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Effects of alkaline extraction on the physicochemical and functional properties of protein concentrates obtained from *Vigna mungo* and *Phaseolus vulgaris* L. legumes

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Abstract

Legumes are well-known as sources of nutritionally desirable plant proteins. The objective of this research was to investigate the effects of alkaline extraction on the functional and antioxidant properties of protein concentrates obtained from Black gram (*Vigna mungo*) and red kidney bean (*Phaseolus vulgaris* L.) legumes. The alkaline extraction at pH 9.0 for soluble protein revealed significantly ($p<0.05$) the highest protein content (71-74%). The total essential and non-essential amino acids of Black gram and red kidney bean proteins increased approximately 3-fold when compared to the amino acid content of the flour. The molecular weight profile of the Black gram (BGP) and red kidney bean (RKBP) protein concentrates ranged from 12 to 100 kDa and 16 to 160 kDa, respectively. Results showed that the BGP and RKBP absorbed soybean oil in the range of 2.49 to 2.67 g of oil/ g protein. The protein concentrates had strong emulsion forming and stabilizing activity. In addition, the antioxidant activities of BGP presented significantly the highest capacity to scavenge $ABTS^{\bullet+}$ with a value of 421.80 mg TEAC/g sample. We conclude that these proteins have the potential to be used as ingredients to formulate functional foods against oxidative stress.

1. Introduction

Animal-based food production has several environmental impacts such as the production of greenhouse gases, land change and degradation and huge amounts of wastewater. Meanwhile, eating unprocessed and/or processed red meat has been connected to the development of several human diseases. Plant proteins are macronutrients that have been trending increasingly in the health food market. Moreover, there is a growing data set of animal studies and clinical evidence, which supports improved human health associated with the consumption of plant proteins at or above normal levels of intake recommendations. Plant-based meat analogues are developed to emulate properties of normal animal meat such as texture, taste, appearance and also nutritional value. Sources of plant proteins commonly found in meat analogues include soy, peas, mung bean and red bean (Michel *et al*., 2021). Legume proteins are widely used as a general source of plant proteins. However, some of the most potent allergenic foods belong to the legume family.

Nowadays, several types of plant sources are researched as better sources of plant proteins due to their high fiber, polyunsaturated fatty acid, oligosaccharide and carbohydrate contents. Legumes are well-known as an important source of nutritional and bioactive proteins and could be claimed as a novel protein source for functional foods (González *et al*., 2020). The benefits of using legumes or beans as an alternative plant protein source are related to their fast and easy-to-grow characteristics, in addition to providing high-quality proteins due to the high contents of essential amino acids, which enable the formulation of high-quality plant -based meat analogues. Black gram (*Vigna mungo* L.) is consumed in several Asian and African countries for its high (24-28%) protein content (Wani *et al*., 2015). Some studies have shown that different environmental conditions affected the protein, amino acid,

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carbohydrate, molecular weight size, functional properties, and bioactive activity of black gram flour and protein concentrate (Kamani *et al*., 2021). The red kidney navy and adzuki beans were found to be sources of functional proteins with good properties such as solubility, water absorption, oil absorption, emulsion and foaming activities (Sai-Ut *et al*., 2009). Additionally, one of the most popular edible beans in Thailand is red kidney bean (*Phaseolus vulgaris* L.), which is used as a snack due to its abundant nutrient components. The total polyphenolic compounds and isolated protein, which were found in dark red beans exhibited antioxidant and antimicrobial properties (Roy *et al*., 2019). Hence, the main goals of this study were to investigate the effect of alkaline extraction of *Vigna mungo* and *Phaseolus vulgaris* L. on the functional and antioxidant properties of the protein concentrates. The legume protein extracts from these edible beans can be developed for use as functional food ingredients to promote human health.

2. Materials and methods

2.1 Material and chemicals

Black gram (BG) and red kidney (RKB) beans used in this research were obtained from a local farm in Sai noi District, Nonthaburi province, Thailand. The dried seed was ground using a high-speed mill and the flour passed through a 100-mesh sieve. All other reagents were of analytical grade and purchased from Ajax Fine Chem, a part of Thermo Fisher Scientific (U and V holding Thailand, Nonthaburi, Thailand).

