine shrimp lethality assay to assess their toxicity levels. The mortality of brine shrimp larvae was recorded for four different concentrations (10 ppm, 100 ppm, 500 ppm, and 1000 ppm) of each sample. Three trials were performed per replicate, with each trial consisting of 10 brine shrimp nauplii, allowing for the computation of percent mortality.

Fig. 2 illustrates the trend of percent mortality at a 6-hour exposure period for the three samples. It was observed that the percent mortality increased with higher concentrations of all three samples across the three replicates. Similarly, Fig. 3 depicts the trend of percent mortality at a 24-hour exposure period, showing even higher mortalities compared to the 6-hour exposure. COCOSHS and DMCHA samples exhibited increasing mortalities, while the CCO sample did not display a clear concentration-dependent pattern. Notably, DMCHA showed the highest percent mortality among the samples.

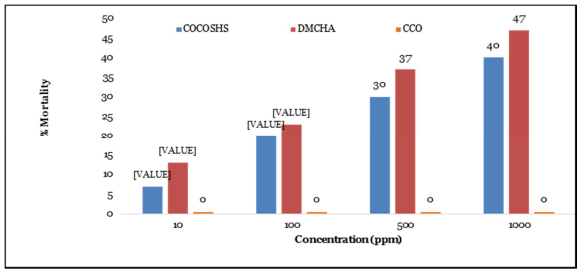

Fig. 2. Percent mortality at 6-hour exposure period.

Of particular interest is the similarity in the pattern of percent mortality between COCOSHS and DMCHA, suggesting the potential retention of DMCHA solvent in the COCOSHS sample. This resemblance in the mortality pattern strengthens the possibility of inefficient solvent removal in the COCOSHS extraction process. The data on DMCHA mortality can serve as an indirect reference for evaluating the

effectiveness of solvent removal in COCOSHS, providing valuable insights into the potential presence of DMCHA in the extracted coconut oil.

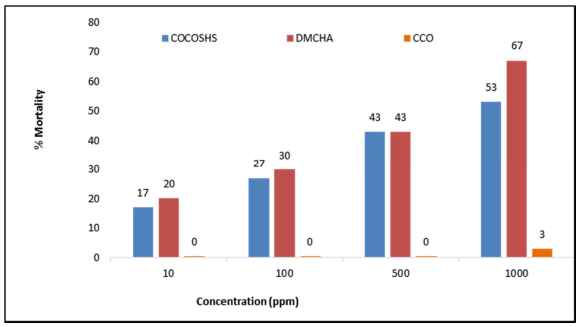

Fig. 3. Percent mortality at 24-hour exposure period.

LC_{50} Values of COCOSHS, DMCHA and CCO

The median lethal concentration (LC50) of each sample (COCOSHS, DMCHA, and CCO) was determined by converting the percent (%) mortality to probit values and plotting them against the log of concentrations. The resulting LC_{50} values for the 6-hour and 24-hour exposure periods were calculated using linear regression analysis and are presented in Fig. 4.

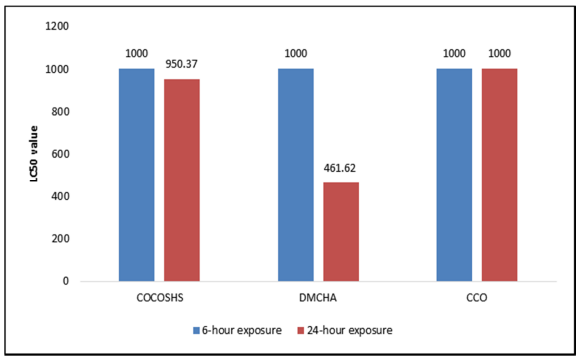

Fig. 4. Average LC_{50} values at 6-hour and 24-hour exposure.

Interpreting the LC_{50} values using Clarkson's Index, it was observed that none of the samples (COCOSHS, DMCHA, and CCO) showed indications of acute toxicity during the 6-hour exposure period, as their LC_{50} values exceeded 1000 ppm. However, at the 24-hour exposure period, only the CCO sample remained above the 1000 ppm threshold, suggesting the non-toxic nature of coconut oil. In contrast, the DMCHA solvent was found to be moderately toxic, while COCOSHS exhibited low toxicity (Table 1).

