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EFFECTS OF DIFFERENT EXTRACTION SOLVENTS ON THE TOXICITY OF *Piper sarmentosum* LEAF EXTRACT IN ZEBRAFISH (*Danio rerio*) EMBRYOS

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Abstract

Piper sarmentosum (P. sarmentosum), is a creeping herb with a pungent odour known as *Kaduk*. It is commonly consumed as raw vegetables (ulam) in Malaysia due to its myriad potential pharmacological activities. Previous studies have shown that the individual aqueous and methanol extracts of *P. sarmentosum* leaf could induce possible adverse effects on zebrafish embryos. However, the effects of different extraction solvents on the toxicity of *P. sarmentosum* leaf have yet to be explored. Hence, the present study evaluated the toxicity effects of *P. sarmentosum* extract procured from the leaves, using four different solvents commonly used to extract active phytochemical compounds against the embryonic development of *Danio rerio* (zebrafish). In the present study, leaf extracts of *P. sarmentosum* were obtained from hexane (HE), dichloromethane (DE), ethyl acetate (EA) and, methanol (ME). ME has the highest percentage yield of 14.73%, followed by EE, HE, and DE with percentage yields of 6.38, 5.50 and 2.30%, respectively. The phytochemical screening by GC-MS revealed the prominent compounds present were asarone and isoelemicin that possess toxicity and contribute to the toxicity effects of the solvent extracts. HE exhibited the highest mortality rate at 96 hpf with LC_{50} value of 23.33 ppm, followed by DE, EE and ME with LC_{50} values of 25.60 ppm, 32.31 ppm and 70.25 ppm, respectively. The present study demonstrated a significant slow heart rate, decreased hatching rate, lack of tail extension and somite formation, with presence of scoliosis and edema in treated embryos, which further postulates the acute toxicity of HE, among other solvent extracts. These findings revealed the toxicity of *P. sarmentosum* leaf extract on living organism, which was highly influenced by the solvents, concentration and time exposure employed.

INTRODUCTION

For the past decades, herbal and botanical supplements were consumed by most of the people for nutrient requirements, especially among patients with chronic diseases. Estimation by the Ministry of Natural Resources and Environment expected that herbal local market in Malaysia would soar to around RM29 billion in 2020 [1]. In Malaysia, herbal supplements can be obtained from the markets majorly in the form of liquid and also in tablet form within a wide range of brands. Since the supplement was a combination of medicinal plants and affordable than synthetic drugs available in the markets, it was used as it is believed to provide fewer side effects, compared to conventional clinical drugs [2]. Although there is scarcity in research on the efficacy and toxicity effects that these herbal supplements have, the products are still in high demands, which raise an alarming concern, as most of the herbal supplements consumed are without prior consultation with the public's general practitioners [1].

One of the herbal supplements that has attracted so much attention with reported myriad biological activities, is *Piper sarmentosum* (*P. sarmentosum*). This wild growing herb from the *Piper* species, with long creep stem, and leaves that are of alternate and cordiform shapes is famously known as *Kaduk* in Malaysia, has been used in ancient medicines. It has also displayed numerous pharmacological activities, such as anti-bacterial, anti-fungal [3], antioxidant [4], anti-inflammatory [5], hypoglycemic [6] and larvicidal activities [7, 8]. Due to all these properties, this herb has high prospects to be commercialized as medicated plant in Southern-East Asia, notably in Malaysia. Today, natural remedies have received high demand in the market around the globe and are commonly used, owing to the cost-effectiveness and the popular belief that these herbs are safe [9, 10].

P. sarmentosum has various applications in numerous ancient medicines. For example, in ancient Chinese drugs, the species was accustomed to treat inflammatory diseases [11]. Within the southern part of Asian countries, the aqueous mixture of *P. sarmentosum* was used to treat diabetic patients as it was found to assist in reducing the blood sugar level of early-stage diabetic patients [12]. *P. sarmentosum* has been used as a carminative to alleviate muscle pain [13] and cough. Rahman et al., 2016 [14] have reviewed that the *P. sarmentosum* leaves and roots aid in relieving headaches when applied to the forehead while the simmering of the plant helps to cure muscle weakness and pain within the bones.

Contrary to the popular belief that herbal supplements are safe and natural, there have been reports on adverse effects following consumption, especially on the toxicity effects against humans' vital organs, such as liver [15], kidney [16], and heart [17], as well as herb-drug interaction [18, 19, 20]. According to the statistical assessment conducted by the Malaysian Adverse Drug Reaction Advisory Committee, together with the National Pharmaceutical Control Bureau and the Minister of Health, there have been a 0.2% from 11,473 of adverse drug reaction cases reported due to herbal consumption, and this number is expected to rise [21]. Despite the extensive study on the pharmacological benefits of *P. sarmentosum* extract, there is scarcity in the research that focuses on the toxicity and adverse effects of the extract, which remains to be explored. Recent study by Zainol Abidin et al., 2020 [22] has shown that the aqueous extract of *P. sarmentosum* leaf exhibited the highest rate of mortality at a concentration as low as 60 µg/mL, with a significant decrease in heart rate and slower embryonic development observed, which results in embryonic coagulation and undeveloped organs. Another study by Omar et al., 2020 [23] has demonstrated the toxicity of *P. sarmentosum* leaf extract from methanol with LC_{50} value of 171.82 µg/mL, but with no further effects on teratogenic of zebrafish embryos. To the best of our knowledge, there is no published research that evaluates the effects of different extraction solvents on the toxicity profile of *P. sarmentosum* extract in any model organisms.

The present study endeavours to evaluate the toxicity effects of *P. sarmentosum* extract procured from the leaves, using four different solvents commonly used to extract active phytochemical compounds from herbal supplements against the embryonic development of *Danio rerio* (zebrafish). Zebrafish has been accepted widely as the vertebrate model to evaluate toxicity. The embryos of zebrafish are found to be similar to humans, structurally and functionally. The zebrafish embryos have a protruding yolk sac that supplies biomolecules, such as proteins, lipids and micronutrients that support the metabolic function and growth. Similar phenomenon could also be observed with human's embryos until the onset of placenta-fetal exchange. The small size and transparency of zebrafish embryos [24] as well as the rapid breeding of zebrafish have further attracted research interest. Additionally, it has also been found that zebrafish embryos share 64 to 100% similarities to mammalian models that are used to evaluate developmental toxicity [25, 26]. Hence from the previous studies stated above, zebrafish is an excellent model to predict the toxicant disruption on humans.

In the present study, extracts of *P. sarmentosum* prepared from different solvents with various degrees of polarity, commonly used for phytochemical extraction, which include HE, DE, EA and ME were obtained from the leaves. The extracts were then analysed for their phytochemical contents using GC-MS analysis, and the toxicity profile of the extracts was further investigated using the zebrafish (*Danio rerio*) embryos model. The indicators for toxicity profile of these extracts were survival rate, heart rate and hatching rate. Additionally, the morphological changes of the embryos upon treatment of different concentrations of the extract were further evaluated by assessing the morphological changes, such as somite formation, tail extension, presence of scoliosis and edema, with a view to understand the relationship of the solvent extracts used, concentration and time dependent effects of *P. sarmentosum* leaf extracts towards the toxicity profile on zebrafish embryos.

