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Kidney therapeutic potential of peptides derived from the bromelain hydrolysis of green peas protein

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ABSTRACT

Objective(s): Kidney disease is a global health problem that needs a solution to its therapy. In the previous study, we found that protein hydrolysate of green peas origin of Indonesia hydrolysed by bromelain (PHGPB) showed improve kidney function in cisplatin-induced nephropathy rats. In this study, we investigated the effect of PHGPB to obtain effective dose that exerts a therapeutic effect on chronic kidney disease (CKD) based on reducing urea and creatinine levels and to elucidate its mechanism of action.

Materials and Methods: Two sets of experiments were conducted: (1) characteristics and proteomic profile of PHGPB, (2) *in vivo* test of PHGPB in gentamycin-induced Wistar rats, including urea and creatinine measurements, activities of antioxidant and kidney-related peptides (ANP, COX-1, and renin).

Results: PHGPB showed three bands under 10 kDa using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and contained 10 identified proteins using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Significant differences in urea and creatinine levels were found between all PHGPB treatments and positive controls (P<0.01). The lowest levels of urea and creatinine that were validated by high super oxide dismutase (SOD) activity and atrial natriuretic peptide (ANP) level were obtained in the 200 mg/day PHGPB treatment. However, the mean renin level was high and cyclooxygenase-1 (COX-1) level did not exceed positive and negative control levels.

Conclusion: PHGPB at dose 200 mg/kgBW shows a potential CKD therapeutic effect that is dose-dependent. Higher PHGPB dose corresponds to better effect on kidney function by increasing antioxidant activity and ANP levels in gentamycin-induced Wistar rats.

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Introduction

Kidney disease is a global health problem with high health care costs. Basic Health Research Data (RISKESDAS, 2013) showed that 0.2% or 2 per 1000 Indonesians are suffering from kidney failure. The leading cause of chronic kidney disease (CKD) in Indonesia is diabetic nephropathy (52%), followed by hypertension (24%) (1).

A Canadian study stated that the hydrolysate protein of yellow peas (*Pisum sativum* L.) alleviates high blood pressure and CKD. Pea protein hydrolysate (PPH) was used as a potential modulator of the renin–angiotensin system (RAS). PPH reduces blood pressure by increasing the levels of cyclooxygenase-1 (COX-1) in renal tissues (2). Hydrolysate proteins are amino acid mixtures obtained from the degradation of hydrolyzed proteins using acids, bases, or proteolytic enzymes to produce peptides and small molecules with high solubility (3). Enzymatic protein hydrolysis improves the function and nutrients of protein sources; it can contain large amounts of efficient peptides without toxic by-products or amino acids (4, 5).

Hydrolysis of green peas proteins produces antioxidant, anti-inflammatory, and hypolipidemic peptides that can be used as supportive therapy in cardiovascular disease (6). Such peptides also display antimicrobial, antihypertensive, immunomodulatory, and anticancer activities (7, 8). The bioactive peptides are released from the protein parent through hydrolysis by digestive enzymes (3). Peptides are utilized as functional foods that demonstrate therapeutic effects and as nutraceuticals to prevent damage and various diseases caused by oxidative stress (6). Several studies proved that PPH has antioxidant and antihypertensive properties (9, 10). Proteolytic enzymes from plants have been extensively studied because of their easy and simple production. Bromelain enzyme is more effective than papain in producing fish protein hydrolysates with desirable bioactivities (e.g., angiotensin-I-converting enzyme (ACE) inhibitory activity and antioxidant activity) and functional properties (e.g., high degree of hydrolysis (DH)) (11).

The kidneys excrete urea and creatinine as waste products of protein metabolism. However, this function

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is impaired when the kidneys are injured. Creatinine is the waste product of the breakdown of creatine phosphate, a compound found in skeletal muscle tissue. Creatinine levels in blood increase when the kidneys are impaired. Therefore, creatinine level is very useful in evaluating renal function. Increased creatinine levels indicate kidney damage characterized by decreased glomerular filtration rate (GFR)(12). Kidney damage is associated with many factors, including hyperactivity of ACE and renin in the renin–angiotensin system (RAS), calmodulin phosphodiesterase 1, and atrial natriuretic peptide (ANP). The main targets of antihypertensive peptides are renin and ACE (13, 14).

ANP is related to hypertension and plasma volume expansion (15). It is a major component in the cardiac–renal axis that participates in hemodynamic decline of the heart and increasing the excretion of sodium to improve blood pressure and vascular function. ANP increases in complicated CKD patients with deteriorating renal function, but the association of plasma ANP levels with CKD damage remains unclear (13, 16). ANP confers protection on the kidney by inhibiting the proliferation of mesangial cells and renal fibrosis (17). However, the predictive value of ANP as a CKD marker in cases of heart disease and blood vessels has not been standardized to date (18).

In Indonesia, yellow peas are rare, and green peas are still not commonly consumed by the public. CKD cases continue to increase every year in this country. Thus, functional foods to prevent kidney damage in CKD patients are needed. In our previous study, we compared the effects of hydrolysate proteins from yellow beans (Canada), pea protein isolates (Canada), Indonesian green peas (*P. sativum*), and Gude seeds (*Cajanus cajan*) hydrolyzed by two enzymatic proteases (neutrase or bromelain) on the renal function of cisplatin-induced rats to produce a hydrolysate protein that can improve kidney function. Results showed that the peptide derived from the bromelain hydrolysis of Indonesian green peas proteins (PHGPB, 25.71% protein, 9.28% fat) showed the lowest levels of urea and creatinine in cisplatin-induced female Wistar rats (19).

PPH contains natural antioxidants that alleviate CKD (10, 20). However, the therapeutic effects and mechanisms of PHGPB for CKD remain to be investigated.

Therefore, the present study aimed to obtain a hydrolysate protein (i.e., PHGPB) that exerts a therapeutic effect on CKD by reducing urea and creatinine levels and to elucidate its mechanism of action. The characteristics, proteomic profile, and superoxide dismutase (SOD) antioxidant activity and kidney-related peptides (ANP, COX-1, and renin) of PHGPB in the kidney of gentamycin-induced Wistar rats were examined.

Materials and Methods

Green peas (*P. sativum* L.) were obtained from Maica leaf, Magelang Plantation, Central Java, Indonesia. Bromelain enzyme was obtained from pineapple stems (*Ananas sativus*) from Subang, North Bandung, Indonesia. Gentamycin (80 mg) for injection/IP was purchased from a local drug store. SOD Activity Colorimetric Assay Kit (BioVision Incorporated, USA K335); ANP (QY-E11002), COX-1 (QY-E10736), and Renin (QY-11096) reagent kits from Qayee-bio for ELISA; 10× radioimmunoprecipitation assay (RIPA) buffer (156034); and protease and phosphatase inhibitor cocktail (ab 201119) were used in the study.

