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In vitro antioxidant and anti-inflammatory activities of whey proteins extracted from fermented camel milk

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Abstract

The fermented camel milk is a processed food little studied and poorly evaluated. The present study aims to assess the effects of spontaneous fermentation on improving the quality of camel milk. Moreover, this was ensured by the *in vitro* study of the antioxidant and anti-inflammatory activity of whey proteins. Samples of the milk were analyzed in the raw state and after a fermentation time (96 hours) at 25 °C. The anti-oxidant activity was performed by 1,1-Diphenyl 2-picrylhydrazyl (DPPH) and β-carotene/linoleic acid (βCLA) assay. While the anti-inflammatory activity was performed by the method of inhibition of albumin denaturation and by the method of membrane stabilization. Follow-up of the fermentation showed that the pH value undergoes a decrease and the lowering of the total and whey proteins levels. Fermented whey proteins (FWP) showed significantly higher DPPH (IC_{50} = 4.29 ± 0.8 mg/ml) and βCLA (IC_{50} = 1.36 ± 0.05 mg/ml) radical scavenging activity than raw whey proteins (RWP) (IC_{50} = 5.36 ± 0.6 and 1.61 ± 0.07 mg/ml) respectively for the two tests. Both methods tested of anti-inflammatory activity showed a good protection against denaturation of proteins, and good stabilization of cell membrane. These results suggest that the spontaneous fermentation of camel milk induced by the development of lactic acid bacteria (LAB) could potentially be used to evaluate a functional food. This may be, by the isolation and selection of potent lactic acid strains for the purpose of their use in food preparations.

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Introduction

Camel's milk (*Camelus dromaderius*) is the most common product for populations raising camels; it is composed by the important and balanced basic nutrients (protein, fat, lactose and vitamins). It is considered a protein-rich source, with potential bioactivity (Benkerroum *et al.*, 2004). It is rich in minerals like iron, magnesium, copper and zinc and rich in vitamins like vitamins A, B2 and C. Through this composition, camel milk is considered an essential nutritional supplement to help immune-deficient patients (Yateem *et al.*, 2008), patients who suffer from inflammatory bowel diseases (Arab *et al.*, 2014). On the other hand, camel milk is characterized by the higher content of immunoglobulins, lysozyme and lactoferrin, which are immunomodulators (El-Agamy, 2009).

Fermented milk is a major food component of traditional food in many parts of Africa. Due to the limitation of cold storage equipment in many rural areas in African countries. The milk is stored at room temperature, allowing them to ferment quickly by the natural lactic flora. Sometimes the fermentation process occurs spontaneously by inoculating raw milk with a small amount of fermented milk previously elaborated. Therefore, the product obtained leads to the domination of the most suitable lactic strains (Heita and Cheikhyoussef, 2014).

Nomads use fermented milk in their daily life not only as a food but also as a remedy for certain health problems like gastric diseases. On the other hand, breeders in urban areas and consumers consider fermented milk to be an altered food, which leads to the rejection of large quantities and subsequently pollution of the environment (Shori, 2013).

Certain dairy products such as cheeses, whey, caseins and certain proteins of these products possessed important antioxidant properties (Ripolles *et al.*, 2015). Milk proteins are an abundant source of bioactive peptides. These peptides are inert in the initial protein of milk (casein and albumins) can be activated by their release in the medium by several processes such as enzymatic hydrolysis by digestive enzymes, or bacterial enzymes during the fermentation of milk by the lactic flora (Mati *et al.*, 2017). Whey proteins (WP) release bioactive peptides during the fermentation process. These peptides can improve certain biological properties in human health, including mineral fixation, growth factors, reducing blood pressure, antioxidant activity, anti-cancer activity (Korhonen and Pihlanto, 2006; Daliri *et al.*, 2017).

Several studies have been done on the physicochemical properties of camel milk fermented spontaneously. While, it remains to know the biological properties of this food widely used as a remedy for certain diseases by the people of Nomads.
To this end, the present study aims to investigate the effects of spontaneous fermentation on improving the biological value of fermented camel milk as a nutrient source poorly explored and poorly valued by the *in vitro* study of the antioxidant and anti-inflammatory activity of camel whey proteins.

Materials and methods

Collection of milk and blood samples

Fifteen camel's milk samples were collected in the region of Ouargla (Algeria) in sterile bottles. Then, there is transported within 2 hours in an ice bag to the laboratory of the Department of Biology, Faculty of Nature and Life Sciences, University of Ouargla for analysis. . Fresh whole human blood (05 ml) was collected from a healthy human volunteer who has not taken any anti-inflammatory drug for two weeks before the experiment in an EDTA tube. This study was approved by the institutional review board of the Kasdi Merbah University (Ouargla, Algeria). Moreover, all participants provided informed consent prior to blood sampling.

