

Nutrient content and toxicity of pumpkin seed flour

^{1,*}Syam, A., ²Zainal, ³Wahiduddin, ⁴Cangara, M.H., ⁵Kurniati, Y. and ¹Hasfiah, N.A.

¹Department of Nutrition, Faculty of Public Health, Universitas Hasanuddin, Jalan Perintis Kemerdekaan 10, Tamalanrea, Makassar, Indonesia

²Food Technology Study Program, Faculty of Agriculture, Universitas Hasanuddin, Jalan Perintis Kemerdekaan 10, Tamalanrea, Makassar, Indonesia

³Department of Epidemiology, Faculty of Public Health, Universitas Hasanuddin, Jalan Perintis Kemerdekaan 10, Tamalanrea, Makassar, Indonesia

⁴Department of Anatomical Pathology, Faculty of Medicine, Universitas Hasanuddin, Jalan Perintis Kemerdekaan 10, Tamalanrea, Makassar, Indonesia

⁵Public Health Study Program, Faculty of Medicine and Health Sciences, Universitas Islam Negeri Alauddin, Sultan Alauddin Street, no. 63, Romangpolong, Gowa, Indonesia

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Abstract

Pumpkin seeds are rarely used as food, despite their potential to become a functional food. Among other ways, pumpkin seeds can be processed into flour. This study aimed to assess the nutrient content, quality, and toxicity of pumpkin seed flour from Indonesia. This study followed an experimental design. Different methods were employed to measure the content, the Luff-Schoorl method for measuring carbohydrates, Kjeldahl method for protein, Soxhlet for fats, and the direct method for ash, crude fiber and moisture. The spectrophotometric method was used to measure vitamin content and mineral content was measured using X-Ray fluorescence. The subchronic toxicity test was conducted using five experimental animal groups, consisting of four intervention groups and one control group. The intervention groups received pumpkin seed flour of 10.8 mg/kg BW, 9 mg/kg BW, 5.4 mg/kg BW and 4.5 mg/kg BW. After 30 days, the experimental animals were dissected to check for liver and kidney damage. Pumpkin seed flour contains water (4.17%), ash (4.02%), proteins (35.30%), fats (36.30%), crude fibers (14.20%), carbohydrates (6.02%), vitamin C (0.10%), vitamin A (65.12 mg/kg), calcium (573.03 ppm), copper (3.10 ppm), iron (104.38 ppm), zinc (68.87 ppm), phosphorus (0.17%), magnesium (0.33%) and manganese (119.48 ppm). In the toxicity test, no death or organ damage occurred among the experimental animals. Therefore, pumpkin seed flour meets the requirements for development into functional food and supplements.

1. Introduction

Pumpkin is widely cultivated in tropical and subtropical countries. It has approximately 825 species (Sood *et al.*, 2012). Pumpkin fruit is consumed mostly as vegetables or processed into cakes. However, the seeds are rarely used (Pawarti, 2012), as most people eat only the flesh and do not consume the seeds. While pumpkin seeds contain many important nutrients for the human body, it is still treated as agro-industry waste. Pumpkin seeds are a good source of protein and have pharmacological activity (Nkosi and Opaku, 2006). They contain good exogenous amino acids and omega 3 and

omega 6 fatty acids, which are important for hormonal balance, brain function and skin health (Patel, 2013).

A study reported that the antioxidant content of pumpkin seed extract can improve fertility and prevent atherosclerosis (hardening of the arteries), high blood pressure and heart disease by improving fat metabolism. Pumpkin seeds are also used as food or functional medicine to treat enterozoa and prostate problems (Kim *et al.*, 2012).

Pumpkin seeds contain very interesting nutraceuticals. The oil of pumpkin seeds is used as a functional food to improve the conditions of patients

*Corresponding author.

Email: amin.gzuh@gmail.com

with hypertension, diabetes and cancer (Montesano *et al.*, 2018). The seed oil has an antihypertensive potential (Zuhair *et al.*, 2000). Pumpkin seed extract supplementation reduces blood pressure (Wong *et al.*, 2019) and is cardioprotective (El-Mossalami *et al.*, 2012). The seeds also contain alkaloids, steroids and hydroquinone phenol compounds—as well as ethyl acetate that can inhibit bacterial growth (Rustina, 2016).