Table 1. Clarkson's toxicity index for LC_{50} values at 6-hour and 24-hour exposure time.

Sample	Period: 6-hour		Period: 24-hour	
	Average LC_{50}	Clarkson's Scale	Average LC_{50}	Clarkson's Scale
COCOSHS	>1000 ppm	Non-toxic	996.37 ±2.39 ppm	Low Toxic
DMCHA	>1000 ppm	Non-toxic	461.62 ±12.07 ppm	Moderately Toxic
CCO	>1000 ppm	Non-toxic	>1000 ppm	Non-toxic

The LC_{50} results provided further support for the previous assumption that COCOSHS contained residual traces of the solvent DMCHA, which likely influenced the determined LC_{50} values. To assess the significance of differences between the samples, statistical tools such as the t-test and F-test were employed. Table 2 presents the calculated values of F_{exp} and t_{exp} for each sample, allowing for comparison of means and variances. At the 6-hour exposure period, the F-test indicated no significant difference between COCOSHS and CCO, as well as between COCOSHS and DMCHA, at a 95% confidence level. Similarly, at the 24-hour exposure period, no significant difference was observed between COCOSHS and CCO, as well as between COCOSHS and DMCHA. These results suggest that the data sets were precise and comparable.

Table 2. Statistical analysis for LC_{50} values at 6-hour and 24-hour exposure time.

	Period: 6-hour			Period: 24-hour		
	COCOSHS & CCO	COCOSHS & DMCHA	Inference	COCOSHS & CCO	COCOSHS & DMCHA	Inference
F_{exp}	0	3.44	Not Significant	0	0.039	Not Significant
F_{crit}	39.00	39.00	-	39.00	39.00	-
t_{exp}	22.58	9.47	Significant Difference	-2.63	75.28	Significant Difference
t_{crit}	2.78	2.78	-	2.78	2.78	-

Additionally, the t-test was utilized to compare the means of the two sets of data, indicating levels of accuracy. The results suggested a significant difference between COCOSHS and CCO, as well as between COCOSHS and DMCHA, at the 6-hour exposure period with a 95% confidence level. Similarly, at the 24-hour exposure period, the calculated t_{exp} values exceeded the critical value (t_{crit}), indicating a significant difference between COCOSHS and CCO, as well as between COCOSHS and DMCHA.

Overall, the combination of brine shrimp lethality assay, LC_{50} values and statistical analyses provides valuable insights into the toxicity levels and differences among the tested samples. The findings contribute to our understanding of the safety considerations and potential risks associated with the use of COCOSHS and also DMCHA as solvent in the extraction of coconut oil. Further investigations and optimization of the extraction process are warranted to minimize any potential adverse effects and ensure the quality and safety of the extracted oil.

Conclusion

In conclusion, this research successfully employed the brine shrimp lethality assay (BSLA) to evaluate the toxicity of coconut oil extracted using switchable hydrophilicity solvent (COCOSHS) and detect any remaining solvent in the extracted oil. The study utilized N,N-Dimethylcyclohexylamine (DMCHA) as a pure switchable hydrophilicity solvent (SHS) and Crude Coconut Oil (CCO) as a reference oil with no SHS traces. The results indicated that all three samples (COCOSHS, DMCHA, and CCO) were non-toxic during the 6-hour exposure period, while exhibiting low toxicity, moderate toxicity, and non-toxicity during the 24-hour exposure period, respectively.

The findings demonstrated the potential of the brine shrimp lethality assay (BSLA) as a simple and cost-effective alternative for qualitatively evaluating the presence of solvent residues in oil products obtained through the SHS extraction process. Furthermore, the determination of LC_{50} values through probit analysis provided valuable insights into the toxicity levels of the tested samples.

This research contributes to our understanding of the safety considerations and potential risks associated with the utilization of switchable hydrophilicity solvents in coconut oil extraction. Further research and process optimization are necessary to minimize any potential adverse effects and ensure the highest quality and safety standards for the extracted oil. The brine shrimp lethality assay, in combination with the determination of LC_{50} values, serves as a valuable tool for assessing the toxicity of COCOSHS, DMCHA, and CCO samples. These findings lay the foundation for future studies and support the development of safer and more efficient extraction processes for coconut oil.

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