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Evaluation of nutritional and other functional qualities as well as dietary safety of pumpkin leaves

Bewertung der ernährungsphysiologischen und anderen funktionellen Eigenschaften sowie der Lebensmittelsicherheit von Kürbisblättern

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Summary

Currently there is a great demand of finding health food resources in the world. Presently, large quantities of pumpkin leaves are underutilized. Their potential of being exploited as a health food resource was therefore evaluated in this study. The experimental results indicated that pumpkin leaves were rich in proteins, all essential amino acids, most vitamins (including B_1, B_2, B_3, B_5, B_6, B_{11}, C, A, E and K) and most essential mineral nutrients (including K, Na, P, Ca, Mg, Fe, Zn and Cu) and that they contained significant amount of other functional compounds including polyphenols, alkaloids, saponins and phytates which contributed significantly to the antioxidative capacity of pumpkin leaf extract. A diet with over 90 % source of dietary proteins attributed to pumpkin leaves can be safely consumed throughout lifetime. Comparing with the growth curve of mice fed on a conventional diet, that of mice tested on the pumpkin leaf diet was identical, but their lifespan was significantly longer. Pumpkin leaf powder was therefore concluded to be nutritious and safe for consumption and rich in some other components beneficial to health. These illustrated the potentiality of pumpkin leaves as a valuable health food resource.

Keywords: Nutrition, Dietary safety evaluation, Pumpkin leaves, Health food resource

Zusammenfassung

Weltweit gibt es einen hohen Bedarf an gesunden Nahrungsmitteln. Derzeit werden große Mengen an Kürbisblättern nicht genutzt. In dieser Studie wurde das Potenzial von Kürbisblättern als Lebensmittelressource bewertet. Die experimentellen Ergebnisse zeigten, dass die Kürbisblätter reich an Proteinen, allen essentiellen Aminosäuren, vielen Vitaminen (B_1, B_2, B_3, B_5, B_6, B_{11}, C, A, E und K) und wichtigsten Mineralstoffen (einschließlich K, Na, P, Ca, Mg, Fe, Zn und Cu) waren. Des Weiteren enthielten die Kürbisblätter signifikante Mengen an sekundären Pflanzenstoffen einschließlich Polyphenolen, Alkaloiden, Saponinen und Phytaten. Diese trugen signifikant zur antioxidativen Kapazität des Kürbisblattextrakts bei. Eine Ernährung, deren Nahrungsprotein mit über 90 % aus Kürbisblättern besteht, kann bedenkenlos lebenslang konsumiert werden. Des Weiteren wurden Wachstumskurven von Mäusen, die mit einer herkömmlichen Diät gefüttert wurden und von Mäusen, die mit einer Kürbisblattdiät gefüttert wurden, verglichen. Die Wachstumskurven waren identisch, jedoch war die Lebensdauer der Mäuse, die mit einer Kürbisblattdiät gefüttert wurden signifikant länger. Kürbisblattpulver wurde daher als nahrhaft und für den Verzehr sicher eingestuft und reich an anderen gesundheitsfördernden Bestandteilen. Dies verdeutlicht das Potenzial von Kürbisblättern als wertvolle Ressource für die Gesundheit.

Schlüsselwörter: Ernährung, Ernährungssicherheit, Kürbisblätter, Nahrungsressource

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Introduction

Currently, there is a great demand for good food (particularly protein) resources in the world (especially in African countries) (FAO, 2009; Godfray et al., 2010; Lawrence et al., 2011; Wu et al., 2014b). The percentage of children experiencing malnutrition has been estimated to be as high as 10 % to 30 % in South Asia and central Africa (Grover and Ee, 2009; Ghosh et al., 2012). Also, considering the continuous growth of the world population from 7.2 billion in 2013 to 8.2 billion by 2025 and to 9.6 billion by 2050 (Wu, 2015; United Nations, 2016), the work of finding sustainable, nutritional or functional new food resources should be urgently emphasized.

Cucurbita moschata (pumpkin) is traditionally grown in many parts of the world. This plant belongs to the family *Cucurbitaceae* that is known for its widespread adaptation including tropical and sub-tropical areas (Schippers, 2000). Young pumpkin leaves have been used as a vegetable in many parts of China and African countries whereas their quality still remains to be not well evaluated.