Subject

Fifty-six healthy male Wistar rats (5–6 weeks) weighing 148–190 g were obtained from the School of Life Sciences and Technology, Bandung Institute of Technology, Indonesia.

Protease preparation

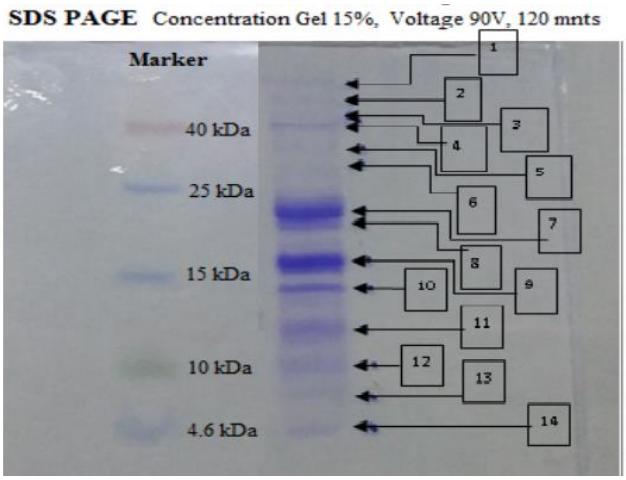
For bromelain production, a solution obtained from pineapple stem (*A. sativus*) was filtered and centrifuged at 4000 rpm for 10 min. The protein concentration of bromelain was determined using Bradford method(21) with tryptophan as standard (22). In addition, the total specific activity of the enzyme and the hydrolysate protein content were measured by Kunitz's method and the bovine serum albumin (BSA) curve (20). Molecular weight of the hydrolysate protein was measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (23).

Protein hydrolysate preparation

Protein hydrolysates were prepared as previously described with modification (9, 24). Dry seeds (500 g) of green peas were mashed, sieved through a 120-mesh sieve, and then dissolved in 2000 ml of water. Bromelain 10% (w/v) was added to each solution (25) and then left for 72 hr (26) on a stirrer at room temperature (25–30 ˚C) (27). After 72 hr, each solution was transferred to a tube and then centrifuged at 6000 g for 10 min. The supernatant was filtered using filter paper. SDS-PAGE was used to separate and determine the molecular weight of the protein hydrolysates (23).

Identification and characterization of protein hydrolysate of green peas by bromelain using LC-MS/MS (28)

Green peas hydrolysate protein was identified and characterized using liquid chromatography-tandem mass spectrometry (LC-MS/MS) on a Q Exactive Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) coupled with a Thermo RSLC nano Ultimate 3000 system (Thermo Scientific, USA). Peptides were analyzed by reversed-phase LC (RP-LC). The RP-LC system was equipped with a trap column (Thermo Scientific, P/N 164649) and an analytical column (75 cm × 15 cm, Easy Spray Column PepMap, Thermo Fisher Scientific). Furthermore, the peptides were sprayed using an emitter together with an analytical column fixed to a nanospray ionization source. The peptides were loaded on the pre-column and rinsed using 0.1% formic acid in water for 2 min. They were then resolved on the analytical column using a gradient starting from 5%–7% (solvent B) for 5 min, 7%–18% for 100 min, 28%–60% for 10 min, and 60%–95% for 5 min, for a total run of 143 min, which includes the washing step at a constant flow rate of 0.300 µl/min. Solvent A was composed of 0.1% formic acid in water, and solvent B was composed of 98% acetonitrile and 0.1% formic acid. The Q Exactive was operated in the data-dependent mode with survey scans acquired at a resolution of 50,000 at m/z 400. The top 10 most abundant isotope patterns



Band	MW (kDa)
1.	57.507
2.	51.428
3.	45.992
4.	41.130
5.	36.782
6.	32.894
7.	24.878
8.	22.878
9.	17.793
10.	14.633
11.	12.035
12.	9.625
13.	8.140
14.	6.156

Figure 1. Result of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of protein hydrolysate of green peas by bromelain (PHGPB)

with charge ≥ 2 from the survey scan were selected with an isolation window of 1.6 and fragmented by higher normalized collision energies of 35. The maximum ion injection times for the survey scan and the MS/MS scans were 20 and 60 msec, respectively. The ion target value for both scan modes was set to 1E6. Dynamic exclusion of the sequenced peptides was set to 30 sec.

LC-MS/MS data were searched using the Sequest search algorithm on Proteome Discoverer (version 2.1, Thermo Scientific) against the protein database of *P. sativum* ID 3888 downloaded from SwissProt (updated 31 July 2017). The search parameters were as follows: a) precursor mass range within 350–8000 Da; b) minimum peak count of 5; c) signal-to-noise threshold of 1.5; d) bromelain as a proteolytic enzyme allowing up to one missed cleavage; e) precursor mass tolerance of 20 ppm and fragment tolerance of 0.1 Da; f) oxidation of methionine as variable modification and carbamidomethylation of cysteine as fixed modification; and g) 0.1% false discovery rate. Ten proteins were identified from this sample, as observed in Table 1.

In vivo test in gentamycin-induced Wistar rats

The 56 male Wistar rats were divided into seven treatment groups: Group 1, 50 mg/day PHGPB; Group 2, 100 mg/day PHGPB; Group 3, 200 mg/day PHGPB; Group 4, negative control; Group 5, gentamycin/positive control; Group 6, comparison control (630 mg/day Ketosteril); and Group 7, vitamin E (200 IU d-α-tocopherol). Except for those in the negative control group, all rats were induced with 80 mg gentamycin IP for 7 days. This experiment has been approved for the ethical clearance from the Ethical Committee of Maranatha Christian University (185/KEP FK UKM-RSI /III/2018).

Sample collection

SOD activity was measured three times (D0, D7, and D35) spectrophotometrically using COBAS ROCHE 311. Urea and creatinine levels were measured six times (D0, D7, D14, D21, D28, and D35). The normal levels of urea and creatinine for 8–16-week-old male rats are 12.3–24.6 and 0.2–0.5 mg/dl, respectively (29). The right kidneys of all rats were prepared to obtain kidney homogenates for the measurement of ANP, COX 1, and renin levels.

