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Assessment of inhibitory activity of hydrolyzed camel whey protein concentrate and its peptides against some fungi

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ABSTRACT— The objective of this study was to investigate the antifungal activity of different concentrations (5, 10 and 20 mg/ml) of camel whey protein concentrate (WPC) and its hydrolysates against *Candida albicans*, *Asperigillus fumigatus*, *Asperigillus niger* and *Asperigillus flavus*Then, the strongest antifungal hydrolysate was further fractionated on fast performance liquid chromatography (FPLC) to separate the potent peptide subfractions at concentration of 1.45 mg/ml. The results revealed that the pepsin-trypsin (P-T) hydrolysate (20 mg/ml) showed the strongest antifungal activity, followed by 20 mg/ml of both unhydrolyzed WPC and pepsin (P) hydrolysate against *C.albicans*, *A.fumigatus*, *A.niger* and *A.flavus*, respectively.However all tested conentrations of trypsin (T) hydrolysate had the lowest activity against all strains.Thus, the potent P-T hydrolysate was subjected to further fractionation and the potent lower molecular weight peptide subfractions (SFs) (< 20kDa) were SF18 against *A.niger*, SF14 against *A.fumigatus* and *A.flavus*, repectively and SF21 against *C.albicans*.Camel´s WPC, its hydrolysates and the potent peptide subfractions (SF18 and SF14) had more hydrophobic amino acids, however SF21 had more hydrophilic amino acids. Consequently, it′s concluded that P-T camel´s WPC hydrolysate can be applied as a natural effective antifungal agent.

KEYWORDS: Camel´s WPC, enzymatic hydrolysis, peptide fractions, antifungal potancy

1. INTRODUCTION

Candida spp. mainly *Candida albicans* is majour human fungal pathogen that can secret toxic metabolites causing candidiasis (Oropharyngeal colonization) and skin lesions in immune compromised persons [1]. *Asperigillus species* mainly *A. flavus* and *A. niger* species can cause spoilage of cheese with production of mycotoxins mainly Aflatoxin B_1 with public health hazards [2].

Camel whey contains higher concentrations of antimicrobial proteins including α-lactalbumin, lactoperoxidase, lysozyme, lactoferrin, serum albumin, immunoglobulins and lactophorin [3]. The antimicrobial activity of the camel milk proteins can be further enhanced through generation of shorter bioactive peptides after enzymatic hydrolysis [4]. The molecular weight (MW) and amino acids of protein hydrolysates and peptide fractions has an influence on their bioactive properties [5].

Additionally, antifungal peptides are promising safe and effective substitutes for antifungal drugs as they have multiple mechanisms of action against broad fungal spectrum [6]. Many bioactive peptides can exert its antifungal activity either through their highly positive charged sequences which bind to negatively charged bacterial membranes or through the hydrophobic nature of antimicrobial peptides help in microbial membrane

disruption [7].

The present study focused on the evaluation of antifungal activity of camel´s WPC and its hydrolysates (P-hydrolysate, T-hydrolysate and P-T hydrolysate) against *C.albicans, A.fumigatus, A.niger* and *A.flavus,*then the strongest antifungal hydrolysate was selected for separation of potent peptide subfractions by FPLC fractionation. Furthermore, the identification of their amino acids and molecular weights camel´s WPC, its hydrolysates and potent peptide subfractions by High performance liquid chromatography (HPLC) and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), respectively.

2. MATERIALS AND METHODS

2.1 Materials

Pepsin enzyme powder from porcine gastric mucosa (EC 3.4.23.1, with activity of 3000 U/g), and trypsin enzyme powder from pancrease (EC 3.4.21.4, with activity of 2000 U/g) were obtained from Sigma-Aldrich Chemical Company, Nasr City, Egypt).

2.2 Preparation of the camel´s WPC

Six litres of fresh camel's milk were obtained from the herd of Sidi-Barani areas, Matrouh Governorate, North West Coast, Egypt. Camel whey protein concentrate was prepared depending on dialysis and freeze drying according to [8]. Camel milk was filtrated through cheese cloth to remove visible dirts and sediment, then skimmed via centrifugation at 12,000 rpm at 4°C for 30 min using a refrigerated centrifuge (SIGMA 3-16KL, Central Lab, Fac.Vet.Med, Benha University). Acetic acid solution (1M) was added to skimmed milk and continously stirred till reached to pH 4.5 (isoelectric point). After centrifugation at 13000 rpm at 4˚C for 30 min, milk was separated into the supernanant layer of whey and the bottom layer of casein sediment. Liquid skimmed whey solution was dialyzed against distilled water using a porous membrane with a molecular weight cut-off (MWCO) of 8 kDa (Sigma-Aldrich Chemical Company, Nasr City, Egypt) for 72 hr at 4°C. The retentate of the dialysis was freeze-dried at -60°C and vacuum pressure of 10 Pa for 24hr **(**using a Biobase Laboratory Vaccum Freeze Dryer, International Scientific Research Center, Dokki, Giza, Egypt).30 gm of camel WPC powder was kept frozen at -20 ºC.

2.3 Enzymatic hydrolysis of the camel´s WPC

The hydrolysis process of camel´s WPC was carried out following the method described by [8]. Camel´s WPC powder was dissolved in to distilled water to obtain WPC suspension of 3.0% (wt/vol) and divided into 3 groups for enzymatic hydrolysis: 1st group of pepsin hydrolysate: Camel´s WPC suspension was acidified to pH 2 using HCl (1M) and pepsin was added at enzyme to substrate ratio (2%(w/w), at 37ºC for 3hr hydrolysis. 2nd group of trypsin hydrolysate: Camel´s WPC suspension was adjusted to pH 7.72 using sodium phosphate buffer (0.1 M), then trypsin was added at same enzyme to substrate ratio (2%(w/w), at 42ºC for 3hr hydrolysis. 3rd group of pepsin-trypsin hydrolysate: Both pepsin and trypsin enzymes were added together to camel´s WPC suspension.

The hydrolysis process of each group was carried out in water bath under constant agitation of 500 rpm (PHOENIX Magnetic Stirrer RSM14HP model, Central Lab, Fac.Vet. Med, Benha University, Egypt). At the end of hydrolysis process, pepsin and trypsin enzymes in each group were inactivated by heating at 85°C for 5 min, then solutions were centrifuged at 10,000 rpm for 30 minutes, then supernatant was immediately freeze dried. The control sample of unhydrolysed WPC was prepared under the same hydrolytic conditions without addition of enzymes.

2.4 Degree of hydrolysis (DH%) and protein concentration %

Degree of hydrolysis (DH) of camel whey protein was estimated by detecting of solubilized protein in 10% (w/v) trichloroacetic acid (TCA), compared to the total protein content of the sample according to [3].
DH % = [Solubilised protein content in 10% TCA (mg)/ Total protein content (mg)] x 100.

2.5 Fractionation of camel´s WPC hydrolysates by Fast Protein Liquid Chromatography (FPLC)

The hydrolysate with the highest antifungal activity was fractionated by FPLC (Chromatographic Lab, International Scientific Research Center, Dokki, Giza, Egypt) according to the method previously developed by [9]. By passing sequentially through three Hitrap CM (carboxymethyl) 5/5 columns (1.5 x 2.5 cm) equilibrated in 20 Mm tris(hydroxymethyl) aminomethane hydrochloride (Tris/HCl) buffer, pH 8.0 containing 0.02% sodium azide. Volumes of 10 mL of whey proteins hydrolysates (10 g L-1 of Tris/HCl buffer) were loaded onto the three columns. Thereafter, the protein-loaded column was washed with 500 mL of the same buffer to remove loosely and unbound sample components. Bound proteins were eluted from the column with a linear gradient of 0- 1M NaCl in the same buffer was applied at flow rate of 1 mL/min and at the ambient temperature. The absorbance of the fractions was determined at 280 nm wavelength. The fraction absorbance was plotted against elution volume using the LP Data View software. Hydrolysate fractions from the column effluent were collected every 5 min in 15-mL tubes using the AKTA Explorer 100 Fraction-Collector 950. After elution of the peptides, the 28 eluted peptide fractions assigned as: F1-F28 (7.5ml each) from hydrolysate were collected and further analyzed for antifungal activity.

The fractions with the highest antifungal activity (F7 and F18) were dissolved in Milli Q water then further continually rechromatographed using the same FPLC conditions till obtain 30 peptide subfractions for each run assigned as: SF1-SF30.The eluted subfractions freeze dried and assayed for the antifungal activity.

