

# Supercritical Fluid Extraction of Herbal Plants for Safe and Halal Enriched Extracts

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## Abstract

In compliance with the halal requirement, herbal processing to produce health beneficial products requires safe and food grade extraction protocols. Supercritical fluid extraction (SFE) technique has been known to produce quality extracts from plants while minimizing the use of organic solvents. In this study, the quality of herbal extracts in terms of component contents and toxicity effects were determined. Three different herbal plants (*Orthosiphon stamineus*, *Phyllanthus niruri* and *Labisia pumila*) were investigated using supercritical CO<sub>2</sub> with food-grade cosolvents. The bioactive compounds in the extracts were analyzed and quantified using high performance liquid chromatography. The component contents (g/ g extract) were found to be: *O. stamineus* (4.69% RA, 0.09% TMF, 0.41% SEN, 0.97% EUP), *P. niruri* (0.37% GA, 2.95% CO, 5.10% EA) and *L. pumila* (0.20% GA, 0.22% MG, 1.05% CA). These results showed comparable or better quality extracts than the previously reported findings. The toxicity test on the zebrafish embryos also showed no toxicity effects by the extracts. Thus, the extraction technique has been proven to be safe for the halal production of enriched extracts from herbal plants.

**Keywords:** Supercritical fluid extraction; Carbon dioxide; Enriched extracts; Marker

compounds; Toxicity test

## Introduction

### Herbal Plants

*Orthosiphon stamineus* (Misai Kucing), *Phyllanthus niruri* (Dukung Anak) and *Labisia pumila* (Kacip Fatimah) are herbal plants belonging to Lamiaceae, Euphorbiaceae and Myrsinaceae genus families, respectively. They are popular folk medicines widely used in Malaysia and Southeast Asia for the treatment of a wide range of diseases. *O. stamineus* leaves are consumed as beverage to improve health and treat a variety of diseases such as kidney disorders, bladder inflammation, gout, diabetes, eruptive fevers, hepatitis, hypertension, syphilis, rheumatism, gonorrhoea and as a diuretic (Akowuah et al., 2004; Ameer et al., 2012). *P. niruri* is extensively used to cure constipation, gonorrhoea, bronchitis, diabetes and jaundice (Ndjonka et al., 2012; Ifeoma et al., 2013; Giribabu et al., 2014; Mao et al., 2016). It is also a popular folk medicine for treating kidney and gallbladder stones as well as wound healing (Shanbhag et al. 2010). Meanwhile, *L. pumila* is known to induce and facilitate childbirth, and to treat rheumatism, gonorea and intestine infection (Wan Ezumi et al., 2007).

Plant phytochemicals have been classified into

six major categories, generally based on their chemical structures and characteristics. These categories are carbohydrate, lipids, phenolics, terpenoids and alkaloids, and other nitrogen-containing compounds. Previous studies reported that the leaves of *O. stamineus* contain high contents of phenolic compounds including lipophilic flavones, caffeic acid derivatives, rosmarinic acid, 2,3-dicaffeoyltartaric acid (Akowuah et al., 2004) and flavonoids such as sinensetin, eupatorin and 3'-hydroxy-5,6,7,4'-tetramethoxyflavone (Yam et al., 2011; Muhammad et al., 2011; Ameer et al., 2012). *Orthosiphon stamineus*'s bioactive compounds such as sinensetin, rosmarinic acid, eupatorin, and 3-hydroxy-5,6,7,4-tetramethoxyflavone exert effects as an antiallergic, antihypertensive, anti-inflammatory, antioxidant (Akowuah et al., 2004; 2005). Scientific research upon *P. niruri* on its phytochemicals composition showed that lignan, glycoside, flavanoid, alkaloid, ellagitannins, terpene and flavanol were contained in leaf, stem and root of the herb (Dhar et al., 1968). Corilagin and geraniin, which are characteristic compounds of ellagitannins, are most extensively obtained from this plant (Mao et al. 2016). These two compounds exhibited multiple activities such as antioxidant and antihyperalgesic activities (Moreira et al., 2013). It was also reported that, gallic acid and ellagic acid from this plant exhibited antioxidant and anti-inflammatory properties (Ndjonka et al., 2012). Previous researches indicate that *L. pumila* constitutes of highly amount of phenolic acid and flavonoid (Karimi & Jaafar, 2011; Chua et al., 2012). It was found to contain gallic acid, caffeic acid, methyl gallate, chlorogenic acid and catechin.