Kidney homogenate making procedure

RIPA buffer (containing 100 mM Tris pH 7.4, 150 mM NaCl, 1 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid), 1 mM EDTA, 1% Triton X-100, and 0.5% sodium deoxycholate) was diluted in 1:9, followed by the dilution of the 1:9 cocktail protease inhibitors. Furthermore, a mixed buffer was prepared, i.e., RIPA buffer ratio: aquabidest: inhibitor = 1: 8: 1. The kidneys of the rats were cut into small pieces on an ice bath, and then chunks of the kidneys were placed in a homogenate mincer equipment (2 ml of mixed solution for each g of kidney). The slurry obtained was centrifuged twice at 4000 rpm for 10 min at 4 ºC. The supernatant was collected to measure the levels of ANP, COX 1, and renin using ELISA. For the final measurement, the supernatant was read by an ELISA reader at 450 nm wavelength.

Statistical analysis

Values were presented as mean±SD. Data obtained were analyzed by ANOVA followed by *post-hoc* LSD test for multiple comparisons. Differences were considered statistically significant ($P \leq 0.05$) and highly significant ($P \leq 0.01$). The mean levels of ANP, COX 1, and renin were interpreted descriptively.

Results

Results of bromelain enzyme measurement

The activity and amount of proteins in bromelain were 51.185.22 MW and 0.3670 mg/ml, respectively. The total specific activity of bromelain was 556.94 U/mg. The protein concentration of PHGPB was calculated by dividing the absorbance of the hydrolysate sample at a 50× dilution (0.02 sample+0.98 distilled water at A280 wavelength) with BSA equation. The result was 48.778 mg/ml, and the pH of PHGPB was 4.9.

Separation and measurement of PHGPB molecular weight

SDS-PAGE (Spectra Multicolor Low Range Protein Ladder paints. No 26628) patterns showed many bands below 14.4 kDa. PHGPB proteins with a molecular weight smaller than 10 kDa had three bands at 9.625, 8140, and 6156 kDa (Figure 1).

Table 1. List of identified protein in green peas hydrolyzed-protein using bromelain by liquid chromatography-tandem mass spectrometry

No	Accession Number	Identified Protein	Molecular Weight (kDa)	Identify
1	P02856	Vicilin, 14 kDa component [OS=*P.sativum*]	14	v
2	P08688	Albumin-2 [OS=*P.sativum*]	26.2	v
3	P02867	Lectin [OS=*P.sativum*]	30.3	v
4	P14594	Legumin B [OS=*P.sativum*]	39	v
5	P05693	Legumin K [OS=*P.sativum*]	39.8	v
6	P13919	Convicilin [OS=*P.sativum*]	46.4	v
7	P02854	Provicilin [OS=*P.sativum*]	46.4	v
8	P13918	Vicilin [OS=*P.sativum*]	52.2	v
9	P15838	Legumin A2 [OS=*P.sativum*]	59.2	v
10	P13915	Convicilin [OS=*P.sativum*]	66.9	v
Total number of identified protein				10

Results of proteomic examination

Ten proteins were identified from PHGPB using LC-MS/MS: vicilin (14 and 52.2 kDa), albumin-2, lectin, legumin B, legumin K, legume A2, convicilin (46.4 and 66.9 kDa), and provicilin (Table 1).

In vivo test results

The SOD activity of rat blood serum was measured by spectrophotometry using COBAS ROCHE 311, and the results were summarized in Table 2. Levenne test analysis to determine the distribution of SOD enzyme data showed that the baseline (D0) data are normally distributed (P>0.05). Kolmogorov Smirnov homogeneity test results showed that the baseline data are homogeneous (P=0.174). Thus, ANOVA can be performed.

After rats were induced with gentamicin 80 mg/kg BW for 7 days, the mean SOD activity in all groups, especially in the positive control group, decreased on D7 (Table 2). However, administration of PHGPB for 28 days increased SOD enzyme levels in all groups.

Results of urea measurements

Normal levels of urea for male rats aged 8–16 weeks are 12.3–24.6 mg/dl (29). All groups on D0 showed normal urea levels. On D7, only the negative control group showed normal urea level. On D14, D21, D28, and D35, all groups (including negative control groups) showed increased urea levels. On D35, the negative control group showed higher urea levels than normal. Group 3 also showed higher urea levels than normal but were lower (31.4 mg/dl) compared to other doses of groups 6 and 7 (Table 3).

Table 2. Superoxide dismutase average level and statistical analysis using analyses of variance

Group	D0	D7	*P**	D35	*P**
	In U/ml		Analysis vs Negative C		Analysis vs Positive C
1. PHGPB 50	88.48±3.13	82.34±3.28	0.000	87.84±3.87	0.000
2. PHGPB 100	90.64±0.71	85.00±1.81	0.052	93.44±0.38	0.000
3. PHGPB 200	91.48±1.17	86.98±1.45	0.684	93.84±0.54	0.000
4. Neg Control	90.22±1.13	87.48±1.39		87.98±1.13	0.000
5. Pos Control/Genta	90.50±1.60	84.76±1.33	0.034	80.98±0.51	
6. Ketosteril	90.48±1.16	84.96±1.40	0.664	91.84±0.50	0.000
7. Vitamin E	90.86±1.13	84.40±1.48	0.018	95.44±0.24	0.000

D0= baseline data before gentamycin induction; D7= result data after gentamycin induction; D14 = result data after 7d gentamycin induction; D21= result data after 14d gentamycin induction; D28 = result data after 21d gentamycin induction; D35 = result data after 28d gentamycin induction; Normal Control/ Healthy Group: The group is not given gentamycin treatment; Negative Control /Sick Group: Gentamycin treated group without protein hydrolysate; Comparison Control: Groups treated with gentamycin and given Vitamin E as comparison

Table 3. Measurement results and statistical analysis of the average urea level of 35 days of treatments

Group	D0	D7	D14	D21	D28	D35
1. PHGPB 50	19.6±1.67	39.6±1.82[a]	39.4±2.88	38.8±3.63	36.4±2.70[b]	36.2±2.05[b]
2. PHGPB 100	20.6±1.14	43.2±1.64[a]	40.6±1.52[b]	39.2±1.64[b]	36.6±1.95[b]	34.6±0.89[b]
3. PHGPB 200	20.6±1.14	41±1.58[a]	37.8±1.30[b]	35.8±1.79[b]	33.6±1.14[b]	31.4±0.55[b]
4. Negative Contr	22.2±1.10	23.8±3.27	25.0±1.87	27.8±2.39[b]	30±2.24[b]	29.6±1.34[b]
5. Positive Contr	19.6±1.82	39±5.00[a]	46.4±5.41[b]	44.6±3.78[b]	42.6±2.07	49.4±2.41[b]
6. Ketosteril	21.6±0.55	41.6±2.07[a]	39.2±2.49[b]	36.6±2.41[b]	34.8±1.30[b]	31.6±1.14[b]
7. Vitamin E	20.8±1.79	42±5.00[a]	39.4±3.51[b]	37.0±2.92[b]	35.4±1.52[b]	35.4±1.95[b]

a = significant different from D0 (P<0,01); b = significant different from D7 (P<0,05)