2.6 Assay for antifungal activity in broth

The antifungal activity of camel´s WPC, its hydrolysates (P-hydrolysate, T-hydrolysate and P-T hydrolysate) and fractionated peptides was assayed against *Candida albicans* and *Aspergillus spp*, such as *A. fumigatus, A.niger* and *A.flavus* (Animal Health Research Institute, Dokki, Cairo, Egypt) as described by [10]. The fungal isolates were propagated in Sabouraud Dextrose Broth then incubated for 48 hr at 25°C. One mL of the culture was serially diluted in 1% peptone water to attain the desired inoculum level (10^3-10^4 cfu/ml) [11].

The concentrations (5,10 and 20 mg/ml) of both camel´s WPC and its hydrolysates were prepared, however the concentration (1.45 mg/ml) of all fractionated peptides was prepared. 100 µl of filter-sterilized antimicrobial stocks and 100 µLof fungal suspensions were added in each sterile well. The optical density at 620 nm (OD_{620}) of each well was measured after 27 hr using an automatic ELISA tray reader (Animal Health Research Institute, Dokki, Cairo, Egypt) at 28ºC for 72 hr. The percentage of inhibition of fungal growth after 72hr of incubation at 28°C was estimated by comparing OD620 nm values measured with the mean values of the negative controls and was calculated according to [12] as follows:

Inhibition% = (OD620 nm control - OD620 nm sample) / OD620 nm control X100.

2.7 Determination of protein concentration

Total protein content of camel´s WPC, its hydrolysates and potent peptide subfractions and subfractions were quantified using Kjeldahl method [13].

2.8 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Molecular weights (MW) of camel´s WPC, its hydrolysates and potent peptide subfractions were analysed by SDS-PAGE (Animal Health Research Institute, Dokki, Cairo, Egypt) by using precast gradient polyacrylamide gel electrophoresis 4-20% (Any kDTM Mini-Protean® TGX gel, Bio-Rad Laboratories Inc.) with coomassie blue staining (Sigma-Aldrich) [14].

2.9 Amino acid profile by High performance liquid chromatography (HPLC)

Total amino acid**s** of camel´s WPC, its hydrolysates and potent peptide subfractions was analysed by HPLC (Chromatographic Lab, International Scientific Research Center, Dokki, Giza, Egypt) according to the method described by [15]**;** The freeze-dried samples were digested using HCl (6M) at 100°C for 24 h under a nitrogen atmosphere. Chromatographic experiments were performed on Agilent 1260 series assembly system after pre-column derivatization OPA and MOC by programming the autosampler C, and analyzed inline by HPLC with DAD detection. Each sample (1 mL) was injected onto a Zorbax 80 A C18 column C18 column (4.6 mm x 250 mm i.d., 5 µm). at 40°C with detection at 338 nm. The mobile phase consisted of buffer (sodium phosphate dibasic and sodium borate), pH 8.2 (A) and ACN: MeOH:H2O 45:45:10 (B) at a flow rate 1.5 ml/min.The amino acid composition was expressed as molar percentage.Triplicate measurements were made and the data were averaged. First, an amino acid mixed standard was prepared, and the corresponding external standard method was established.

2.10 Statistical analysis

All the experiments were conducted in triplicate and the results were expressed using one-way ANOVA analysis. Differences among means were tested for significance ($P<0.05$) as described by [16]. Statistical analysis of the data was carried out employing analysis of variance (ANOVA).

3. RESULTS AND DISCUSSION

Data presented in Figure,1 showed the protein concentration and degree of hydrolysis (DH%) of camel´s WPC and its hydrolysates. The mean value of protein concentration in camel´s WPC was 25.23%± 0.64, which gradually increased to 28.74% ± 0.01, 28.93% ± 3.04 and 30.72% ± 3.16 ($P< 0.05$) in T-hydrolysates, P-hydrolysates and P-T hydrolysates, respectively. However, the P-T hydrolysates had the highest mean value of DH% (34.06% ± 1.53). This value was significantly different ($p<0.05$) from the mean values of 16.91% ± 1.75 and 14.93% ± 1.50 of T- hydrolysates and P-hydrolysates, respectively. However, the DH% of unhydrolyzed camel´s WPC was 8.47%±2.06 which may be attributed to the mild denaturation and hydrolysis during the heat inactivation [17]. On contrast, [18] reported that tryptic hydrolysates of camel whey protein had 14% (w/w) of protein content and 25.41% of DH%. These differences may be related to the difference in the protein concentration methods and conditions of hydrolysis applied [19].

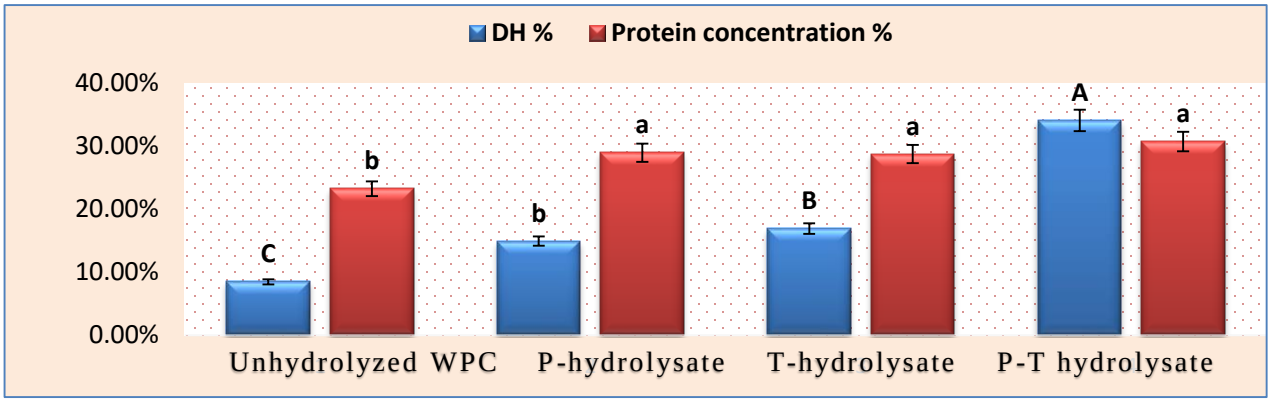

Figure. 1: Protein concentration% and degree of hydrolysis (DH%) of unhydrolyzed camel´s WPC and its

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hydrolysates (P-hydrolysates {pepsin generated camel´s WPC hydrolysates};T-hydrolysates {trypsin generated camel´s WPC hydrolysates};P-T hydrolysates [pepsin and trypsin generated camel´s WPC hydrolysates}. Values are expressed as means ± SD of 3 independent determinations. Different letters indicate a significant difference among different hydrolysates ($p < 0.05$).

3.1 Antifungal activity in broth

The present study evaluated the effect of different concentrations (5, 10 and 20 mg/ml) of camel´s WPC and its hydrolysates (P-hydrolysate, T-hydrolysate and P-T hydrolysate against tested fungal strains (*C.albicans* , *A.fumigatus, A.niger and A.flavus*). As shown in Figure (2.A), the mean values of inhibition % of camel´s WPC and its hydrolysates against *C.albicans* varied from 16.33% ± 0.21 to 97.94% ±0.02. The P-T hydrolysate had the highest activity at 20 mg/ml with mean value reached to 97.94% ±0.02 followed by mean values of 91.00 % ±0.19 and 70.32% ±0.15 at concentrations (10 mg/ml and 5 mg/ml) of P-T hydrolysates, respectively ($P > 0.05$). The lowest activity was observed by 5 mg/ml of T-hydrolysate with mean value of 16.33 ±0.22, respectively.