The camel milk is fermented spontaneously by storing milk samples at room temperature (25°C) for 96 hours (04 days) without adding any lactic leaven. The fermentation is occurred by the development of the endogenous lactic flora (*Streptococcus* and *Lactobacillus*).

Separation of WP

The raw or fermented milk was centrifuged at 5000×g for 30 min at 10 °C, to remove the fat components. The separation of WP is made by the separation of whey and casein from skimmed milk after adjusting pH to 4.3 by the addition of 1M HCl and centrifugation at 5000×g for 30 min at 20 °C. All last operations were repeated twice for the reason to remove any casein and fat residues. The supernatant was then dialyzed for 48 hours and freeze-dried (Si Ahmed *et al.*, 2013).

Physicochemical analyzes

The pH value of samples (raw or fermented camel milk) is measured at + 22 °C using a pH meter (HANNA Instrument, Romania) after calibration by two points calibration using an alkaline buffer (pH 10 usually) and acidic buffer (pH 4). The Dornic Acidity is assayed by titration using an N/9 Sodium Hydroxide solution in the presence of Phenolphthalein (Vignola, 2002). The concentration of total and WP is determined according to Lowry *et al.*, (1951).

Enumeration of Lactic acid bacteria (LAB)

The enumeration of LAB was performed on each sample of raw or fermented milk. Three successive dilutions of samples in a physiological saline solution were included in the MRS agar (Man Rogosa Sharpe). The Petri dishes were incubated at 30 °C for 48 hours (Marchal *et al.*,1987).

Determination of antioxidant activity

Antioxidant activity by the DPPH test

The antioxidant activity of camel WP was carried out by the DPPH test described by (Parejo *et al.*, 2000). The DPPH solution is prepared by dissolving DPPH in ethanol to obtain a concentration of 100 µM. 1ml of camel WP solution of raw or fermented camel milk by different concentration is mixed with 1 ml of the DPPH solution. The blank solution is also prepared by adding 1 ml of DPPH with 0.5 ml of distilled water and 0.5 ml of ethanol. After homogenization, the mixture is incubated for 30 minutes at room temperature (25 °C) and in a dark room. The absorbance of each sample was measured at 517 nm using a UV-Visible spectrophotometer *(SCHIMADZU, Japon)*. The antioxidant activity was expressed as percentage inhibition of the DPPH radical and calculated according to the following equation:

$$\%\ \text{DPPH Inhib} = \left(1 - \frac{\text{ATS}}{\text{ABS}}\right) \times 100$$

ATS: Absorbance of test solution.

ABS: Absorbance of blank solution.

Antioxidant activity by βCLA test

The antioxidant activity by βCLA test was cited by several authors (Kelen and Tepe, 2007; Tosun *et al.*, 2009; Ueno *et al.*, 2014). Approximately 10 mg of β-carotene was dissolved in 10 ml of chloroform. The β-carotene-chloroform solution (0.2 ml) was pipetted into a boiling flask containing 20 mg of linoleic acid and 200 mg of Tween 80. The chloroform was removed using a rotavapor at 40 °C for 5 min, and then 50 ml of distilled water was slowly added to the residue resulting from evaporation with vigorous agitation to form an emulsion. A series of test tubes of different concentrations of WP solution was prepared. In each tube, 1 ml of the emulsion was added with 40 µl of the WP solution. The blank solution is prepared by adding 1 ml of the emulsion with 40 µl of distilled water. The absorbance of the blank solution is read immediately at 470 nm against the distilled water. All tubes (blank solution and test solution) are placed in a water bath at 50 °C and in the dark for 120 min. The absorbance is read at 470 nm against the distilled water. The antioxidant activity was expressed as percentage inhibition of the β-carotene radical and calculated by the following formula:

$$\%\ \text{Inhib}\ \beta\text{CLA} = \frac{\text{ATS120} - \text{ABS120}}{\text{ABS0} - \text{ABS120}} \times 100$$

ATS 120: Absorbance of the test solution after 120 minutes.

ABS0 : Absorbance of the blank solution at 0 minutes.

ABS120 : Absorbance of the blank solution after 120 minutes.