Previous studies have reported the potential benefit of pumpkin seeds, as seeds from different pumpkin types will have different content and biological activities (Ceili *et al.*, 2006). Most importantly, pumpkin seed flour can be processed into a supplement at affordable prices (Syed *et al.*, 2019). In addition, the nutraceutical content in pumpkin seeds allows their development into various new products (Lestari and Meiyanto, 2018). However, studying its toxicity is important to ensure safe consumption (Doho and Chacha, 2020). This study aimed to determine the nutrient content and subchronic toxicity of pumpkin seed flour.

2. Materials and methods

2.1 Pumpkin seed flour preparation

The pumpkin seed flour was prepared at the Culinary Laboratory of the Faculty of Public Health, Universitas Hasanuddin. The pumpkin seeds were collected from the local market, washed and cleaned. Then, they were dried in the sun for 7 hrs and in an oven at 70–75°C for 3 hrs. Finally, the pumpkin seeds were blended until fine and sieved through a 70-mesh sieve.

2.2 Measuring carbohydrate content

The Luff–Schoorl method was used to determine carbohydrate content. The pumpkin seed flour sample was carefully weighed (5 g) into a 500 mL Erlenmeyer flask. Then, 200 mL of 3% HCl was added and boiled for 3 hrs in an upright cooler. Subsequently, the solution was cooled and neutralized with 30% NaOH solution (tested qualitatively with litmus paper or phenolphthalein). Thereafter, a total of 3% CH₃COOH was added. The contents were then transferred to a 500-mL volumetric flask and distilled water was added to the mark and then filtered. Then, 10 mL of the filtrate was pipetted into a 500-mL Erlenmeyer flask and 25 mL of the Luff–Schoorl solution was added. Next, 15 mL of distilled water and a few boiling stones were added. The mixture was heated with a constant flame. Distilled water was added until the 100-mL line mark, was shaken, left to stand for 30 mins, continued to simmer for 10 mins and then chilled with ice in a tub. After cooling, 15 mL of 20% KI and 25 mL of H₂SO₄ were added slowly. It was then titrated immediately with 0.1 N Na₂S₂O₃ solution (use 0.5% starch indicator). The

calculation was as follows:

$$\% \text{ Carbohydrates} = (\text{mg glucose} \times \text{dilution factor}) / (\text{sample weight (mg)}) \times 100\%$$

2.3 Determination of protein content

The Khjedhal method was used to measure protein content. Pumpkin seed was weighed (0.5 g) and placed into a 100-mL Kjeldahl flask. Thereafter, approximately 1 g of selenium was mixed with 25 mL concentrated H₂SO₄. The Kjeldahl flask and its contents were shaken until all was moistened with H₂SO₄. Then, it was subjected to a destruction process in the acid chamber. The process employed gradual temperature increase until a clear yellow solution was produced. The solution was allowed to cool, poured into a 100-mL volumetric flask, rinsed with distilled water and placed up to the mark of the volumetric flask. It was then transferred into a container with 10 mL of 2% H₃BO₃. The sample solution was pipetted by 10 mL each time into the distillation flask. Then, 10 mL of 30% NaOH was added gradually using a dropper (sample solution until the color turned red), followed by 100 mL of distilled water. The distillation process was continued until the reservoir volume became approximately 50 mL. Subsequently, the sample in the flask was titrated with a 0.0171 N H₂SO₄ solution. The protein content was then calculated by the following formula:

$$\text{Protein content} = \text{N content} \times \text{correction factor}$$

$$\text{N content} = (V \times N \times 14 \times 6.25 \times P) / (\text{mg sample weight}) \times 100\%$$