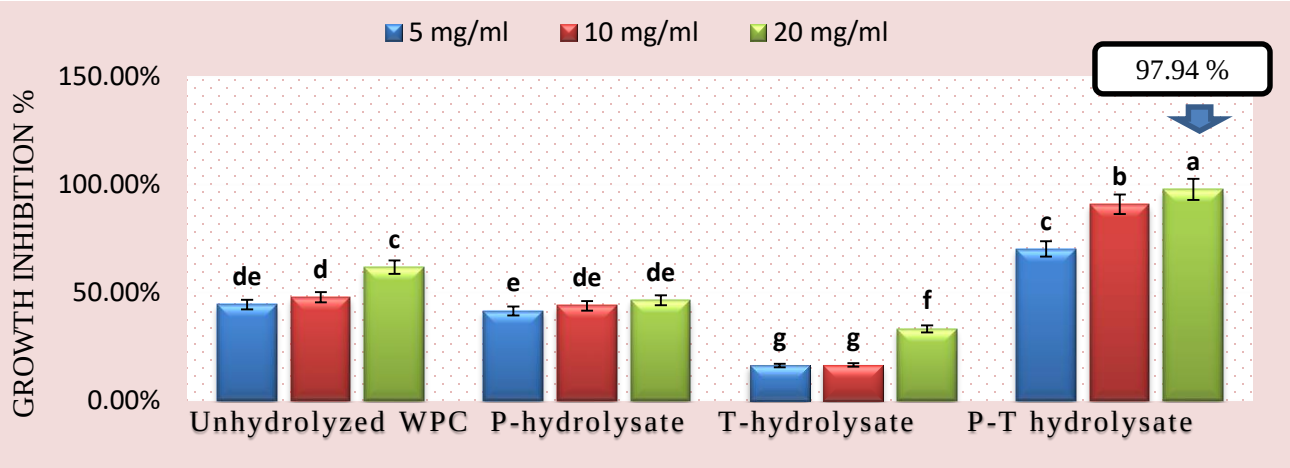

Figure. 2A: Antifungal activity of three concentrations (5, 10 and 20 mg/ml) of camel´s WPC (at 0hr of hydrolysis) and its hydrolysates (P-hydrolysates {pepsin generated camel WPC hydrolysates}; T-hydrolysates {trypsin generated camel WPC hydrolysates};P-T hydrolysates [pepsin and trypsin generated camel WPC hydrolysates} against A: *Candida albicans* (10^3 - 10^4cfu/ml) after incubation at 28 °C in microplate wells for 72hr. Values are expressed as means ± SD of 3 independent determination. Different litters indicate a significant difference among different hydrolysates ($p < 0.05$).

With respect to *Asperigillus spp.*, Figure (2.B) showed the P-T hydrolysate at 20 mg/ml had the highest activity with mean value of 95.84% ±0.02 against *A.fumigatus* followed by mean values of 94.88% ±0.02 and 91.36% ±0.07 at concentration (20 mg/ml) of unhydrolyzed WPC and P- hydrolysate, respectively with non-significant differences ($P > 0.05$). However, T-hydrolysate (5 mg/ml) had the lowest activity with mean value of 33.33% ±0.30 against *A.fumigatus*.

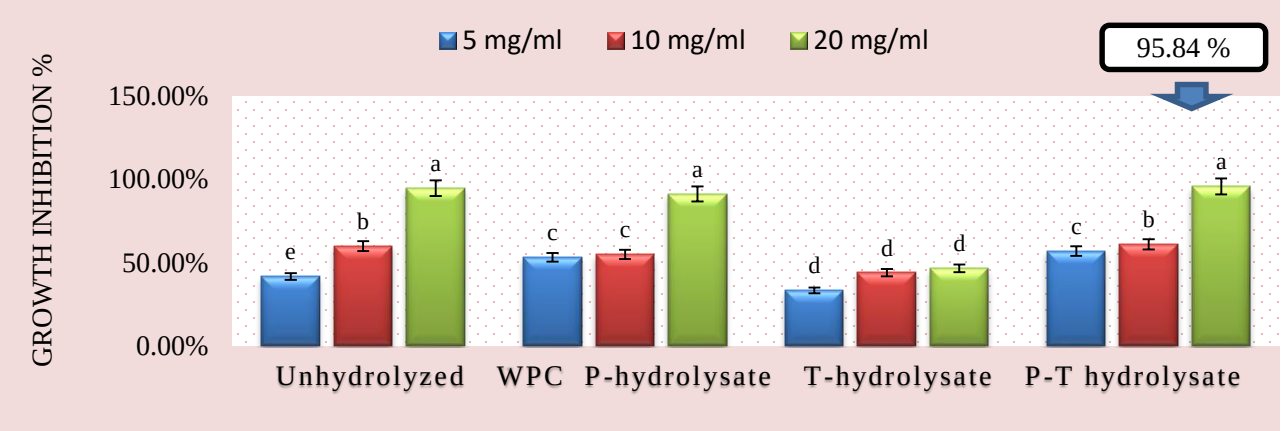

Figure. 2B: Antifungal activity of three concentrations (5, 10 and 20 mg/ml) of camel´s WPC (at 0hr of hydrolysis) and its hydrolysates (P-hydrolysates {pepsin generated camel WPC hydrolysates}; T-hydrolysates {trypsin generated camel WPC hydrolysates};P-T hydrolysates [pepsin and trypsin generated camel WPC hydrolysates} against A: *Asperigillus fumigatus* (10^3 - 10^4cfu/ml) after incubation at 28 °C in microplate wells for 72hr. Values are expressed as means ± SD of 3 independent determination. Different litters indicate a significant difference among different hydrolysates ($p < 0.05$).

In addition, variable antifungal activities against *A.niger* were observed in Figure (2.C). P-T hydrolysates at 20 mg/ml had the highest inhibition % with mean value of 63.88% ± 0.07, however T- hydrolysate at 5 mg/ml had the lowest activity with mean value of 23.10% ± 0.17 against *A.niger*. On other hand, Figure (2.D) revealed that both, 10 and 20 mg/ml of P-T hydrolysate had the highest antifungal activities with mean values of 53.70%± 0.27 and 57.77 % ± 0.30, respectively against *A.flavus* ($P > 0.05$). The lowest mean value of 20.11% ± 0.02 was detected by 5 mg/ml of T-hydrolysate.

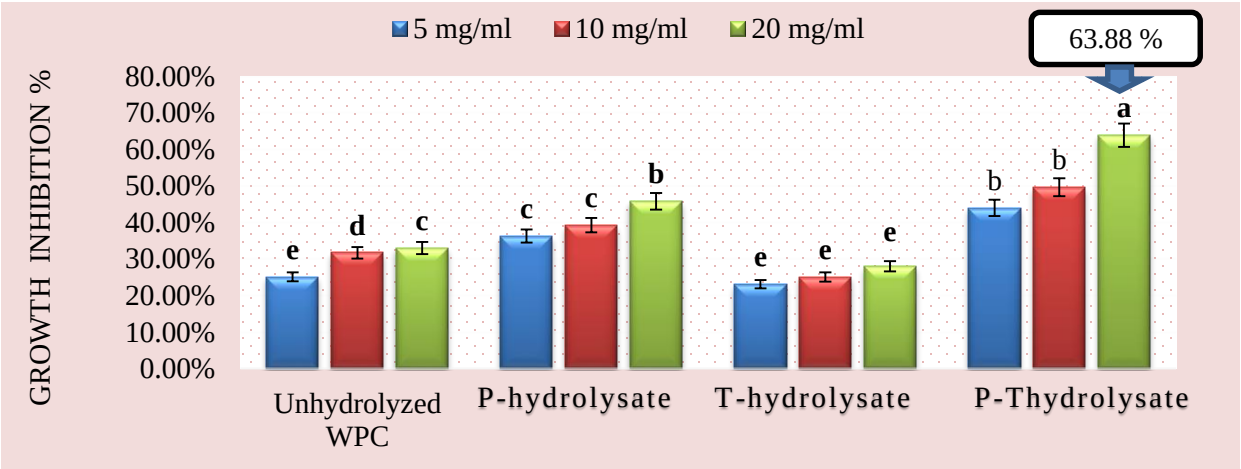

Figure. 2C: Antifungal activity of three concentrations (5, 10 and 20 mg/ml) of camel´s WPC (at 0hr of hydrolysis) and its hydrolysates (P-hydrolysates {pepsin generated camel WPC hydrolysates}; T-hydrolysates {trypsin generated camel WPC hydrolysates};P-T hydrolysates [pepsin and trypsin generated camel WPC hydrolysates} against A: *Asperigillus niger* (10^3 - 10^4cfu/ml) after incubation at 28 °C in microplate wells for 72hr. Values are expressed as means ± SD of 3 independent determination. Different litters indi a significant difference among different hydrolysates ($p < 0.05$).