Determination of anti-inflammatory activity

Inhibition of albumin denaturation method

The *in vitro* anti-inflammatory assay is performed by using the method of inhibition of protein denaturation sited by many authors (Kar *et al.*, 2013; Srikanth *et al.*, 2015). The protein used is bovine serum albumin (BSA). The test is done by making

0.5 ml of test solution; 0.4 ml of BSA (0.5%) and 0.1 ml of camel WP with different concentrations and 0.5 ml of a blank solution was prepared (0.4 ml of BSA (0.5%) and 0.1 ml of distilled water).

All the tubes are incubated at 37°C for 30 minutes and then incubated at 50°C for 30 min. 2.0 ml of saline phosphate are added to all the solutions and the absorbance is read at 278 nm. The percentage inhibition of albumin denaturation is calculated according to the following equation:

$$\% \text{ Inhib Denaturation} = (1 - \frac{ATS}{ABS}) \times 100$$

Membrane stabilization test using human red blood cells

The *in vitro* anti-inflammatory assay is performed by membrane stabilization test, is the one described by several authors (Sakat *et al.*, 2010; Leelaprakash and Mohan Dass, 2011; Nagaharika *et al.*, 2013).

Fresh human blood (05 ml) collected in the EDTA tube is centrifuged at 3000 rpm for 10 min. The precipitant cells were washed with normal saline and a 10% v/v suspension of human red blood cells (HRBC) was prepared. The reaction mixture (4.5 ml) consisted of various concentrations of camel WP (1 ml), 1 ml of phosphate buffer (0.15 M, pH 7.4), 2 ml of hyposaline (0.36%) and 0.5 ml of HRBC suspension. The blank solution consisted of distilled water instead of hyposaline. The assay systems were incubated at 37 °C for 30 min and centrifuged at 3000 rpm for 20 min. The hemoglobin (Hb) concentration of the supernatant solution was estimated by reading of absorbance at 560 nm. The percentage of stabilization of the HRBC membrane was calculated according to the following equation:

$$\% \text{ of Stabilization} = (1 - \frac{ATS}{ABS}) \times 100$$

Statistical analysis

Statistical analysis was performed by using SPSS 17 for Windows. All the analysis was run in triplicate. The data are presented as Mean ± Standard error. For the comparison between two variants, the single factor analysis of variance (ANOVA) is used by the Tukey test in order to estimate the significant differences at the 5% probability threshold.

Results and discussions

Physicochemical analyzes and enumeration of LAB

The variations in physicochemical parameters and the LAB count after fermentation are summarized in Table 1. After fermentation, the pH value shows an extremely significant decrease ($p < 0.05$) from the initial value in the raw state. At the same time, Dornic acidity shows an increase of 80 °D after 96 H of fermentation. On the other hand, during the fermentation, the LAB shows an exponential growth of 3.76 Log10 cfu/ml ($p < 0.05$). This growth is provided by the hydrolysis of glucose as an energy source and at the same time the release of organic acids that leads to acidification of the medium (Rahmawati and Suntornsuk, 2016; Ayyash *et al.*, 2017).

Table 1. Physicochemical parameters and enumeration of LAB in raw and fermented camel milk [a b] Mean within rows having same superscripts do not differ at $p<0.05$.

Parameters	Raw milk	Fermented milk
pH	6.26 ± 0.23[a]	4.15 ± 0.16[b]
Dornic acidity (°D)	18 ± 1.2[a]	98 ± 3.05[b]
Total proteins (g/l)	35.2 ± 1.5[a]	30.8 ± 1.6[b]
WP (g/l)	6.11 ± 1.2[a]	5.8 ± 0.68[a]
Caseins (g/l)	29.1 ± 0.65[a]	25.1 ± 1.38[b]
LAB Log10 cfu/ml	3.92[a]	7.68[b]

Total proteins and caseins show a significant decrease ($p<0.05$) after fermentation. While WP show a decrease but not significantly ($p>0.05$) (Table 1). Several authors have reported the phenomenon of proteolysis of milk proteins (caseins) during fermentation such as Gjorgievski *et al.*, (2014); Bahobail *et al.*, (2014); Soleymanzadeh *et al.*, (2016); Rahmawati and Suntornsuk, (2016).

Table 2. The IC50 values of ascorbic acid and camel WP for different test [a b] Mean within rows having same superscripts do not differ at $p<0.05$.

Test	IC50 (mg/ml)		
	Raw WP	Fermented WP	ascorbic acid
DPPH	5.36 ± 0.6[a]	4.29 ± 0.8[b]	0.092 ± 0.01[c]
β-carotene	1.61 ± 0.07[a]	1.36 ± 0.05[b]	0.142 ± 0.08[c]
Albumin denaturation	8.23[a]	5.54[b]	//
Membrane stabilisation	3.54[a]	2.65[b]	//

This phenomenon is due to the action of exo-proteinases synthesized by LAB which leads to the release of peptides and amino acids as a nitrogen source essential for their growth (Virtanen *et al.*, 2007; Soleymanzadeh *et al.*, 2016).