2.4 Determination of fat content

The Soxhlet method was used to determine fat content. The pumpkin seed flour was weighed (1 g) and then put in a 15-mL test tube. Chloroform was added, and the tube was closed. It was covered tightly, shaken, and then left overnight. Later on, the sample was filtered through a filter paper into a test tube. Then, 5 mL of the sample was pipetted into a cup of known weight (A g). The sample was then placed into an oven at 100°C for 4 hrs. Next, it was taken out, put into the desiccator for 30 mins, and then weighed (B g). Fat content was calculated using the following formula:

$$\% \text{ Fat content} = (P \times (B - A)) / (\text{sample weight}) \times 100\%$$

$$P = \text{Dilution (10/5)}$$

2.5 Determination of ash content

Ash content was measured using the direct method (oven). A clean porcelain cup was put in the oven at 105°C for 2 hrs. Then, the cup was removed from the

oven, cooled in a desiccator for 30 mins and then weighed (A g). After that, 1 g of pumpkin seed flour was put into the porcelain and weighed (B g). The porcelain cup filled with pumpkin seed flour was then put in an electric furnace, with the temperature set to 600°C. After 3 hrs, the seeds were turned into ashes (speed up the ashing process, once the furnace is opened). The sample was left to cool a bit, put in the desiccator for one hr, and then weighed (C g). The calculation formula is as follows:

$$\text{Ash content (wb)} = (C-A)/(B-A) \times 100\%$$

2.6 Determination of crude fiber content

The direct method was used to measure the crude fiber content of pumpkin seed flour. A 0.5-g sample was placed into an Erlenmeyer flask. Then, 30 mL of 0.3 N H₂SO₄ was added. The mixture was heated for 30 mins, added with 15 mL of NaOH 1.5 N and heated again for 30 mins. The mixture was then strained into sintered glass no. 1 while sucked using a vacuum pump. It was then washed with 50 mL of hot water, 50 mL of 0.3 N H₂SO₄, 50 mL of hot water and 50 mL of acetone. The sample was then dried in an oven for 8 hrs or left overnight. Thereafter, it was cooled in the desiccator for 1 hr and then weighed (A g). It was made into ashes in an electric furnace for 3 hrs at 500°C, allowed to cool, placed in a desiccator for one hr, and then weighed (B g). The crude fiber content was roughly calculated using the following formula:

$$\% \text{ Crude fiber} = (A-B)/(\text{sample weight (mg)}) \times 100\%$$

If the crude fiber content exceeds 1%, it is filtered with ashless filter paper, after obtaining a fixed weight of residue, then ashing until a fixed weight of ash is obtained.

$$\text{Residue weight} = (\text{Residual weight} - \text{Ash weight}) / (\text{Sample weight (mg)}) \times 100\%$$

2.7 Determination of moisture content

Ash content was determined using the direct method (oven). A clear porcelain cup was put in the oven at 105°C for 2 hrs. The cup was then removed from the oven, cooled in a desiccator for 30 mins, and then weighed (A g). After that, 1 g of pumpkin seed flour was put into the porcelain and weighed (B g). The porcelain filled with pumpkin seed flour was then put in an electric furnace set to 600°C and left for 3 hrs until turned into ashes (speed up the ashing process, once the furnace is opened). The sample was left to cool, put in the desiccator for 30 mins, and then weighed (C g). Ash content (wb) = (D-A)/(B-A) × 100%.

2.8 Determination of vitamin A content

Vitamin A content was measured using the spectrophotometer method. Pure beta-carotene (vitamin A) was weighed as much as 25 mg and then dissolved in 250 mL of petroleum ether. The mixture was then diluted to 20–100 mL by adding petroleum ether. The mixture was then pipetted as much as 5, 10, 15, 20, 25 and 30 mL into each 100-mL volumetric flask containing 3 mL of acetone. The mixture was then diluted to the mark with petroleum ether, with concentrations of 0.5.10⁻³, 1.10⁻³, 3.1.5.10⁻³, 2.10⁻³, 2.5.10⁻³ and 3.10⁻³ mg/mL. Then, the optical density of the solution was measured at 460–480 nm using 3% acetone in petroleum ether as a blank. Next, 20 g of the sample was weighed, added with 70 mL of acetone and 15 mL of water and adjusted with petroleum ether in a 100-mL volumetric flask. The extraction results were filtered using Whatman filter paper no. 1. The volume of the filtered beta-carotene was then determined using a measuring cup. Furthermore, the sample was put into a tube for centrifugation at 4000 rpm. The pigment content of beta-carotene was then analyzed using a spectrophotometer at a wavelength of 460–480 nm.