Although the beneficial effects of utilizing some other plant leaves have been extensively reported in the literature (Wu et al., 2014a; Szymczyk et al., 1995; Yang et al., 2004; Santhi and Annapoorani, 2009; Santhi et al., 2010; Satoh et al., 1995; Liang et al., 2010; Nudelman et al., 2001), that of pumpkin leaves has not been well studied. What are the nutritional and other functional qualities of pumpkin leaves? Only the following information can be found in the literature. Depending on growing conditions and maturity, pumpkin leaves may contain more than 30 % proteins. This may mean that pumpkin leaves are superior to soybean in terms of production of protein per hectare. Furthermore, previous studies indicated that pumpkin leaf proteins have good chemical score and digestibility (Huang and Wu, 2010; Shen and Wu, 2017). The ethanol extract of pumpkin leaves was found to have significant antioxidative activity (Shen and Wu, 2017).

The aim of this study is to comprehensively evaluate the nutritional and other functional qualities as well as the dietary safety of pumpkin leaves. The experimental works include the test of nutritional quality and dietary safety of pumpkin leaf proteins by feeding mice a diet with over 90 % source of dietary proteins attributed to pumpkin leaves, determining the content of vitamins, mineral nutrients, crude fat, crude protein, carbohydrates and some functional components such as total phenols, alkaloids, phytates, saponins as well as estimating their functional properties.

Materials and Methods

Sample preparation

Fresh pumpkin leaves were collected from a vegetable farm in Beibei, Chongqing, PRC, in the early July before plants were at the flower-bud stage and washed with clean water to remove the burr on their surfaces. The cleaned leaves were dried at 100 °C in a hot air oven, and then ground. The dried sample powder was named Pumpkin Leaf Powder Preparation (PLPP).

Chemical analysis of PLPP

The method of determination of crude protein, ash and heavy metals was the same as that reported by Wu et al. (2012). Crude fat content was determined by using the Soxhlet method. Total alkaloids and phenolic compounds were determined according to the method reported by Wu and Sun (2011). Vitamins A (retinol) and E (α-tocopherol) were determined by reversed phase high-performance liquid chromatography. The operation procedures of these methods were the same as that reported in GB 5009.82-2016. High-performance liquid chromatography was used for measuring vitamin B_1 (thiamine; GB 5009.84-2016), vitamin B_2 (riboflavin; GB 5009.85-2016), vitamin B_6 (pyridoxine; GB 5009.154-2016) and vitamin C (ascorbic acid), respectively. Microbial method was used for measuring vitamin B_3 (niacin; GB 5009.89-2016), vitamin B_5 (pantothenic acid; GB 5009.210-2016) and vitamin B_{11} (folate; GB 5009.211-2016), respectively. The determination of vitamins K was conducted according to GB/T 26442-2010. Total phytate content was measured according to the method reported by Kwanyuen and Burton (2005).

For the determination of mineral nutrients, the pumpkin leaf powder (1 g) was ashed in a muffle oven at 560 ± 25 °C. The ash produced was then dissolved in 2 N HCl (10 ml) and the solution was adjusted to a volume of 100 ml as the sample solution. Mineral nutrients in the sample solution were measured by an atomic absorption spectrophotometer.

Analysis of protein amino acids

Pumpkin leaf protein concentrate was prepared by the method reported by Shen and Wu (2017). The protein concentrate was incubated with 5 % (w/v) pepsin in 0.1 N HCl at 37 °C for 48 h. The mixture was then centrifuged and washed three times. The supernatant was quantitatively collected. The residue was then incubated with trypsin (0.5 % of weight of the original protein concentrate sample) at pH 8.0 and 23 °C for 16 h. After centrifugation, the two enzymatic extracts were mixed and the enzymes in the mixture were completely removed by boiling followed by centrifugation. The extracted proteins (hydrolyzed into polypeptides) were completely hydrolyzed in 6 mol/L HCl at 110 ± 1 °C for 24 h under vacuum condition. For measuring tryptophan, the proteins (hydrolyzed into polypeptides) were completely hydrolyzed in 4 mol/L NaOH solution at 110 ± 1 °C for 20 h under vacuum condition. After the final hydrolysate was adjusted to pH 5–6, the composition and content of amino acids were analyzed by using L-8800 Hitachi Amino Acid Analyzer.

