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FULL PAPER

Nutritional composition, phytochemicals and acute toxicity of herbal mixture (lemon, apple cider, garlic, ginger and honey) in zebrafish embryo and Wistar rat

[1]Abu Bakar Sajak, A., [1,*]Azlan, A., [2]Abas, F. and [3]Hamzah, H.

[1]Department of Nutrition and Dietetics, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[2]Laboratory of Natural Products, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[3]Department of Veterinary Medicine, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

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Abstract

This study was aimed to provide the reference frame for the safe dose design of polyphenol-rich herbal mixture, which consist of lemon, apple cider, garlic, ginger and honey (PRM) for the future efficacy study. Prior to this, the nutritional composition was first conducted and the identification of metabolites that present in PRM was determined using proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. The acute toxicity of the PRM was then evaluated in zebrafish embryo and Wistar rats following The Organisation for Economic Co-operation and Development (OECD) guidelines. The PRM nutritional composition and sugar profile showed it was high in carbohydrate, ash and protein and the main sugar is fructose. It also contains metabolites such as fructosefuranose, lactic acid, ascorbic acid, acetic acid, cycloalliin, pyruvate, 5-hydroxymethylfurane, α- and β-glucose. From the zebrafish embryo acute toxicity result, the lethal concentration, LC50 of PRM was at 487.50 µg/mL. Meanwhile, in Wistar rats' model, no lethality was observed in the group treated with PRM at the end of the study (14 days). No changes were also observed from the behavioural and appetite as well as the biochemical parameters (AST, ALT, total cholesterol, triglyceride, LDL, HDL, total protein and creatinine) of the treated group. Therefore, the safe dose for PRM can be up to 2000 mg/kg b.w. in Wistar rats and below 487.50 µg/mL in zebrafish embryo model.

1. Introduction

Herbal mixture is still heavily used by the population in the countries such as India, China, Chile and Africa for maintaining health condition (Amole, 2012). Commonly, a mixture of herbs or plants is rich with primary and secondary metabolites such as organic acids, sugars, amino acids, phenolic compounds, alkaloids and tannins (Chien *et al.,* 2016).

Synergistic effects of these metabolites could have a potential in reducing the risk and treating metabolic diseases (Shin *et al.,* 2014; Chien *et al.,* 2016). Nonetheless, it could also increase the risk of side effects and toxicity (e.g. hepatotoxicity and nephrotoxicity), which eventually can lead to death (Shin *et al*., 2014). A systemic review by Jatau *et al*. (2016), found the prevalence rate of complementary and alternative medicine (CAM) in the emergency department (ED) ranged between 1.40-68.10%, where most of these cases are from the herbal therapy usage.

Therefore, several countries such as the European Unions and the United States of America have taken several regulatory measures in controlling the quality of herbal remedies production. Generally, the production of herbal remedies must meet the specific standards of safety and quality for the general public to consume (Ekor, 2013). Among the common test being held prior to clinical study or for the general consumption is the toxicity study. Acute toxicity study is important in determining the safety doses of a new substance or sample before being released for general public consumption (Colerangle, 2017).

In determining acute toxicity of a substance or compound, the most commonly used model is the murine model such as rats. This model is small, inexpensive,

*Corresponding author.
Email: *azrinaaz@upm.edu.my*

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easy to handle and most importantly, it is a mammal (Gad, 2014). However, a longer time is needed to determine the teratogenic effect of the sample on the mammalian animal model. This is because embryonic development in the mammal is slower and complex. Furthermore, the parents (mother) need to be sacrificed for this type of study (Adenan *et al.,* 2018).

Thus, zebrafish (*Dario rerio*) embryo acute toxicity has been proposed as an alternative model for drug discovery and toxicology studies. Nonetheless, both of the models (murine and zebrafish embryo) have their own advantage and disadvantage. Aside from their physiology, factors such as the rate of absorption or diffusion of the sample also play a major role in determining the toxicity effect (Klüver *et al.,* 2015). Therefore, it would be an added advantage to conduct acute toxicity test in different type of model.