Antioxidant activity

In the present study, the percentage of DPPH and β-carotene-linoleic acid inhibition increases significantly ($p<0.05$) with the increase in the concentration of camel WP and ascorbic acid (fig 01, 02 and 03). Furthermore, the antioxidant activity of FWP is significantly higher ($p<0.05$) than that of RWP.

This is shown by the low IC50 value of FWP, but it is not lower than that of ascorbic acid (Table 02), the reason why it is a pure and strong antioxidant.

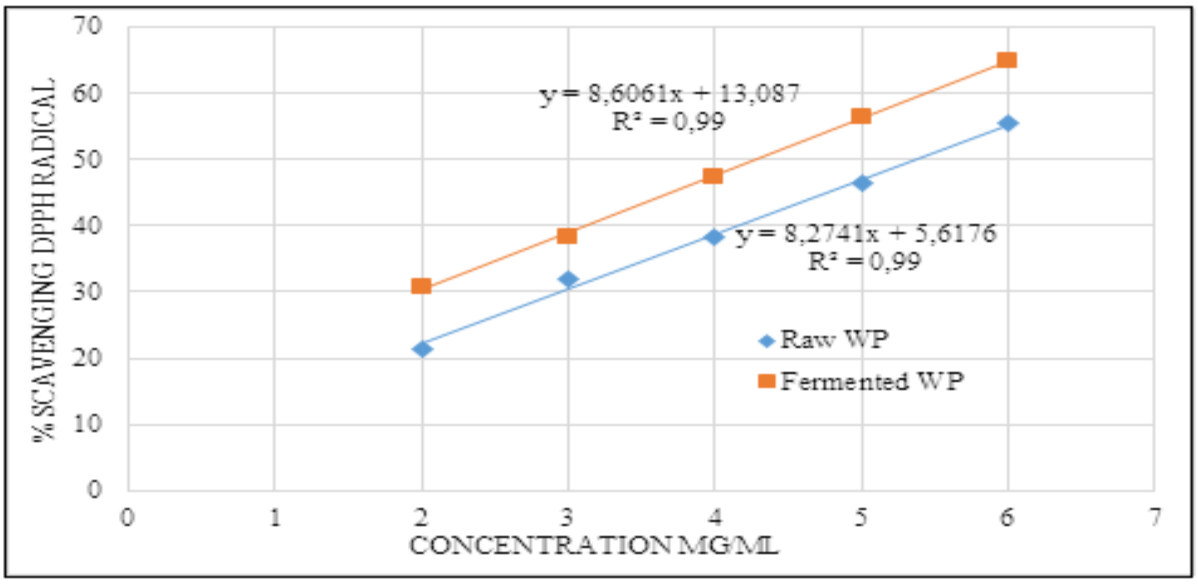

Fig. 1. Scavenging of DPPH radical by camel WP as a function of concentration.

Maryam *et al.*, (2013) gave an IC50 value of DPPH inhibition close to that of this study. On the basis, that they tested the inhibition of DPPH by the whey of cow's milk fermented by two lactic acid strains, *Lactobacillus plantarum* and *Leuconostoc mesenteroides* of 2.92 and 8.81 mg/ml respectively as IC50 value. On the other hand, Homayouni-Tabrizi *et al.*, (2016) gave a very low IC50 value of scavenging of DPPH radical by two polypeptides (0.04 and 0.02 mg/ml) extracted from camel milk. The first contains

13 amino acids and the second contains 15 amino acids. This result can be explained by the purity of polypeptides by contribution to the proteins mixture of camel WP in the case of our study.

Shori, (2013) and Shori and Baba, (2014) noted that fermentation increases the inhibition of DPPH during the manufacture of camel milk yoghurt. Similarly, Balakrishnan and Agrawal (2014) noted a significant increase in the inhibition of DPPH by whey of three types of milk (cow, goat and camel) after fermentation by *Pediococcus pentosaceus*, knowing that the whey from camel milk gave a significant percentage of initial inhibition (before fermentation) compared to other types of milk. Salami *et al.*, (2010); Moslehishad *et al.*, (2013) and Soleymanzadeh *et al.*, (2016) have shown that the antioxidant activity of camel milk and their hydrolysates is significantly higher than that of bovine milk. This may be due to the difference in the amounts of the antioxidant amino acid residues present and the peptides resulting from the enzymatic hydrolysis during fermentation by the lactic flora for camel milk compared to the bovine milk. Furthermore, Farah *et al.*, (1992) showed that the vitamin C in camel milk is three times higher than that of cow's milk, which gives an important antioxidant activity of raw camel milk rather than raw bovine milk.