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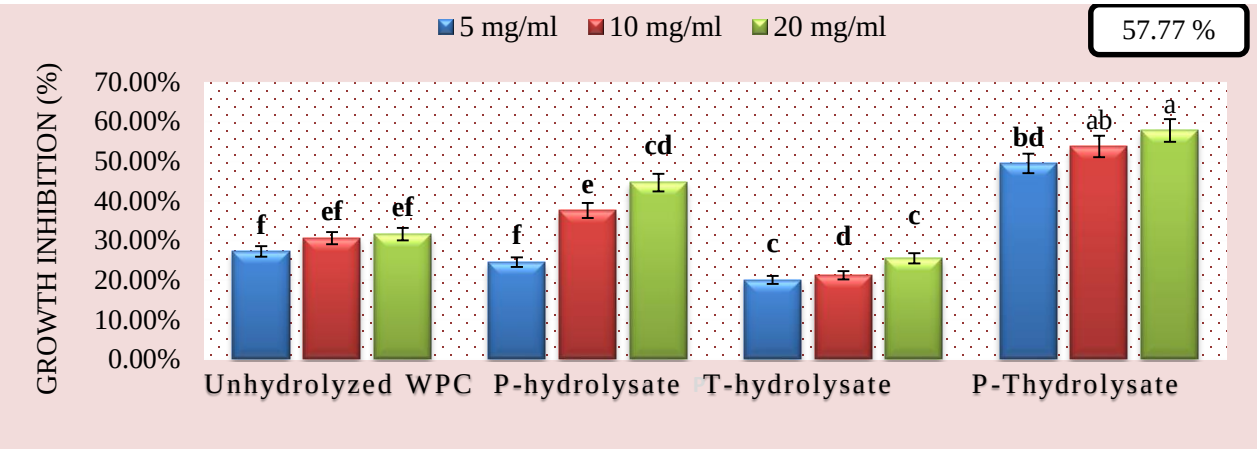

Figure. 2D: Antifungal activity of three concentrations (5, 10 and 20 mg/ml) of camel´s WPC (at 0hr of hydrolysis) and its hydrolysates (P-hydrolysates {pepsin generated camel WPC hydrolysates}; T-hydrolysates {trypsin generated camel WPC hydrolysates};P-T hydrolysates [pepsin and trypsin generated camel WPC hydrolysates} against A: *Asperigillus flavus* (10^3 - 10^4cfu/ml) after incubation at 28 °C in microplate wells for 72hr. Values are expressed as means ± SD of 3 independent determination. Different litters indi a significant difference among different hydrolysates ($p < 0.05$).

It was declared that the P-T hydrolysate had the highest antifungal activity. Thus, this hydrolysate was fractionated on FPLC and the antifungal activity of each eluted fraction was evaluated at concentration of 1.45 mg/ml. Figure,3 revealed that P-T hydrolysate was fractionated in to 28 eluted peptide fractions (F1-F28) depending on their net charges. Most of the peptide fractions had variable inhibitory effects depending on peptide sequences and fungal strain.

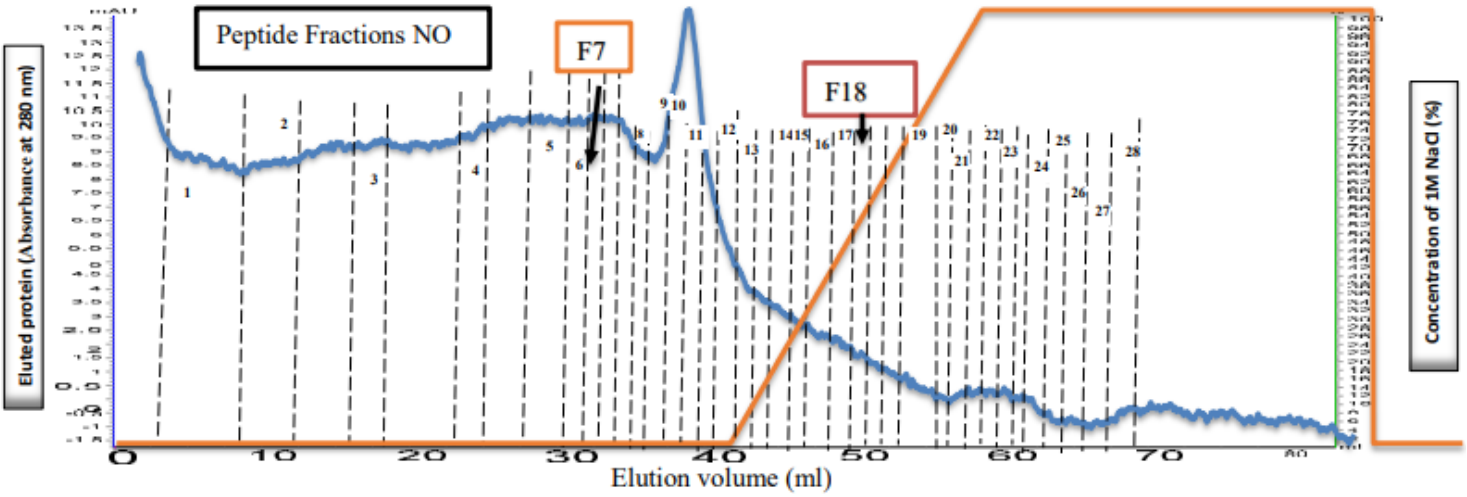

Figure. 3: Chromatographic elution profile of 28 peptide fractions obtained from P-T hydrolysates (pepsin and trtpsin generated WPC hydrolysates) according to net charge by cation-exchange fast protein liquid chromatography (FPLC). Elution was monitored by absorbance at 280 nm, at different elution volumes (ml). F1-F28 refer to pooled peptide fractions, which were collected as idicated with number. The most potent fraction s was named F7 and F18 showed by a square.

With respect to Figure.4, F7 showed the highest inhibitory activity against *A. fumigatus*, *A.flavus* and *A.niger* with mean values of 59.43 ± 2.00 %, 52.84 ± 1.33 % and 51.94 ± 2.26%, respectivly. However, F18 exhibited

The highest inhibitory activity against *C.albicans* with mean value of 48.00 ± 1.72% (Figure,5).

Therefore, these potent fractions (F7 and F18) were further re-fractionated by FPLC in to 30 eluted peptide subfractions (SF1-SF30) as shown in Figure.6.A and Figure.7.A. From all subfractions eluted from F7, the highest growth inhibition was observed in SF18 with mean value of 76.45 ± 0.09% against *A.niger* followed by SF14 with mean values of 63.25 ± 0.94 % and 58.00 ± 1.08 % against *A.fumigatus* and *A.flavus*, respectively(Figure, 6.B). However, SF21 eluted from F18 was the most effective against *C.albicans* with mean value of 26.53 ± 0.58 % (Figure, 7.B).

Figure (8) revealed the peptide concentrations in potent antifungal subfractions with of mean values of 1.26 ± 0.37 %, 3.15 ± 0.26 % and 2.10 ± 0.03% for SF18, SF14 and SF21, respectively. It was observed that the highest antifungal activity of SF18 against *A.niger* was not correlated with the elevation of peptide concentration, which a greed with [20] who stated that the antimicrobial activity of peptide fractions depends not only on peptide concentration but also on the concentration of effective fragments in peptide fraction and fungal species.

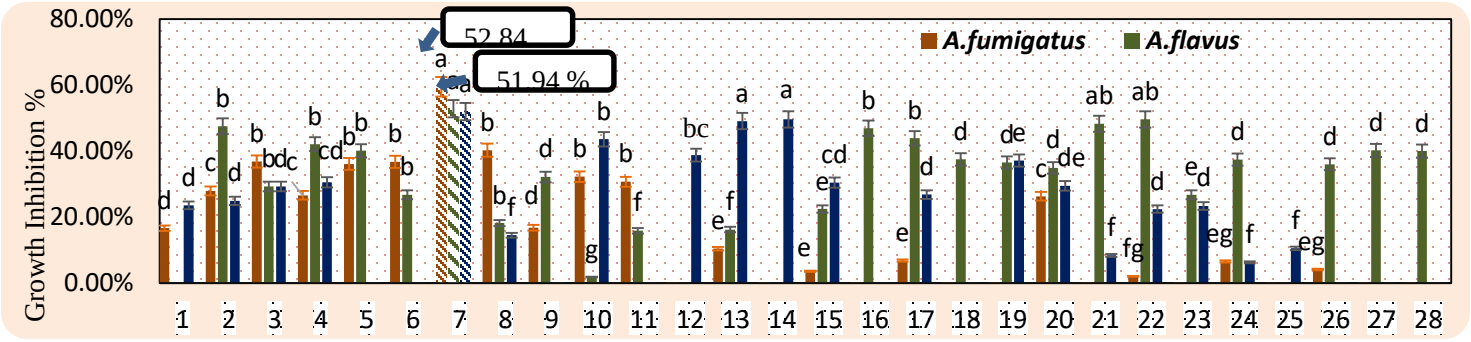

Figure. 4: Growth inhibitory activity (%) of 28 peptide fractions (1.45mg/ml) obtained from P-T hydrolysates (pepsin and trtpsin generated WPC hydrolysates) against three Asperigillus Species (103 - 104cfu/ml) after incubation at 28 °C in microplate wells for 72h. Bars represent mean of triplicate determinations. The most potent fraction was named F7 and showed by a strained bar. Values are expressed as means ± SD of 3 independent determinations. Different litters indicate a significant difference among different hydrolysates ($p < 0.05$).