2.9 Determination of vitamin C content

Vitamin C content was measured using the titration method. Here, 10 g of sample was put into a 100-mL volumetric flask. The solution was boiled within 3 mins (using a stopwatch) and simmered for 10 mins. The solution was then filtered. A total of 5–25 mL of the filtrate was pipetted into a 125-mL Erlenmeyer flask. Then, 2 mL of 1% starch was added, followed by 20 mL of distilled water, if necessary. Finally, the solution was titrated with 0.01 N iodine.

2.9 Determination of mineral content

The X-ray fluorescence (XRF) method was employed to determine the mineral content. The pumpkin seed flour sample was put into an XRF container. The container was then fed into the XRF machine for mineral analysis of pumpkin seed-based biscuits. The analysis was conducted using XRF software on a computer.

2.11 Subchronic toxicity test

The subchronic toxicity test was conducted at the Biopharmaceutical Laboratory of the Faculty of Pharmacy, Universitas Hasanuddin. This study consisted of one control group and four intervention groups, each of which consisted of six rats. The intervention groups received doses of pumpkin seed flour of 10.8 mg/kg BW, 9 mg/kg BW, 5.4 mg/kg BW, and 4.5 mg/kg BW for 30 days. On day 1, the experimental animals were dissected

using a series of surgical instruments that had been prepared and cleaned using 70% alcohol and distilled water.

Histological preparations of the liver and kidneys were stained using the hematoxylin–eosin (HE) method. Histopathological analysis was conducted to see abnormalities or damage to the liver and kidneys as a result of the administration of pumpkin seed powder. Several parameters of liver cell damage were assessed, including congestion, fat degeneration, and necrosis.

3. Results

As shown in Table 1, macronutrient contents of pumpkin seed flour are 36.30% fat (highest) and 6.02% carbohydrates (lowest). This study also found that pumpkin seed flour contained a considerable amount of vitamins and minerals. Specifically, the pumpkin seed flour contained 0.01% vitamin C and 65.12 mg/kg vitamin A. Minerals found in the pumpkin seed flour were calcium, iron, copper, zinc, phosphorus, manganese, and magnesium, which are important for the human body.

The given dose was the dose from a human weighing 70 kg converted to a dose of male Wistar rats weighing 200 g multiplied by 0.018. The doses for the subchronic toxicity test based on the weight of the powder in human capsule sizes were 00, 0.1, and 2 with a powder weight of 600, 500, 300, and 250 mg, which were the middle

doses (Araujo *et al.*, 2016). These were then converted to rat doses: 250 mg to 4.5 mg/kg BW rats (PS1), 300 mg to 5.4 mg/kg BW (PS2), 500 mg to 9 mg/kg BW (PS3), and 600 mg to 10.0 mg/kg BW (PS4).

The results of the quantitative observations of rat deaths after the first 3 hrs to 30 days following the tests are shown in Table 2, which showed that none of the rats died from all groups of experimental animals.

As shown in Table 3, the body weight increased in both the intervention and control groups. The highest weight change occurred in PS4 (dose of 10.8 mg/kg BW) and the lowest weight change occurred in the control group.

Observation data on liver histopathological images of the control group and treatment groups (PS1 4.5, PS2 5.4, PS3 9, and PS4 10.8) are presented in Figure 1. On microscopic observations of rat liver treated with 4.5 and 5.5 mg/kg BW of pumpkin seed powder, changes/damages to the liver tissue were in the form of congestion in 1–25% of the visual field, which is considered a mild level of change. Meanwhile, the treatment with larger doses, i.e., 9 mg/kg BW and 10.8 mg/kg BW, did not lead to a change/damage to the liver tissue, whether in the form of congestion, fat degeneration and necrosis. Likewise, the liver of the control group did not sustain changes/damages to its histopathological structure.

