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ORIGINAL RESEARCH PAPER

EVALUATION OF BILE ACIDS-BINDING CAPACITY AND TYROSINASE INHIBITOR POTENCY OF NOVEL PEPTIDES PREPARED FROM AGRICULTURAL WASTES *VIA* ENZYMATIC HYDROLYSIS

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Abstract: The objective of this study was to prepare bile acids-binding and tyrosinase inhibitory peptides for industrial potential application. Proteins were extracted from *Brassica olercea* (cauliflower) midribs, *Corchorus olitorius* (corchorus) stems and *Vicia faba* (broad bean) leaves then were subjected to hydrolysis by immobilized lettuce protease. For maximum peptides production, the influence of incubation temperatures, and substrate concentrations on the hydrolysis processes were investigated. Peptides were fractionated by ultrafiltration into three fractions according to their molecular weight. Corchorus (fractions F_2 and F_1) and broad bean (fraction F_1) peptides showed bile acids-binding higher than that bind by cholestyramine resin. They may have potential application as a cholesterol-reducing agent for hypercholestermic patients. Cauliflower (F_2 and F_1) and corchorus (fraction F_1) peptides showed tyrosinase inhibitor potency higher than that by kojic acid. They may have potential application as a whitening agent.

Keywords: *bioactive peptides, broad bean leaves, cauliflower midribs, corchorus stems, extraction, hydrolysate, protein digestion, ultrafiltration*

INTRODUCTION

Cauliflower midribs, corchorus stems and broad bean leaves (agricultural wastes) have been widely consumed in the Middle East. They contain valuable amount of carbohydrates, protein, vitamins and minerals [1 – 3]. Plant proteins such as lupin, rape seeds and potato are known to be good sources of protein [4 – 6], they are inexpensive and rich resources of active compounds that can be utilized as bioactive compounds [7]. Peptides derived from digestion of proteins by protease have been reported to carry specific bioactivities. Antimicrobial, antihypertensive, antioxidant, anticoagulant peptides, derived from food proteins, have been previously identified [8 – 11]. They enhanced the treatment of various disease and disorders.

Hypercholesterolemia may be prevented by the use of hypolipidemic drugs such as bile acid sequestrants such as cholestyramine [12]. Binding of bile acids is believed to promote their excretion and hence to reduce the serum cholesterol level in human. Bile acids are acidic steroids that are biosynthesized from cholesterol in the liver and reabsorbed by the terminal ileum. Researchers have demonstrated that proteins and their hydrolysates of lupin, rapeseed, lentil and soy can bind bile acids *in vitro* [13].

The key enzyme in melanin biosynthesis is tyrosinase (EC 1.14.18.1). It catalyzes hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and also catalyzes oxidation of *o*-diphenols to *o*-quinone (diphenolase activity). *o*-quinone is transformed into melanin by series of *non*-enzymatic reaction [14]. Tyrosinase inhibitors agents are used topically for treating hyperpigmentation in humans and plants. They are used in cosmetic products for maintaining skin whiteness [15]. Arbutin is a natural product and reduced or inhibits melanin synthesis by inhibition of tyrosinase [16]. Kojic acid is believed to inactivate tyrosinase by chelating copper ions of tyrosinase and reduce melanin formation [17]. It is used as whiteness agent.

The purpose of this study was to prepare bile acids-binding and tyrosinase inhibitory peptides from three agricultural wastes, *Brassica olercea* (cauliflower) midribs, C*orchorus olitorius* (corchorus) stems and *Vicia faba* (broad bean) leaves), using immobilized lettuce protease. Maximum peptides production was investigated. The prepared peptides solutions (hydrolysates) were fractionated by ultrafiltration according their molecular weights. Bile acids-binding capacity and tyrosinase inhibitor potency were evaluated.