2.2 Extraction of Black gram (*Vigna mungo*) *and red kidney bean* (*Phaseolus vulgaris* L.) *protein*

BG and RKB flours were each mixed with distilled water at a ratio of 1:10 for 15 mins, adjusted to pH 9, 10, 11 and 12 with 2 mol/L NaOH, then stirred at 650-700 rpm for 2 hrs at room temperature. After centrifugation, the supernatant was collected and adjusted to the isoelectric point of pH 4.5 using 2 mol/L HCl followed by centrifugation at 7,000 rpm, 4°C and for 30 mins. The precipitate was dispersed in distilled water at the ratio of 1:3 (w/v), adjusted to pH 7.0 with 2 mol/L NaOH, and then freeze-dried (Brishti *et al*., 2017). The freeze-dried samples were analyzed for physiochemical composition.

2.3 Measurements of physiochemical parameters

The yield of BG and RKB protein extracts was calculated as the ratio of the final weight of the dried protein to the initial weight of the flour used for extraction (Wang *et al*., 1999). The dried protein concentrates were analyzed for color in CIE Lab scale (L*, a* and b*), water activity (aw), moisture content and protein content (Dumas Combusion, Dumatec 8000 LECO, FP-528, USA).

2.4 Amino acid profile

Amino acid profiles of BG and RKB flour and protein concentrated were determined using a high-performance liquid chromatography (HPLC) amino acid analyzer (Shimadzu, model LC-20A). The samples (0.5 g) were digested using 6 M HCl at 110°C for 22-24 hrs. Digested samples were diluted with 10 ml of 0.1 mol/L HCl and the solution was then filtered with a membrane disc polytetrafluoroethylene (PTFE) filter prior to analysis. The amino acids were derivatized using post-column derivatizer (Shim-pack ISC-07/S 1504 Na) with three reagents, a reaction solution and *O*-pthaladehyde (OPA). The mobile phases consisted of three eluents, eluent A filled with 0.2 mol/L sodium citrate (containing 7% EtOH, pH 3.2), eluent B filled with 0.6 mol/L sodium citrate in 0.2 mol/L boric acid, pH 10 and eluent C filled with 0.2 mol/L sodium hydroxide at a flow rate of 0.3 mL/min. The amino acids were detected and quantified using a fluorescence detector at an excitation wavelength of 348 nm and emission at 450 nm.

2.5 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

The BG and RKB protein concentrates (10 mg each) were mixed with 1.0 ml deionized water followed by addition of 4 µL SDS sample buffer 4× (0.5 mol/L Tris HCl, pH 6.8)), glycerol (30% v/v), 8% (w/v) SDS, 0.5% (w/v) bromophenol blue, and 50 mmol/L dithiothreitol (DTT). The protein samples were boiled at 95°C for 10 mins, cooled and loaded onto the gel. A 4% stacking gel was prepared to contain 15% (v/v) separating polyacrylamide gel. A 20 µL sample solution was loaded on a Mini-Protein II cell apparatus (Atto Co., Japan) and run for 1 hr and 20 mins at 150 V. Protein bands were visualized following Coomassie Brilliant Blue R250 staining. The molecular weight range of the protein pattern standard was 11-245 kDa (Schägger and Jagow, 1987).

2.6 Functional properties of black gram and red kidney bean protein concentrates

2.6.1 Oil absorption capacity

Oil absorption capacity was analyzed by following a previous method (Brishti *et al*., 2017) but with minor modifications. The BG and RKB protein concentrates (1 g each) were mixed with 10 mL of soybean oil in a 15 mL centrifuge tube, and then stirred by vortex for 2-3 mins. The tube was allowed to stand for 30 mins at room temperature (RT) and then centrifuged at 3,000 rpm for 30 mins. The supernatant was discarded, the tubes inverted for 25 mins, and then OAC was calculated and reported following the Brishti *et al*. (2017) method.

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2.6.2 Emulsion activity and emulsion stability

Emulsion activity (EA) was determined by using the method described by Brishti *et al*. (2017) and Yasumatsu *et al*. (1972) with minor modifications. BG and RKB protein concentrates (1 g each) in 10 mL of distilled water were homogenized (3,000 rpm) for 1 min and then, soybean oil (10 mL) was added to the protein solution. The tubes were remixed using a homogenizer for 1 min and agitated at RT for 20 mins. The suspension solution was centrifuged at 3,000 rpm for 5 mins and an emulsion layer was noticed. The emulsion activity was calculated following a previously described method Yasumatsu *et al*. (1972) Emulsion stability (ES) was further investigated by subjecting the emulsions to water bath heat treatments at 80°C for 30 mins and centrifuging again. ES was calculated by using the same equation as Yasumatsu *et al*. (1972).