The herbal mixture consists of ginger, garlic, apple cider, lemon and honey (PRM) was previously reported to reduce triglyceride levels in hyperlipidemia-induced rats and had a hepatoprotective effect on the liver (Naseem *et al*., 2016). In addition, this mixture was shown to reduce postprandial glucose in non-diabetic human females, alone or together with exercise (Ishak *et al*., 2018). Even though there were few studies conducted on this herbal mixture, there is still a lack of information in regard to its safety dosage and its phytochemicals that may contribute to their bioactivity. Thus, this study is aimed to provide the reference frame for the safe dose design of PRM as well as their toxic and adverse effects in the animals for the future efficacy study.

2. Materials and methods

2.1 Sample preparation

Preparation of polyphenol-rich mixture (PRM) was done according to Ishak *et al.* (2018), with a slight modification on the ratio. Lemon, garlic, ginger was first juiced using a juicer (ELBA EJE71A043lWH, Kuala Lumpur, Malaysia). Next, 250 mL juice of each ingredient was mixed with 250 mL of apple cider vinegar, which total up to a volume of 1000 mL. Then, the mixture was simmered (85°C) for 30 mins until it reduced to three quarter (750 mL) of the original volume (1000 mL). Tualang honey was added in 1:1 ratio into the mixture after it being left to cool in the room temperature. The PRM mixture was kept in the chiller (4°C) prior to use.

2.2 Proximate analysis of PRM

For the proximate analysis of PRM, the sample was sent to accredited laboratory analysis, Malaysian Agricultural Research and Development Institute (MARDI), Malaysia. The proximate analysis of PRM was done in duplicate. Standard method by AOAC (1990) was used to determine the energy, moisture, ash and crude fibre. Meanwhile, the protein was determined using Kjeldahl (Buchi 412) and fat using Soxhlet (Gerhardt Soxtherm 2000) according to Pearson *et al.* (1981). Carbohydrate was calculated by difference, which is by subtracting the value of protein, moisture, crude fibre, fat and ash from 100 (AOAC, 1993).

2.3 Sugar profile

Determination of sugar profile was sent to accredited laboratory analysis, MARDI, Malaysia. Sugar profile (fructose, galactose, maltose and sucrose) was determined using high-performance liquid chromatography (HPLC) according to previous method 89 by AOAC international (1993), Will and Greenfield (1984) and Waters (1987). PRM was first freeze-dried and extracted with 85% aqueous ethanol prior to the analysis. For the standard sugar solution, each 2% of individual sugar (fructose, galactose, maltose and sucrose) solution and 2% sugar mixtures were prepared for retention time and quantitative determination. All the samples were filtered with 0.45 µL ultrafilter prior to injection. The chromatographic separation of sugars standards and from the sample was achieved using Waters HPLC system in an isocratic mode consist of acetonitrile: distilled water: ethanol in 82: 17.5: 0.5. For separation, Hypersil (APS2) NH2, 5 µm (Thermo Hypersil-Keystone) with guard column was used. The column temperature was set to 25-30°C, flow rate of 1.5 mL/min and using RI detector. Both standard and PRM (10 µL) were injected to chromatographic column and areas or peak height of each sugar was measured.

2.4 Phytochemicals identification using ^{1}H NMR measurement

Prior to ^{1}H NMR measurement, two samples of PRM were prepared (with and without the addition of honey). This due to the high concentration of sugars in honey might over masked the other metabolites in the mixtures. In brief, 20 mg of PRM sample was dissolved in 1 mL of D_2O – solvent and this solution were then mixed with phosphate buffer in D_2O (pH 6.0), containing 0.1% TSP in 1:1 ratio and centrifuged. The 0.6 mL of the supernatant was transferred into a NMR tube for ^{1}H NMR analysis.