MATERIALS AND METHODS

Reagents

L-Tyrosine, *L*-dihydroxyphenylalanine (DOPA), tyrosinase and Kojic were obtained from Sigma-Aldrich Co. (St. Louis, Mo, USA). Colorimetric enzymatic Kits for bile acids determination with standard bile acids reagent was purchased from BEN Biochemical Enterprise (Italy). All chemicals used in this study were of analytical grade.

Plant materials

Dry lettuce seeds (*Lactuca sativa*), family *Asteracea*, were bought from local markets (Cairo, Egypt). Fresh *Brassica olercea* (cauliflower) midribs, family *Brassicaceae* and *Corchorus olitorius* (corchorus) stems, family Jew's mallow) were purchased from local markets, Cairo, Egypt, while *Vicia faba* (broad bean) leaves, family *Leguminoseae* was collected from field. They were oven dried at 60 °C to inactivate the enzymes present. They were grinding and then stored in plastic bags at -20 °C until they were used for hydrolysate preparation.

Preparation of the proteins

Proteins were extracted from cauliflower midribs, corchorus stems and broad bean leaves (Figure 1). Dried wastes (5 g) were soaked with 100 mL distilled water and left overnight at 4 °C. The homogenate was filtered by gauze followed by centrifugation at 3,500 g for 10 min. The resulted supernatants were saturated with ammonium sulphate to a concentration of 70 % and left overnight at 4 °C. The precipitates were collected by centrifugation at 3,500 g for 10 min then dialyzed overnight against distilled water with dialysis bag molecular weight cut off 10 - 14 KDa to remove peptides, amino acids and small molecular weight. The supernatant was stored at -4 °C for further use as proteins.

Cauliflower midribs

Corchorus stems

Broad bean leaves

Figure 1. *Plant wastes used*

Determination of protein concentration

The concentration of extracted protein was determined by method of Lowry *et al.* [18], using bovine albumin as standard. Protein concentration was expressed as $\mu g \cdot g^{-1}$ dry waste.

Preparation of immobilized protease

Immobilized protease was prepared followed procedure described in literature [19]. Crude protease was extracted from dry lettuce seeds with 0.1 M Tris-HCl buffer, *p*H 10.0. Free protease was precipitated from the prepared crude enzyme by ammonium sulphate at 60 % saturation. Calcium alginate beads were prepared according to the method of Ortega *et al*. [20] with some modifications. Alginate-glutaraldehyde beads were prepared by dropping 50 mL of 2 % sodium alginate into 100 mL of 0.2M calcium chloride with continuous stirring and stored at 9 °C for 24 h prior to use. Alginate beads were activated by adding to 6.5 % glutaraldehyde in 0.2 M Tris-buffer at 25 °C with stirring for 2 h. Two g of calcium alginate activated beads were mixed with 1.5 mL of the free enzyme followed by adding 1.5 mL distilled water to ensure full immersion of beads in enzyme solution. The loading process was performed for 1 h under continuous shaking at 9 °C.

Protein digestion

Production of peptides was accomplished through enzymatic hydrolysis of the extracted protein, using immobilized lettuce protease. Immobilized protease beads were added to 0.1 mL of the extracted protein followed by 2 mL 50 mM Tris-HCl buffer, *p*H 10. The tubes were incubated for 1 hour in a water bath at 70 °C. The undigested protein was precipitated with trichloroacetic acids and the amount of protein split products (peptides and amino acids) was separated, named "peptides solution" and stored at -10 °C to be further used.

Determination of peptides concentration

The peptides solution was estimated with phenol reagent which gives a blue color [21], using tyrosine as standard. To 5 mL of the supernatant and 5 mL 0.5 mM NaOH mixed well, then 0.5 mL Folin reagent was added drop wise while shaking. After standing for 10 minutes at room temperature, the absorbance was measured at 660 nm.