Table 1. Results of the analysis of pumpkin seed flour in 100 g.

No	Parameter	Unit	Pumpkin seed flour sample			Average
			I	II	III	
1	Water	%	4.24	4.11	4.15	4.17
2	Ash	%	3.98	3.98	4.1	4.02
3	Protein	%	35.13	35.45	35.31	35.30
4	Fat	%	36.11	36.03	36.75	36.30
5	Coarse fiber	%	14.33	13.65	14.62	14.20
6	Carbohydrates	%	6.21	6.78	5.07	6.02
7	Vitamin C	%	0.098	0.102	0.097	0.10
8	Vitamin A	mg/kg	64.51	65.68	65.16	65.12
9	Calcium	Ppm	571.81	547.4	599.87	573.03
10	Copper	Ppm	2.72	3.61	2.96	3.10
11	Iron	Ppm	105.05	108.02	100.06	104.38
12	Zinc	Ppm	69.62	68.68	68.32	68.87
13	Phosphorus	%	0.149	0.164	0.194	0.17
14	Magnesium	%	0.324	0.353	0.302	0.33
15	Manganese	Ppm	120.98	117.86	119.61	119.48

Table 2. Number of deaths of experimental animals

Description	Treatment				
	Control	PS1 4.5	PS2 5.4	PS3 9	PS4 10.8
Number of samples	6	6	6	6	6
Number of dead rats	0	0	0	0	0
Percentage of dead rats (%)	0	0	0	0	0

Table 3. Changes in the body weight of the experimental animals in all groups

Rats	Body weight (g)/Group (Dose)														
	PS1 (4.5 mg/kgBW)			PS2 (5.4 mg/kgBW)			PS3 (9 mg/kgBW)			PS4 (10.8 mg/kgBW)			K (Standard)		
	pre	post	Δ	pre	post	Δ	pre	post	Δ	pre	post	Δ	pre	post	Δ
T1	151	166	15	159	173	14	155	180	25	154	175	21	156	180	24
T2	150	167	17	153	170	17	152	170	18	150	169	19	152	170	18
T3	164	200	36	155	168	13	151	167	16	156	180	24	155	172	17
T4	158	180	22	172	220	48	159	181	22	160	180	20	150	160	10
T5	160	180	20	167	200	33	161	185	24	155	180	25	164	187	23
T6	152	170	18	150	161	11	160	190	30	159	190	31	160	185	25
	Average		21.3	Average		22.6	Average		22.5	Average		23.3	Average		19.5
	p		0.001	P		0.013	p		0.000	p		0.000	p		0.000