2.7 Antioxidant activities of black gram and red kidney bean protein extract

Determination of *in vitro* antioxidant activities using a spectrophotometric method (Thaipong *et al*., 2006). Sample aliquots of 10 µL were reacted with 2,2-diphenyl -1-picrylhydrazyl radical (DPPH), 10 mM 2,4,6-tri (2-pyridyl)-S-triazine 1,1-diphenyl-2-picrylhydrazyl, 20 mM $FeCl_3$ and 0.3 M acetate buffer, pH 3.6 mixed at a ratio of 1:1:5 (FRAP reagent) and 190 µL 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS), then the reaction mixture was incubated at room temperature in the dark for 30 mins (DPPH) and 15 mins (FRAP and ABTS assay) before the absorbance was read at 515 nm, 593 nm and 734 nm, respectively. The standard curve was linear between 25 and 600 mM Trolox. Results are expressed in mg Trolox equivalents (TEAC)/g sample.

2.8 Statistical analysis

Data are presented as the mean ± standard deviation (SD) of the determination made for each sample. Data were tested by one-way analysis of variance (ANOVA) and the data for functional properties and antioxidant activities of BGP and RKBP were subjected to independent t-test using SPSS version 16.0.

3. Results and discussion

3.1 Physiochemical characteristics of black gram and red kidney bean protein concentrated

In this study, protein concentrates were extracted from BG (24.19±0.56% protein content) and RKB (21.46±0.20% protein content) flours. BG and RKB protein are base extraction consisting of pH 9, 10, 11, and 12 then acid precipitation which has an impact on physiochemical properties. The effect of pH value on the protein extraction yield and protein yield for both legumes is displayed in Table 1. The results showed that the alkaline environment at pH 11 had the highest percent yield while pH 12 and pH 9 produced the lowest yields for BG and RKB, respectively. The highest protein solubilized during the alkaline extraction was achieved for BG and RKB flours at pH 9 ($p<0.05$), namely 71.61±1.79% and 74.88±0.25% of dry weight basis. These observations relate to high net charge for proteins in alkaline environments, which would increase hydrophilic surface and high repulsive forces to help reduce protein-protein interactions and increase protein-water interactions (Kramer *et al*., 2012). The moisture content of all the dried protein extracts ranged from 8.60±0.19% to 13.12±0.69% while aw was about 0.21±0.01 to 0.42±0.01 (Table 1). Color parameters (L*, a* and b*) of BGP and RKBP are presented in Table 1. L* values ranged from 63.58 to 76.91 for BGP and 60.29 to 73.99 for RKBP, while a* values ranged from 3.34 to 4.94 for BGP and 5.84 to 8.52 for RKBP. All the protein extracts displayed positive b* values (yellowness), that ranged from 13.60 to 16.25 for BGP and 7.41 to 21.77 for RKBP. Kidney bean isolates from high-pressure treatments reported L*, a* and b* values that ranged from 61.30 to 66.32, 9.11 to 10.66 and 25.13 to 26.89, respectively (Ahmed *et al*., 2018). Thus, the variations in

Table 1. Physiochemical composition of BG and RKB protein extracts.

Sample	Alkaline pH	%Yield	% Protein	Aw	Color		
					L*	a*	b*
BGP	pH 9	15.66	71.61±1.79^{a}	0.37±0.03^{b}	76.18±0.15^{a}	3.34±0.05^{c}	16.25±0.33^{a}
	pH 10	13.54	64.06±0.83^{b}	0.42±0.01^{a}	76.91±0.74^{a}	4.00±0.12^{b}	15.45±0.33^{b}
	pH 11	18.92	57.16±0.35^{d}	0.39±0.03^{ab}	75.66±0.74^{a}	4.88±0.13^{a}	15.49±0.28^{b}
	pH 12	10.25	59.92±1.43^{c}	0.21±0.01^{c}	63.58±2.67^{b}	4.94±0.59^{a}	13.60±0.42^{c}
RKBP	pH 9	7.14	74.88±0.25^{a}	0.38±0.03^{b}	73.12±0.37^{a}	6.25±0.16^{bc}	21.77±0.60^{a}
	pH 10	9.90	74.28±0.18^{a}	0.37±0.02^{b}	73.20±0.68^{a}	5.84±0.05^{c}	18.89±0.08^{b}
	pH 11	13.95	58.98±0.15^{c}	0.44±0.01^{a}	73.99±1.11^{a}	6.59±0.23^{b}	19.54±0.70^{b}
	pH 12	10.75	68.93±1.74^{b}	0.24±0.01^{c}	60.26±0.24^{b}	8.52±0.41^{a}	7.41±0.37^{c}

Values are presented as mean±SD. Values with different superscripts within the same column are statistically significantly different ($p<0.05$) using ANOVA method in the physicochemical properties.