Optimization catalytic conditions

The conditions used for the digestion protein were tailored in order to maximum peptides production. Effect of different protein concentrations and different temperatures (60 and 70 °C) on peptides production was studied at *p*H 10.0. Affinity of protease enzyme towards isolated proteins was investigated by calculated relative µg peptides production per µg protein.

Fractionation of the prepared peptides by ultrafiltration

Peptides solutions were successively ultrafiltrated according to the method of Tsomides [22]. The ultrafiltration was performed using ultrafiltration centrifugal units (Amikon, Millipore, USA), with molecular weight cut-off 100 and 10 KDa. Peptides concentrations of each fraction (F_3, F_2 and F_1) were estimated. They were stored at -4 °C to be further used.

Ultraviolet spectrum analysis

Extracted proteins and peptides solutions were analyzed for their UV spectrum using Agilent Technologics, Cary, 100 Series UV-Visible spectrophotometer, Victoria, 3170, Australia.

Determination of tyrosinase inhibitor potency

Tyrosinase inhibitor potency of extracted proteins (P), the prepared peptides solutions (H) and ultrafiltration fractions F_3, F_2, F_1 was determined as described by Fawole *et al*. [23] with some modifications. *L*-tyrosine and *L*-DOPA were used as the substrate to determine the monophenolase and diphenolase activities of tyrosinase, respectively. Kojic (tyrosinase inhibitor drugs) was used as positive control. In a 96-well micro plate, 50 µL of sample was mixed with 30 µL of tyrosinase in phosphate buffer, *p*H 6.5. After 5 min incubation, 0.1 mL of 1 mM substrate was added to the reaction mixtures and incubated further for 10 to 30 min at 37 °C. Absorbance values of the wells were then determined at 492 nm using a micro plate reader. Tyrosinase inhibitor potency was represented as µg kojic equivalent per µg protein or peptides (µg KE·g^{-1}). Amount of tyrosinase inhibitory peptides yield from one gram dry waste also was calculated (mg KE·g^{-1} dry waste).

Determination of bile acids-binding capacity

In vitro, bile acids-binding capacities were determined according to the method of Kahlon and Woodruff [24]. Cholestyramine resin, a drug that binds bile acids, was also evaluated for its ability to bind bile acids as a positive control for this analysis. Aliquots of the samples were dissolved in 0.1 M sodium phosphate buffer at *p*H 7. Subsequently, 100 µL of each sample preparation was added to 900 µL of 1.5 mM standard bile acids solution in the same buffer as the samples. The bile acids/sample mixtures were incubated at 37 °C for 1 h, with subjecting of each sample to centrifugation at 10,000 g then collecting the supernatant. The amounts of bile acids bound to the samples were analyzed spectrophotometrically at 405 nm using Ben bile-acids analysis kit. The intensity of color at the reaction conditions was directly proportional to the bile acids concentration in the sample. Then the amount of bile acids remaining (unbound with the samples) was calculated. Bile acids-binding capacity was expressed as µg cholestyramine resin equivalent per µg protein or peptides (µg CE·g^{-1}). Amount of tyrosinase inhibitory peptides yield from one gram dry waste also was calculated (mg CE·g^{-1} dry waste). All analyses were at least performed in triplicate.

Statistical analysis

The results were expressed as a mean ± SD, n = 3 (standard deviation) for each analysis. Data was analyzed statistically using Student's t-test (2 tailed) by SPSS program. All analyses were at least performed in triplicate. Mean assigned with * and ** denote a statistically significant difference at $p < 0.05$ and $p < 0.001$, respectively.