### Extraction of Herbal Plants

Conventional extraction methods were carried out on these herbal plants such as reflux, soxhlet and maceration (Saidan et al., 2015; Kamarudin et al., 2016). These extraction methods are susceptible to thermal degradation of compounds due to prolonged heating and it is also time consuming (Pang et al., 2017). The

conventional methods of extraction also utilize harmful and expensive solvents yet require multiple fractionation-purification steps. In addition, these conventional extraction methods using liquid solvents are very slow and usually need several hours or sometimes even days to obtain complete extractions. In recent years, modern technology offers a more efficient and environmentally-friendly method to increase the production of the desired compound of interest (Easmin et al., 2014).

Supercritical fluid extraction (SFE) by carbon dioxide is one of the most common and competitive eco-friendly extraction methods for herbal products. Supercritical fluid extraction (SFE) has great advantages compared to conventional methods due to the reduction of solvent consumption, low temperature operations and short extraction times. Supercritical fluid technology is advantageously positioned as a sustainable and safe extraction alternative for the preparation of plants extracts and has been widely studied in many applications during the last decade (de Melo et al., 2014). In SFE, the usage of large amounts of solvents can be avoided, hence improving yield/selectivity of useful products and can be achieved only by manipulating temperature and pressure or by a cosolvent combination. The method of SFE with cosolvent application was demonstrated on Malaysian indigenous herbal plants, *Phyllanthus niruri* Linn (dukung anak) and *Polygonum minus* Huds (kesum) (Markom et al. 2007; Markom et al., 2012). However, the limitation of SFE is that the main extractant (carbon dioxide, CO<sub>2</sub>) behaves as a nonpolar solvent that only dissolves the water-insoluble nonpolar compounds such as vegetable oils, butter, and fats. In order to overcome this problem, a more polar cosolvent is introduced in SFE. The solvation features of supercritical CO<sub>2</sub> can be modified by the addition a food-grade organic solvent such as ethanol (acts as cosolvent in CO<sub>2</sub>) or an aqueous alcohol mixture. The SFE with a food grade cosolvent is a promising alternative for safer extraction and environmentally friendly method. This is

important in order to meet the halal/thayyiban criteria (safe, clean, nutritious, quality) in food products.

### Safety of Herbal-based Products

Despite the growing research by scientists on herbal plants, there are still concerns associated with not only their use, but their safety. Only less than 10% of herbal products in the world market are truly standardised to known active components. Besides, strict quality control measures are not always thoroughly obeyed (Winston & Mimes, 2007). There is no known information on the active and/or toxic constituents of most of these herb products used. In many countries, herbal dietary supplements are not subjected to the same regulatory standards as orthodox drugs in terms of efficacy and safety. This raises the concern on their safety and implications for their use as dietary supplements. Toxicity testing can reveal some of the risks that may be associated with use of herbs, therefore preventing potential harmful effects when consumed as dietary supplement or medicine.

Toxicity study is essential to assess potential harmful effects to humans through laboratory animals. Conventional *in-vivo* animal models like rats and mice are very important to toxicology research. However, utilisation of these model organisms in toxicology research are very expensive, time consuming, and raises ethical concerns. A previous study demonstrated that the utilisation of rodents can be replaced by zebrafish as an alternative model organism as they found that models, zebrafish and mice showed similar core features of behavioural alterations after developmental exposure to a toxicant (Spulber et al., 2014). The zebrafish has appeared as an ideal experimental model animal for a large scale research on vertebrate neurodevelopment and behaviour (Kalueff et al., 2014; Guo et al., 2015), cost saving and reducing the use of mammals (Bal-Price et al., 2010; Crofton et al., 2012). The zebrafish embryo toxicity test is a robust test which is

very economical, fast and convenient and has always been considered as an important animal model. Zebrafish embryo is also efficiently applied in screening systemic toxicants and complex mechanisms.