Mouse feeding test guidelines followed

The experimental protocol followed the main features of OECD Guideline for Testing of Chemicals, No.420 (2001): Acute Oral Toxicity-Fixed Dose Procedure (OECD, 2001), No. 452 (2009): Chronic Toxicity Studies (with the exceptions that total numbers of mice studied were 104 instead of 80, and that visual observation, tissue histopathology, general dissection, internal organ investigation and hematology analysis were carried out after 30-day, 90-day, 180-day and 12- month feeding, respectively, instead of after 12-month feeding alone) (OECD, 2009) as well as Toxicological Evaluation Procedures and Methods of Food Safety (National Standards of PRC, 2003), and OECD Principles of Good Laboratory Practice (OECD, 1998). All experimental mice were treated by following Guide for the Care and Use of Laboratory Animals (NRC, 2011).

Ingredient composition (g/100 g) of the diets for 30-day, 90-day, 180-day and 12-month mouse feeding tests

Two kinds of diets were prepared in accordance with the composition of source materials and the daily nutrient

requirements, which were named Control and PLPP, respectively. Details of each feedstuff formula are shown in Table 1. After all the components of formulated feedstuffs of each group were well mixed together, the mixture was pressed into sticks by employing a screw extrusion presser. The assay diet contained 14.18 % proteins, 11.96 % fats (1.63 % from PLPP, 0.33 % from wheat bran) and 6.08 % ashes (5.74 % from PLPP, 0.34 % from wheat bran) in addition to 44.35 % starches.

Mice for 30-day, 90-day, 180-day and 12-month mouse feeding tests

The experimental mice, i. e. 52 male Kunming mice weighing 17–22 g and 52 female Kunming mice weighing 17–22 g (Certificate No. of Approval: 0003255) were obtained from Laboratory Animal Center of Chongqing Medical University. They were randomized into 2 groups. Each group included 26 male mice and 26 female mice, respectively. Mice were housed individually in stainless steel cages (only ≤4 mice kept in each cage) in an air-conditioned room at 22 ˚C and 50–55 % relative humidity.

Observations on 30-day, 90-day, 180-day and 12-month mouse feeding tests

Each group (52 mice) was fed either control or diet containing PLPP (5 g per day for each mouse), from 4 weeks of age. Water was provided ad libitum. After the mice were fed for 30 days, or 90 days, or 180 days, 2 male and 2 female mice were selected randomly from each group, respectively. Body weight was measured, and blood samples were collected. The mice were then sacrificed, and their livers, kidneys, and spleens were eviscerated and weighed. Tissue samples were collected at necroscopy for histopathological evaluation following OECD Guideline for Testing of Chemicals No. 452. The rest of mice in two groups (Control and PLPP testing group) was also weighed and continuously fed for a 12-month observation. After they were fed for 12 months, all the mice of each group were picked out from housing cages, respectively. The following steps were the same as those for the 30-day or 90-day, or 180-day observation. During the whole experiment period, the visual investigation of the behavior and any signs of illness of the mice were also undertaken every day.

Hematology analysis

All blood samples were analyzed by using XFA6100 Auto Blood Analyzer. The parameters that were measured included white blood cell count (WBC), red blood cell count (RBC), haemoglobin (HGB), lymphocyte (LYM, %) and neutrophilic granulocyte (GRA, %). Clinical biochemical analysis was carried out by using BTS-240 Auto Blood Biochemistry Analyzer. The parameters that were measured included alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CRE), blood urea nitrogen (UN), glucose (GLU) and cholesterol (CHOL).

Test on lifespan

Mice (100, 4 weeks of age) divided into two groups (Control and PLPP testing group) was continuously fed till dying a natural death for lifespan estimation. Each group includes 25 males and 25 females.

Determination of LD50

The control diet was the same as that for the repeated dose study (see Table 1, footnote c, for the composition). All diet components were mixed together and pressed into sticks by employing a screw extrusion presser. Mice prepared for the experiments included 20 male mice weighing 17–20 g and 20 female mice weighing 17–20 g. They were randomized into 4 groups. Each group included 5 male mice and 5 female mice. After stopping to supply the normal feedstuff for 12 h, four groups of the mice were fed with 5, 10, 15 and 20 g/kg body weight PLPP [designed after preliminary experiments following OECD Guideline for Testing of Chemicals, No.420 (2001): Acute Oral Toxicity-Fixed Dose Procedure] by using an intra-gastric syringe. For the dosage of ≥ 15 g/kg body weight, the tested material was prepared by mixing 1 part PLPP with 2 parts salad oil and the mice were fed in 2 times in 2 h by using an intra-gastric syringe. Observations were made to check for any deaths within 24 h of acute dosing, with special attention given during the first 4 h, and daily thereafter, for a total of 14 days. Then, the normal feedstuff sticks and water were fed *ad libitum* to mice throughout the experimentation.