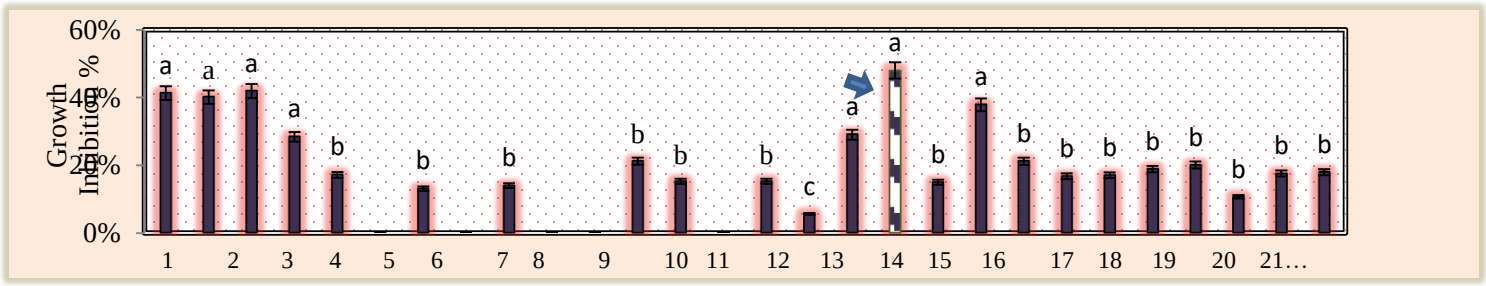

Figure. 5: Growth inhibitory activity (%) of 28 peptide fractions (1.45 mg/ml) obtained from P-T hydrolysates (pepsin and trtpsin generated WPC hydrolysates) against candida albicans (10^3 - 10^4cfu/ml) after incubation at 28 °C in microplate wells for 72h. Bars represent mean of triplicate determinations; The most potent fraction was named F18 and showed by a strained bar. Values are expressed as means ± SD of 3

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independent determinations. Different litters indicate a significant difference among different hydrolysates ($p < 0.05$).

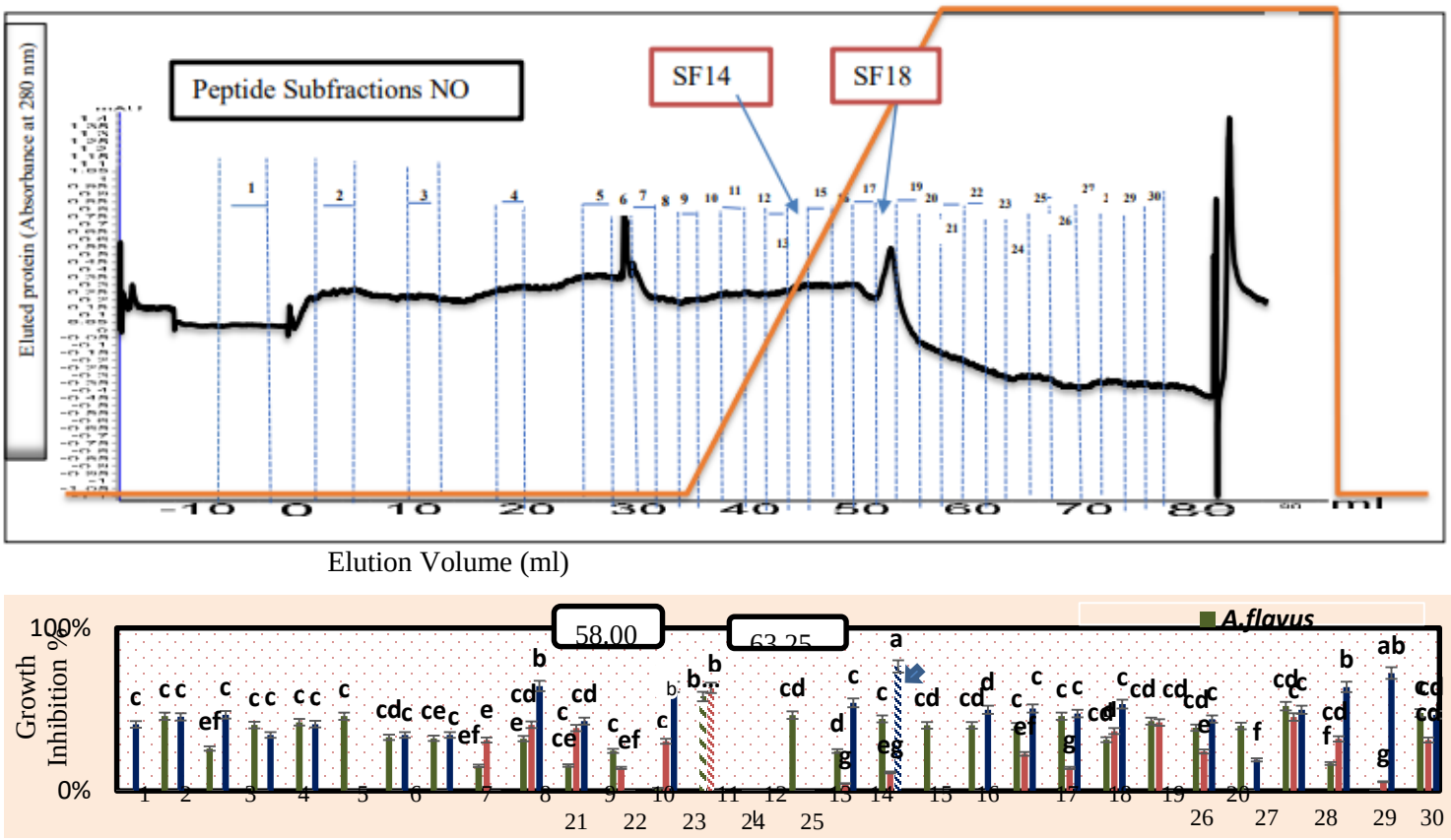
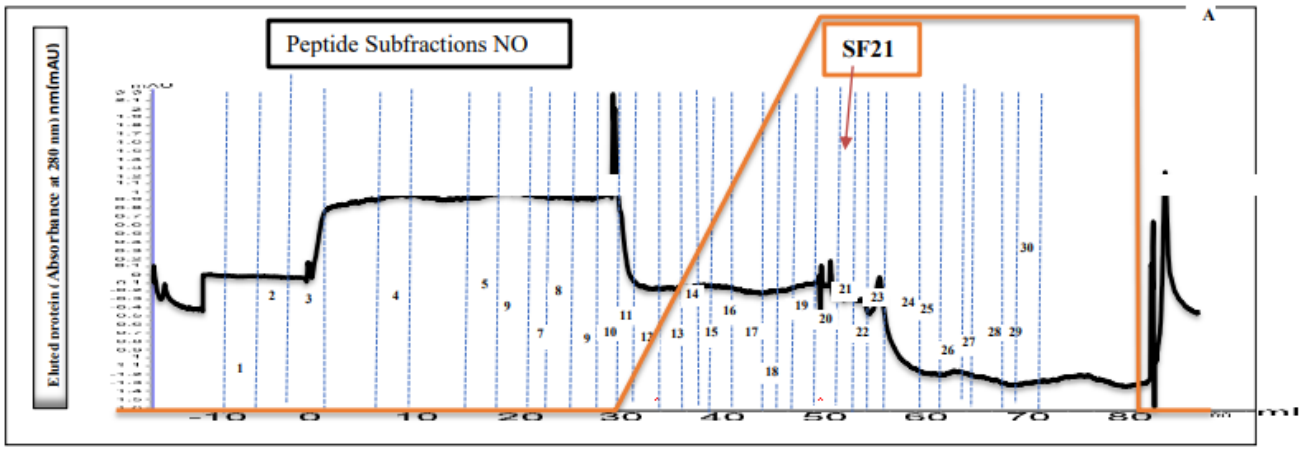


Figure 6 Separation of antifungal peptides by FPLC.A) Chromatographic elution profile of F7 obtained from P-T hydrolysates (pepsin and trtpsin generated WPC hydrolysates) according to net charge by cation-exchange fast protein liquid chromatography (FPLC). Elution was monitored by absorbance at 280 nm, at different elution volumes (ml). SF1-SF30 refer to pooled peptide fractions, which were collected as idicated with number. The most potent subfractions was named SF14 against *A.fumigatus* and *A.flavus* and SF18 against *A.niger* (10^3 - 10^4cfu/ml) showed by a square. B) Antifungal activities of subfractions (at 1.45 mg/ml) purified from F7 fraction. The potent fractions showed by a strained bars. Bars represent mean of triplicate determinations. Values are expressed as means ± SD of 3 independent determinations. Different litters indicate a significant difference among different hydrolysates ($p < 0.05$).