All the ^{1}H-NMR measurements were performed according to a previous method by Abu Bakar Sajak *et al.* (2016). For ease in metabolite identification, additional support from a two-dimensional (2D) NMR-*J*-resolved was also performed. The 2D NMR spectra were

processed using ACD/NMR Processor Academic Edition software (v. 12.0, ACDLABS). Assignment of metabolites was done by comparing the data Human metabolome database (HMDB) (Wishart *et al.* 2009) and previously reported assignments (Kim *et al*., 2010; Kim *et al.,* 2011; Boffo *et al.,* 2012; Fotakis *et al.,* 2013; Liang *et al.,* 2015; Campo *et al.,* 2016; Abu Bakar Sajak *et al.,* 2016).

2.5 Zebrafish embryos acute toxicity model

Zebrafish embryos acute toxicity was conducted according to Ismail *et al*. (2017) with a slight modification. Briefly, the healthy zebrafish embryos (24-hour post-fertilization (hpf)) were transferred one each into each well in the 96-well-plate using a pasteur pipette. Prior to this, 100 µL of PRM in different concentration was aliquoted into each well. The total of embryo for each concentration is n = 5. The PRM in different concentration was prepared by dissolving the PRM with the embryo media. The embryo development was monitored after 24 hrs, 48 hrs and 72 hrs of treatment. Three morphological characteristics such as hatching rate, survival rate and heartbeat (beats/minute) were recorded and analysed under an inverted microscope. Paracetamol at 1000 µg/mL was used as a positive control for this experiment.

2.6 Murine acute toxicity model

Acute toxicity study was conducted according to OECD Guidelines for Testing Chemicals 423 (2001), on male and female Wistar rats (n = 6 each sex) of 150-220g (Institutional animal ethics approval: AUP-R012/2017). The rats were acclimatized for 1 week and place in plastic cages in groups (male, female separated) with stainless steel covers at room temperature (26-28° C) under a 12 hrs dark/12 hrs light cycle. The rats were fed with normal rat chow and filtered water ad libitum during the acclimatization period.

After one week, the rats were separated into two groups, control (n = 6, 3 male and 3 female) and treatment (n = 6, 3 male and 3 female). The control group was given 1 mL of filtered water, while the treatment group was given PRM at 2000 mg/kg body weight. The rats were observed for any changes in breathing, behaviour or mortality for the first 2 hrs, 4 hrs, 6 hrs and throughout within the 14 days after the single dosage.

2.6.1 Bodyweight measurement

The weight of each rat was taken at 0 week (on the day of the arrival), day 1 (the day after acclimatization, right before sample administration), 1st week and the 2nd week after the dosing.

2.6.2 Organ weight measurement

Exsanguination under ether anaesthesia was conducted on the rats after the 14th day of the first dosing. Organs such as kidney, liver, stomach, and spleen were weighed after rinsed clean (with 0.09% normal saline) and blot dried with tissue paper. The relative organ weight was calculated as follows:

$$\text{Relative organ weight (ROW)} = \frac{\text{Absolute organ weight (g)}}{\text{Bodyweight of rat on saccrifice day (g)}} \times 100$$

2.6.3 Serum Biochemistry

Serum biochemistry was measured for lipid profile (total cholesterol (TC), triglyceride (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL)), kidney function (creatinine), total protein and liver profile (AST and ALT) using automatic biochemical analyser (Hitachi 902, Roche, Germany). All the values are expressed as the mean ± standard deviation.

2.7 Statistical Analyses

The results are presented as the mean ± standard deviation. The statistical significance of the difference was evaluated using one-way ANOVA with Tukey's post hoc test $p<0.05$ is considered significant.