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color characteristics amongst BGP and RKBP could possibly be due to the differences in the original color of the flours used to prepare them.

3.2 Amino acid composition

Legumes are considered an alternative source of proteins and essential amino acids. The amino acid profile (mg/g of sample) of BG, BGP, RKB, and RKBP were, therefore, determined (Table 2). The highest amino acid content of BG and RKB was observed for glutamic acid (35 to 49 mg/g), aspartic acid (27 mg/g), leucine (18 to 20 mg/g) and phenylalanine (13 mg/g) while the lowest values belonged to cysteine (0.4 to 0.6 mg/g). The major amino acids of BGP and RKBP seem to be the same group but their contents increased 3-fold when compared to the initial content in the flour (Table 2). The present study revealed that glutamic acid accumulated 18% of total amino acid composition in both BGP and RKBP, which explains the unique monosodium glutamate (MSG)-like taste products from legume proteins (Azad and Ping, 2021). The most abundant total non-essential amino acids in all legume proteins were Ser, Gly, Glu, Pro, Ala, Tyr, Arg and Asp. The values of amino acid profile in BG and RKB in this study are similar to undehulled black gram from Enugu State (Nwosu *et al*., 2019). Moreover, BGP and RKBP were found to contain interesting groups of amino acids, which belonged to high hydrophobic amino acids (HAA, 260 and 318 mg/g), negatively charged amino acid (NCAA, 216 and 247 mg/g), branched chain amino acid (BCAA, 116 and 133 mg/g), and positively amino acid (PCAA, 111 and 105 mg/g), which could have a positive effect on oil emulsification activity. The differences in amino acids found in the same legume species may be a consequence of the genetic variation and cultivation process applied in commercial practices, which was also revealed by the work of Mendez *et al*. (2005).

Table 2. Amino acid profile of BG and RKB flour and protein concentrates.

AA profile	Amino acid content (mg/g sample)			
	BG	RKB	BGP	RKBP
Thr	7.92	9.92	23.70	29.70
Met	2.97	1.45	8.52	7.61
Phe	12.89	13.76	43.85	55.23
His	6.42	6.52	19.99	21.90
Lys	15.48	15.70	45.65	42.38
Val	10.04	12.38	31.11	43.56
Ile	8.13	10.33	25.43	29.92
Leu	18.31	20.76	59.50	69.25
Trp	2.08	1.91	5.72	4.75
Cys	0.42	0.61	2.52	2.67
Ser	13.27	15.26	43.52	56.36
Gly	9.17	9.11	24.66	27.46
Glu	40.31	35.70	127.68	141.50
Pro	9.49	8.76	30.20	26.98
Ala	9.85	9.77	29.19	27.53
Tyr	7.74	5.79	24.61	32.73
Arg	12.22	11.00	46.18	41.71
Asp	27.68	27.50	88.42	105.89
TEAA	84.65	93.35	265.99	297.97
TNEAA	129.74	122.88	414.46	460.16
HAA	81.92	85.52	260.65	318.70
PCAA	34.12	33.22	111.81	105.99
NCAA	67.99	63.19	216.10	247.39
AAA	22.71	21.47	74.18	92.70
SCAA	3.39	2.06	11.04	10.29
BCAA	36.47	43.47	116.04	133.73

EAA: essential amino acid, TEAA: total essential amino acid, NEAA: non-essential amino acid, TNEAA: total non-essential amino acid, NCAA: negative charge amino acid (Asp/Glu), PCAA: positive charged amino acid (His/Lys/Arg), HAA: hydrophobic amino acid (Val/Ala/Ile/Leu/Try/Phe/Trp/Pro/Met/Cys), AAA: aromatic amino acid (Phe/Trp/Tyr), BCAA: branch chain amino acid (Val/Leu/Ile), SCAA: sulphur containg amino acids (Cys/Met), Thr:Threonine, Met:Methionine, Phe:Phenylalanine, His:Histidine, Lys:Lysine, Val:Valine, Ile:Isoleusine, Leu:Leucine, Trp:Tryptophan, Cys:Cysteine, Ser:Serine, Gly:Glycine, Glu:Glutamic acid, Pro:Proline, Ala:Alanine, Tyr:Tyrosine, Arg:Arginine, Asp:Aspartic acid.