Statistical analysis

The results were statistically analyzed by using one way analysis of variance (ANOVA) and they were expressed as mean ± standard deviations. To determine statistical significance between the results of Control and PLPP, t- tests were performed. The calculated t-value higher than $t_{0.05}$ from t-table was considered to be statistically significant. SPSS 17.0 was used for the statistical analysis.

TABLE 1: *Ingredient composition (g/100g) of the experimental diets.*

Groups	Corn starch (g)	Protein sources (g)[a]	Soy oil (g)	Wheat bran (g)[b]	Vitamins + minerals (g)	Total (g)
Control[c]	–	–	–	–	–	100
PLPP	44.35	PLPP 36.12	10.00	8.20	1.33[d]	100

[a] Protein content in PLPP group was adjusted to 14.18 % (12.89 % from PLPP and 1.29 % from wheat bran).

[b] Wheat bran (100 g) contains 14.3 g water, 15.7 g protein, 4.0 g fat, 31.2 g dietary fiber, 4.2 g ash, 400 I.U. Retinol (VA), 6.7 I.U. α-Tocopherol (VE), 0.3 µg Thiamine (VB1), 0.3 mg Riboflavin (VB2), 9.8 mg Fe, 2.1 mg Cu, 6.0 mg Zn, 10.8 mg Mn, 7.15 µg Se, 681.0 mg P, 207.1 mg Ca, 384.1 mg Mg, 12.3 mg Na and 6.8 mg K.

[c] The Control (100 g) for the mouse feeding tests consists of 35 g corn powder, 5 g soybean powder, 15 g de-oiled soya powder, 15 g wheat flour, 2 g yeast powder, 2.5 g bone powder, 3 g de-oiled sesame powder, 4 g fish powder, 2 g milk powder, 0.44 g salt, 0.06 g Chinese Viduo (vitamin + mineral mixture), 1 g salad oil and 15 g wheat bran.

[d] 1.33g Chinese Viduo contributes 4000 I.U. Retinol (VA), 400 I.U. Cholecalciferol (VD), 30 I.U. α-Tocopherol (VE), 1.5 mg Thiamine (VB_1), 1.7 mg Riboflavin (VB_2), 3.13 mg Pyridoxine (VB_6), 60 mg Ascorbic Acid (VC), 6 µg Cobalamin (VB_{12}), 25 µg Phylloquinone (VK_1), 30 µg Biotin (VH), 400 µg Folic Acid (VB_{11}), 20 mg Niacin (VB_3), 10 mg Pantothenic Acid (VB_5), 17.75 mg Fe, 2 mg Cu, 6.24 mg Zn, 2.5 mg Mn, 150 µg I, 25 µg Cr, 25 µg Mo, 25 µg Se, 5 µg Ni, 10 µg Si, 4 µg Li and 10 µg V. to the experimental diet (100 g)

TABLE 2: *Content of nutrients in 100 g raw pumpkin leaves.*

Nutrients	Content	Nutrients	Content	Nutrients	Content
K	436.18 mg	Pantothenic acid	0.299 mg	Retinol	1943 IU
Na	10.98 mg	Total ascorbic acid)	11.11 mg	Vitamins K	1.12 mcg
P	104.31 mg	Niacin	0.931 mg	Crude fat	0.41 g
Ca	38.95 mg	Pyridoxine	0.208 mg	Crude protein	2.74 g
Mg	38.15 mg	Riboflavin	0.129 mg	Carbohydrate	2.34 g
Fe	2.24 mg	Thiamin	0.095 mg	Energy	19.11 kcal
Zn	0.21 mg	Folate (DFE)	35.99 µg	Ash	1.16 g
Cu	0.028 mg	α-Tocopherol	1.07 mg	Dry matter	16.01 g

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Results and Discussion

Nutritional evaluation

The content of nutrients including crude proteins, mineral nutrients, vitamins, carbohydrate, energy, ashes and total dry mass in fresh pumpkin leaves are shown in Table 2. It is obvious that the pumpkin leaves studied were quite rich in these nutrients. Especially, they contained large quantities of proteins which were found to be 35.68 % (N x 6.25) on a dry matter basis.