MATERIALS AND METHODS

Materials

All chemicals and reagents used were of analytical grade. Methanol, ethyl acetate, dichloromethane, hexane (R&M Chemicals, United Kingdom) and dimethyl sulfoxide (DMSO) (Merck, Germany) (PP: 99.8%, AR), Follin Ciocalteu reagent (Merck, Germany), were procured and used as purchased without further purification. The Danio Assay Kit for toxicity assessment was purchased from the Danio Assay Laboratories (Danio Assay Laboratories Sdn. Bhd, UPM, Malaysia), which was equipped with live zebrafish embryos, 96 well plates, 500 mL of Danio-embryo media containing 0.1% DMSO, and manual instruction. The

wild-type Zebrafish (AB strain) was maintained by the Danio Assay Laboratories according to standard in a recirculation system, and under the permission of the Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia (UPM/IACUC/AUP No. R024/2014).

Methods

Plant Materials: Similar size of fresh *P. sarmentosum* leaves (Specimen voucher: MFI 0149/20) were collected from Taman Pertanian Universiti, Universiti Putra Malaysia. The leaves were then washed thoroughly under running tap water in order to decrease contamination from microbes and to remove any unwanted debris. The leaves were then spread on trays evenly and oven-dried at 50°C for 3 days [8]. Then, by using blender (Panasonic, Malaysia), the leaves were ground into powder, and stored in glass bottles at room temperature, until further use.

Preparation of *P. sarmentosum* Solvent Extracts

P. sarmentosum leaves underwent maceration with four different solvents which were methanol (ME), hexane (HE), dichloromethane (DE) and ethyl acetate (EE). Ground plant material (5 g) was soaked in 50 mL of each solvent. The mixture was then softened by macerating it twice for every 3 days at room temperature and kept away from the light, to prevent sample degradation. The crude extract was then filtered using filter paper (No.1, Whatman, England) and was further concentrated using rotary evaporator (Buchi, Switzerland) operating at 37°C until constant weight was recorded. Following that, appropriate amount of methanol or DMSO was added into the dried crude extracts to yield 20 mg/mL of stock extracts. Extracts were then diluted into several concentrations ranging from 0-100 ppm in Eppendorf tube (Sartorius, Germany) with 0.5% DMSO or 0.5% MeOH to be used throughout the assay. Extracts were stored at 4°C, in a dark condition to minimize sample degradation.

Gas chromatography – Mass Spectrometry (GC-MS) Analysis

Phytochemical analysis of the four extracts obtained was conducted using the gas chromatography-mass spectrometry (GC-MS) system, equipped with a BPX5 fused silica capillary column with ZB-5MS 30 mm x 0.25 mm inner diameter x 0.25 µm film thickness (GC-MS QP2010 Plus, Shimadzu, Japan). All extracts dissolved in methanol at 5 µg/mL were filtered through the 0.45 µm PTFE membrane filter (Millipore, USA) prior to analysis. The prepared samples were injected at into the injection port in a split mode, which was then heated at 320 ºC to a point where the sample vapourized immediately. As the vapourized sample passed through the column in a linear velocity at 36.6 cm/sec with 53.5 kPa, the oven where the column resides, gradually increased in temperature (50ºC) through a pre-programmed method. The total flow was 10.0 mL/min with column flow of 1.00 mL/min. After reaching the end of the column, the compounds then hit a detector. The proportional peaks of each chemical component were recorded on a chromatogram. The GC-MS injection volume was 0.5 µL and the analysis was conducted for 7 to 20 min. Mass spectrometer equipped with ACQ detector, set at 240 °C for ion source temperature and 300 °C of interface temperature with m/z (mass scan) of 35–450 amu, was used to identify the compounds present. The peak areas and retention times were measured by electronic integration. The relative amount of individual components was expressed as a percentage by area normalization. The compounds present in the extracts were identified and interpreted by using the mass spectral data library from the National Institute of Standards and Technology (NIST) 08.

Toxicity Assessments of Zebrafish (Danio Rerio) Embryos

Zebrafish embryos were supplied together with the Danio Assay Acute Toxicity Kit by the Danio Assay Laboratories Sdn. Bhd., UPM. The embryos were provided in a petri dish containing Danio-SprintM Embryo Media. Any embryos that appear milky, white, and opaque were considered as dead, and were removed and separated from the healthy ones. Within a short period of time, the embryos were carefully transferred into 96-well plate with 1 embryo per well. After that, each embryo was treated with different concentrations (0-100 ppm) of all solvent extracts of *P. sarmentosum*. Following that, the embryos were also treated with either 0.5% of methanol or DMSO, which act as the vehicle control (n=3, 8 replicates for each concentration). The embryos in the plate were incubated overnight at 28 ± 2°C with a 10-h light/14-h dark cycle to allow the embryos to acclimatize to the environmental/laboratory conditions. The developmental changes and morphological abnormalities were observed at each 24 h interval starting from 24 up to 96 hpf. For the development assessment, the mortality, heart rate and hatching rate were examined. For mortality rate, dead and alive embryos were denoted as “1” and “0”, respectively at every 24 h interval. For heart rate, it was observed under an inverted microscope (Nikon, Japan) and recorded by using stop watch during the early 15 s at 96 hpf. After obtaining the number of heart beats, it was multiplied by 4 in order to standardize it into beats per minute (bpm) [27]. On top of that, for the hatching rate, it was observed by indicating the hatched embryos and non-hatched embryos with “1” and “0”, respectively from 24 to 96 hpf.

Evaluation of Morphological Abnormalities

At each 24 h interval starting from 24 up to 96 hpf, each embryo was observed for its morphological abnormalities. The observation was conducted by using an inverted microscope at 40X magnification, followed by image capturing and video recording by DinoCapture 2.0 software (Dino-Lite, USA). The parameters observed were the somite formation, tail extension, presence of scoliosis and edema. All of the abnormalities observed on each embryo was indicated by "1", meanwhile embryos with no sign of abnormalities were indicated as "0".

Statistical Analysis

Statistical analysis was performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). All data were analysis using One-way ANOVA (Analysis of Variance), with post-hoc Tukey or Dunnet test applied to evaluate the significance of the toxicity effect of the treatment solutions at different concentrations exposure in comparison to the respective control group, unless otherwise stated. Data represent mean ± standard deviation (SD) and were considered significant when p value is lower than 0.05 (p<0.05).