3. Results

Nutritional composition (Table 1) showed that PRM is high in carbohydrate (43.40±1.84 g/100 g), followed by protein (0.75±0.07 g/100 g) and ash (0.30±0.00 g/100 g) with the total energy of 176.50±7.78 kcal/100 g. The moisture content of PRM was 55.55±1.90 g/100 g sample, which indicates half of the sample is consist of water. Meanwhile, the analysis of the sugar (Table 1) showed that fructose was the primarily sugar component in PRM with 18.60±0.57%, followed by glucose 10.70±0.57% and maltose 1.00±0.28%.

Table 1. Nutritional composition and sugars profile of herbal mixture (PRM)

Nutritional composition (per 100g)		Sugars Profile	(%)
Energy	176.50±7.78 kcal	Fructose	18.60±0.57
Fat	0.00±0.00 g	Glucose	10.7±0.57
Protein	0.75±0.07 g	Maltose	1.00±0.28
Moisture	55.55±1.90 g	Sucrose	0.00±0.00
Ash	0.30±0.00 g		
Carbohydrate	43.40±1.84 g		

Identification of metabolites in PRM was performed using proton nuclear magnetic resonance, ^{1}H NMR. Since honey has a higher concentration of carbohydrates (e.g. glucose, fructose, maltose), two PRM samples (with and without honey) were run separately to avoid other

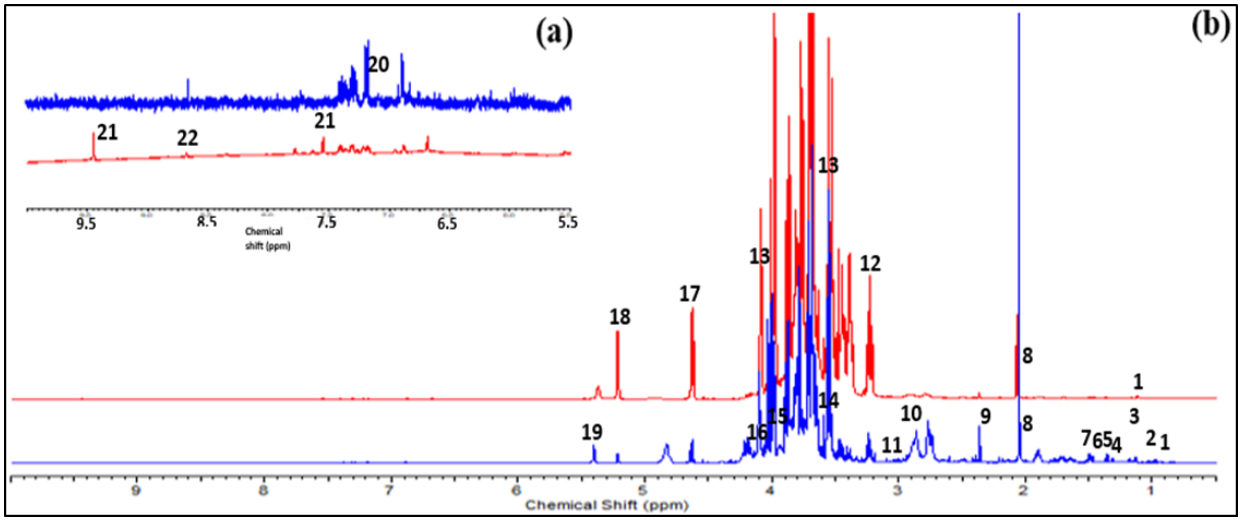

Figure 1. Representative ^{1}H NMR spectra for polyphenol-rich mixture (PRM) with (red) and without (blue) the addition of honey from 0.50 – 10.00 ppm, where (a) extended region form 5.50 – 10.00 ppm, (b) the full spectra. Different number on spectra represents: 1, valine; 2, isoleucine; 3, ethanol; 4, threonine; 5, lactic acid; 6, alanine; 7, cycloalliin; 8, acetic acid; 9, pyruvate; 10, asparagine; 11, gamma amino butyric acid (GABA); 12, β-glucopyranose; 13, fructosefuranose; 14, glycerol; 15, ascorbic acid; 16, fructose; 17, β-glucose; 18, α-glucose; 19, sucrose; 20, tyrosine; 21, 5-hydroxymethylfurfural (5-HMF); 22, formic acid.