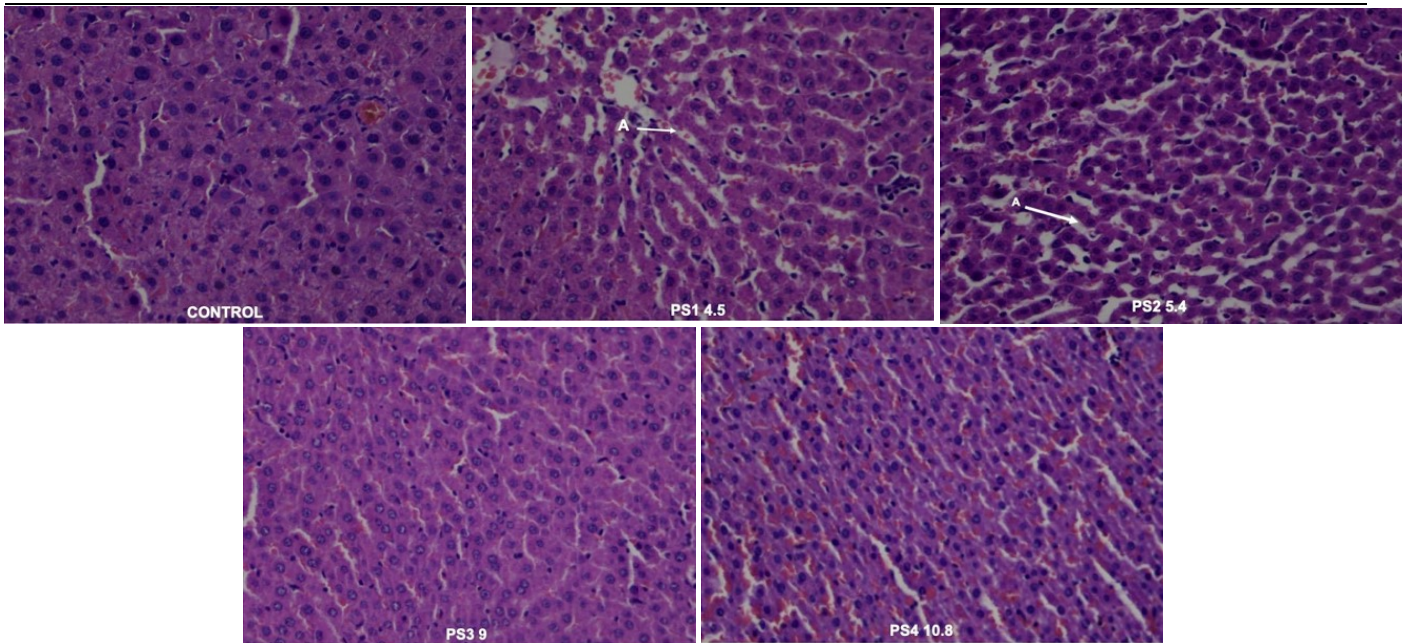


Figure 1. Histopathological structure of the liver mice. Control group: no vascular and sinusoidal congestion, PS1 4.5 group: vascular and sinusoidal congestion (A), PS2 5.4 group: vascular and sinusoidal congestion (A), PS3 9 group: no vascular and sinusoidal congestion, PS4 10.8 group: no vascular and sinusoidal congestion.

Observation data on kidney histopathological images of the control group and treatment groups (PS1 4.5, PS2 5.4, PS3 9, and PS4 10.8) are shown in Figure 2. On microscopic observations of rat kidneys treated with 4.5 mg/kg BW of pumpkin seed powder, changes/damages to the kidney tissue were in the form of hydrophilic degeneration with a score of 1 and intratubular protein with a score of 1, i.e., 1–25% histopathological changes in the visual field, which is considered a mild level of change. Meanwhile, treatment doses of 5.4 mg/kg BW and 9 mg/kg BW led to changes/damages to the kidney tissue, in the form of hydrophilic degeneration with a score of 2, i.e., 26–50% of histopathological changes occurred in the visual field, which are considered a moderate level of change, intratubular protein with a score of 1 and necrosis with a score of 1, which corresponded to histopathological changes of 1%–25% of the visual field and are considered a mild level of change. As for the treatment dose of 10.8 mg/kg BW, changes/damages to the kidney tissue were in the form of hydrophilic degeneration, intratubular protein, and

necrosis with a score of 1, which corresponded to histopathological changes of 1–25% of the visual field and are considered a mild level of change. In the control group, no such change/damage to the histopathological structure of the rat kidney occurred.

4. Discussion

This study found that pumpkin seeds have the highest content of fat. Fat types present in pumpkin seeds are linoleic, oleic, stearic, and palmitic, which meet the needs for approximately 95% of the total fatty acid requirement and 75% of them are unsaturated fats (Bialek et al., 2017). Small concentrations of arachidic and linolenic acids were also reported (Jafari et al., 2012). The results of this study are consistent with those of another study (Montesano et al., 2018), which reported that pumpkin seeds mostly contained oleic acid, with sterols as the highest component.