The essential amino acids of pumpkin leaf proteins and their comparison with that of other food proteins as well as FAO/WHO reference pattern are shown in Table 3. From this table it can be seen that the limiting amino acid of pumpkin leaf proteins was found to be Ileu (isoleucine), but its chemical score was 91. All other food proteins which are listed in this table also have limiting amino acids. Oat proteins have three amino acids with their content lower than FAO/WHO reference pattern and their chemical score is only about 67 %. The chemical score of green bean or sesame proteins is lower than that of pumpkin leaf proteins.

Antioxidative components

The water or ethanol extract of pumpkin leaves has antioxidative capacity (Table 4). From Table 4, it can also be seen that the analyzed pumpkin leaves contained significant amount of polyphenols, alkaloids, saponins and phytates. These compounds should be soluble in the water or ethanol extract of pumpkin leaves. Therefore, they should partly contribute to the oxidative capacity of the extract of pumpkin leaves. In addition, vitamins, amino acids, carbohydrates, etc. may also significantly contribute to the oxidative capacity of pumpkin leaves.

TABLE 3: *Comparison of essential amino acids in PLPP and in other food materials (g/100g dry basis).*

Essential amino acids	Pumpkin leaf proteins	Beef proteins	Oat proteins	Soy proteins	Green bean proteins	Sesame proteins	FAO/WHO reference pattern
Ileu	3.64	5.20	3.81	5.01	3.89	3.61	4.0
Leu	9.24	8.21	7.21	7.89	8.81	6.71	7.0
Lys	8.40	9.01	3.70	5.71	6.79	2.70	5.5
Met+Cys	5.32	3.62	4.41	3.21	2.61	4.59	3.5
Phe+Tyr	7.56	8.71	8.31	8.91	8.29	7.49	6.0
Thr	4.20	4.72	3.31	4.21	4.71	3.61	4.0
Trp	1.54	0.89	1.31	1.21	1.01	1.29	1.0
Val	6.72	5.61	5.11	5.51	4.51	4.61	5.0
Total	46.62	45.97	37.17	41.66	40.62	34.61	36.0

TABLE 4: *Content of functional compounds in 100 g PLPP (dry basis) and their antioxidative capacity.*

Functional components	Content	Antioxidative capacity[11]	
		Hydroxyl radical-scavenging activity (%)	Reducing power equivalent to Vc (mg/mL)
Polyphenols	10.94 mg	+, not determined.	+, not determined.
Alkaloids	7.34 mg	+, not determined.	+, not determined.
Saponins	35.05 mg	+, not determined.	+, not determined.
Phytates	15.11 mg	+, not determined.	+, not determined.
Ethanol extract[a]	–	47.75	0.475
Water extract[a]	–	42.75	0.425

+: means that these compounds have antioxidative capacity.
[a]: Shen and Wu (2017).

Dietary safety evaluation

The heavy metals, i. e. Cd, Cr, Pb, Pt, Pd, Sn, Hg, Ba, Ag, Sd, and Al in PLPP were analyzed. The concentrations of all these metals in PLPP were found to be within their maximum limits in food additives reported by WHO (1989).

Polyphenols, saponins, phytates and alkaloids in the tested PLPP could have antinutritional properties, but this should be dependent upon their concentration. Their concentrations in 100 g testing diet were 3.95 mg (10.94 mg x 36.12 %), 2.65 mg (7.34 mg x 36.12 %), 12.66 mg (35.05 mg x 36.12 %) and 5.46 mg (15.11 mg x 36.12 %), respectively (calculated according to the data shown in Table 4). Mouse test proved that these compounds with their concentrations up to these values did not cause any health problem, which is discussed in the following text.