metabolites being masked or overlap by honey spectra. Figure 1 shows the spectra of PRM with and without honey. A total of 22 metabolites were identified (Table 2). These include amino acids (valine, isoleucine, threonine, asparagine, tyrosine and alanine), organic acids (acetic acid, lactic acid, formic acid and ascorbic acid), sugars (*α*-glucose, *β*-glucose, sucrose, fructose, fructosefuranose and *β*-glucosepyranose) and others (5-hydroxymethylfurfural (5-HMF), gamma-aminobutyric acid (GABA), pyruvate, glycerol, ethanol and cycloalliin).

There are many models that currently being used to determine the toxicity level of compounds or substance. Fish embryo toxicity test has been proposed as one of the alternatives to determine substance or compound with low to moderate toxicity effect. Figures 2a and 2b, show that the survival rate and hatching rate were dose-dependent (780 - 6.09 µg/mL). The zebrafish embryo started to hatch after 72 hrs being exposed to the treatment. From the result, the LC_{50} of PRM was 487.50 µg/mL. At the highest concentration, 780 µg/mL, only 20% of the embryos hatched and survives after 72 hrs.

The heart rate (Figure 2c) was calculated at the end of the study (96 hrs after exposure with PRM). From the result, the average heart rate of zebrafish embryo treated with PRM was 121.71 beats/min. Lower to medium PRM concentration (48.75-6.09 µg/mL) significantly (p<0.05) increased the heartbeat compared to the positive control, paracetamol (111.20±1.60 beat/min). Meanwhile, there was no significant difference (p>0.05) for the higher concentration (390.00-97.50 µg/mL) when compared to the positive control, paracetamol.

As there was no acute toxicity study previously conducted on PRM, the selection of dosage for this