RESULTS AND DISCUSISON

The present study was conducted to investigate the possible preparation and separation of bile binding and tyrosinase inhibitory peptides from some agricultural wastes for industrial potential application. Increasing interest in the recycling of plant wastes is reflected by the number of reports for their re-utilization and production bioactive peptides. Fresh cauliflower midribs, corchorus stems and broad bean leaves were chosen based on containing appreciable amount of protein content. They are abundant, low cost, available and with continuous regeneration. Their proteins content were depending on the cultivar and growing conditions [25]. They were subjected to extract their proteins by salting out method. This method avoided harmful effect of irreversible protein denaturation as previously reported [26].
For production of bioactive peptides, the extracted proteins did undergo hydrolysis by the immobilized lettuce protease. Immobilized lettuce protease was selected based on its high specific activity [19].
Evaluation of the bile acids-binding capacity and tyrosinase inhibitor potency of extracted proteins and their hydrolysates (peptides solutions) were investigated.

Evaluation of bile acids-binding capacity

Bile acids binders help in reeducation of cholesterol level in the blood. Human bile acids mixture was used for bile acid binding experiments, which is being simulating the condition in the human body. This was recommended by Yoshie-Stark & Wäsche [27]; Kongo-Dia-Mukala *et al.* [28]. This gives our results reliability and offers a final better choice for future *in vivo* testing. Cholestyramine was used as positive control and the results were expressed as the amount of bound bile acids relatively to cholestyramine activity (µg CE·g^{-1}). The bile binding capacity of cauliflower midribs hydrolysate was equal to that of its parent protein (Table 1). This is in agreement with that previously reported [27 – 30].
Bile acids-binding capacity of corchorus protein was improved by enzymatic hydrolysis by 1.75, while hydrolysis of broad bean protein lead to appearance of bile acids-binding capacity by its hydrolysate. Similarly, whey proteins and bovine milk proteins hydrolysates (peptides) showed higher or appearance of angiotensin-1-converting enzyme inhibitory activities than their parent proteins [31, 32]. The difference between different protein types and hydrolysates bioactivities could be thus related to the amino acid contents. Kahlon & Woodruff [24] reported that the variability in the bile acid binding between various treatments used (soy protein, pinto beans, black beans and wheat gluten) may relate to differences in anionic, cationic, physical and chemical structure. Hydrophobic amino acids have been reported to strongly bind bile acids via hydrophobic reactions with lipids (bile acids, cholesterol, others lipids and others sterols) [28].

Evaluation of tyrosinase inhibition activity

Tyrosinase (diphenolase) inhibitor potency of cauliflower midrib and corchorus stems proteins was improved by enzymatic hydrolysis by 1.44 and 1.46 times, respectively,

while monophenolase tyrosinase inhibitor potency of broad bean was improved but less than kojic activity (Table 1).

Table 1. *Bile acids-binding capacity and tyrosinase inhibition potency of the isolated proteins (P) and their hydrolysates (H)*

Waste names		Bile acids-binding capacity		Tyrosinase (diphenolase) inhibition potency		Tyrosinase (monophenolase) inhibition potency	
		[µg CE·µg^{-1}]	[mg CE·g^{-1} dry waste]	[mg KE·g^{-1} dry waste]	[mg KE·g^{-1} dry waste]	[µg KE·µg^{-1}]	[mg KE·g^{-1} dry waste]
Cauliflower midribs	P	4.8±0.01	0.055	2.39±0.02	0.027	0.0	0.0
	H	4.8±0.1	1.561	3.43±0.03**	1.15	0.43±0.0**	0.139
Corchorus stems	P	15.79±0.0	0.162	1.82±0.01	0.0186	0.11±0.01	0.001
	H	27.64±0.01*	4.798	2.66±0.05**	0.461	0.0	0.0
Broad bean leaves	P	0.0	0.0	1.01±0.01	0.562	0.56±0.2	0.311
	H	4.4±0.0**	26.690	1.07±0.10	4.490	0.61±0.2	3.700

*Mean assigned with * and ** denote a statistically significant difference at p <0.05 and p <0.001, respectively*

Thus, corchorus and broad bean hydrolysates showed higher bile acids-binding capacity than its parent proteins while cauliflower and corchorus showed high tyrosinase inhibitor potency than its parent proteins.