The testing material, dose and diet composition were carefully designed in order to take control of PLPP so that it was the only variable of dietary toxicity. The ingredient composition of the diet assay (PLPP) included 36.12 % PLPP which contained proteins (12.89 %), corn starch, soya oil, wheat bran and vitamins plus minerals. The testing dose of PLPP in the diet assay was designed to predominantly supply experimental mice with enough diet protein and obtain results applicable to food or feeding practice. The ingredient composition of control included conventional food materials commonly employed as daily foods. Control was designed to prevent errors of damages to experimental mice by the lack of essential nutrients.

LD_{50} of PLPP

The results of determination of LD_{50} indicated that all of the mice tested were alive at the doses of 5, 10, 15 and 20 g/kg body weight of PLPP, respectively. This means that the LD_{50} of PLPP is more than 20 g/kg body weight. According to National Standards of PRC (2003), if the LD_{50} of a chemical compound is >15 g/kg body weight, it should be considered to be acutely non-toxic. Therefore, it is suggested that PLPP be not acutely toxic.

Observations on 30-day, 90-day, 180-day and 12-month mouse feeding test

The result of the visual observation on samples selected from two testing groups after feeding the mice for 30 days, 90 days, 180 days and 12 months showed neither deaths nor adverse changes in general appearance, respectively. Visual observations on the behavior of the mice also did not indicate any evidence of toxicity. Tissue histopathology, general dissection and internal organ investigation also found that the diets (Control and PLPP) after feeding the mice for 30 days, 90 days, 180 days and 12 months resulted in no abnormality, respectively. These results indicated that all of the mice appeared clinically healthy during the experimental period.

The results of viscera investigation of the mice after 30 days, 90 days, 180 days and 12 months feeding are present in Table 5. Statistical analysis (t-test) of the average liver/body weight ratio values shown in this table indicated

TABLE 5: *Results of viscera investigation at the end of 30, 90 or 180 days and 12 months feeding time.*

Feeding time	Sex	Groups	BW[a] (g)	Liver		Kidney		Spleen	
				Weight (g)	Liver/body weight Ratio (%)	Weight (g)	Kidney/body weight Ratio (%)	Weight (g)	Spleen/body weight Ratio (%)
30 days (P > 0.05)[b]	Male	Control	33.12	1.6196	4.89±0.971	0.5200	1.57±0.511	0.0629	0.19±0.032
		PLPP	28.12	1.3469	4.79±0.723	0.4724	1.68±0.552	0.0506	0.18±0. 013
	Female	Control	31.87	1.3704	4.30±0.953	0.3856	1.21±0.431	0.1020	0.32±0. 071
		PLPP	29.09	1.2567	4.32±0.881	0.3462	1.19±0.353	0.0640	0.22±0. 043
90 days (P > 0.05)[b]	Male	Control	39.94	1.4578	3.65±0.693	0.4234	1.06±0.311	0.0799	0.20±0.094
		PLPP	39.49	2.1956	5.56±0.504	0.3870	0.98±0.212	0.0750	0.19±0.013
	Female	Control	38.54	1.5686	4.07±1.232	0.3276	0.85±0.151	0.1156	0.30±0.042
		PLPP	39.08	1.4264	3.65±0.913	0.3439	0.88±0.252	0.0899	0.23±0.054
180 days (P > 0.05)[b]	Male	Control	43.56	2.1127	4.85±0.614	0.4312	0.99±0.214	0.0784	0.18±0.074
		PLPP	45.34	2.3531	5.19±0.641	0.5305	1.17±0.312	0.1043	0.23±0.044
	Female	Control	43.08	1.7103	3.97±1.282	0.3705	0.86±0.195	0.0603	0.14±0.025
		PLPP	42.89	1.8657	4.35±1.104	0.4546	1.06±0.173	0.0901	0.21±0.051
12 months ($x \pm \delta_{n-1}$, n = 23; P > 0.05)[b]	Male	Control	48.90	2.3276	4.76±0.651	0.5770	1.18±0.106	0.1223	0.25±0.098
		PLPP	47.56	2.4065	5.06±0.583	0.5945	1.25±0.304	0.0713	0.15±0.025
	Female	Control	48.23	2.4838	5.15±0.461	0.5064	1.05±0.316	0.1061	0.22±0.094
		PLPP	47.29	2.3163	4.50±0.517	0.4170	0.88±0.165	0.0670	0.14±0.015

[a]: BW-body weight.