In the present work, the quality of herbal extracts in terms of component contents and toxicity effects were determined. Supercritical fluid extraction (SFE) of bioactive compounds from different herbal plants was employed using CO<sub>2</sub>-cosolvent mixture. The toxicology of the extract was conducted with the use of zebrafish (*Danio rerio*) embryos to determine the toxicity effects of the extracts.

### Materials and Methods

#### Sample preparation

Dried leaves of *O. stamineus* and *P. niruri* samples were purchased from a local supplier (Herbagus Naturally Healthier, Penang, Malaysia) while *L. pumila* samples were purchased locally from Batu Pahat, Johor. The samples were packaged into a nylon-liner low density polyethylene pouch covered with aluminium foil upon arrival at the laboratory. The samples were grounded and sieved into 0.5 mm particle size using a milling machine (Fritsch, Germany) and kept in a dark environment at room temperature until they were used.

#### Supercritical fluid extraction

Supercritical Fluid Extraction (SFE) system (Figure 1) comprises a carbon dioxide pump model PU-2080 (JASCO Corporation, Japan), series 111 solvent pump (Lab Alliance, USA), BP 1580-81 model back pressure regulator, BPR (JASCO Corporation, Japan), extractor vessel enclosed in a FX2-2 model air circulating oven (Sheldon Manufacturing, USA), 682-8 model pressure transmitter (Dwyer Instrument, USA) and a sample collector. A chiller (Protech Electronic, Malaysia) was used to retain the liquid state of liquefied carbon dioxide at -4 °C before the extraction process started.

Crude extracts were collected after the sample underwent drying process by using oven at 45 °C overnight. All the extractions from the extracts were conducted in replicates.

In this extraction mode, the samples were placed in the extraction vessel, which was in the oven. The total flow rates for liquid CO<sub>2</sub> and cosolvent were fixed at 4.0 mL/min; temperature was set at 60 °C, pressure of 200-225 bar and using ethanol in water (co solvent) with 10 % (v/v) cosolvent content. Approximately 2-5 g (±0.05) of samples were placed into the extractor vessel. 30 to 60 min static extractions were allowed, followed by 4 hours dynamic extraction. Each fraction was collected every 20-30 minutes. The extracts were then dried in an oven at 45 °C and the dried extracts were kept in -20 °C before undergoing HPLC analysis. For ZET (Zebrafish Embryotoxicity Test), the extracts with 3 different concentrations; 25 ppm, 50 ppm and 100 ppm were prepared.

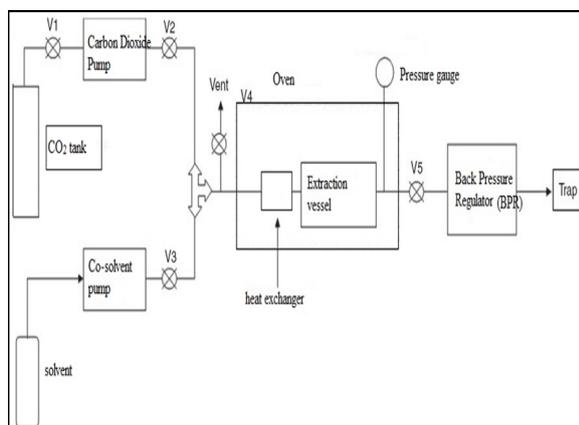


Figure 1: Schematic diagram of SFE system

## Quantification of bioactive compounds

Analyses on *O. stamineus* extracts were performed on a Waters e2695 Separation Module High Performance Liquid Chromatography (HPLC) system (Waters Corporation, USA) equipped with Waters 2998 Photodiode Array detector, an auto sampler, a quaternary pump, a degasser and a column oven. The column used was a reverse phase C18, Chromolith (100

mm x 4.6 mm, 5 µm particle diameter). An acetonitrile/water/trifluoro acetic acid mobile phase system was used for the chromatographic separation. For *P. niruri* and *L. pumila* extracts, the component separation and determination were performed using High Performance Liquid Chromatography (HPLC) technique equipped with an auto sampler and a UV/vis detector (Agilent Technologies, Germany). The column used was a reverse phase C18, Kinetex (250 x 4.6 mm, 4 µm). The mobile phase used were 0.1 % phosphoric acid in water (solvent A) and acetonitrile (solvent B) and injected with gradient conditions for each sample. Chromatographic separation was conducted at 35 °C and wavelength of 270 nm with 20 µL injection volume.