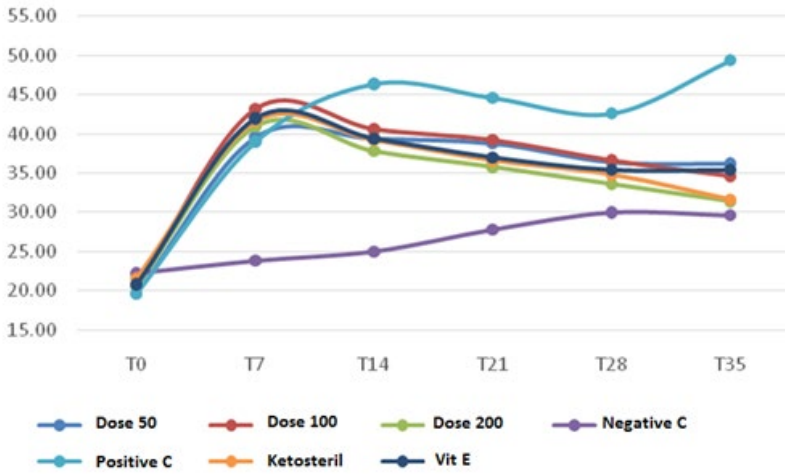


Figure 2. Graph of trend in urea mean level of 35 days of protein hydrolysate of green peas by bromelain (PHGPB) treatments
Note: x: urea mean level; y: days of PHGPB treatments

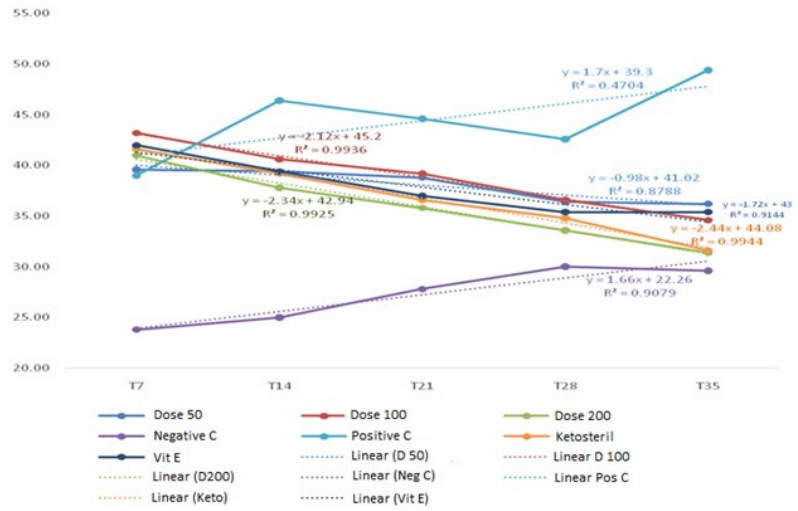


Figure 3. Graph of change in urea mean level based on time delivery to show the effect of protein hydrolysate of green peas by bromelain (PHGPB)

Results of data processing between D0 and D7 with paired t-test showed that induction with 80 mg/kg BW gentamicin for 7 days significantly increased urea level (P<0.01) in all groups, except in the negative control group. This result suggests that kidney damage induced by gentamycin was successful. In gr oup 1, urea levels decreased on D21 after the administration of PHGPB. In groups 2 and 3, urea levels significantly decreased on D7. Similar results were observed in groups 6 and 7 (Table 3).

Test results of paired t-samples showed a significant decrease on D7 (P<0.01) in all groups. Urea levels significantly decreased in groups 1, 2, 3, and 7 (P<0.01). Among the treatment groups, group 3 showed the largest decrease in urea levels. In the positive control group, urea levels continued to increase (Table 4, Figure 2 and 3).

Table 4. Comparison of urea mean level based on time delivery of protein hydrolysate of green peas by bromelain (PHGPB)

Group	Δ 14-7	Δ 21-7	Δ 28-7	Δ 35-7
1. PHGPB 50	-0.20±1.30*	-0.80±2.17*	-3.20±1.64*	-3.40±1.95*
2. PHGPB 100	-2.60±1.14*	-4.00±1.00*	-6.60±1.34*	-8.60±1.14*
3. PHGPB 200	-3.20±0.84*	-5.20±2.05*	-7.40±1.95*	-9.60±1.82*
4. Negative Con	1.20±1.79*	4.00±1.41	6.20±2.17	5.80±2.59*
5. Positive Con	7.40±1.52	5.60±1.95	3.60±4.28	10.40±4.04
6. Ketosteril	-2.40±0.55*	-5.00±0.71*	-6.80±1.48*	-10.00±1.73*
7. Vitamin E	-2.60±1.82*	-5.00±2.74*	-6.60±4.04*	-6.60±3.78*

* significant different from positive control group (P<0,01)
Mark - (negative) showed decreased urea level

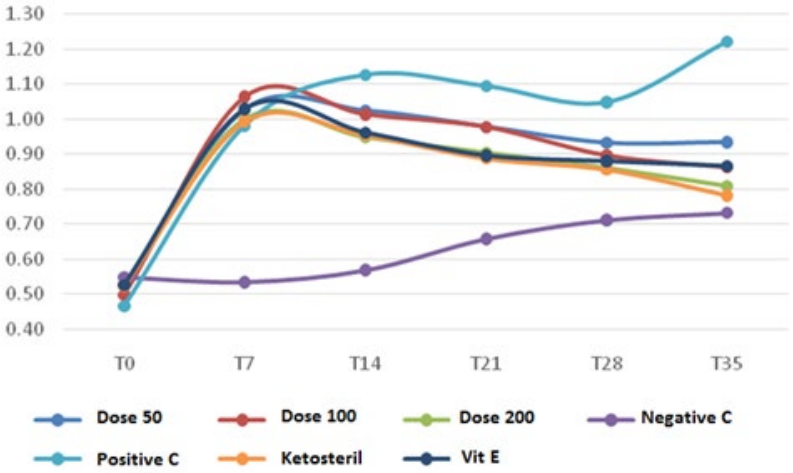


Figure 4. Graph of trend in creatinine mean level of 35 days of protein hydrolysate of green peas by bromelain (PHGPB) treatments
Note: x: urea mean level; y: days of PHGPB treatments

Results of creatinine measurement

At the end of the treatment period (D35), the serum creatinine level in the positive control group was 167% and 150.6% higher than those in the negative control group and group 3, respectively (Table 5). On the other hand, the mean serum creatinine levels between negative control and PHGPB were not significantly different (P<0.01). Normal creatinine levels for rats aged 8–16 weeks are 0.2–0.6 mg/dl. On D0, all groups showed normal creatinine levels. Only the negative groups showed normal levels of creatinine on D7 and D14 (Table 5).