Two types of essential fatty acids are important for humans, i.e., linoleic and alpha-linolenic fatty acids,

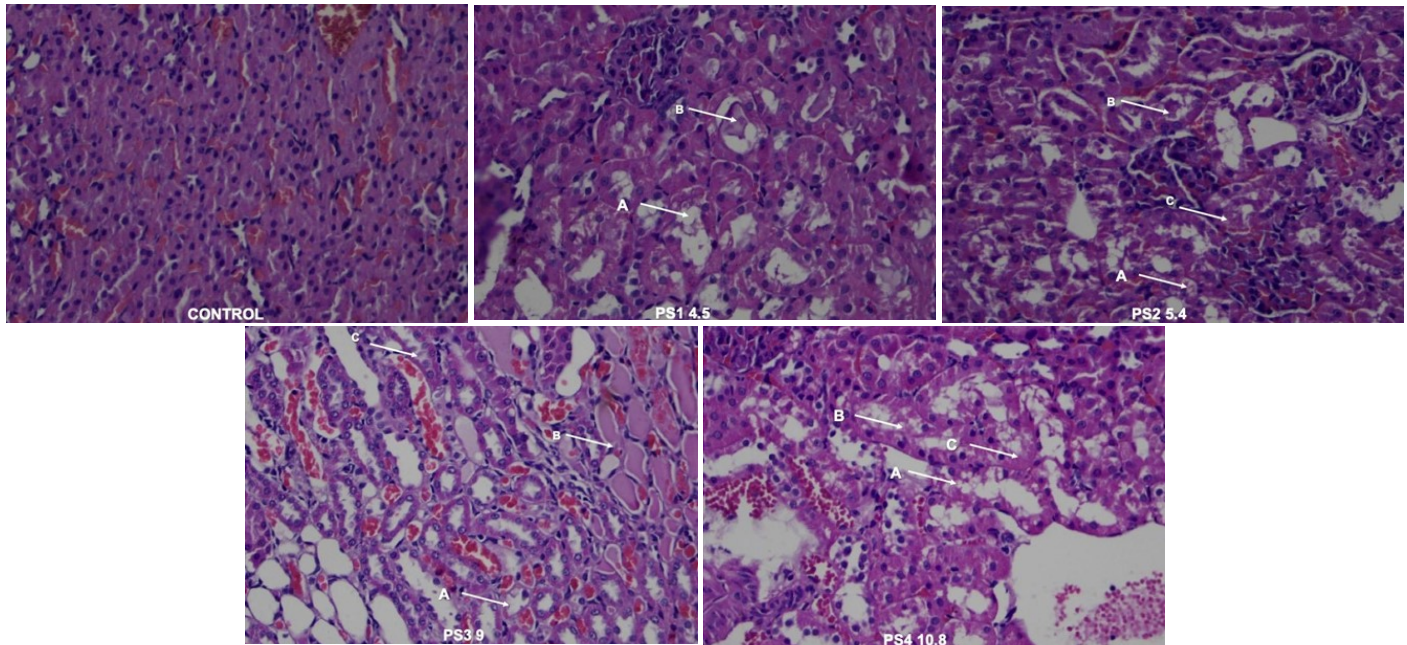


Figure 2. Histopathological structure of the kidney mice. Control group: no hydrophilic degeneration, intra tubular protein and necrosis, PS1 4.5 group: hydrophilic degeneration (A) and intra tubular protein (B), PS2 5.4 group: hydrophilic degeneration (A), intra tubular protein (B) and necrosis (C), PS3 9 group: hydrophilic degeneration (A), intra tubular protein (B) and necrosis (C), PS4 10.8 group: hydrophilic degeneration (A), intra tubular protein (B) and necrosis (C).

because these two fatty acids cannot be produced by the body; therefore, they must be obtained from food (Meru *et al.*, 2018). Unsaturated fatty acids are useful for protection against heart disease. Unsaturated fatty acids are also needed for the growth and development of the brain and a healthy nervous system. It also can prevent coronary heart disease, hypertension, and arthritis.