RESULTS AND DISCUSSIONS

Extraction is the initial step in phytochemicals analysis from therapeutic plants, since it is important to extract out secondary metabolites for further isolation and identification. The selection of the solvent system for extraction relies on the chemical features of the bioactive compounds being aimed. Hydrophilic compounds are preferably extracted by using polar solvents such as ethanol, methanol or ethyl-acetate and for lipophilic compounds, dichloromethane is the most potential solvent to be used [28]. In this study, the maceration method was applied promptly due to its simplicity and cost-efficiency though longer time was required to complete the extraction process. In the present study, the effect of employing different solvents for extraction was evaluated by using solvents such as methanol, ethyl acetate, dichloromethane and hexane [7, 29-31], which have been widely used and accepted as the preferred solvents for extraction of bioactive compounds from plants. ME obtained the highest percentage yield of 14.73%, followed by EE, HE, and DE with percentage yields of 6.38, 5.50 and 2.30%, respectively (Table 1). The percentage yield of the present study was highly comparable to the previous study [7] that obtained a percentage yield of 2.31% for DE, but extracted from the roots and aerial parts of *P. sarmentosum*. As for methanol extract, our data shows a prominently higher percentage yield when compared to a previous study by Azlina et al., 2011 [29] that has also utilized methanol for the *P. sarmentosum* leaf extract, with only 6.00% of yield recorded, a 2.5-fold lower to the present study. The difference in the percentage yield obtained was due to the different polarity of each solvent. Methanol is a polar protic solvent, while dichloromethane and ethyl acetate are polar aprotic solvents, while hexane is a non-polar solvent. The result showed that the extraction yield was higher in ME, due to the presence of polyphenols or intermediate polar compounds in the leaves, while yield of HE was much lower as hexane is commonly used to extract out chlorophylls in plant extract [28] or non-polar compound from plant materials. The non-polar property of hexane allows it to extract fatty materials of plant compounds. This result is in agreement with Hematpoor et al., 2016 [7] that reported the percentage yield of successive extractive values for root from *P. sarmentosum* using hexane extract has the lowest yield of 0.98%. The results obtained in the present study were congruent with previous research that reported that the percentage yields of extract from plant materials are highly dependent on many factors, which include the solvent used [31], the nature of the samples and the extraction process [32].

Table 1. Percentage yield of different solvent extracts of *P. sarmentosum* leaf in comparison to published studies.

Part used	Percentage yield (%)	Solvent extract	References
Whole plant	5.30	Ethanol	[50]
Root	1.64	Methanol	[7]
	2.31	Dichloromethane	
	0.98	Hexane	
Stem and leaf	0.32	Essential oil	[51]
Leaf	6.00	Methanol	[29]
Leaf	14.73	Methanol (ME)	Present study
	6.38	Ethyl acetate (EA)	
	2.23	Dichloromethane (DE)	
	5.50	Hexane (HE)	

Phytochemical profiling of *P. sarmentosum* leaves extracts was analysed by GC-MS, which allows a wide range of analytical needs that include compound characterization, quality control, accurate mass measurement, quantitative analysis, structural elucidation, impurity profiling and metabolite identification. According to GC-MS analysis, ME of *P. sarmentosum* (Table 2A) yielded 23 peaks which were then further identified and listed. Caryophyllene and (Z)-β-Asarone were the first (RT of 10.87 min) and last (RT of 18.57 min) compounds detected. With peak areas in the percentage of 26.12% and 26.32% respectively, isoelemicin and asarone were the most significant phytocompounds identified from this analysis. Neophytadiene took up the next place with 4.69% of peak area and 13.39 of RT. Next, GC-MS analysis for EE of *P. sarmentosum* revealed a lower number of peaks which was 21, with the first compound identified in EE (Table 2B) was found to be similar with ME that was caryophyllene (1.55%). Asarone and isolemicin were again found to be as the most significant phytocompounds with the highest peak area in percentage, 29.91 and 27.10%, respectively. GC-MS analysis of both DE and HE showed a significantly reduced number of compounds extracted, which was in consensus with the lower percentage yields obtained from both solvents as presented in Table 1. There were only 14 compounds identified for DE (Table 2C) and 12 compounds for HE (Table 2D), but the most significant compounds extracted in these solvents were also found to be isoelemicin and asarone, albeit at higher percentages (38.68% of isoelemicin and 34.08% of asarone in DE, while 44.82% of isoelemicin and 35.23% of asarone). Asarone and isoelemicin are natural alkenylbenzyls often isolated from aromatic plants as the essential oil fractions [33- 34], and the extraction percentage of these two compounds were observed to be increased with the decrease in the polarity of solvents. Ether and methyl, are the main functional groups of asarone, which are considered as non-polar that results in higher affinity of this compound towards non-polar solvents, and in this case, asarone was extracted at higher percentages in HE, followed by DE, EE and lastly ME.

Table 2. Chemical composition of (A) methanol extract (ME), (B) ethyl acetate extract (EE), (C) dichloromethane extract (DE) and (D) hexane extract (HE) of *P. sarmentosum* leaf by GC-MS analysis.

(A)

No.	Retention time (min)	Compounds	Peak area (%)
1.	10.87	Caryophyllene	1.20
2.	11.54	Phenol-2,4-bis(1,1-dimethylethyl)-	3.29
3.	11.65	1,3-Benzodioxole-4-methoxy-6-(2-propenyl)	2.80
4.	11.87	Elemicin	0.39
5.	11.93	(Z)-Isoelemicin	26.12
6.	12.31	Asarone	26.32
7.	12.44	1-naphthalenol	0.72
8.	12.52	Apiol	0.84
9.	12.53	Cyclopentanecarboxylic acid	1.51
10.	12.78	1-Tetradecene	2.47
11.	12.85	Decane-1-iodo	1.60
12.	13.22	2-Butyloctanol	1.12
13.	13.39	Neophytadiene	4.69
14.	13.50	Hexahydrofarnesyl acetone	0.66
15.	13.83	3-methyl-2-(3,7,11-trimethyldodecyl) furan	0.37
16.	13.89	Palmitic acid	4.21
17.	13.92	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy	4.88
18.	14.23	1,6-heptadiene-2-methyl-6-phenyl-	2.64
19.	14.75	(Z)-6-Octadecenoic acid, methyl ester	3.10
20.	14.85	Octadecanoic acid, methyl ester	4.53
21.	14.90	Phytol	4.70
22.	14.99	1,5-diphenyl-2-pentene	1.35
23.	18.57	(Z)-β-Asarone	0.49

(B)

No.	Retention time (min)	Compounds	Peak area (%)
1.	10.87	Caryophyllene	1.55
2.	11.44	Bicyclogermacrene	0.08
3.	11.55	Phenol-2,4-bis(1,1-dimethylethyl)	2.21
4.	11.59	δ-Cadinene	0.61
5.	11.67	Myristicin	3.23
6.	11.85	Cyclohexanemethanol	0.25
7.	11.87	Elemicin	0.32
8.	11.94	(Z)-Isoelemicin	27.10
9.	12.31	Asarone	29.91
10.	12.44	α-Muurolol	0.62
11.	12.53	Apiole	0.94
12.	12.54	Dillapiole	1.43
12.	12.78	1-Dodecanol-3,7,11-trimethyl	0.42
13.	13.38	Neophytadiene	8.55
14.	13.84	3-methyl-2-(3,7,11-trimethyldodecy)	0.45
15.	13.91	Palmitic acid	5.14
16.	14.24	1,6-Heptadiene-2-methyl-6-phenyl	1.76
17.	14.76	(Z)-6-Octadecenoic acid, methyl ester	2.80
18.	14.86	Methyl isostearate	3.52
19.	14.90	Phytol	7.87
20.	14.98	Piperidine	0.77
21.	18.58	(Z)-β-Asarone	0.47

(C)