[b]: There is no statistically significant difference between Control and PLPP.

no significant differences ($p > 0.05$) between Control and PLPP at each feeding time except the male mice fed Control for 90 days. Significant differences ($p < 0.05$) of the average liver/body weight ratio values between Control and PLPP after feeding for 90 days were indicated by statistical analysis (t-test). Further investigation is needed to find the reasons for this result. This difference could be caused by individual variation, given the small numbers of mouse samples selected from each group. It should be noted that the liver/body weight ratio of the mice fed Control neared to the lower limit of the normal range while that of the mice fed PLPP neared to the upper limit. Furthermore, the results of visual observations on the behavior of the mice tested, the appearance of liver, and longer feeding time than 90 days showed no abnormality. This table also indicates that the treatment did not adversely affect the kidney/body weight and spleen/body weight ratios of the mice of all diet groups fed for 30 days, 90 days, 180 days and 12 months since statistical analysis (t-test) indicated no significant differences ($p > 0.05$) between Control and PLPP at each feeding time, respectively. Others un-shown in this table including the average heart/body weight and lung/body weight ratio values were also normal.

TABLE 6: *Results of haematology analysis at the end of 30, 90 or 180 days and 12 months feeding time.*

Feeding time	Sex	Groups	HGB (g/l)	RBC (10^9 /ml)	WBC (10^6 /ml)	LYM (%)	GRA (%)
30 days (P > 0.05)	Male	Control	150.01±4.32	10.71±1.87	4.92±1.03	73.1±3.46	14.5±2.07
		PLPP	137.03±3.24	9.48±1.69	4.71±0.84	68.9±4.21	18.1±3.35
	Female	Control	154.01±4.50	10.06±2.08	4.15±0.75	70.5±5.07	16.5±2.54
		PLPP	145.04±4.32	9.45±1.17	4.55±0.56	73.6±5.25	13.3±3.19
90 days (P > 0.05)	Male	Control	165.07±0.78	7.84±1.03	4.52±0.54	78.5±3.63	13.6±0.97
		PLPP	160.18±6.31	9.78±2.45	10.86±2.27	62.9±2.12	20.5±3.58
	Female	Control	141.04±5.07	9.27±1.55	7.81±0.91	75.9±2.73	12.6±1.25
		PLPP	137.11±5.45	9.14±2.36	8.75±2.17	79.4±1.93	13.7±3.17
180 days (P > 0.05)	Male	Control	150.03±4.32	10.01±2.08	5.91±1.03	73.1±5.07	12.3±1.15
		PLPP	155.33±5.09	10.17±1.42	11.80±2.47	77.0±3.46	12.5±1.20
	Female	Control	154.02±4.51	10.72±1.08	9.32±0.75	70.5±3.09	14.1±1.12
		PLPP	147.67±5.35	8.86±1.01	9.25±1.41	84.7±3.01	14.2±1.13
12 months ($x \pm \delta_{n-1}$, n = 23; P > 0.05)	Male	Control	156.14±1.73	9.39±0.52	9.44±0.81	78.5±2.09	13.5±1.09
		PLPP	128.45±1.37	8.86±1.03	4.62±0.55	78.5±1.89	13.6±1.05
	Female	Control	133.01±1.16	9.18±1.42	8.20±0.66	76.4±1.91	17.0±1.02
		PLPP	122.31±1.21	9.74±1.11	4.97±0.38	76.4±1.81	13.8±1.06
Normal Values*			148 (100–190)	9.3 (7.7–12.5)	8 (4–12)	74 (54–85)	23 (12–44)

*: measured values of blood samples from 30 healthy Kunming mice.

**: There is no statistically significant difference between Control and PLPP.

Some blood parameters that were measured by hematology analysis of the mice after feeding for 30 days, 90 days, 180 days and 12 months are present in Table 6. This table indicates that HGB, RBC, WBC, LYM and GRA of mice of all diet groups were normal. Other blood parameters un-shown in this table including ALT, AST, CRE, UN, GLU and CHOL obtained by clinical biochemical analysis were also normal. It is therefore suggested that all components in PLPP should not acutely, sub-chronically and chronically harm the blood system of mice.