In this study, the identification and quantification of bioactive compounds were carried out by comparing HPLC retention time to those of the standards (rosmarinic acid, 3-hydroxyl-7,3',4',5'-tetramethoxyflavone, sinensitin, eupatorin, gallic acid, ellagic acid, corilagin, methyl gallate and caffeic acid). Each sample and standard were filtered with nylon syringe filter (pore size of 0.45 µm).

## Toxicity Test on Zebrafish Embryos

The fish husbandry, embryos collection and zebrafish embryo developmental toxicity evaluation methodologies were a modification of Abu Bakar *et al.* (2017) (Institutional Animal Care and Use Committee of Universiti Putra Malaysia (UPM) (IACUC/AUP-R024/2014) Wild-type short fin adult zebrafish (*Danio rerio*) were procured from a local petshop (AquaMart, Kajang) and were allowed to adapt to Laboratory of Natural Products, Institute of Bioscience, Universiti Putra Malaysia conditions in a freshwater aquarium equipped with recirculating system. The animals were kept in the aquaria with the water temperature maintained at 25–27 °C, on a ratio of 14 hours light: 10 hours dark of light cycle. A tray made up of artificial aquatic plants and brown yarn was used to collect the fertilised zebrafish eggs.

Approximately 30 minutes after lights on, the embryos were collected and after that the eggs were rinsed with distilled water. The zebrafish embryos were exposed to different range of extract concentrations (25, 50 and 100 ppm) and control consisting of egg buffer solution. Thirty-six zebrafish embryos were exposed in a 96-well microplate containing 250  $\mu$ L of exposure solution. All experiments were performed in triplicates and repeated at least three times.

The egg buffer solution containing 15 mM NaCl, 0.5 mM KCl, 1 mM  $MgSO_4$ , 0.15 mM  $KH_2PO_4$ , 0.05 mM  $Na_2HPO_4$ , 1 mM  $CaCl_2$ , 0.7 mM  $NaHCO_3$  (pH 7.0) was used during the incubation period at 28 °C (Nasiadka and Clark, 2012). The examination of the eggs was done with the aid of a standard light microscope (Axiovert 25, Carl Zeiss, New Zealand) at 4 hours post fertilization (hpf) in order to choose a normal fertilized embryo that have intact chorion membranes and have reached the gastrulation stage (50% epiboly) (Li et al., 2011). Any dead or unfertilized eggs were removed. The data were presented as mean  $\pm$  standard deviation (SD) unless otherwise stated.

## Results and Discussion

### Effects of SFE on Extraction and Component Yields

From the total extraction and component yields, it is shown that the addition of cosolvent (polar organic solvent) usually causes higher polarity of the extractant, which later increases the solubility of the extract and tend to extract different polarity compounds simultaneously. The presence of water in ethanol will increase extract yield since non-polar and polar components are extracted together. According to Kamarudin, Markom & Latip (2016), the type, polarity and molecule structure of the solvent play significant roles in extracting the polar phenolic compounds. It can be deduced from this study that the main components present in the herbal plants are hydrophilic and polar compounds.

Figure 2 shows the overall extraction yield of the three herbs as a function of time in SFE. After 4 hours, the yield has achieved its exhaustive state. Due to the limited solubility of polar organic compounds in SFE, quantitative extraction of these compounds with pure supercritical carbon dioxide is not possible. In this study, the addition of ethanolic or aqueous ethanol cosolvent in SFE gave high extraction efficiency for bioactive compounds.