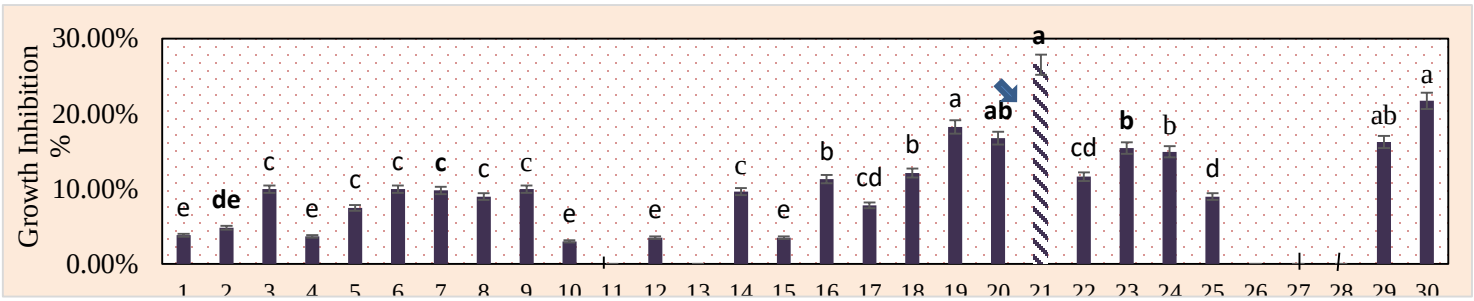


Figure. 7: Separation of antifungal peptides by FPLC.A) Chromatographic elution profile of F18 obtained from P-T hydrolysates (pepsin and trtpsin generated WPC hydrolysates) according to net charge by cation-exchange fast protein liquid chromatography (FPLC). Elution was monitored by absorbance at 280 nm, at different elution volumes (ml). SF1-SF30 refer to pooled peptide fractions, which were collected as idicated with number. The most potent sub fractions was named SF 21 against *C.albicans* (10^3 - 10^4cfu/ml) showed by a square. B) Antifungal activities of subfractions (at 1.45 mg/ml) purified from F18 fraction. The potent fractions showed by a strained bar. Bars represent mean of triplicate determinations. Values are expressed as means ± SD of 3 independent determinations. Different litters indicate a significant difference among different hydrolysates ($p < 0.05$).

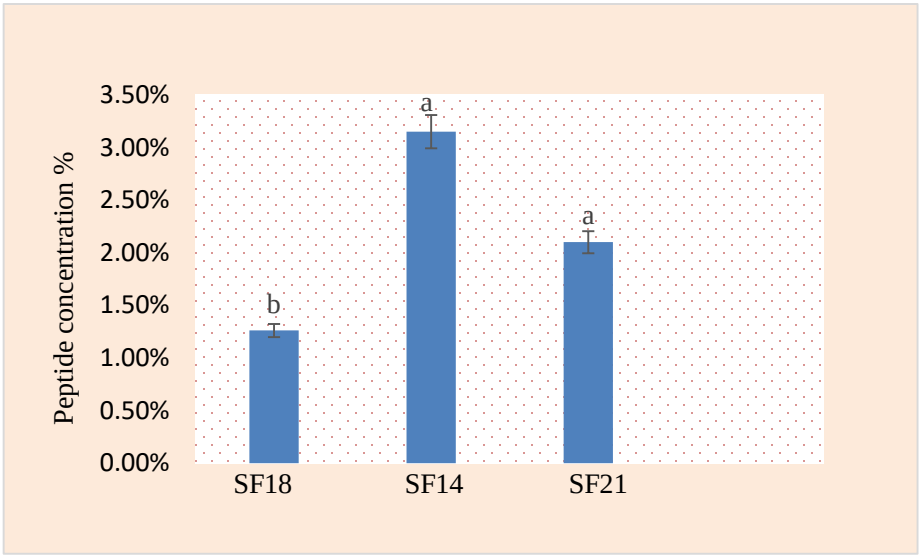


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Figure. 8: The peptide concentration of the most active peptide subfractions against *Asperigillus fumigatus, Candida albicans, Asperigillus flavus,* and *Asperigillus niger*. Values are expressed as means ± SD of 3 independent determinations. Different letters indicate a significant difference among samples ($p < 0.05$).

3.2 Molecular weight (MW) and amino acid profile of antifungal camel WPC and its hydrolysates

The camel´s WPC and its hydrolysates were compared with the standard molecular weight marker on SDS-PAGE (Figure 9 and Table 1). The electrophoretic pattern showed that unhydrolyzed WPC had scattered and thick bands at various molecular weights appeared in lane A. However, the gradual disapearance of major proteins and decrease in intensity and height of bands in the P-hydrolysate, T-hydrolysate and P-T hydrolysate in lanes B, C and D, respectively which indicates successful hydrolysis of camel´s WPC and appearance of peptide bands with lower molecular weights not be seen in the gel.With respect to the most active antifungal peptide subfractions (SF18, SF14 and SF21), although the differences in MW profile of these subfractions as shown in Figure (9), thick bands were migrated on gel below 20 kDa. SF18 with MW ranged from 19.6 - 2.5 kDa. However, it ranged from 14.98 - 4.5 kDa and 12.67 - 3.02 kDa for SF14 and SF21, repectively.

Table (2) and Figure (10) showed the differences in amino acid profile of camel´s WPC, its hydrolysates and most active peptide subfractions. The hydrophobic amino acids represented 98.23% of amino acids in P-T hydrolysate followed by 92.48%, 65.36% and 54.91% in P-hydrolysate, unhydrolyzed WPC and T-hydrolysate, respectively. However, these amino acids represented 53.65% and 57.34% in subfractions SF18 and SF14, respectively. In contrast, SF21 showed a significant decrease in hydrophobic amino acid content (37.81%) and increase in hydrophilic (62.19%). Among hydrophobic amino acids, proline was determined to be the major cyclic cationic amino acid with the highest concentration in P-T hydrolysates (87.61%)followed by 81.49%, 45.13%, 8.47%, 27.23% and 19.88% in P-hydrolysates, unhydrolyzed WPC, T-hydrolysates, SF18 and SF14, respectively (Table.2). As shown in Table (2), the presence of methionine in the peptide sequences of camel´s WPC, its hydrolysates and most active peptide subfractions may be responsible for inhibition of DNA synthesis and prevent cell replication in DNA cycle in microbial cell [21].