On D21, D28, and D35, all groups (including the negative control group) showed elevated creatinine levels. On D35, the negative control group showed higher levels of creatinine than normal, whereas group 3 showed the lowest creatinine levels (0.81 mg/dl) among all groups, although the levels were not yet normal. The graph in Figure 4 and 5 shows that creatinine levels continued to decline over time. Thus, we assumed that

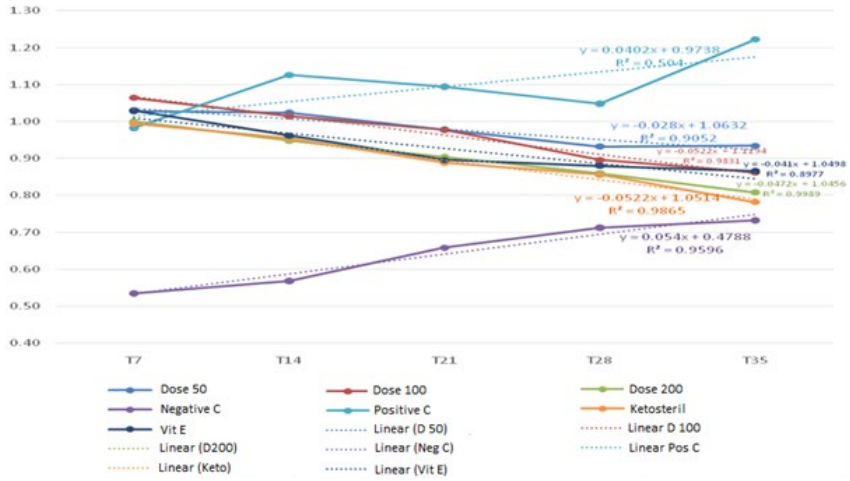


Figure 5. Graph of change in creatinine mean level based on time delivery to show effect of protein hydrolysate of green peas by bromelain (PHGPB)

Table 5. Measurement result and statistical analyses of creatinine mean level

Group	D0	D7	D14	D21	D28	D35
1. PHGPB 50	0.53±0.05	1.03±0.05[a]	1.02±0.03	0.98±0.07	0.93±0.05[b]	0.93±0.04[b]
2. PHGPB 100	0.50±0.03	1.06±0.07[a]	1.01±0.06[b]	0.98±0.06[b]	0.90±0.03[b]	0.86±0.02[b]
3. PHGPB 200	0.53±0.02	1.00±0.12[a]	0.95±0.08	0.90±0.06[b]	0.86±0.06[b]	0.81±0.02[b]
4. Negative Con	0.55±0.04	0.53±0.04	0.57±0.04[b]	0.66±0.08[b]	0.71±0.08[b]	0.73±0.04[b]
5. Positive Con	0.47±0.05	0.98±0.17[a]	1.13±0.14[b]	1.09±0.12[b]	1.05±0.06	1.22±0.04[b]
6. Ketosteril	0.52±0.03	0.99±0.06[a]	0.95±0.05[b]	0.89±0.05[b]	0.86±0.04[b]	0.78±0.05[b]
7. Vitamin E	0.53±0.04	1.03±0.07[a]	0.96±0.07[b]	0.90±0.06[b]	0.88±0.02[b]	0.87±0.05[b]

a= significant different from D0 (P<0,01); b= significant different from D7 (P<0,05)

Table 6. Comparison of creatinine mean level based on time delivery of protein hydrolysate of green peas by bromelain

Group	Δ 14-7	Δ 21-7	Δ 28-7	Δ 35-7
1. PHGPB 50	0.004±0.03*	-0.05±0.05*	-0.10±0.04*	-0.09±0.04*
2. PHGPB 100	-0.05±0.02*	-0.09±0.02*	-0.17±0.05*	-0.20±0.05*
3. PHGPB 200	-0.05±0.05*	-0.10±0.07*	-0.14±0.08*	-0.19±0.11*
4. Negative C	0.03±0.02*	0.12±0.07	0.18±0.08*	0.20±0.05
5. Positive C/Genta	0.14±0.03	0.11±0.06	0.07±0.12	0.24±0.15
6. Ketosteril	-0.04±0.02*	-0.11±0.02*	-0.14±0.04*	-0.21±0.03*
7. Vitamin E	-0.07±0.02*	-0.13±0.04*	-0.15±0.06*	-0.16±0.07*

* significant different from positive control group (P<0,01); Mark - (negative) showed decreased creatinine level

Table 7. Results of atrial natriuretic peptide, cyclooxygenase—1 and renin mean level measurement

Group	ANP (pg/ml)	COX-1 (pg/ml)	Renin (pg/ml)
1. PHGPB 50	222.10	193.10	24123
2. PHGPB 100	281.30	202.97	362.80
3. PHGPB 200	345.26	211.77	405.17
4. Negative C	262.70	247.80	295.73
5. Positive C/Genta	290.47	232.80	354.93
6. Ketosteril	305.73	233.27	339.43
7. Vitamin E	346.83	320.90	419.60

prolonging the treatment can yield better results.

Pair t-test analysis of data processing between D0 and D7 showed that induction with 80 mg/kg BW gentamicin for 7 days significantly increased creatinine levels in all groups, except in the negative control group (P<0.01) (Table 6). This result suggests that kidney damage induced by gentamycin was successful. In group 1, the creatinine levels decreased from D21 after the administration of PHGPB. In group 3, the creatinine levels significantly decreased from D14 after the administration of the test substance. Significant reduction in groups 2, 5, and 7 occurred from D7 after the administration of PHGPB (Table 6).

Measurement results of kidney-related peptide levels

Measurements of ANP, COX-1 and renin levels were performed on right renal mouse homogenates using ELISA with the Qayee-bio production kit in triplicates. Because of the limited tools used in our study, the kidney tissue was measured per group so that the average results of data can only be calculated per group as much as three times. These data could not be statistically analyzed, but could be analyzed descriptively (Table 7).

The ANP levels in the negative and gentamycin control groups were 262.70 and 290.47 pg/ml, respectively. The Groups 6 and 7 showed good ANP levels, with 305.73 and 346.83 pg/ml, respectively. The treatment groups showed good results. Results of groups 2 and 3 increased with dose. Group 3 showed the highest mean ANP levels among the three treatment groups (345.26 pg/ml), but they were slightly lower than those in group 7 (346.83 pg/ml) (Table 7, Figure 6).