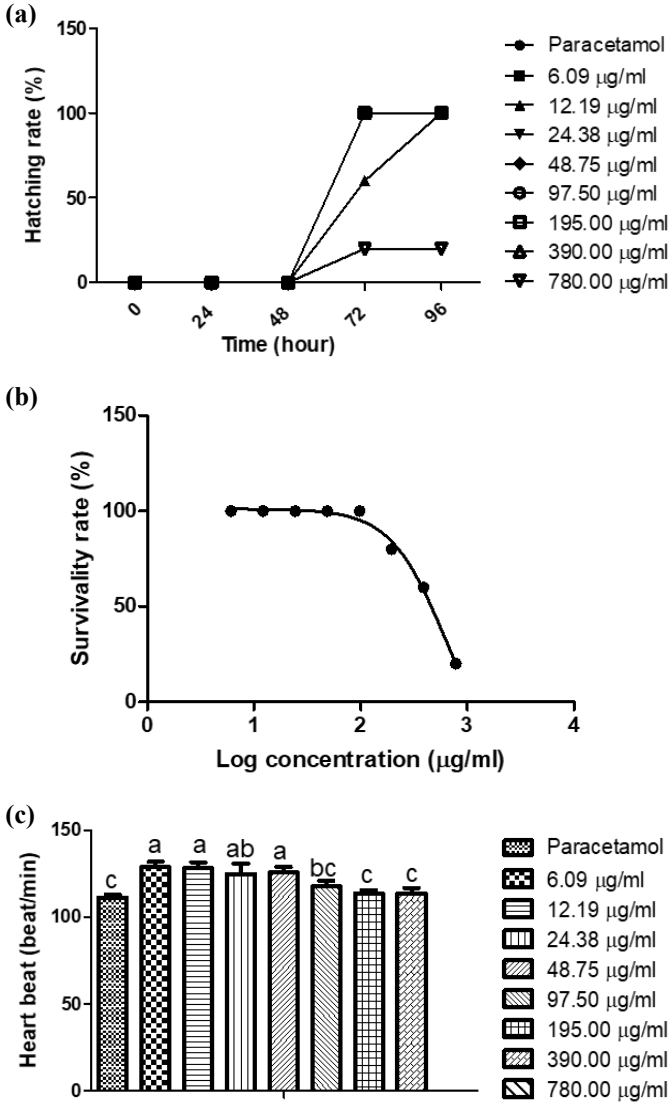

Figure 2. Hatching rate curve (a), survival curve (b) and heart rate (c) when tested with PRM at different concentration (6.09 -780 µg/mL), where (a) is hatching curve of zebrafish embryo from 0 – 96 hrs. Survival curve (b) and heart rate (c) were reading at 96 hrs.

Table 2. Assignment of ^{1}H NMR spectra peaks of polyphenol-rich mixture (PRM)

Metabolites	^{1}H (ppm) and multiplicity	References
Amino acids		
Alanine	1.47 d	Abu Bakar Sajak *et al.* (2016)
Asparagine	2.86 dd, 2.95 dd	Kim *et al.* (2010)
Isoleucine	1.01 d	Fotakis *et al.* (2013)
Threonine	1.32 d	Campo *et al.* (2016)
Tyrosine	6.89 d, 7.18 d	Abu Bakar Sajak *et al.* (2016)
Valine	0.98 d, 1.03 d	Kim *et al.* (2010)
Sugars		
Fructose	3.89 dd, 4.01 m	Fotakis *et al.* (2013)
Fructosefuranose	3.52 - 3.55 m, 4.06 - 4.09 m	Boffo *et al.* (2012)
Sucrose	5.40 d	Abu Bakar Sajak *et al.* (2016)
α-glucose	5.22 d	Abu Bakar Sajak *et al.* (2016)
β-glucopyranose	3.23 t	Boffo *et al.* (2012)
β-glucose	4.63 d	Abu Bakar Sajak *et al.* (2016)
Organic acids		
Acetic acid	2.07 s	Campo *et al.* (2016)
Ascorbic acid	3.68 dd, 3.70 d, 3.98 m	Kim *et al.* (2011)
Formic acid	8.34 s	Liang *et al.* (2015)
Lactic acid	1.37 d	Boffo *et al.* (2012)
Others		
5-hydroxymethylfurfural (5-HMF)	6.68 dd, 7.54 dd, 9.44 s	Boffo *et al.* (2012)
Cycloalliin	1.50 d	Liang *et al.* (2015)
Ethanol	1.17 t	Liang *et al.* (2015); Campo *et al.* (2016)
Gamma-amino butyric acid (GABA)	3.08 t	Kim *et al.* (2010)
Glycerol	3.55 m	Boffo *et al.* (2012)
Pyruvate	2.36 s	Wishart *et al.* (2009)

Table 3. Relative organ weight (ROW) of control and polyphenol-rich mixture (PRM) group

Group	Weight	Liver	Kidney		Stomach	Spleen
			Left	Right		
Control	203.67±30.44	3.47±0.41	0.44±0.08	0.43±0.07	0.71±0.10	0.31±0.09
PRM (2000mg/kg b.w)	198.00±40.81	3.26±0.71	0.380.04	0.39±0.04	0.61±0.07	0.25±0.08

Where* shows significant differences (p<0.05) between the two groups using T-test.

murine acute toxicity study was determined by comparing with previous studies conducted on the individual ingredients (garlic, honey, ginger, apple cider, and lemon). The lowest dosage for acute toxicity tested was found in lemon and garlic, where it was tested up to 2000 – 2500 mg/kg body weight (Chijok e-Nwauche and Dede, 2010; Lawal *et al.,* 2016).