Optimization of peptides production from protein

For maximum production of peptides from protein, optimal hydrolysis conditions including incubation temperature and protein concentration per reaction mixture were investigated. The results showed that the highest peptides production was at 70 °C (Table 2) as similarly reported by Adebiyi *et al.* [33] for rice bran hydrolysis. Results showed that the extracted proteins were differed in the affinity towards the immobilized protease (protease digestibility). Difference in the amount of peptides released was related to the substrate concentration and consequently to the affinity of the enzyme towards the different proteins types (Figure 2).
Maximum peptides production for cauliflower midribs, corchorus stems and broad bean leaves were found at 0.34, 0.63 and 12.28 µg / R.M. (reaction mixture), respectively (Table 3).

Table 2. *Effect of different incubation temperature on peptides production*

Samples	Protein concentration	Peptides concentration	
		70 °C	60 °C
	[µg protein·g^{-1} dry waste]	[µg peptides· g^{-1} dry waste]	[µg peptides·g^{-1} dry waste]
Cauliflower midribs	11.57±1.02	325.32±25.1*	224.32±20.1
Corchorus stems	10.26±0.09	173.6±8.5*	143.47±8.5
Broad bean leaves	556.8±6.5	6066±341.9*	5097.47±341.9

*Mean assigned with * denote a statistically significant difference at p <0.05*

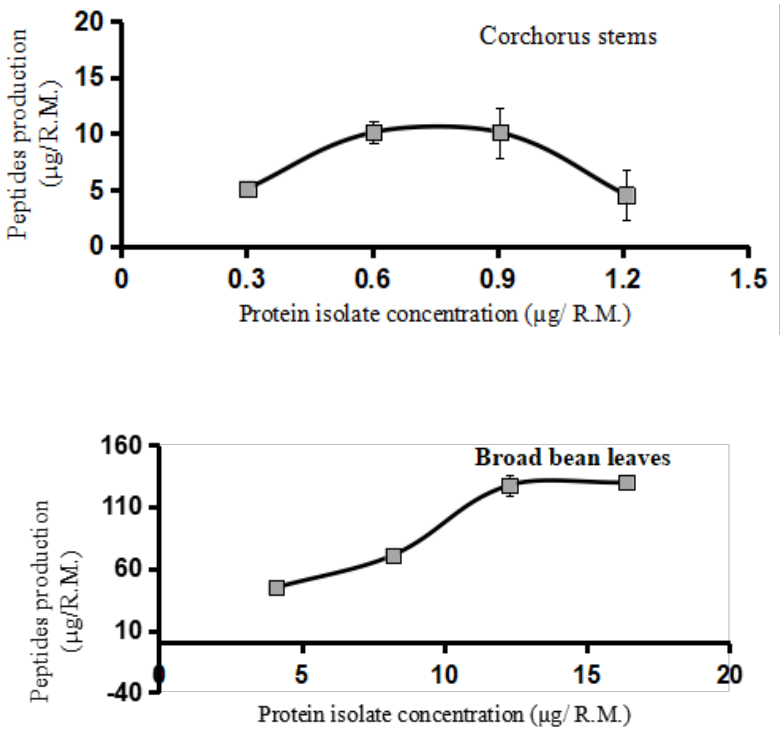

Figure 2. *Effect of different protein concentrations on peptides production*

The extracted proteins from cauliflower midribs, corchorus stems and broad bean leaves were successively hydrolysis by the immobilized lettuce protease with relative peptides production 28.11, 16.93 and 10.88 µg peptides / µg protein, respectively (Figure 3). Relative peptides production indicates the affinity of towards the isolated protein. However, the protein extraction from cauliflower midribs and corchorus stems by alkaline-isoelectric method, previously reported by El-Sayed *et al.* [34], were difficult hydrolysis by the immobilized lettuce protease with relative peptides production only 0.071 and 1.28 µg peptides / µg protein, respectively.