The negative control group showed a COX-1 level of 247.8 pg/ml, whereas the gentamycin control showed a lower yield of 232.80 pg/ml. Comparison group showed good results, especially group 7 (320.90 pg/ml), whereas group 6 showed only 233.27 pg/ml. Among the treatment groups, group 3 exhibited the highest result (211.77 pg/ml) (Table 7, Figure 7).

Renin level was 295.73 pg/ml in the negative control group, while 354.93 pg/ml in the positive control/ gentamycin group. Among the three treatment groups, group 1 showed appropriate renin level (241.23 pg/ml), which was lower than those in the negative and positive

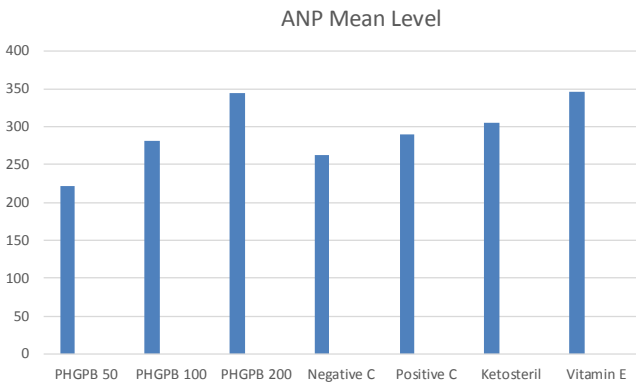


Figure 6. Results measurements of atrial natriuretic peptide mean level on day 35 of homogenate kidney of gentamycin-induced Wistar rats. The result were obtained from 3 times of ELISA measurements

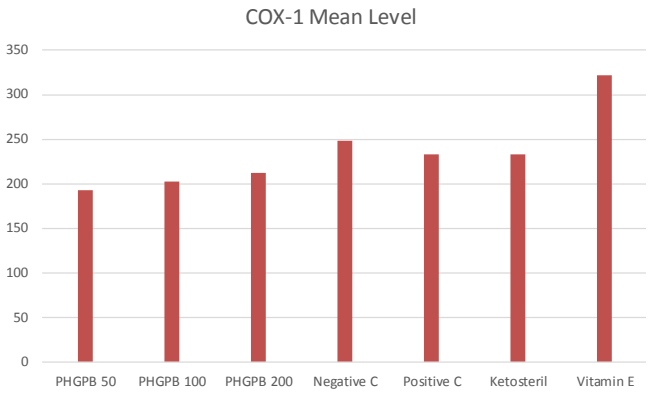


Figure 7. Results measurements of cyclooxygenase—1 mean level on day 35 of homogenated kidney of gentamycin-induced Wistar rats. The result were obtained from 3 times of ELISA measurements

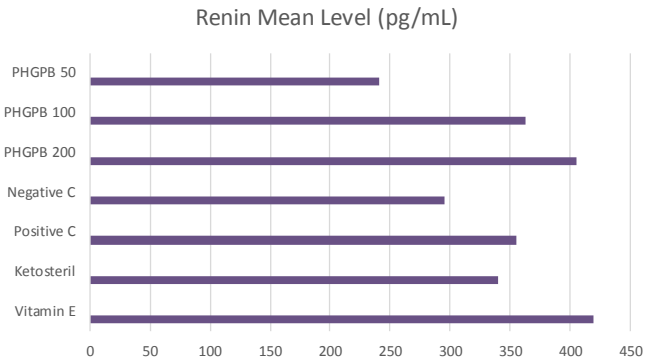


Figure 8. Results measurements of renin mean level on day 35 of homogenated kidney of gentamycin-induced Wistar rats. The result were obtained from 3 times of ELISA measurements
Horizontal: renin mean level; Vertical: groups of treatment

controls. Group 3 showed a high level of renin (405.17 pg/ml), whereas group 6 showed poor results (339.43 pg/ml) and group 7 showed greater level of renin than the positive control group (419.60 pg/ml) (Table 7, Figure 8).

Discussion

We prepared PHGPB using enzymatic hydrolysis with a readily available enzyme, bromelain. Bromelain shows stronger inhibitory effects on ACE, antioxidant properties, and high DH than papain (11). The process of hydrolysis in this study was simple and followed the procedures described by Restriani, who found that the bromelain enzyme concentration of 10% yields the highest DH in Nyamplung seed protein (*Calophyllum inophyllum*) (25).

The hydrolysis process was left for 72 hr, and the proteolytic activity of the bromelain solution remained relatively stable at room temperature (26). The enzymatic activity of bromelain decreases gradually from 25 ˚C to 95 ˚C but remains stable at room temperature (26, 30). This simple method of enzymatic hydrolysis by bromelain aims to produce PHGPB for CKD therapy.

Peel–Protein–Permeate hydrolysate containing a small-molecular-weight peptide (<10 kDa) with 2–6 amino acids exerts a strong inhibitory effect on renin and angiotensin. This antihypertensive potential is due to the easy absorption of hydrophilic peptides with small molecules (31). The smaller molecular weight of the peptide, the easier it will be to be absorbed by the body. Protein hydrolysate fraction composed mainly of low-molecular-weight peptides (generally < 3 kDa) is more effective as a renin and ACE inhibitor than larger peptides. In general, proline-containing peptides, branched-chain amino acids, and aromatic amino acids have strong inhibitory activity against renin and ACE (14). In the present study, the types of amino acids and peptides from PHGPB were not examined.

The PHGPB is suggested to contribute to glomerular cell injury by nephrotoxic substances because gentamycin is mostly excreted through glomerular filtration (37). Results showed a decrease in creatinine levels due to improvement of glomerular filtration; glomerular cell damage caused by gentamycin can be mitigated by the antioxidant activity of PHGPB. A clear difference in outcome was observed between the three doses of PHGPB groups (levels of SOD activity continued to increase) and the positive control group (levels of SOD activity continued to decline) (Table 2). Pea hydrolysate protein and their fractions have therapeutic potential for CKD preceded by oxidative damage (10). The hydrolysate protein with molecular weight of < 10 kDa obtained from fermented peas purified by *Lactobacillus rhamnosus* BGT10 had the highest antioxidant activity (38).

The highest average SOD activity and the lowest urea and creatinine levels were observed in group 3 (PHGPB 200 mg/day). Higher dose possibly corresponds to better kidney protection and antioxidant activity. Comparative analysis revealed significant increases in PHGPB groups 1, 2, and 7 (doses of 100, 200, and vitamin E), respectively. Antioxidants from natural sources have been widely used as a therapy for many diseases because they are safe, effective, and include the most common adjuvants used to treat several diseases. For example, Camel whey protein (CWP) is proven to be a powerful natural antioxidant because it can reduce oxidative stress, improve the function of immune system, and increase glutathione levels (39).