No.	Retention time (min)	Compound	Peak area (%)
1.	10.86	Caryophyllene	3.28
2.	11.57	Phenol-2,4-bis (1,1-dimethylethyl)	0.30
3.	11.60	δ-Cadinene	0.32
4.	11.66	Myristicin	6.33
5.	11.88	Cyclohexanemethanol	0.33
6.	11.88	Elemicin	0.30
7.	11.94	(Z)-Isoelemicin	38.68
8	12.31	Asarone	34.08
9.	12.45	α-Muurolol	0.63
10.	12.52	Apiol	1.12
12	12.54	Apiole	1.61
12.	12.79	4,8-dimethylnonanol	0.25
13.	13.39	Neophytadiene	6.78
14.	14.90	Phytol	5.99

(D)

No.	Retention time (min)	Compounds	Peak area (%)
1.	10.87	Caryophyllene	0.59
2.	11.62	Myristicin	9.03
3.	11.87	Elemicin	0.59
4.	11.92	(Z)-Isoelemicin	44.82
5.	12.31	Asarone	35.23
6.	12.45	α-Muurolol	0.96
7.	12.52	Apiol	2.09
8.	12.55	Apiole	2.10
9.	13.42	Neophytadiene	2.17
10.	13.60	Hexahydrofarnesyl acetone	0.69
11.	14.90	Phytol	0.84
12.	18.60	2-Propenoic acid, 3-(3,4-dimethoxyphenyl	0.89

Following the identification of the phytochemicals in the previous sections, the toxicological profile of the respective solvent extracts was assessed in zebrafish embryogenesis assay. The toxicity of different concentrations of each *P. sarmentosum* extract was evaluated, by observing the developmental changes and morphological abnormalities of the treated zebrafish embryos. For the developmental changes, there were several parameters observed, which include mortality rate, hatching rate and heart rate, meanwhile for the morphological abnormalities, they include somite formation, tail extension, presence of scoliosis and edema. Over the past few years, zebrafish has emerged as an ideal model organism for carrying out high-throughput screening and toxicity assessment [27, 35-36]. Zebrafish has been favoured by researchers over the conventional animal model used previously which are rodents, such as mice and rats as it is more cost-saving. Moreover, issues raised by many non-government organisations that support the animals' welfare, as well as animal ethics committee have prompted many researchers to turn to zebrafish as an experimental model in conducting their screening and toxicity analysis. Zebrafish as a model organism has attracted the interest of many researchers due to its unique characteristics, which are small size, transparent and short breeding cycle that promote high throughput chemical screening [37]. In addition, 70% of zebrafish genes have been found to be homologous to human genes [38] and it also exhibits similar physiological responses, especially during the development of chronic diseases [39]. Therefore, it is possible to predict the toxicity effects of one toxicant towards human through toxicity assessment of this toxicant in zebrafish model.

Survival rate was one of the developmental parameters observed and it was demonstrated by the total of embryos alive upon exposure to a range of different concentrations (0-100 ppm) of *P. sarmentosum* extracts. Figure 1 shows the survival rate of embryos treated with respective samples. All solvent extracts exhibited concentration- and time-dependent effects towards the survival rate of zebrafish embryos. HE demonstrated the lowest survival rate, followed by DE, EE and lastly, ME. As a comparison, the embryonic survival rate was shown to be 100% when exposed to only solvents (0.5% of methanol or 0.5% of DMSO) for 96 hpf. ME recorded only 29.17% of survival rate at the highest concentration employed (100 ppm) at 96 hpf, while no embryos were observed to be alive at 96 hpf for other solvent extracts at concentrations >40 ppm. Embryos demonstrated a significantly lower survival rate in EE, DE, and HE, when compared to ME at 40 ppm, with only 41.67%, 20.83% and 16.67% survival rate reported, respectively. Survival rate was then reflected by the LC_{50} values as presented in Figure 2. ME recorded the highest LC_{50} values at all time points, and was not significantly affected by the increase in the time exposure (Table 3). On the other hand, all solvent extracts (EE, DE and HE) exhibited a significantly lower LC_{50} values compared to ME. The LC_{50} values tabulated for ME, EE, DE and HE at 96 hpf were 70.25 ppm, 32.31 ppm, 28.86 ppm and 22.33 ppm, respectively. There was no significant difference in the LC_{50} values recorded between EE, DE and HE (Figure 2). The polarity of the solvents used for extraction and the method of extraction play vital roles in determining the efficiency and efficacy of toxicology activities [40]. GC-MS analysis of all solvent extracts revealed an interesting fact, which indicates the presence of two prominent compounds in each *P. sarmentosum* extract, which are isoelemicin and asarone, that could trigger specific toxicity following interactions with biological systems, despite the widely studied pharmacological activities. In a recent study by Shang et al., 2020 [36], they observed a markedly increase in the