Rosmarinic acid (RA), TMF, sinensetin (SEN) and eupatorin (EUP) were successfully detected and quantified in *O. stamineus* extract as can be seen in Figure 3. In *P. niruri* extraction, SFE with the addition of 50% ethanol-water as cosolvent gave a high extraction yield of bioactive compounds. This result agreed well with other finding (Yaneth et al. 2016) that reported the effect of  $CO_2$ /ethanol/water mixture on the extraction of phenolics from purple corn cob (*Zea mays* L.) and found that 70% ethanol-water mixture as cosolvent was able to produce the highest phenolic content. Three ellagitannins (gallic acid, corilagin and ellagic acid) were successfully detected and quantified in this extract as shown in Figure 4. As can be seen in Figure 5, HPLC profile of the *L. pumila* extract confirms that gallic acid, its derivative (methyl gallate) and caffeic acid are the main phenolics present in the *L. pumila* species. Many researchers had recognized gallic acid as the core compound of *L. pumila* (Karimi & Jaafar 2011, Mohd Azrie et al., 2016). Gallic acid and its derivative, methyl gallate are well studied plant phenolics. The presence of gallic acid and caffeic acid in all the three varieties of *L. pumila* had also been previously reported (Karimi et al., 2011).

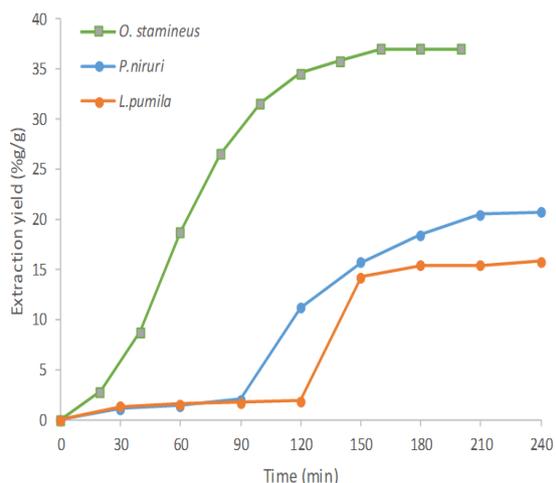


Figure 2: Overall extraction curve as a function of time for the three herbs.

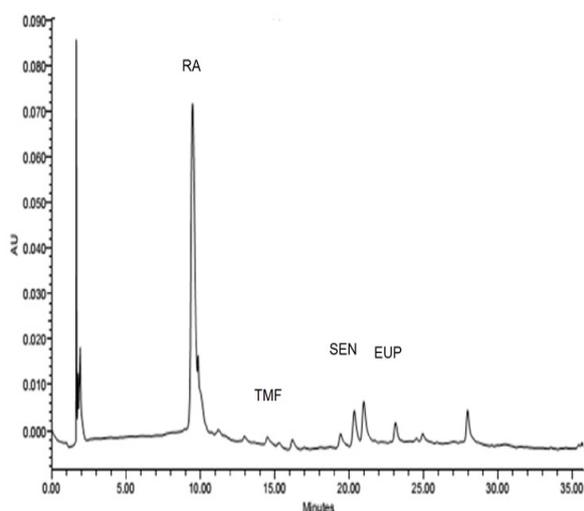


Figure 3: HPLC profile of *O. stamineus* extract. RA is rosmarinic acid, TMF is 3-hydroxyl-7,3',4',5'-tetramethoxyflavone, SEN is sinensetin and EUP is eupatorin.

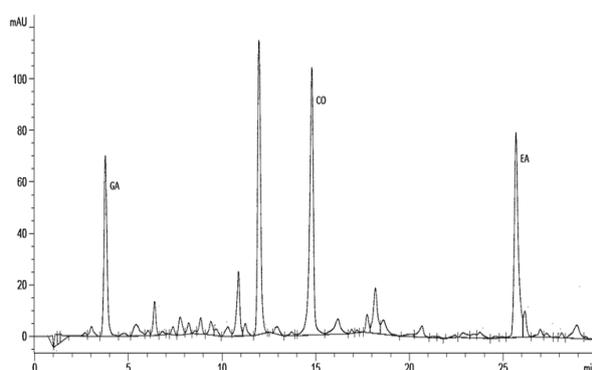


Figure 4: HPLC profile of *P. niruri* extract. GA is gallic acid, CO is corilagin and EA is ellagic acid