Proteomic analysis of PHGPB using LC-MS/MS identified 10 proteins. Common types of specific proteins found in the hydrolysate proteins of peas include vicilin, convicilin, provicilin, and legume. Vicilin and convicilin are major allergens of nuts (32). Legumin or vegetable casein is commonly found in peas, lentils, vetches, and other legumes; it is soluble in water, dissolves in very weak acids and alkalis, and is not coagulated by heat (33). This fact shows that peas possess a good nutritional content for a balanced diet and may even prevent degenerative diseases, such as type II diabetes and cardiovascular disease (34). Chemical substances that are responsible for the renal-protective effects, mechanisms, and nutritional properties of the constituent elements (proteins, carbohydrates, ether extracts, and fibers) of green peas remain unclear and need further investigation (35).

Proteins in legume seeds represent about 200 g/kg (dry weight) in peas (35). Proximate analysis showed that the crude protein content in PHGPB was 25.71%. Peas are a gluten-free, easy-to-obtain, and cheap protein source that is high in antioxidants and energy, micronutrients, antidiabetic and anticancer properties, fiber and low in cholesterol, fat, and glycemic index (36). Globulin 7S and 11S from peas and legumes are named vicilin and legumin, respectively. Vicilin fraction contains small amounts of soluble and high soluble non-starch polysaccharides of isoleucine, leucine, phenylalanine, and lysine. Measurement *in vitro* showed that the protein digestibility values for peas are the same or even higher than that for control protein (Lactalbumins and Casein) (35).

Increased creatinine levels indicate a decrease in GFR (12). However, the levels of urea and creatinine can only detect the occurrence of CKD when the decline in kidney function expressed in GFR is less than 50%. The Cockcroft–Gault formula for human GFR, which is calculated based on blood creatinine levels, is widely used for calculating GFR for humans (40). However, the formula for calculating GFR in experimental animals

is not yet standardized. On the last day of treatment in this study (D35), all treatment groups (including negative control, ketosteril, and vitamin E groups) showed higher mean creatinine levels compared to the normal creatinine level. This result was probably due to the fact that the induction of gentamycin caused severe renal impairment, but treatment for 28 days showed an improvement (pair t test results, all treatments showed a significant difference from the positive control (P <0.01), although the levels had not reached normal creatinine levels. These results could be achieved by extending treatment time. In regression analysis of creatinine, coefficient of determination R^2 was more than 0.9 for groups 1, 2, 3, 4, 6, and 7. Groups 7 and 3 even showed an R^2 value almost 1.0, indicating that the effect of treatment on outcomes was very strong.

Although there were some limitations in the study, we try to explain our findings; results of ANP, COX-1 and renin measurements. Due to limitations of data, mean level of ANP, COX-1 and renin results were interpreted descriptively. However, a controversial result was that the ANP positive control level was higher than the negative control level. Considering the limitations of our study, we cannot explain the mentioned controversial result. Compensatory mechanisms are possibly present in the body to reach blood pressure homeostasis, which agree with the findings of Ogawa *et al.*, that ANP increases in complicated CKD patients with deteriorating renal function. However, the association of plasma ANP levels with CKD damage still needs to be investigated (17)but the relationship between the plasma level of ANP or BNP and the future development of CKD is unclear. Methods: We measured the plasma ANP and BNP levels of 294 local residents without CKD in a Japanese community (56.5 Â± 10.4 years, mean Â± S.D.. Treatment with PHGPB increased ANP levels in a dose-dependent manner. Group 3 showed ANP levels of 131.42% and 118.86% greater than negative and positive controls, respectively. However, a controversial result was that the ANP level of positive control was higher than the negative control level. Considering the limitations of our study, we cannot explain this controversial result. We consider the possibility of a body compensation mechanism in an effort to maintain blood pressure homeostasis.

In general, COX-1 examination showed poor results, and three treatment groups showed lower results than the positive control/gentamycin group. This result may be due to the short duration of PHGPB administration or molecular weight of the peptide greater than 3 kDa in PHGPB. Further research is needed for clarification.

Renin result measurement of group 1 showed slight good results, lower than negative and positive control (81.57%) compared to the negative and positive control (67.96%). Nevertheless, whether higher PHGPB dose increases the renin level needs further verification. The same results were found in comparison of groups 6 and 7. The mean renin level in the 200 mg/day PHGPB treatment (405.17 pg/dl) was similar to that in the comparative control vitamin E (419.60 pg/dl). This is probably due to the short time of treatment. By the end of the treatment time, the body was still in the phase of adaptation response against kidney damage as a result of gentamicin induction.

Mechanism of improved renal function by antioxidant activity is also due to ANP and low renin level but not through the COX-1 mechanism. ANP has a very important function in the inhibition of renin in the RAS system (41, 42). The high mean ANP level in the gentamycin group signifies a compensatory mechanism of the body to restore blood pressure (13, 16). ANP levels need to be measured several times to evaluate the patterns and trends in the body's response to healthy kidney and injury states.

Conclusion

PHGPB dose 200 mg/kgBW shows a potential CKD therapeutic effect that is dose-dependent. In specific, higher PHGPB dose corresponds to better kidney protection and antioxidant activity through the mechanistic action of antioxidant activity and ANP levels in gentamycin-induced Wistar rats.

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Conflicts of Interest

All contributing authors declare no conflicts of interest.

References

1. Badan penelitian dan pengembangan kesehatan indonesia. Riset Kesehatan Dasar (RISKESDAS). Health Res Dev Agency Indonesia 2013; 2013:1-384.
2. Hoskins I. Pea protein may prevent kidney disease [Internet]. www.cabi.org. 2017. Available from: http://www.cabi.org/nutrition/news/19303.
3. Korhonen H, Pihlanto A. Food-derived bioactive peptides-opportunities for designing future foods. Curr Pharm Des 2003; 9:1297-1308.
4. Malomo SA, Aluko RE. A comparative study of the structural and functional properties of isolated hemp seed (*Cannabis sativa* L.) albumin and globulin fractions. Food Hydrocoll 2015; 43:743-752.
5. Tavano OL. Protein hydrolysis using proteases: An important tool for food biotechnology. J Mol Catal B Enzym 2013; 90:1-11.
6. Pownall TL, Udenigwe CC, Aluko RE. Amino acid composition and antioxidant properties of pea seed (*Pisum sativum* L.) Enzymatic protein hydrolysate fractions. J Agric Food Chem 2010; 58:4712-4718.
7. Shahidi F, Zhong Y. Bioactive peptides. J AOAC Int 2008; 91:914-931.
8. Kim SK, Wijesekara I. Development and biological activities of marine-derived bioactive peptides: A review. J Funct Foods 2010; 2:1-9.
9. Li H, Aluko RE. Identification and inhibitory properties of multifunctional peptides from pea protein hydrolysate. J Agric Food Chem 2010; 58:11471–11476.
10. Pownall TL, Udenigwe CC, Aluko RE. Effects of cationic property on the *in vitro* antioxidant activities of pea protein hydrolysate fractions. Food Res Int 2011; 44:1069-1074.
11. Gajanan PG, Elavarasan K, Shamasundar BA. Bioactive and functional properties of protein hydrolysates from fish frame processing waste using plant proteases. Environ Sci Pollut Res