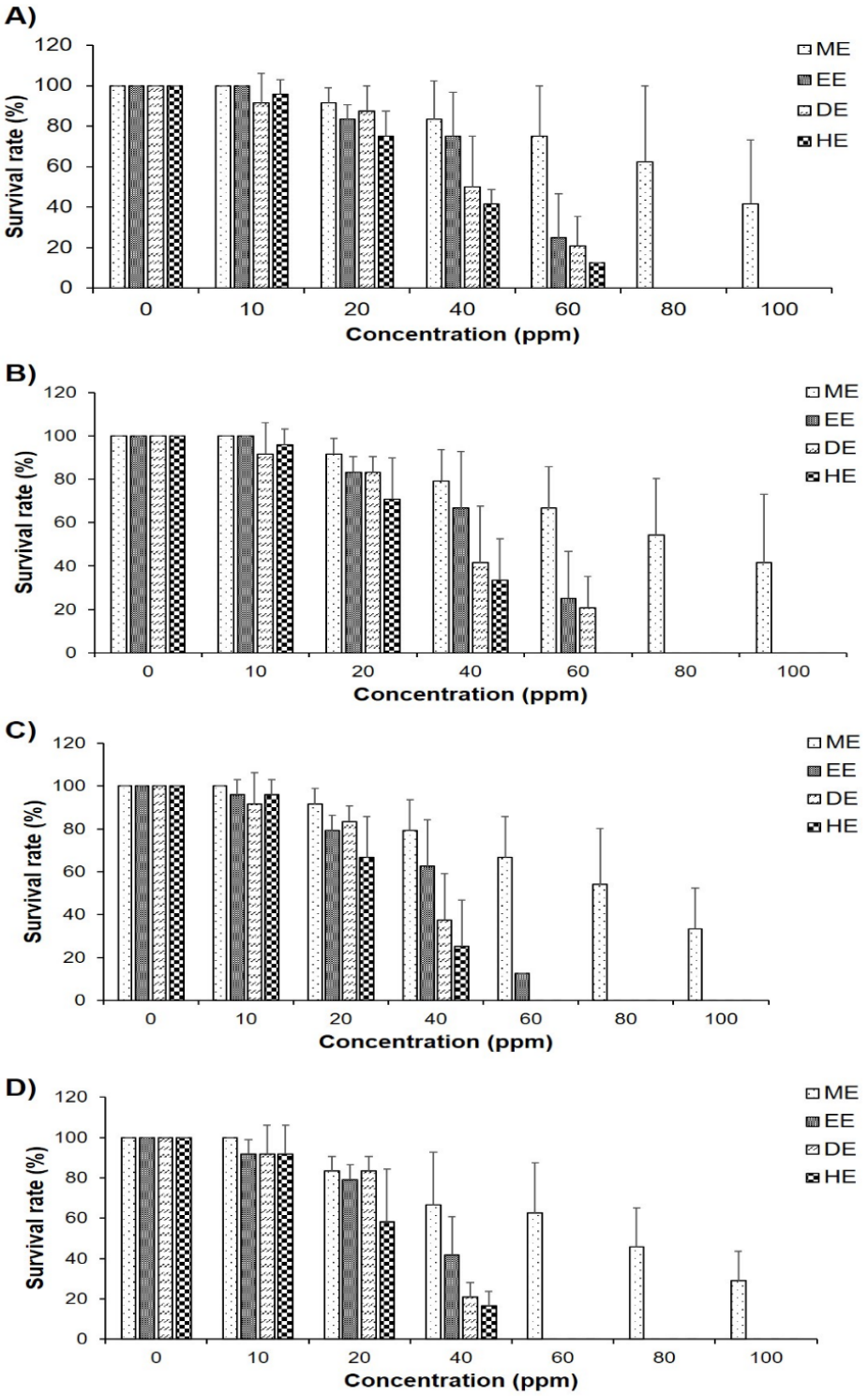

Figure 1: Effects of different treatments on survival of zebrafish (*Danio rerio*) embryos upon exposure at 24 to 96 hpf. Embryos of zebrafish were exposed at different concentrations of either (A) methanol extract (ME), (B) ethyl acetate extract (EE), (C) dichloromethane extract (DE) or (D) hexane extract (HE) of *P. sarmentosum* at concentrations of 0-100 ppm. Distilled water containing 0.5% DMSO or 0.5% methanol was used as the vehicle control. Data were averaged from three independent experiments and are shown as mean ± SD (n=3).

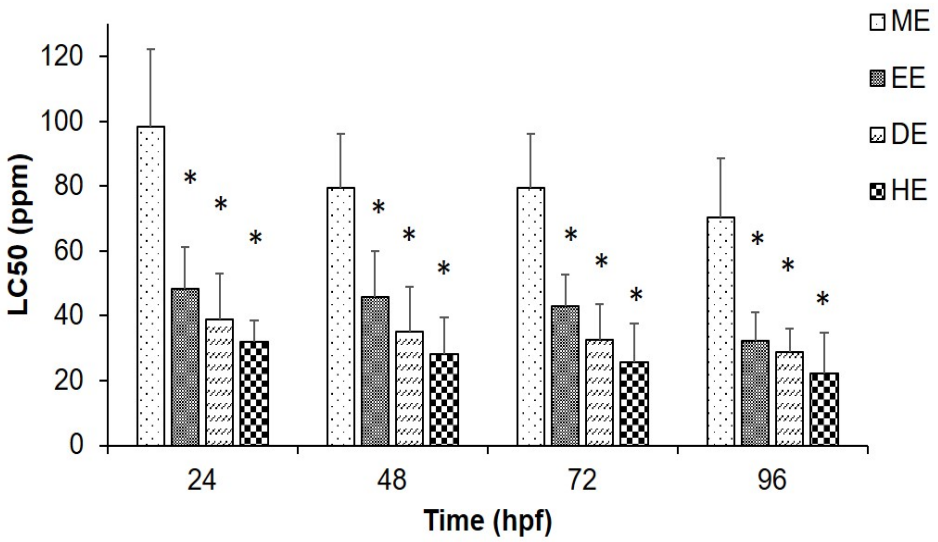

Figure 2: Lethal concentration 50 (LC_{50}) of zebrafish embryos of all samples at 24 to 96 hpf. Data represent the value of mean ± SD (n=3). Significant difference to ME is denoted by "*" (One-way ANOVA, $p < 0.05$).

Table 3. Comparative LC_{50} values of different solvent extracts of *P. sarmentosum* leaf against *Danio rerio* (zebrafish) embryos.

Extract (ppm)	#LC_{50} values (95% CI, ppm)			
	24 hpf	**48 hpf**	**72 hpf**	**96 hpf**
Methanol extract (ME)	98.41 (83.54-111.4)	79.64 (74.10-86.42)	79.64 (74.10-86.42)	70.25 (63.49-78.41)
Ethyl acetate Extract (EE)	48.44 (46.53-50.40)	45.72 (43.29-47.99)	42.92 (40.64-44.57)	32.31 (30.55-34.17)
Dichloromethane Extract (DE)	38.97 (36.45-41.41)	35.01 (32.68-37.39)	32.64 (30.76-34.65)	25.6 (27.87-29.89)
Hexane Extract (HE)	32.10 (30.85-33.38)	28.11 (26.47-29.85)	25.6 (24.15-27.15)	22.33 (21.08-23.66)

Note. #Mean value ± *SD*, (N=24)

mortality rate of zebrafish embryos when exposed to 30 µM of α-asarone from 24 to 192 hpf, while asarone has also been identified to induce hepatoxicity in THLE-2 human hepatocytes cells [33], with LC_{50} values ranging from 40 to 46 ppm. A study by Hematpoor et al., 2006 [7] showed that asarone was one of the active phenylpropanoids isolated from the root extract of *P. sarmentosum*, observed to be highly potent against the *Aedes* larvae. This particular compound caused up to 100% of mortality rate at 15 µg/mL and was identified as a neurotoxic compound due to its high acetylcholinesterase (AChE) inhibition. On the other hand, isoelemicin is commonly known to have a formal link with myristicin to amphetamines which causes hallucinogenic effects [41] and has been shown to induce cellular toxicity in HepG2 human liver cells, that was associated with multiple cytochrome P450 enzymes, such as CYP1A1, CYP1A2 and CYP3A4 [34]. Further precaution of the usage of elemicin in food, essential oils, as well as flavouring agents has been issued by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2008, which warrants future research on the possible risk of elemicin to human health [34]. The solvent extracts employed in the present study contains 26 to 35% of asarone and 26 to 45% of isoelemicin, which is postulated to cause synergistic toxicity that results in 100% mortality of zebrafish embryos at concentrations of ≥ 60 ppm in EE, DE and HE at 96 hpf.

Heart rate was also one of the toxicological end-points in zebrafish development that was evaluated following exposure to *P. sarmentosum* extracts. This was conducted to assess for any signs of cardiac disruption that might indicate potential toxicity effects. Transparent embryos during early stages of development allow for direct visual inspection of heart morphology, as well as easy assessment of the heart rate [42]. Normal cardiac rhythm is vital for proper development and growth of zebrafish. The heart rate was recorded during toxicity assay of the *P. sarmentosum* extracts for a solid 15 s at 96 hpf and the number obtained was multiplied by 4 to standardize it into beats per minute

(bpm). During this hour, embryos have fully developed a visibly regular heart beat and the normal embryonic heart rate of zebrafish was measured at 120-180 bpm, which is closer to the normal human heart rate compared to conventional animal model such as mouse [43]. Based on Figure 3, there was a significant sign of bradychardia (slow heart rate) for embryos treated with all solvent extracts at concentrations as low as 10 ppm. The heart rate was expressed as the heart rate measurements of the surviving embryos in different concentrations. Other than that, the heart rate was also influenced by deformity such as edema which lowered down the heart rate simultaneously. It has been reported that asarone employed at a lower concentration (1-15 μM) could contribute to the high incidence of heart defects, which indicates that the point target for toxicity of asarone is heart, which is the earliest organ developed during embryogenesis [36]. There was however no study reported on the toxicity of elemicin on the heart.