This study found that pumpkin seeds have a protein content of 35.50%. This result is higher than that revealed in previous studies which reported 25.40% (Gohari-Ardabili *et al.*, 2011) and 33.92% (Rezig *et al.*, 2013) of protein content. Proteins are broken down into several amino acids that play an important role in cell building. Adequate intake of amino acids is needed to support normal body functions. The protein contained in pumpkin seeds is similar to the amino acid content in soybean seeds given their high bioavailability (Rezig *et al.*, 2013). However, proteins isolated from pumpkin seeds contain more promising antioxidants (Yang *et al.*, 2019), and they are a good source of protein for making new food products (Pham *et al.*, 2017).

Another study (Devi *et al.*, 2018) examined the nutrient content of pumpkin seeds and reported that the water and carbohydrate content found in that study was higher than the results of the present study. However, the levels of protein, fat, fiber and ash in the present study are higher than those reported in that study.

Pumpkin seeds also contain small amounts of minerals and fiber. Despite the low levels, they play an important role in synergizing the various benefits of pumpkin seeds (Caili *et al.*, 2006). The nutrient content

of pumpkin seeds can be influenced by the method of planting them. A study conducted in Turkey found that variations in the amount of water used to irrigate pumpkin plants led to differences in the oil content of pumpkin seeds (Kernak *et al.*, 2019). However, other nutrients, such as protein, fatty acids and vitamin E are not significantly different (Kernak *et al.*, 2019).

For the subchronic toxicity tests, body weight data before and after treatment, mortality of experimental animals, and histopathological observations of the liver and kidneys, which included scoring of histopathological changes in the form of congestion, fat degeneration, and necrosis in the liver of experimental animals and hydrophilic degeneration, intratubular protein, and necrosis in the kidneys of experimental animals, were observed. The subchronic toxicity test is performed to determine the adverse effects of repeated daily doses of the drug or exposure to these substances, which lasts about 10% of the entire lifespan of the animal. However, some researchers use shorter durations, for example, 14 and 28 days of substance administration (Djojsumarto, 2008).

In the subchronic toxicity test, test preparations in several dose levels are administered daily to several groups of experimental animals with one dose per group. During the administration of the test preparations, animals should be observed daily to determine the occurrence of toxicity. Animals that die during the administration period, if they have not passed the rigor mortis (rigid) period, are immediately autopsied, and the organs and tissues are observed macropathologically and histopathologically. After the administration period, all

living animals were autopsied, followed by macropathological observations and then histopathological observations of each organ. The purpose of the subchronic toxicity test is to observe the occurrence of toxic effects that were not detected in acute toxicity tests; information on the possibility of a toxic effect after repeated exposure to the test preparations for a certain period; obtain information on doses that do not cause toxic effects (no observed adverse effect level); and examine the cumulative effect and reversibility effect of these substances. Observations for toxic effects include clinical symptoms, body weight changes, mortality in each test group, and organ histopathology.

Toxicity test observation data are divided into two categories: qualitative and quantitative data. Qualitative data are obtained from observations of clinical symptoms obtained from vital functions, whereas quantitative data are obtained using lethal dose 50 (LD₅₀), which is a statistically derived quantity to state a single dose of a compound that can cause toxic effects or death in 50% of the experimental animals after treatment (Jenova, 2009).

Physiological responses to toxic symptoms in humans and animals can occur in the liver (Araujo et al., 2017). The kidneys are also the target of toxicity tests (Konan et al., 2007). Changes (increase or decrease) in the body weight of experimental animals may indicate physiological disturbances or may be caused by disorders of major organs, such as the liver or kidneys. Furthermore, weight loss can be caused by impaired absorption of nutrients as a result of the intervention.

4. Conclusion

Pumpkin seed flour from Indonesia contains beneficial nutrients, both macronutrients (carbohydrates, proteins, and fats) and micronutrients (vitamins and minerals). Regarding quality, pumpkin seed flour meets the requirements for moisture content and compaction density tests. Results of the subchronic toxicity test from animal experiments also show that pumpkin seed flour is safe for consumption. These results indicate that pumpkin seed flour from Indonesia can be processed into functional food and supplements.

Conflict of interest

All authors state that there is no conflict of interest.

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