Although the results obtained after 30, 90 and 180 days may not give a definite conclusion because of small numbers of mice investigated, that obtained after 12 months should indicate that the liver, kidney, spleen, heart, lung and blood of mice would not acutely and chronically be harmed by any component in PLPP or Control. The results obtained 30, 90 and 180 days were only assistant evidence. The numbers of mice after 12 months were 92 (46 for each group) which were more than that required by the OECD guideline. This should give the major evidences that can make a reliable conclusion.

All the results described above indicate that pumpkin leaves are very safe for consumption for a long term. When applying this conclusion in practice, it should be noted that the concentration of toxic heavy metals in pumpkin leaves may be affected by their content in growing soil.

Effect on mouse growth

The growth curve of the mice feeding for 30 days to 12 months is given in Figure 1. From this figure, it is obvious that the body weight of all the mice fed Control and PLPP gradually increased from 30-day to 12-month feeding time.

Journal of Food Safety and Food Quality 70, Heft 1 (2019), Seiten 1–30

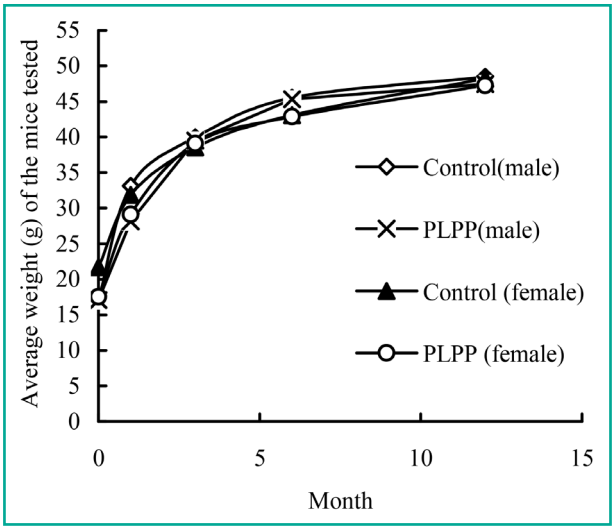

FIGURE 1: *Growth curve of mice fed on different diets. There is no statistically significant difference between Control and PLPP ($P > 0.05$).*

The growth curve of all the mice including male and female fed Control or PLPP is very similar to each other. All the mice fed Control or PLPP rapidly grew from 30-day to 90-day feeding time. Their growth rate slowed down from 90-day to 180-day feeding time. Their growth rate further slowed down from 180-day to 12-month feeding time. At the end of 12-month feeding time, the average absolute body weight of all the mice fed Control or PLPP was between 45 to 50 g. So, there were no significant and adverse effects of the treatment on body weight gain or absolute body weight of all the mice fed Control or PLPP accept for those fed control were a little bit fat according to subjective assessment of the carcass.

Impact on the lifespan of mice

The mouse feeding test till dying a natural death showed that the average lifespan of the mice fed PLPP was 538 days while that of the mice fed the Control was 500 days. Significant differences ($p < 0.05$) of the lifespan between Control and PLPP groups were indicated by statistical analysis (t-test). However, the lifespan of the mice fed Control or PLPP was in the normal range of the lifespan of Kunmin mice fed a normal (or conventional) diet (Liu et al., 2004). Therefore, it may be a little bit of early to rule out that this range of longer lifespan is just within the range of biological variation between individuals. However, the results obtained in this study should indicate that the pumpkin leaf protein as a predominantly dietary protein source was good enough to feed mice well throughout their lifetime.

Conclusion

PLPP was found to be rich in proteins, vitamins and essential mineral nutrients. Especially, the essential amino acids of PLPP were well balanced. It also contained significant amount of other functional compounds including poly phenols, alkaloids, saponins and phytates. These compounds should contribute significantly to the antioxidative capacity of pumpkin leaf extract.

The results of mouse feeding test proved that PLPP was not acutely or sub-chronically and chronically poisonous to mice. This indicated that the concentration of the functional compounds in the PLPP was not high enough to cause adverse effect on health. As comparing with a conventional feedstuff, consumption of pumpkin leaf proteins (over 90 % of dietary protein supply) throughout lifetime did not adversely affect the growth or health of mice while it resulted in a comparable or even significantly longer lifespan. This result also indicates that pumpkin leaves as a predominant protein source are enough to support the growth of mice. Therefore, pumpkin leaves should be considered as good agricultural products (protein resources) for food or feed industries or consumption as a leafy vegetable.

Conflict of interest

Authors declare no conflict of interest.

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