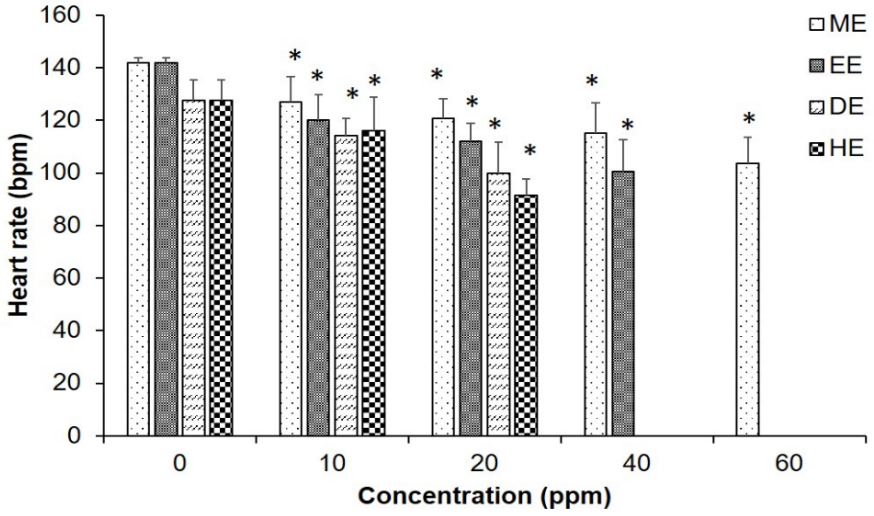

Figure 3: Effects of different treatments on the heart rate of zebrafish (*Danio rerio*) embryos upon exposure at 24 to 96 hpf. Embryos of zebrafish were exposed at different concentrations of either (A) methanol extract (ME), (B) ethyl acetate extract (EE), (C) dichloromethane extract (DE) or (D) hexane extract (HE) of *P. sarmentosum* at concentrations of 0-100 ppm. Distilled water containing 0.5% DMSO or 0.5% methanol was used as the vehicle control. Data were averaged from three independent experiments and are shown as mean ± SD (n=3). Significant difference compared to control treatment (0 ppm) is denoted by "*" (One-way ANOVA, $P<0.05$).

Based on the zebrafish embryonic developmental stage, period of 48 until 72 hpf is called as hatching period. Whether or not an embryo has hatched, its development is still progressing hour by hour. In this period, morphogenesis of many of the organ rudiments is now rather complete and slows down considerably, with some notable exceptions including the gut and its associated organs. Some of the easy-to-see developments are pectoral fins, the jaws and the gills [44]. Based on Figure 4, it is visible that ME has the highest hatching rate at 72 hpf, **(**Figure 4A) followed by EE (Figure 4B), DE (Figure 4C) and HE (Figure 4D). Since ME is the least toxic solvent, this could be attributed to the ability of the embryos to fully develop and hatch normally. In addition, it could be assumed that there was a permanent delayed-hatching involving the embryos for ME at ≥ 40 ppm, EE at ≥ 10 ppm, as well as DE and HE at ≥ 20 ppm. In a previous study, it has been shown that asarone was able to cause a significant decrease in the hatching rate of zebrafish embryos at 30 μM [36].

The formation of somite (Figure 5A) is one of the stages in the embryonic development. The overall process of zebrafish development is similar to that in amphibians, birds, and mammals [45]. In zebrafish, gastrulation is first visible when the shield is established on the dorsal side of the embryo. The first somite forms shortly after the end of gastrulation. At the end of the first day of development, somite formation is complete and somite patterning is close to completion [46]. A normal developed embryo would show spontaneous movements which is side-to-side contractions, while treated embryos with toxicant showed no or permanent delayed of somite formation (Figure 5B). Based on Figure 6A, since somite formation is favourable in the zebrafish embryonic development, the somite formation exhibited a significant decrease in the increasing concentrations starting from 40 ppm at 96 hpf when compared to the control group, with only 33.33% and 20.83% of embryos were found to have somite for 40 ppm DE and HE, respectively, which were significantly lower than ME.