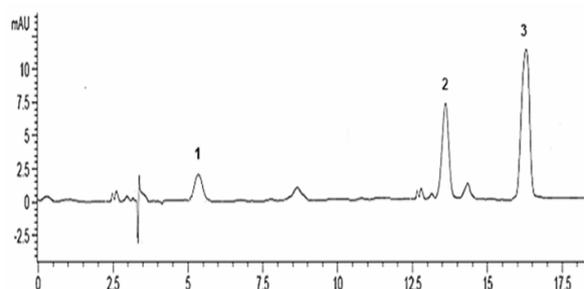


Figure 5: HPLC profile of *L. pumila* extract. (1) gallic acid (GA), (2) methyl gallate (MG), (3) caffeic acid (CA).

The characteristics of an ideal extraction method should be rapid, able to yield quantitative recovery of target solute without degradation, and the solvent should be easily separated from the solute. Tables 1-3 indicate the total extraction yields and component contents obtained at some of the best extraction conditions. The results are compared to the available standardized extracts and it can be observed that the enriched extracts from this study are comparable or better in terms of the component contents especially for the marker compounds. Thus, the SFE successfully yielded high quality enriched extracts from the three herbal plants.

Table 1: Comparison of *O. stamineus* extractions

| Method   | T<br>(°C) | P<br>(bar) | Cosolvent/<br>Solvent          | Extraction<br>Time<br>(hr) | Total Yield<br>(% g/g) | Component Content<br>(% g/g extract) |   |                     |                    |
|--|-----------|------------|--------------------------------|----------------------------|------------------------|--------------------------------------|---|---------------------|--------------------|
|  |           |            |                                |                            |                        | Rosmarinic<br>Acid<br>(RA)           | 3-hydroxy-7,3',4',5'-<br>tetramethoxyflavone<br>(TMF) | Sinensetin<br>(SEN) | Eupatorin<br>(EUP) |
| This study<br>(SFE)                                | 60        | 225        | 75%<br>Ethanol                 | 3.3                        | 37.1                   | 4.69                                 | 0.09  | 0.41                | 0.97               |
| Saidan et al.<br>(2015)<br>(solvent<br>extraction) |           |            | Ethanol,<br>methanol,<br>water | na                         | na                     | 1.87-3.15                            | 0.17-0.27   | 0.18-0.40           | 1.46               |

Table 2: Comparison of *P. niruri* extractions

| Method                           | T<br>(°C) | P<br>(bar) | Cosolvent/<br>Solvent | Extraction<br>Time<br>(hr) | Total Yield<br>(% g/g) | Component Content<br>(% g/g extract) |                   |                      |
|----------------------------------|-----------|------------|-----------------------|----------------------------|------------------------|--------------------------------------|-------------------|----------------------|
|                                  |           |            |                       |                            |                        | Gallic Acid<br>(GA)                  | Corilagin<br>(CO) | Ellagic Acid<br>(EA) |
| This study<br>(SFE)              | 60        | 200        | 50% Ethanol           | 4.0                        | 20.76                  | 0.37                                 | 2.95              | 5.10                 |
| HEPAR-P™<br>(solvent extraction) |           |            | na                    | na                         | na                     | 0.21                                 | 2.64              | 4.17                 |

Table 3: Comparison of *L. pumila* extractions

| Method  | T<br>(°C) | P<br>(bar) | Cosolvent/<br>Solvent | Extraction<br>Time<br>(hr) | Total Yield<br>(% g/g) | Component Content<br>(% g/g extract) |                           |                      |
|---|-----------|------------|-----------------------|----------------------------|------------------------|--------------------------------------|---------------------------|----------------------|
|   |           |            |                       |                            |                        | Gallic Acid<br>(GA)                  | Methyl<br>Gallate<br>(MG) | Caffeic Acid<br>(CA) |
| This study<br>(SFE)                             | 60        | 200        | 50% Ethanol           | 4.0                        | 15.8                   | 0.20                                 | 0.22                      | 1.05                 |
| Karimi et al.<br>(2011)<br>(solvent extraction) |           |            | Methanol              | na                         | na                     | 0.02                                 | -                         | 0.004                |