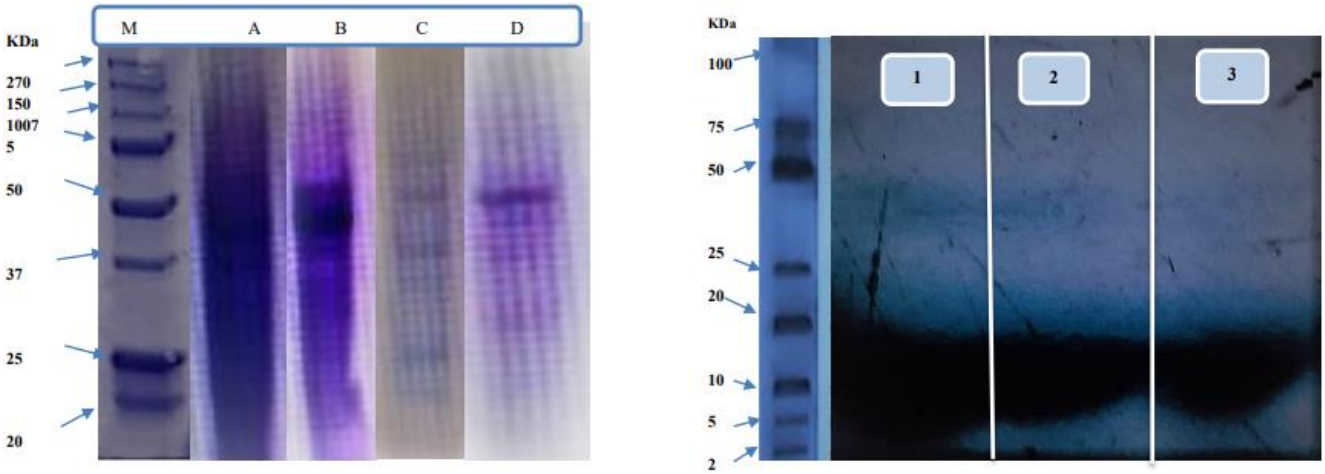

Figure. 9: SDS-PAGE profile of camel whey protein samples and most bioactive peptide subfractions isolated from P-T hydrolysates (pepsin and trypsin- generated hydrolysates) by FPLC;; Lane M: molecular mass standards; lane A: un hydrolyzed camel WPC, lane B: P-hyydrolysates (pepsin treated camel WPChydrolysates), lane C: T-hdrolysates (trypsin treated camel WPC hydrolysates), lane D: P-T hydrolysates (pepsin-trypsin treated camel WPC hydrolysates) , lane 1: SF18 (subfraction 18); lane 2: SF14 (subfraction 14) and lane 3: SF21 (subfraction 21), respectively.

Table 1: Molecular wheight, concentration intensity and peak height of protein bands. The numbers 1–6 refer to protein subunits of intact camel WPC as shown in the second column. Lf: lactoferrin; SA: serum albumin; IgG-HC: heavy chains of IgG; IgG-LC: light chains of IgG; PGRP: peptidoglcan recognetion protein; α-La: α-lactalbumin.

Band Number		Intact WPC		P- WPC hydrolysates		T- WPC hydrolysates		P-T WPC hydrolysates	
		MW(kDa)	Peak height	MW(kDa)	Peak height	MW(kDa)	Peak height	MW(kDa)	Peak height
1	LF	3.536	188.7186	58.004	222.5121	44.248	155.6965	51.068	51.2985
2	SA	59.758	218.4251	49.347	83.5016	35.112	167.8914	-	-
3	IgG-HC	48.266	205.7307	13.097	173.9021	17.047	160.8695	-	-
4	IgG-LC	41.667	210.9221	-	-	-	-	-	-
5	PGRP	21.554	198.0916	-	-	-	-	-	-
6	α-La	12,667	200.8255	-	-	-	-	-	-

SF18		SF14		SF21	
MW (kDa)	Peak height	MW (kDa)	Peak height	MW (kDa)	Peak height
19.621	441.1553	14.981	656.4032	12.674	339.1235
14.420	536.2784	9.422	406.5861	5.901	521.9601
5.991	542.9266	4.530	357.6283	3.022	327.1103
2.511	759.1808	-	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-

Table 2: The quantitative analysis (HPLC) of camel whey protein samples and most bioactive peptide subfractions isolated from P-T hydrolysates (pepsin and trypsin- generated hydrolysates) by FPLC; [a]Hydrophobic amino acids (HAA);[b] Hydrophilic amino acids;[c]Essential amino acids;[d] Neutral amino acids(NAA);[e]Acidic amino acids (negatively charged) (NCAA);[f]Basic amino acids(positively charged) (PCAA); [g]Aromatic amino acids;[h]Cyclic amino acids and [I]non essential amino acids. ND – not detected. Notes: Values represent the means ± standard deviation of triplicates. Means in the same row with different letters as superscripts are significantly different ($p < 0.05$). P-hydrolysates (pepsin generated camel WPC hydrolysates), T-hydrolysates (trypsin generated camel WPC hydrolysates) and P-T hydrolysates (pepsin and trypsin generated camel WPC hydrolysates). SF18 (subfraction 18); SF14 (subfraction 14) and SF21 (subfraction 21).

NO	Amino acids	Relative molecular mass (g/mol)	Conc (umoles/ml)	Conc (Moler percentage %) Unhydrolyzed WPC	P-hydrolysate	T-hydrolysate	P-T hydrolysate	SF18	SF14	SF21
1	L-Aspartic acid[b,e,I]	133.11	0.5	21.93±0.5[a]	3.05±0.03[b]	18.04± 0.19[a]	0.29±0.06[c]	4.57±0.4[a]	3.29±0.07[b]	1.70±0.2[c]
2	L-Glutamic acid[b,e,I]	147.13	0.5	3.7±0.3[a]	1.72±0.14[b]	2.88± 0.84[a]	0.49±0.08[c]	24.56±0.3[a]	17.16±0.04[c]	22.78±0.1[b]
3	L-Serine[b,d,I]	105.09	0.5	1.07±0.1[a]	0.32±0.1[b]	0.73± 0.15[a]	0.08±0.09[b]	4.19±0.3[a]	3.94±0.1[a]	3.09±0.1[b]
4	L-Histidine[b,c,f]	155.16	0.5	0.53±0.1a	0.19±0.03[a]	0.14± 0.04[a]	0.15±0.03[a]	0.85±0.1[b]	1.83±0.1[a]	0.71±0.1[c]
5	L-Glycine[a,d,I]	75.07	0.5	2.62±0.1[b]	0.30±0.02[bc]	18.35± 0.17[a]	0.25±0.03[c]	11.53±0.3[b]	13.56±0.4[a]	2.75±0.1[ab]
6	L-Theronine[b,c,d]	119.12	0.5	1.41±0.02[b]	0.19±0.02[c]	10.78± 0.03[a]	0.06±0.02[d]	2.99±0.1[a]	2.18±0.2[b]	1.86±0.02[b]
7	L-Arginine[b,c,f]	174.2	0.5	1.76±0.003[b]	0.20±0.01[c]	10.29± 0.01[a]	0.15±0.04[c]	1.58±0.1[c]	3.24±0.2[b]	11.8±0.13[a]
8	L-Alanine[a,d,I]	89.09	0.5	0.52±0.1[b]	0.18±0.04[c]	3.06± 0.04[a]	0.13±0.04[d]	2.43±0.3[a]	1.64±0.3[a]	3.59±0.1[b]
9	L-Tyrosine[b,g,I]	181.19	0.5	2.84±0.1[b]	1.00±0.16[c]	1.32±0.34[a]	0.22±0.03[d]	2.23±0.1[b]	12.79±0.1[b]	14.8±0.12[a]
10	L-Cystine[b,I]	240.3	0.5	ND	ND	ND	ND	ND	ND	ND
11	L-Valine[a,c,d]	117.15	0.5	1.41±0.04[c]	1.03±0.04[b]	12.43± 0.04[a]	1.53±0.30[b]	12.84±0.1[a]	2.60±0.2[c]	6.31±0.1[b]
12	L-Methionine[a,c]	149.21	0.5	3.24±0.14[a]	1.04±0.04[b]	0.27± 0.31[c]	1.01±0.10[b]	0.44±0.01[c]	1.37±0.3[a]	2.17±0.1[b]
13	L-Phenylalanine[a,c,g]	165.19	0.5	4.90±0.16[b]	1.83±0.3[c]	9.31± 0.03[a]	0.84±0.10[c]	2.10±0.1[c]	2.32±0.1[a]	7.23±0.1[b]
14	L-Isoleucine[acd]	131.17	0.5	2.17±0.03[a]	0.49±0.04[c]	0.07± 0.06[d]	0.79±0.03[b]	2.75±0.2[a]	2.31±0.1[a]	1.21±0.11[b]
15	L-Leucine[a,c,d]	131.17	0.5	5.37±0.7[a]	6.12±0.06[a]	2.95± 0.16[b]	6.07±0.10[a]	5.57±0.2[a]	2.62±0.1[b]	13.64±0.2[a]
16	L-Lysine[b,c,f]	146.19	0.5	1.40±0.01[a]	0.85±0.15[b]	0.91± 0.26[b]	0.33±0.05[c]	1.69±0.2[a]	1.80±0.1[a]	5.45±0.2[b]
17	L-Proline[a,h,I]	115.13	0.5	45.13±3.2[b]	81.49±2.22[a]	8.47± 0.34[c]	87.61±0.31[a]	19.88±0.1[b]	27.23±0.3[a]	0.91±0.1[c]