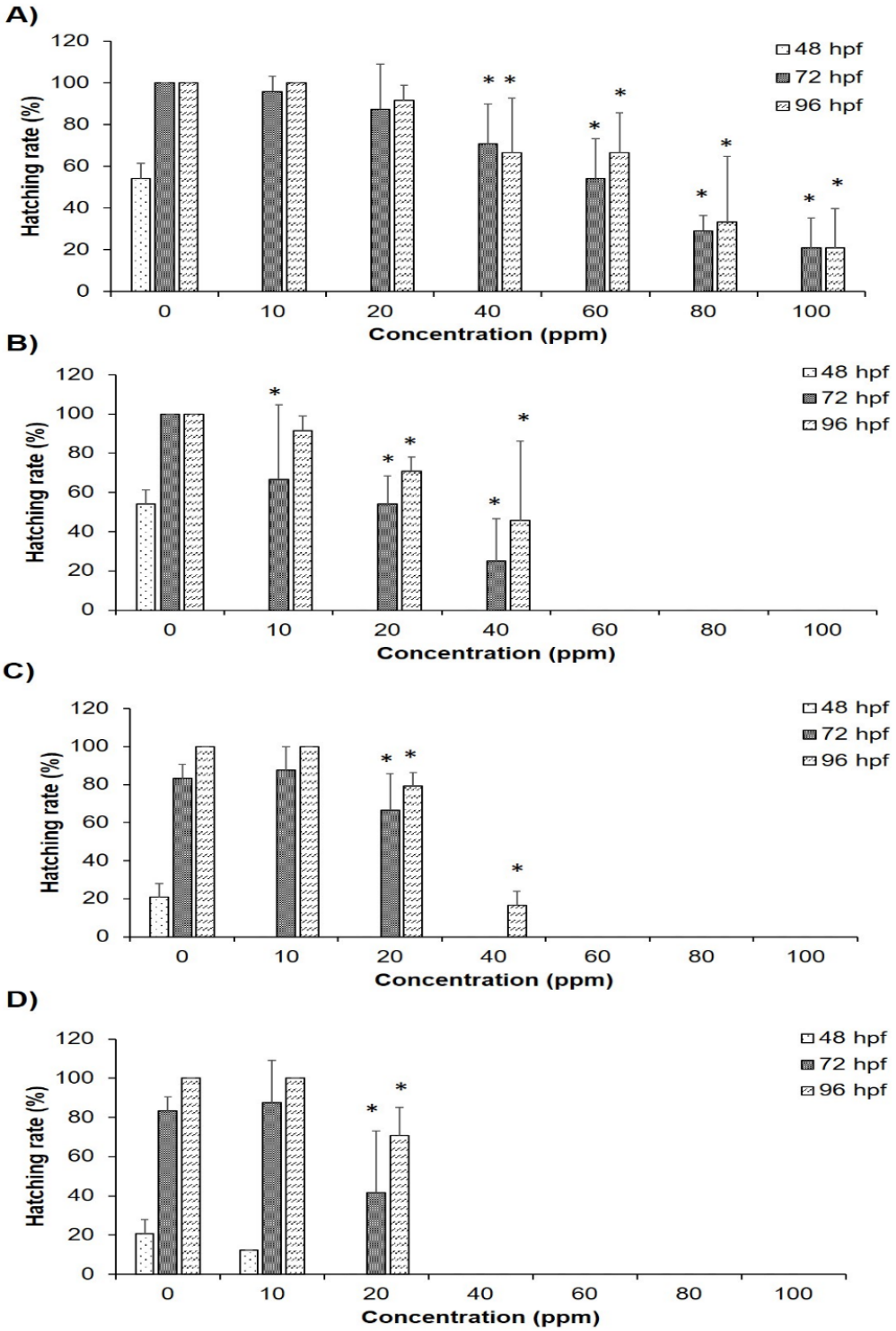

Figure 4: Hatching rate (%) of zebrafish (*Danio rerio*) embryos exposed to different concentrations of either (A) methanol extract (ME), (B) ethyl acetate extract (EE), (C) dichloromethane extract (DE) or (D) hexane extract (HE) of *P. sarmentosum* at concentrations of 0-100 ppm. Distilled water containing 0.5% DMSO or 0.5% methanol was used as the vehicle control. Data were averaged from three independent experiments and are shown as mean ± SD (n=3). Significant difference compared to control treatment (0 ppm) is denoted by "*" (One-way ANOVA, $p<0.05$).

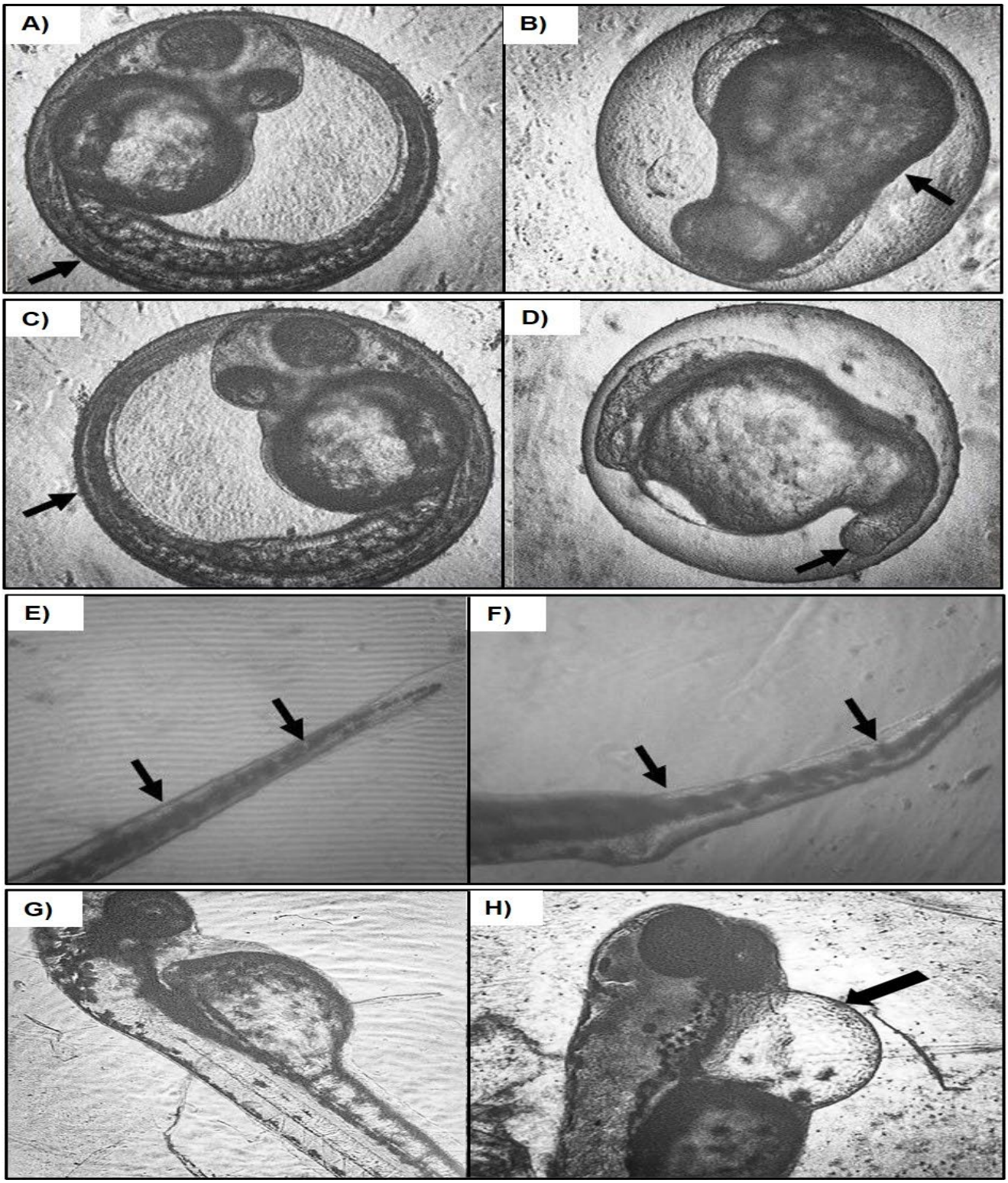

Figure 5: Microscope images showing incidence of malformations in zebrafish embryos. The comparison between the abnormalities was denoted by arrows Somite formation in **(A)** normal developing embryo (non-treated embryo) and **(B)** treated embryo. Figure shows **(C)** normal developing embryo with tail extension (as indicated by arrow) and **(D)** embryo with no tail extension (as indicated by arrow), **(E)** normal developing embryo and **(F)** embryo with presence of scoliosis. **(G)** Normal developing embryo and **(H)** embryo with presence of pericardial edema as indicated by arrow.