na - data not available

### Toxicity Test on Enriched Extract

For toxicity test, the total percentage of mortality were represented by dead and unfertilized embryos which exhibited coagulated embryos, lack of somite formations, non-detachment of the tail and absence of heartbeat (OECD, 2013). Table 6 shows the total number of the dead embryo/deformities in the zebrafish larvae until hatching (24–72 hpf). Under observation, zebrafish eggs remained transparent from fertilization to when the tissues become dense and pigmentation was started at 72 hours post fertilization (hpf), which allowed open observations of the general main morphological changes up to and beyond pharyngulation. The results showed no mortality, therefore the median lethal dose (LD50) was not determined at 25, 50 and 100 ppm of the extract concentrations.

The normal zebrafish embryonic heart rate is between 140–180 beats per minute (bpm) (Sarmah & Marrs, 2013). At different extract concentrations, 25, 50 and 100 ppm exposed to embryo groups showed no significant difference with p-values greater than 0.05 as compared to the control group (Table 3). Therefore, the exposure of extracts did not cause any abnormality on the heart beats. The cardiac toxicity was also assessed by manually counting individual larval heart beat within 1min from 48 hpf to 50 hpf in response to different range of extract exposures (Zhang et al., 2014).

The total percentage of mortality and morphological deformities were observed and recorded at 24, 48 and 72 hpf (hours post fertilisation). The formation of the morphological deformities after exposure to sample treatment on the zebrafish embryo include impairments in the fin folds and tail primordium, bent body axis (kink in tail, lordios or scoliosis), no heartbeat, abnormal eye and shape of the yolk. Table 4 shows that no abnormalities and no death detected after 72 hpf exposure to the extracts. The embryos are considered dead when it exhibits coagulation of fertilized eggs, lack

of somite formation, lack of detachment of tail-bud from the yolk sac and no heart beat (OECD, 2013). The controls hatched, swam fast throughout the wells and appeared to be developing normally. Figure 5 is an example of tail detachment of the embryos after exposure to *O. stamineus* extract for 72 hours (72 hpf). There were no abnormalities recorded on tail detachment and other parts of the embryos showing that the extracts did not cause any harm to the embryos at concentration <100 ppm in terms of abnormalities to heartbeat, percentage of mortality and general morphology.

Table 6: Median Lethal Dose (LD50)

| Sample concentration (ppm) | Number of the dead embryo / deformities |
|----------------------------|---|
| 25                         | 0/36                                    |
| 50                         | 0/36                                    |
| 100                        | 0/36                                    |



Control



25 ppm

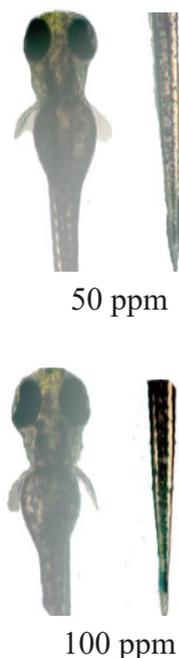


Figure 6: Detachment of tail observed after 72 hours exposure of zebrafish embryos to *O. stamineus* extract (72 hpf)

## Conclusion

In this study, supercritical carbon dioxide extraction with aqueous ethanol cosolvent successfully extracts bioactive compounds from three local herbs with higher or comparable contents with previously reported data. SFE with ethanol-modified carbon dioxide can be considered as 'green technology' because it used only a small amount of organic solvents and reduced energy consumption through reduced processing time. The evaluation of toxic effects of the *O. stamineus* extract (concentration < 100 ppm) conducted on zebrafish embryos proved that extract was not contaminated with the residual solvent and thus, proved the safety of the extract. This satisfies one of the halal criteria for the herbal-based products. The findings of this study will expedite the use of SFE for safe and halal herbal-based products due to the high quality and no significant toxicity effects in its products.

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