Table 3. *Optimum hydrolysis conditions of the extracted proteins by immobilized lettuce protease for maximum peptides production*

Parameters	Optimum condition		
	Cauliflower midribs	**Corchorus stems**	**Broad bean leaves**
Incubation temperature [°C]	70	70	70
Incubation time [min]	60	60	60
Protein concentration [µg / R.M.]	0.34	0.603	12.28
Peptides concentration [µg / R.M.]	9.56±0.64	10.11±0.96	129.56±2.57

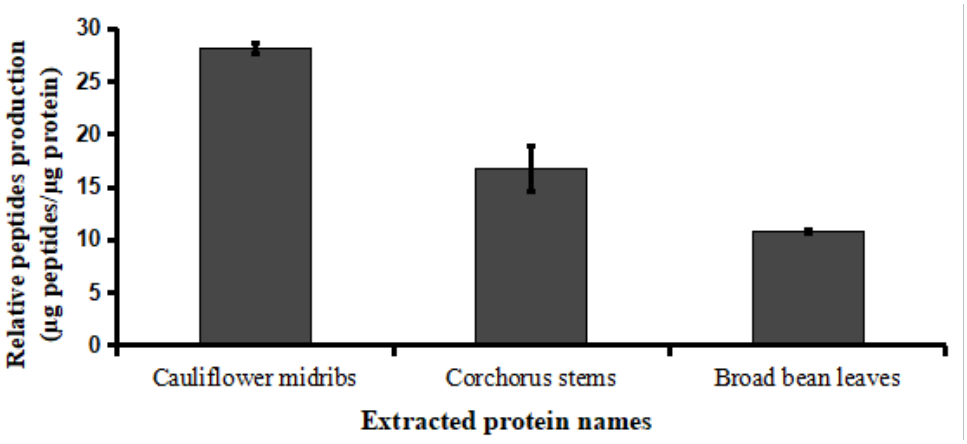

Figure 3. *Relative peptides production yield from dry wastes using immobilized protease at 70 °C*

Peptides were separated by ultrafiltration. They were labelled as F_3 (>100 KDa), F_2 (10-100 KDa) and F_1 (<10 KDa). The results showed different distribution percent of molecular weight according to the degree of hydrolysis and the type of the protein used (Table 4). They possibly depended on the catalytic mechanism and substrate affinity to the immobilized enzyme. Corchorus stems F_3 showed the highest relative percentage (55 %) then that of broad bean leaves (26 %) and cauliflower midribs (26 %). Cauliflower midribs fraction F_2 showed the highest relative percentage (40.3 %) then that of broad bean leaves (20.4 %) and corchorus stems (11.2 %). Fraction F_1 of broad bean leaves hydrolysate showed the highest relative percentage (53.6 %), corchorus stems (44 %) and cauliflower midribs (33.72 %).

Fractions (F_2 and/or F_1) of corchorus stems with molecular weight lower than 100,000 KDa and Broad bean leaves F_1 with molecular weight lower than 10 KDa showed higher bile acids binding capacity than the other fractions (Table 5). The yield of bile acids-binding peptides of corchorus and broad bean F1 were 3.54, 8.44 and 30.5 mg CE/g dry waste, respectively. Similarly, corn protein hydrolysate with molecular weight below 10,000 KDa was the major contributor for the higher bile binding capacity of flavourzyme hydrolysate [29]. Peptide (VAWWMY) Soystatin enzymatically prepared from soybean protein was previously reported to have potent bile acid binding *in vitro* using taurocholate and *in vivo* using Wistar rats strain [35].