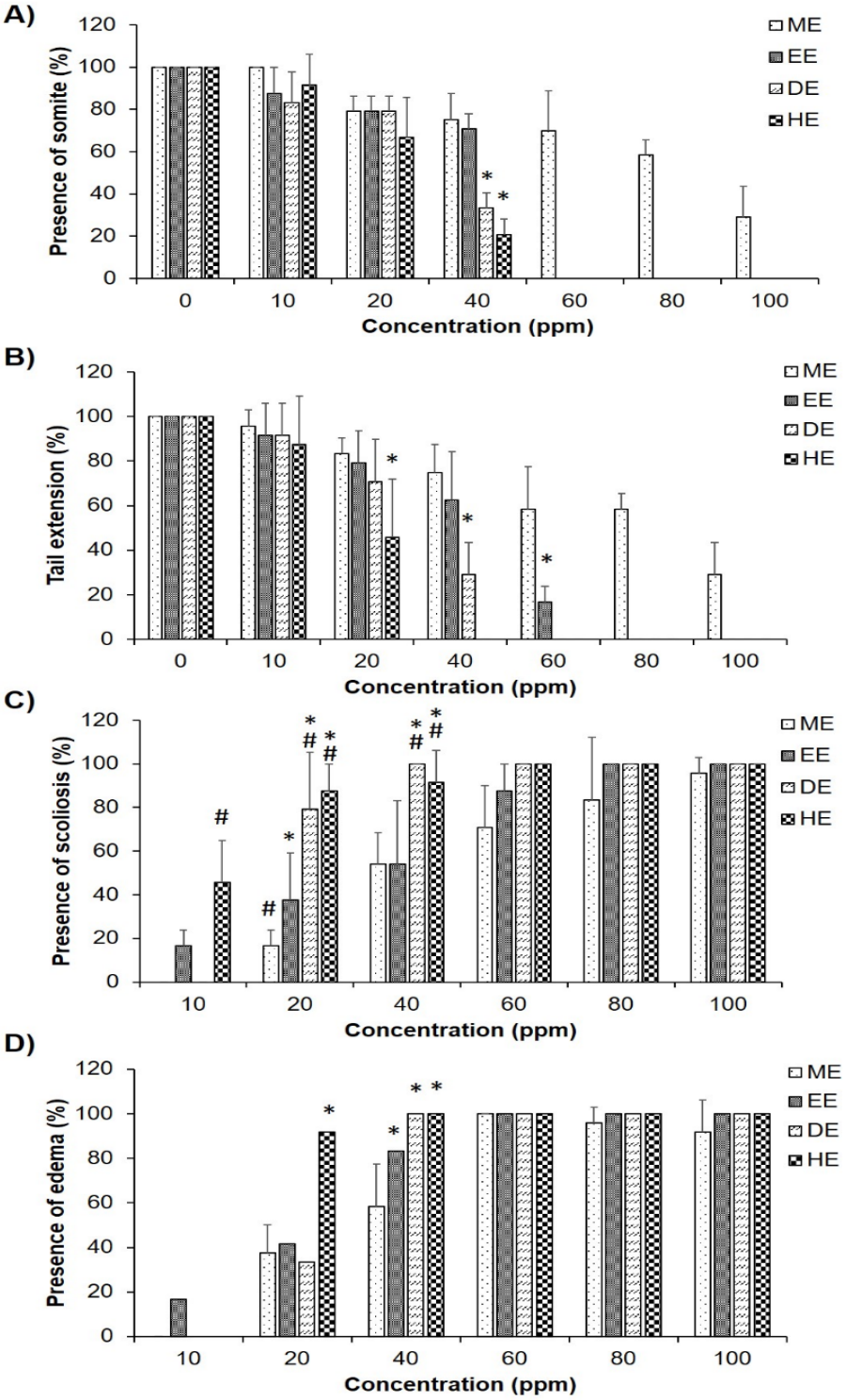

Figure 6: Morphological changes presented in zebrafish (*Danio rerio*) embryos following treatments with methanol extract (ME), ethyl acetate extract (EE), dichloromethane extract (DE) and hexane extract (HE) of *P. sarmentosum* leaf at different concentrations (0-100 ppm) as indicated by the presence of (A) somite, (B) tail extension, (C) scoliosis and (D) edema. Data were averaged from three independent experiments and are shown as mean ± SD (n=3). * and # indicate significant difference to ME and EE, respectively. (One-way ANOVA, $p<0.05$).

In a normal developing zebrafish embryo, detachment of tail from the yolk (Figure 5C) is observed following posterior elongation of the embryonic body. Tail pigmentation starts at 36 hpf during the pharyngula stage. Figure 5D demonstrates the malformation of zebrafish embryo at 96 hpf, with no tail extension was observed when exposed to 40 ppm of HE. HE and DE showed a lower incidence of tail extension at 20 ppm and 40 ppm when compared to both control and ME, which is in congruent with the toxicity effects of HE and DE (Figure 6B). Embryos treated with ME exhibited a higher incidence of tail extension, which indicates that treatment of embryos with HE and DE would exacerbate the malformations in embryos compared to ME. ME demonstrated a concentration-dependent decrease of tail extension, with a decrease in tail extension to 83.33% at 20 ppm and a significant drop to 29.17% at 100 ppm, the highest concentration employed in the present study. Meanwhile, EE showed a markedly decrease of tail extension formation at 16.67% for 60 ppm exposure.

Scoliosis is an abnormal curvature of the spine when the spine is curved from side to side or in an “S” or “C” shape [47] as shown in Figure 5F, when compared to a normal developing embryo (Figure 5E). As the presence of scoliosis is unfavourable in the embryo, it tallies with the mortality rate. Higher mortality rate contributes to higher presence of scoliosis observed [48]. Based on Figure 6C, HE, DE and EE exhibited 100% of scoliosis formation in the treated embryos at concentrations as low as 40 ppm. On the other hand, embryos treated with ME were only observed to have 95.83% of scoliosis at 100 ppm, which was not significantly different compared to other solvent extracts.

Unlike somite and tail extension, edema (Figure 5H) is a form of deformity and unfavourable property in embryonic development. For normal embryos (Figure 5G), they develop fully functional kidney unit within 72 h, and the presence of pericardial edema is one of the signs that indicates kidney failure. Occurrence of pericardial edema is believed to be attributed to high accumulation of fluid in the body cavity, which eventually leads to swollen of the heart causing heart failure [49]. Based on Figure 6D, it can be seen that at 60 to 100 ppm, all of the solvent extracts experienced more than 90% of edema formation. On the other hand, HE showed the highest percentage of edema occurrence at a lower concentration of 20 ppm (91.67%), which further confirms that HE was not only detrimental to the development changes of embryos, but also increased the malformation incidence. This was followed by DE, with 100% of edema was observed to be present at 40 ppm, followed by EE and ME at 60 ppm. The results obtained for the malformation incidence in zebrafish embryos was in accordance with the toxicity effects of all the solvent extracts, which demonstrated that HE was the most toxic extract, followed by EE, DE and lastly, ME. The phenomenon observed was in agreement with the phytochemical analysis of all solvent extracts as observed by the GC-MS, which further confirms the presence of two prominent compounds that have been investigated for their toxicity in biological systems; asarone and isoelemicin. Asarone has been shown to increase heart malformation rate and pericardial edema even at lower concentrations [36], and was able to induce hepatoxicity [33], which could further elevate the toxicity effects of the *P. sarmentosum* extract. The synergistic effects caused by the presence of other bioactive compounds, such as elemicin could further bring about the adverse effects of *P. sarmentosum* extract, which has been commonly used as vegetable staples in Asian countries, such as Malaysia and Thailand. In conclusion, the collective data gathered from the present study has provided a fundamental understanding of the toxicity effects of *P. sarmentosum* extract, which was largely affected by the solvents and concentrations of the extracts employed. However, further study is warranted to evaluate the toxicity effects of the *P. sarmentosum* extract on other *in vivo* models, such as rodents to determine and correlate the administration peroral dose, with the effective therapeutic concentration, to avoid any adverse effects following consumption.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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