From the observation in Wistar rats model, no lethality, behavioural changes, and abnormality in breathing were observed after the first 2, 4, 6 hrs of administration and throughout the 14 days of PRM administration at 2000 mg/kg body weight. There were no significant changes (p>0.05) in weight between the control and PRM group after 14 days of PRM administration (Figure 3). Relative organ weight also showed no significant difference (p>0.05) between those two groups (Table 3). Serum biochemistry results also showed there were no apparent changes (p>0.05) in the lipid profiles, liver function enzymes, kidney function (creatinine) and total protein level of rats administered with PRM (Table 4).

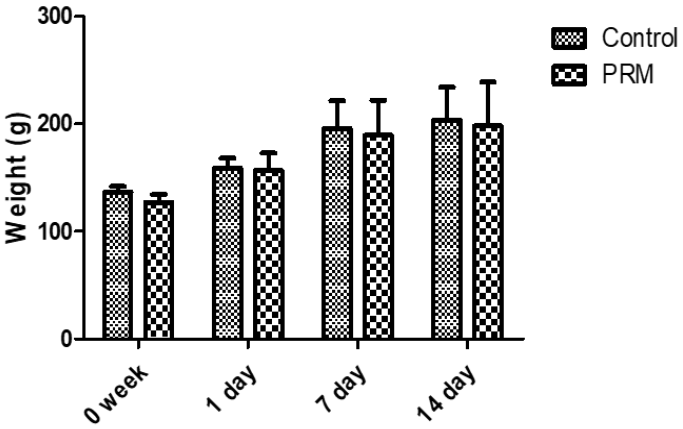

Figure 3. Weight for control and polyphenol-rich mixture (PRM) treated group at 0 week (acclimatization), 1st day, 7th day and 14th day. Where * showed significant differences (p<0.05) between the two groups using T-test

Table 4. Serum biochemistry for control and polyphenol-rich mixture (PRM) treated rats

Test	Protein (g/L)	Kidney function	Liver function (U/L)		Lipid profile (mmol/L)			
Group	Total Protein	Creatinine	AST	ALT	Cholesterol	Triglyceride	LDL	HDL
Control	75.24±11.81	39.00±7.00	201.00±78.23	27.40±6.43	2.02±0.44	0.84±0.36	0.52±0.20	1.42±0.35
PRM	70.14±5.65	36.80±7.12	189.60±43.59	24.20±3.11	2.10±0.27	0.90±0.26	0.43±0.13	1.50±0.15

Where AST is aspartate aminotransferase, ALT is alanine aminotransferase, HDL is high density lipoprotein and LDL is low density lipoprotein. Symbol* shows significant differences (p<0.05) between the two groups.

4. Discussion

As far as we have known, this is the first report on the nutritional composition of PRM. The physicochemical analysis showed that the nutritional composition of PRM is mostly consisting of carbohydrate, ash and protein. Where, most of this carbohydrate probably came from honey, that known to be rich with sugars. This result is supported by the previous study on honey by Ouchemoukh *et al.* (2010), where they found that the monosaccharide (fructose and glucose) are the majority in the Algerian honey samples.

To identify the phytochemicals in PRM, ^{1}H NMR analysis has been done on the PRM sample. The usage of ^{1}H NMR spectra has been performed for identification and quantification of metabolites in foodstuffs such as honey, herbs, meat and many more (Marcone *et al.,* 2013; Campo *et al.,* 2016). From the spectra, the major signal in PRM came from acetic acid, followed by sugar group such as fructose, glucose and sucrose.

Due note, there was a little bit of differences in sugar profile such as sucrose and maltose in PRM analyzed with HPLC and ^{1}H NMR. The differences in this result are probably due to the sensitivity of the analytical instruments. Previous research showed that metabolites such as ascorbic acid, cycloalliin, 5-hydroxymethyfurfural (5-HMF) have a good anti-oxidant, anti-ageing and anti-inflammatory activities (Superko and Krauss, 2000; Akhtar *et al.,* 2012; Ahmed and Othman, 2013). The presence of these metabolites makes PRM a suitable candidate for bioactives mixture in preventing and treating metabolic diseases.