3.3 Evaluation of SDS-PAGE patterns of black gram and red kidney bean protein concentrated

The SDS-PAGE profile of the BGP and RKBP is shown in Figure 1. The molecular weight (Mw) bands of the BGP were 12-30, 46-63 and 100-160 kDa. Among them, the band with ~20 kDa had the highest intensity, which is related to the acidic and basic subunits of legumin (Verma *et al*., 2014). The BGP displayed 46 to 63 kDa polypeptides, which originated mainly from vicilin. This pattern is somewhat consistent with the electrophoretic bands observed for untreated black gram protein (Verma *et al*., 2014) and modification of protein extracted from black gram (Azad and Ping, 2021). Electrophoresis profile of the RKBP revealed molecular weight (Mw) bands of 16, 20, 26, 32, 48, 100 and 160 kDa (Figure 1). These subunits along with 55 kDa might be ascribed to vicilin, which was reported to be an oligomeric protein consisting of three polypeptide subunits (α, β and γ) with Mw between 43 and 53 kDa (Romero *et al*., 1975). Moreover, the subunits of 50 and 30 kDa with minor components of lower Mw (19-12.5 kDa) have been ascribed to vicilin protein fraction while those of ~40 and ~20 kDa might be due to the legumins (Gatehouse *et al*., 1982). Additionally, the polypeptide with an estimated molecular weight of approximately 12-30 kDa, 43-63 kDa and 50-55 kDa which may correspond to legumin, 7S vicilin, and vicilin were

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detected in the BGP and RKBP, which are close to the reported sizes of 19-12.5 kDa, 41-47 kDa and 53 kDa (Romero *et al*., 1975; Gatehouse *et al*., 1982; Ahmed *et al*., 2018).

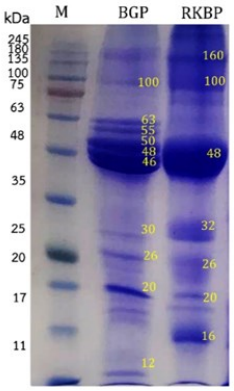

Figure 1. Pattern of protein obtained from black gram protein (BGP) and red kidney bean protein (RKBP). M is molecular weight of standard marker range from 11-245 kDa.

3.4 Functional properties of black gram and red kidney bean protein concentrated

The functional properties of food ingredients such as solubility, water adsorption, emulsion, foaming, and gelification are key measures of the potential for the development of food products. In this study, pH value was investigated as an extrinsic factor of protein solubility. As shown in Table 3, BGP (2.49 g oil/g protein) and RKBP (2.67 g oil/g protein) were not significantly different ($p>0.05$) in oil absorption capacity (OAC) as established by the independence t-test. These OAC results were affected by intrinsic factor which is Mw (12 to 160 kDa), protein conformation (α, β, γ) and high HAA of protein (Table 2) as they can promote oil holding on protein surface because the hydrophobic surface interacts with the non-polar oil. In this research, the OAC capacity had a higher ability to adsorb oil than previously reported chickpea flour and black gram flour (Solanke *et al*., 2021). A similar result for OAC has been reported for extrinsic (ultrasonicated) modification of protein extracted from black gram by-product (Kamani *et al*., 2021) and the reported OAC of 2.2 to 7.2 g oil/g for Bambara groundnut protein isolates (Adebowale *et al*., 2011). In contrast, the OAC from Indian black gram (*Phaseolus mungo* L.) showed a higher ability with values of 6.5 to 7.1 g/g (González *et al*., 2020). From the results, RKBP showed the highest values for both emulsifying capacity and stability, when compared with BGP ($p<0.05$). The emulsification activity of RKBP was close to that reported for red kidney beans from the Royal Project Shop, Chiang Rai, Thailand (Sai-Ut *et al*., 2009). The emulsification of proteins is influenced by solubility and surface hydrophobicity (Voutsinas and Nakai 1983).

Table 3. Functional properties of BG and RKB protein concentrates.