Table 4. *Peptides concentration of the hydrolysate (H) and ultrafiltration fractions (F)*

Sample	Cauliflower midribs		Corchorus stems		Broad bean leave	
	[%]	[mg·g^{-1} dry waste]	[%]	[mg·g^{-1} dry waste]	[%]	[mg·g^{-1} dry waste]
H	100	0.325	100	0.174	100	6.066
F_3	25.99	0.084	54.59	0.095	26.00	1.717
F_2	40.30	0.131	11.19	0.019	20.38	1.236
F_1	33.72	0.109	43.79	0.076	53.62	3.253

Table 5. *Bile acids-binding capacity of the hydrolysate (H)and the ultrafiltration fractions (F)*

Waste name		Bile acids-binding capacity	
		[µg CE·µg^{-1}]	[mg CE·g^{-1} dry waste]
Corchorus stems	H	27.64±0.05	4.798
	F_3	42.86±0.01**	4.06
	F_2	182.64±1.46**	3.54
	F_1	111.11±0.66**	8.444
Broad bean leaves	H	4.4±0.05	26.690
	F_3	2.15±0.02	3.69
	F_2	0.0	0.0
	F_1	9.38±0.04**	30.50

*Mean assigned with ** denote a statistically significant difference at p <0.001, respectively*

Tyrosinase inhibitors like combination of ascorbic and citric acids or 4-hexylresorcinol are used as antibrowning agents in food industry [36], while kojic acid are used as skin whitening components in cosmetic industry [37]. The use of proteins and peptides as tyrosinase inhibitors was consider safe than the use of acids agents like ascorbic, citric and kojic acids. Fractions F_2 and F_1 of cauliflower midribs also were showed higher diphenolase inhibitor potency than hydrolysate by 3.5 times. Fraction (F_1) for corchorus stems showed highest diphenolase inhibitory activity (Table 6) than hydrolysates by 4.1 times. The yield of the diphenolase inhibitor of F_2 and F_1 of cauliflower midribs and F_1 of corchorus stems were 0.98, 1.084 and 56 mg EK/g dry waste, respectively. Schurink *et al.* [38] reported screening results for octameric synthetic peptides library synthesized from different industrial protein sources, such as milk (*β*-casein, *β*-lactoglobulin and *α*-lactalbumin), wheat (glycinin, *β*-conglycinin and gliadin) and egg (ovalbumin). The strongest inhibition performed from *β*-lactoglobulin and *α*-lactalbumin with 96.8 and 90.8 % respectively. To our knowledge this is the first report to test the inhibition of tyrosinase activity by the extracted proteins and their hydrolysates.

The inhibition of the monophenolase activity of tyrosinase enzyme also was tested. Our results showed that fractions showed no monophenolase tyrosinase activity after ultrafiltration (unpublished data).

Table 6. *Tyrosinase inhibition potency of the hydrolysate (H) and ultrafiltration fractions (F)*

Waste name		Tyrosinase (diphenolase) inhibition potency	
		[µg KE·µg^{-1}]	**[mg KE·g^{-1}dry waste]**
Cauliflower midribs	H	3.43±0.04	1.115
	F_3	3.6±0.06*	0.30
	F_2	7.5±0.27**	0.98
	F_1	9.89±0.24**	1.084
Corchorus stems	H	2.66±0.29	0.461
	F_3	0.35±0.02**	0.03
	F_2	0.45±0.01**	0.08
	F_1	7.408±0.33**	56.25

*Mean assigned with * and ** denote a statistically significant difference at p <0.05 and p <0.001, respectively*

Ultraviolet spectrum analysis

UV spectrum analysis for protein and their hydrolysates were performed. Generally, all the samples showed absorption below 300 nm (Figure 4 a, b, c). The UV spectrum of protein and hydrolysate showed disappearance and appearance of new peaks. These results may be due to enzymatic hydrolysis of the parent protein. The absence of absorbance of spectra in the visible region, suggests the absence of chromophore and nucleotides. They showed presence of absorption peaks at 277 and 252 nm for H. Disappearance (at 264 nm) and appearance of new peaks (at 252 and 277 nm) may be due to successively digestion of the protein by the immobilized protease.