Acute toxicity studies are conducted to test the short-term adverse effects of a substance in single or multiple of dose (Gad, 2014). Findings from this studies is important, as it can provide the information for the possible acute toxicity in human, to estimate the safe dose before the efficacy study, to determine the appropriate dosage for multiple-dose toxicity, to estimate the time course for the drug-induced clinical observation and to determine the target organ toxicity produced by the drugs (Colerangle, 2017).

In this study, we conducted two acute toxicity study using two different models. The relevance in conducting these two different models for the acute toxicity study is because the different model has different adaptability and sensitivity to toxicity (Adenan *et al.,* 2018). The differences in toxicity level in the different model are mostly due to their physiology, behavioural and environment. So far, there is no standard dose equivalence of zebrafish embryo to rats. Therefore, this study aims to provide information on the toxicity dose of PRM for both models.

The feasibility of using zebrafish embryo as preliminary indicator for teratology and embryological toxicity makes it one popular animal model (He *et al.,* 2014). Not to mention, 75% of genes that encodes the proteins and organs for zebrafish are similar to human (Hsu *et al.,* 2007). In this study, zebrafish embryo that been used is 24 hours post-fertilization (hpf), which is the organ development stage. The lethality of zebrafish embryo is identified from the hatching rate and the absence of heartbeats. The un-hatched embryos can be due to the formation of coagulation, which disrupts the embryo development and eventually leads to the lethality of the embryo (Ali, 2007).

From the result, the lethality of the zebrafish embryo is dose-dependent. A similar result also found by Adenan *et al.* (2018), where they found that lethality rate in zebrafish embryo treated with kelulut honey is dose-dependent. In addition, heartbeat (cardiac rate) of PRM increased as the PRM concentration lower when compared to the positive control, paracetamol. However, this heartbeat is considered as normal. The normal embryonic cardiac rate is more or less similar to human, which is 120-180 beat per minutes (De Luca *et al.,* 2014). This result means that PRM in higher concentration (780-97.50 µg/mL) and paracetamol (1000 µg/mL) can cause abnormalities in cardiac rate count (≤ 120 beats per minutes). The cardiac rate is mostly influenced by the ventricular contraction (Antkiewicz *et al.,* 2005). Therefore, a high concentration of PRM and paracetamol can decrease the ventricular contraction in the zebrafish embryo.

According to The Organisation for Economic Co-operation and Development (OECD) guideline for the murine model, only moderately toxic doses should be

used in acute toxicity study and the doses that can produce pain, distress and lethality should be avoided (Colerangle, 2017). From the weight, relative organ weight (ROW) and serum biochemistry results, it can be concluded that single-dose administration of PRM at 2000 mg/kg body weight in Wistar rats is safe. No apparent toxicity or adverse side effects were seen in the rats. Previous acute toxicity study conducted by Lawal *et al.* (2016), noted the rats given garlic at 5000 mg/kg were weak and lethargic but no death was recorded. Therefore, the doses chosen for this study is still appropriable and no apparent side effects from the interaction of the ingredients of the mixtures.

4. Conclusion

In conclusion, the PRM toxicity effect on zebrafish embryo model is dose/concentration-dependent. The highest concentration of PRM, can reduce the hatching, survival and cardiac rate of the embryo. Meanwhile, in the murine model, no toxicity effect was observed in the rats. Therefore, the safe dose design for PRM can be up to 2000 mg/kg. Since no adverse effects and lethality was observed in the study, the lethal dose (LD_{50}) for this model cannot be determined. Up to 22 metabolites manage to be identified using ^{1}H NMR. Identification of metabolites in PRM showed that it contains metabolites such as fructosefuranose, lactic acid, ascorbic acid, acetic acid, cycloalliin, succinate, 5-hydroxymethylfurane, α- and β-glucose.

Conflict of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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