Functional properties	BGP	RKBP
Oil absorption capacity (g of soybean oil/g protein)	2.49±0.14[a]	2.67±0.12[a]
Emulsion activity (%)	20.32±0.28[a]	28.10±0.51[b]
Emulsion stability (%)	15.31±1.19[a]	27.90±0.57[b]

Values are presented as mean±SD. Values with different superscripts within the same row are statistically significantly different ($p<0.05$) using T-test method in the functional properties.

3.5 Antioxidant activities of black gram and red kidney bean protein concentrated

Antioxidants are used to inhibit or retard the generation of reactive oxygen species (ROS), thereby preventing oxidative stress. In recent years, the antioxidant activity of bioactive peptides derived from the digestion of various proteins has attracted much attention (Kou *et al*., 2013). Many antioxidant properties of plant protein hydrolysates have been reported, including their abilities to retard ROS, scavenge free radicals, chelate pro-oxidation transition metal and/or prevent the peroxide radicals of lipid oxidation (Kou *et al*., 2013; Torres-Fuentes *et al*., 2015). The ability of BGP and RKBP to inhibit ABTS•+, DPPH• and Fe^{3+} is presented in Figure 2. BGP showed the highest capability ($p<0.05$) to scavenge ABTS•+, DPPH•, and Fe^{3+} radicals when compared to RKBP. There were significant differences between the ABTS scavenging activity of BGP (421.80 mg TEAC/g sample) and RKBP (158.57 mg TEAC/g sample) compared to other scavenging pathways as well as Trolox. The presence of hydrophobic/aromatic amino acids such as Val, Ala, Ile, Leu, Try, Phe, Trp, Pro, Met and Cys that possess an ability to transfer electrons to the free radicals can contribute to increased antioxidant properties of the protein concentrates (Udenigwe and Aluko, 2011). The results exhibited that *Vigna mungo* (black) can retard DPPH radical (22.7%), FRAP (11.80 µg AAE/mg of the

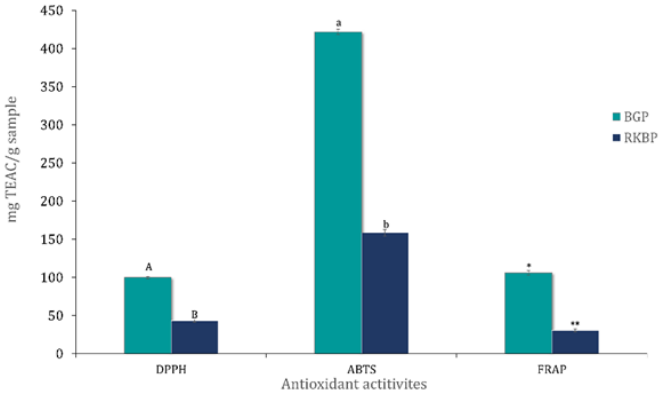

Figure 2. DPPH, ABTS and FRAP activities of black gram protein (BGP) and red kidney bean protein (RKBP). Data are expressed as mean±SD. Bars with different notations are statistically significantly different ($p<0.05$) using t-test method among the antioxidant activity.

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sample) and reducing power activity (14.90 µg AAE/mg of the sample). Moreover, *Phaseolus vulgaris* (brown and light pink) protein also showed higher DPPH activity (39.1%), FRAP (18.90 µg AAE/mg of sample) and reducing power activity (28.40 µg AAE/mg of sample) when compared to other Indian legume proteins (Petchiammal and Hopper, 2014). Thus, the factors that influence the antioxidant activity of food proteins are the type of material, the structural properties, conformation, the presence of specific amino acids and their specific positioning in the sequence of the polypeptides.

4. Conclusion

Proteins from black gram and red kidney bean were isolated with the recovery of most of the protein components with alkaline extraction followed by isoelectric precipitation, which led to a protein content that is 3 to 4-fold that of the flour. Hydrophobic amino acids were the major composition in both the flour and protein concentrates. All the protein concentrates of black gram and red kidney bean consisted of polypeptides with medium to high molecular weights of 16 to 160 kDa. Red kidney bean protein had higher oil absorption capacity, emulsion activity (%) and emulsion stability (%) than that of black gram protein. Interestingly, the isolated black gram protein possessed an excellent ability to neutralize ABTS•+ radicals. Red kidney bean protein was also found effective in delaying the ABTS, DPPH and FRAP activities. Therefore, the present study reveals that the polypeptides in the isolated proteins have significant potential to be used as an excellent functional agent and also as an antioxidant in food systems.

Conflict of interest

The authors declare no conflict of interest.

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