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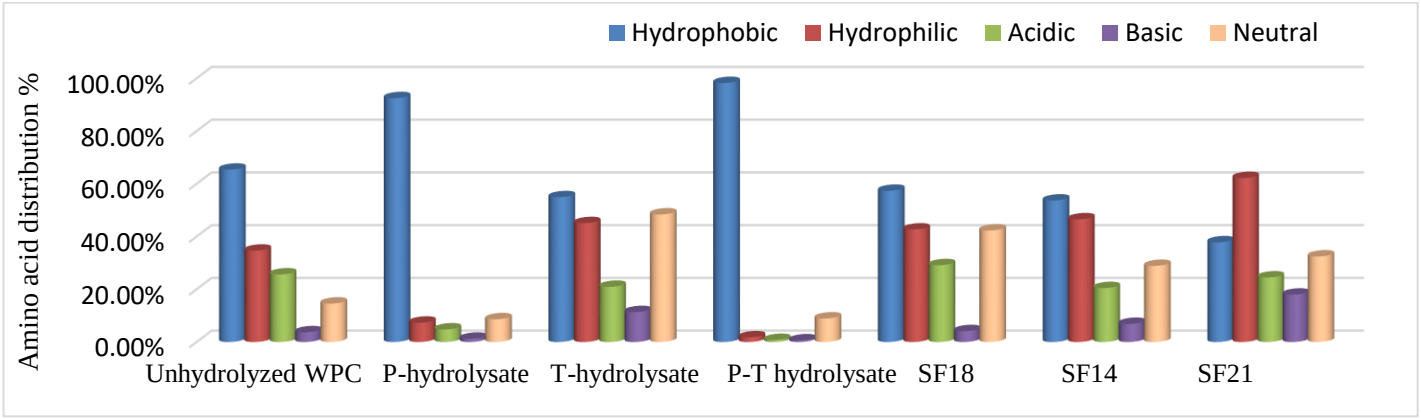

Figure. 10: The distribution of total amino acids (%) of camel whey protein samples and most bioactive peptide subfractions isolated from P-T hydrolysates (pepsin and trypsin- generated hydrolysates) by FPLC.

4. DISCUSSION

The DH% serves as indicator of hydrolysis efficiency and it can affect the bioactivity of generated hydrolysates [22]. The greater DH% of P-T hydrolysate might be attributed to greater suseptibility of cleavage sites and the breakdown of more peptide bonds in camel´s WPC by both pepsin and trypsin enzymes [23].

Camel whey proteins and their generated peptides have broad spectrum antifungal activity [24]. This study revealed that all different concentrations of camel´s WPC and its hydrolysates exhibited various degrees of growth inhibition against the tested fungal strains and the inhibitory effect was concentration and strain dependent. The reduction was greatest for *C.albicans* followed by *A. fumigatus*. However, *A.niger* and *A.flavus* were the least sensitive strains. This result may be attributed to the higher concentrations of camel whey proteins, which are effective against *C.albicans, A.fumigatus* and *A.niger* either by inhibition of spore germination of fungi or iron sequestration [25].

The strongest activity against *C.albicans* may be related to the greater candidicidal activity of camel lactoferrin and its hydrolysates which are more effective against *C.albicans* [26]. In contrast, the lower sensitivity of *Aspergillus spp.* may releated to some resistance factors released from the cell wall of *Aspergillus spp.* which act against many antimicrobial agents [27].

The antifungal activity of un hydrolyzed WPC may be releated to some bioactive peptides liberated by the endogenous proteolytic enzymes normally present in raw camel milk [28]. However, the highest antifungal potency of P-T hydrolysate at 20 mg/ml might be adue to the synergism between higher concentrations of different peptides of various molecular weights and amino acid sequences with improved antifungal activity [29]. The camel lactoferricin peptides in P-T hydrolysate can interact with the plasma membrane of the blastoconidia and with surface ATP binding sites, resulting in a reduction in the mitochondrial function, pore formation and cell death. In addition, 20% reduction in the internal thiol levels of *C.albicans* [30]. All these previous results are in harmony with [31], [32].

These greater antifungal activity of peptide fractions eluted from P-T hydrolysate may be attributed to a greater antifungal activity of several camel lactoferrin peptides mainly lactoferricin (fragment 17-42) and lactoferrampin (fragment 265-284) as revealed by [33]. The differences in antifungal activities of peptide fractions may related to the variations in their amino acid sequences and effective peptide concentrations [34].

This is in harmony with [8] who extracted several antimicrobial peptide fragments from camel whey hydrolysates. Also, the further FPLC fractionation of P-T hydrolysate enhanced the antifungal activities of peptides against *Asperigillus spp*, however, decreased their effictivness against *C.albicans*. This may be attributed to the ability of FPLC fractionation to separate, concentrate the active peptides more effective against *Asperigillus spp* [35], which agreed with [36]. On contrast, there was potent synergistic effect of polypeptides in camel whey hydrolysates against *C.albicans* similar to those stated by [37].

A significant difference in protein composition between milk of camel and other species is the main cause to monitor protein hydrolysis and to characterize the peptides liberated from whey protein hydrolysates by using recent proteomic SDS-PAGE, and HPLC [38]. Also, the antimicrobial efficacy of the bioactive peptides is highly dependent on their specific amino acid composition, sequences and hydrophobicity as revealed by [39].The current result revealed the higher bands intensity of P-hydrolysate (lane B) on SDS, which may be due to the compact globular structure of some camel whey proteins which hides its their peptic cleavage sites [40], while the thinner and lower bands intensity observed in P-T hydrolysate and its potent peptide subfractions demonstrated that all whey protein bands were completely hydrolysed in to lower molecular wheight peptides after synergistic pepsin-trypsin hydrolysis. This result agreed with [41] who revealed the greater antimicrobial effect of whey peptide fractions with lower molecular wheight below 20 kDa.Similar results were reported by [42], [43].

The differences in amino acid compositions of camel´sWPC and its hydrolysates could be attributed to enzyme specificity, substrate affinity, temperature, pH and time of hydrolysis [44]. It was observed that hydrophobic amino acids were the predominant amino acids which may be responsible for the highest antifungal activity of camel´s WPC, its hydrolysates and potent peptide subfractions. This may be attributed to greater affinity of both pepsin and trypsin enzymes to hydrolyze protein at hydrophobic residues in the peptide bond [45]. This agreed with [46] who reported that the richness of camel WPC hydrolysates with hydrophobic amino acid residues play a vital role in their antimicrobial activity. The interaction of these amino acids particularly tryptophan and arginine of lactoferrin peptides with the microbial membrane lipids causing pore formation and cell destruction [47]. Similar findings revealed by [48], [8] who stated that both hydrophobic and hydrophilic peptides may act synergistically for enhancing antifungal activity of whey protein hydrolysates. Also, the richness of camel´sWPC, its hydrolysates and potent peptide subfractions with proline might be due to construction of peptide bonds between proline and other amino acids by both pepsin and trypsin enzymes [49]. This agreed with [50] who revealed that proline-rich peptides can interact with the ribosome and disrupt protein synthesis in microbial cell causing death of microorganisms. Similar findings were carried out by [51] who purified proline rich peptide sequences from antimicrobial whey hydrolysates.

5. CONCLUSION

All tested concentrations (5,10 and 20 mg/ml) of camel´s WPC and its hydrolysates (P-hydrolysate, T-hydrolysate and P-T hydrolysate) showed variable inhibitory activities against *C.albicans, A.fumigatus, A.Niger* and *A.Flavus* in broth. The antifungal activity was concentration and strain dependent. The P-T hydrolysate (20 mg/ml) had the highest antifungal activity against all strains. *C.albicans* and *A.fumigatus* was the most sensitive strain. Several peptide fragments (1.45 mg/ml) with potent antifungal activity can be generated from P-T hydrolysate. The potent peptide subfractions include SF18 against *A.niger* followed by SF14 a gainst *A.fumigatus* and *A.flavus* and finally, SF21 against *C.albicans*.consequently; Further studies should be applied for further purification and sequencing of these peptides to be applied as natural effective antifungal agents in dairy products.

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