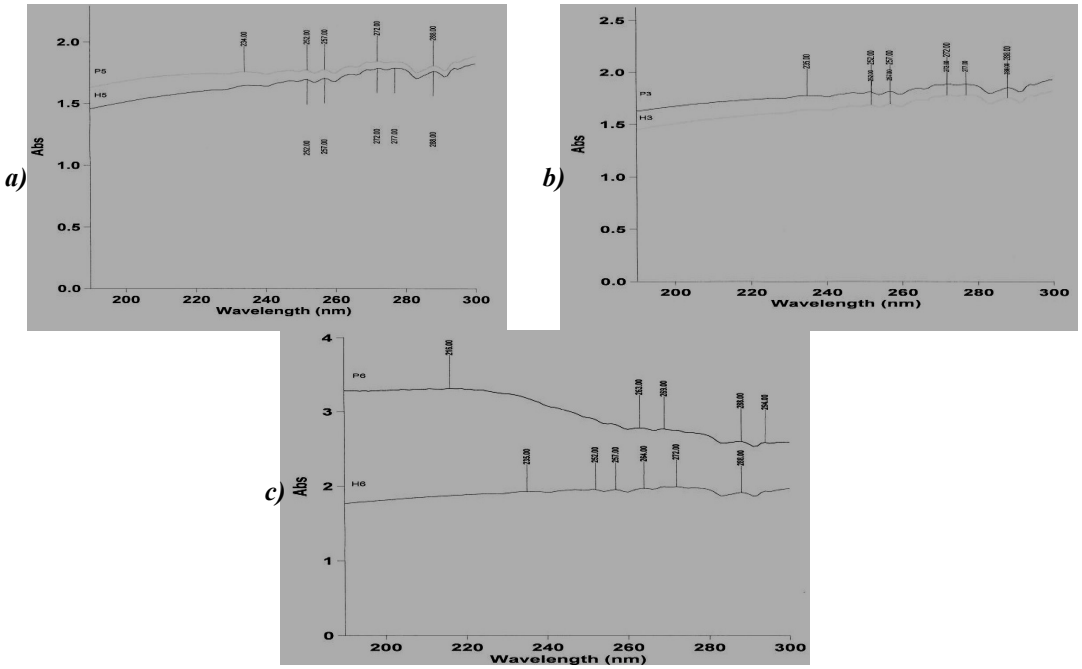

***Figure 4**. UV spectrum*
a) of cauliflower midribs protein P and hydrolysate H
b) of corchorus stems protein P and hydrolysate H
c) of broad bean leaves protein P and hydrolysate H

CONCLUSION

Proteins were extracted from the cauliflower midribs, corchorus stems and broad bean leaves. Immobilized protease was successfully used to improve or produce bile acids-binding and tyrosinase inhibitory peptides from the extracted proteins. The methods applied for protein extraction and peptides production were simple and inexpensive to be scaled-up. Corchorus and broad bean peptides showed high bile binding potency and novelty relative to cholestyramine (cholesterol lowering drug). The test of bile acids-binding activity was performed by using serum bile acids mixture which turned the results more reliable for future possible *in vivo* application. Bile acids-binding peptides F_1 of corchorus and broad bean were 8.44 and 30.5 mg CE/g dry waste, respectively. Cauliflower midribs and corchorus stems peptides showed high tyrosinase diphenolase

inhibitor potency. The yield of the prepared potent diphenolase inhibitor of F_2 and F_1 of cauliflower midribs and F_1 of corchorus stems with 0.98, 1.084 and 56 mg EK/g dry waste, respectively. Hyperpigmentation of the skin and the market need for safe whitening agent can make this study of valuable importance for cosmetic industry. Thus, broad bean leaves and corchorus stems hydrolysate may have potential application as a cholesterol-reducing agent for hypercholestermic patients, while cauliflower midribs and corchorus stems may have potential application as a whitening agent.

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