2016; 23:24901-24911.
12. Sherwood L. Human Physiology: From Cells to Systems. 7th ed. Human Physiology. Yolanda Cossio; 2010. p 766.
13. Santos-Araújo, C., Leite-Moreirab,A., Pestana M. Clinical value of natriuretic peptides in chronic kidney disease. Nefrologia 2015; 35:227-233.
14. Aluko RE. Structure and function of plant protein-derived antihypertensive peptides. Curr Opin Food Sci 2015; 4:44-50.
15. Levin ER, Gardner DG, Samson WK. Natriuretic peptides. N Engl J Med 1998; 339:321-328.
16. Saito Y. Roles of atrial natriuretic peptide and its therapeutic use. J Cardiol 2010; 56:262-270.
17. Ogawa N, Komura H, Kuwasako K, Kitamura K, Kato J. Plasma levels of natriuretic peptides and development of chronic kidney disease. BMC Nephrol 2015; 16:171-177.
18. de Chatel R, Mako J, Toth M, Barna I, Lang RE. Atrial natriuretic peptide (ANP) in patients with chronic renal failure on maintenance haemodialysis. Int Urol Nephrol 1991; 23:177-183.
19. Hidayat M. Preparation and Examination of Hydrolysate Protein of Green Peas by bromelain for Improvement Kidney Function. Indonesia; EC00201810615, 2018.
20. Kunitz M. Crystalline Deoxyribonuclease I. Isolation and general properties spectrophotometric method fo the measurement of desoxyribonuclease activity. J Genet Physiol 1950; 33:349-362.
21. Harlow E, Lane D. Bradford Assay. Cold Spring Harb Protoc [Internet]. 2006;2006(6):pdb.prot4644-pdb.prot4644. Available from: http://www.cshprotocols.org/cgi/doi/10.1101/pdb.prot4644
22. Alu'datt MH, Rababah T, Alhamad MN, Alodat M, Al-Mahasneh MA GS. Molecular characterization and bio-functional property determination using SDS-PAGE and RP-HPLC of protein fractions from two Nigella species. Food Chem 2017; 230:125-134.
23. Laemmli UK. Cleavage of structural proteins during the assembly of bacteriophage T4. Nature 1970; 227:680-685.
24. Li H, Prairie N, Udenigwe CC, Adebiyi AP, Tappia PS, Aukema HM, *et al*. Blood pressure lowering effect of a pea protein hydrolysate in hypertensive rats and humans. J Agric Food Chem 2011; 59:9854-9860.
25. Restriani R. Hidrolisis secara enzimatis protein bungkil biji ryamplung (*Calophyllum inophyllum*) menggunakan bromelain. Biota 2015; 1:86-91.
26. Hale LP, Greer PK, Trinh CT. Proteinase activity and stability of natural bromelain preparations. Int Immunopharmacol 2005; 5:783-793.
27. Poh SS and Abdul Majid F. Thermal stability of free bromelain and bromelain-plyphenol complex in pineapple juice. Int Food Res J 2011; 18:1051-1060.
28. Carrillo E, Rubiales D, Castillejo MA. Proteomic Analysis of Pea (*Pisum sativum* L.) Response During Compatible and Incompatible Interactions with the Pea Aphid (*Acyrthosiphon pisum* H.). Plant Mol Biol Rep 2014; 32:697-718.
29. Giknis MLA CC. Clinical Laboratory Parameters for Crl: WI (Han). Montreal: Charles River Accelerating drug development; 2008. p 9.
30. Pavan R, Jain S, Shraddha, Kumar A. Properties and therapeutic application of bromelain: a review. Biotechnol Res Int 2012; 2012:976203-976209.
31. Girgih AT, Nwachukwu ID, Onuh JO, Malomo SA, Aluko RE. Antihypertensive properties of a pea protein hydrolysate during short- and long-term oral administration to spontaneously hypertensive rats. J Food Sci 2016; 81:1281-1287.
32. Sanchmonge R, Lopez-Torrejon G, Pascual CY, Varela J, Martin-Esteban M SG. Vicilin and convicilin are potential major allergens from pea. Clin Exp Allergy 2004; 34:1747-1783.
33. Gilman DC, Peck HT CF. Legumin. In: Rines GE, editor. The Encyclopedia Americana . New York: Dodd, Mead; 1920.
34. Leterme P. Recommendations by health organizations for legume consumption. Br J Nutr 2002; 88:239-242.
35. Rubio LA, Pérez A, Ruiz R, Guzmán MÁ, Aranda-Olmedo I, Clemente A. Characterization of pea (*Pisum sativum*) seed protein fractions. J Sci Food Agric 2014; 94:280-287.
36. Maphosa, Y., Jideani V. Chapter 6: The role of legumes in human nutrition. In: Functional food – improve health through adequate food. I 2017. Intechopen Publisher, pp 103-121.
37. Lopez-Novoa JM, Quiros Y, Vicente L, Morales AI, Lopez-Hernandez FJ. New insights into the mechanism of aminoglycoside nephrotoxicity: An integrative point of view. Kidney Int 2011; 79:33-45.
38. Stanisavljević NS, Goran N. Vukotić GN , Pasto FT3, Sužnjević D JZ. antioxidant activity of pea protein hydrolysates produced by batch fermentation with lactic acid bacteria. Arch Biol Sci 2015; 67:1033-1042.
39. Badr G, Ramadan NK, Sayed LH, Badr BM, Omar HM, Selamoglu Z. Camel whey protein as a new dietary approach to the management of free radicals and for the treatment of different health disorders. Iran J Basic Med Sci 2017; 20:338-349.
40. Douglas C. Eaton JPP. Vanders Renal Physiology. 8th ed. Lange -Mc Graw Hill; Cited: October 30, 2018.
41. Kasama S, Furuya M, Toyama T, Ichikawa S, Kurabayashi M. Effect of atrial natriuretic peptide on left ventricular remodelling in patients with acute myocardial infarction. Eur Hear J 2008; 29:1485-1494.
42. Gutkowska J, Jankowski M, Antunes-Rodrigues J. The role of oxytocin in cardiovascular regulation. Am J Physiol Regul Integr Comp Physiol 2007; 293:267-275.