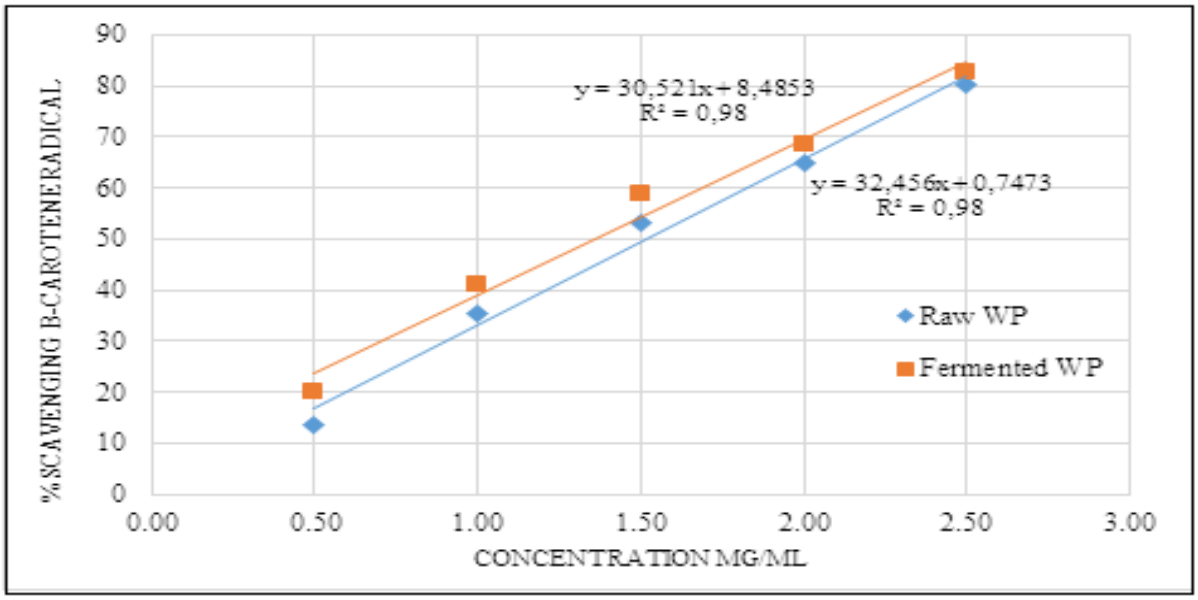

Fig. 2. Scavenging of β-carotene-linoleic acid radical by camel WP as a function of concentration.

During fermentation, there is an increase in the number of LAB (Table 1), at the same time, there is the accumulation of bioactive primary metabolites such as lactic acid, acetic acid and especially the peptides derived from proteolysis, which leads to an increase in antioxidant activity (Gjorgievski *et al.*, 2014; Khan *et al.*, 2019). Clearly, which was confirmed in the present study by the hydrolysis of caseins and the increase in antioxidant activity after fermentation.

Kumar *et al.*, (2016) have noted that the increase in the time of enzymatic hydrolysis, that is, the increase in the degree of hydrolysis, induces the increase of the antioxidant activity evaluated by different tests, which explain the significant difference between the antioxidant activity of fermented milk and raw milk.

Anti-inflammatory activity

In this study, the percentage inhibition of albumin denaturation and stabilization of the erythrocyte membrane increased significantly with the increase in camel WP concentration (fig 04 and 05). Both tests showed that fermented camel WP exhibits significantly higher inhibition than raw camel WP. This is justified by the significant low IC50 value ($p<0.05$) of FWP comparing to RWP (table 02). However, it is not lower than that of diclofenac

sodium, which exhibits an inhibition of 93.2% for a concentration of 0.1 mg/ml according to Rahman *et al.*, (2015), because it is pure and strong anti-inflammatory. The denaturation of proteins by alteration of the bonds such as electrostatic, hydrogen, hydrophobic and disulfide bonding, this alteration causes the loss of their tertiary and secondary structure as well as their biological function. This denaturation in some inflammatory autoimmune diseases leads to the production of autoantibodies which lead to the onset of chronic inflammatory diseases such as rheumatoid diseases (Grant *et al.*, 1970). As a result, it can be stated that hydrolysis of camel milk protein by fermentation was able to inhibit protein denaturation and give protection against the inflammatory problem.

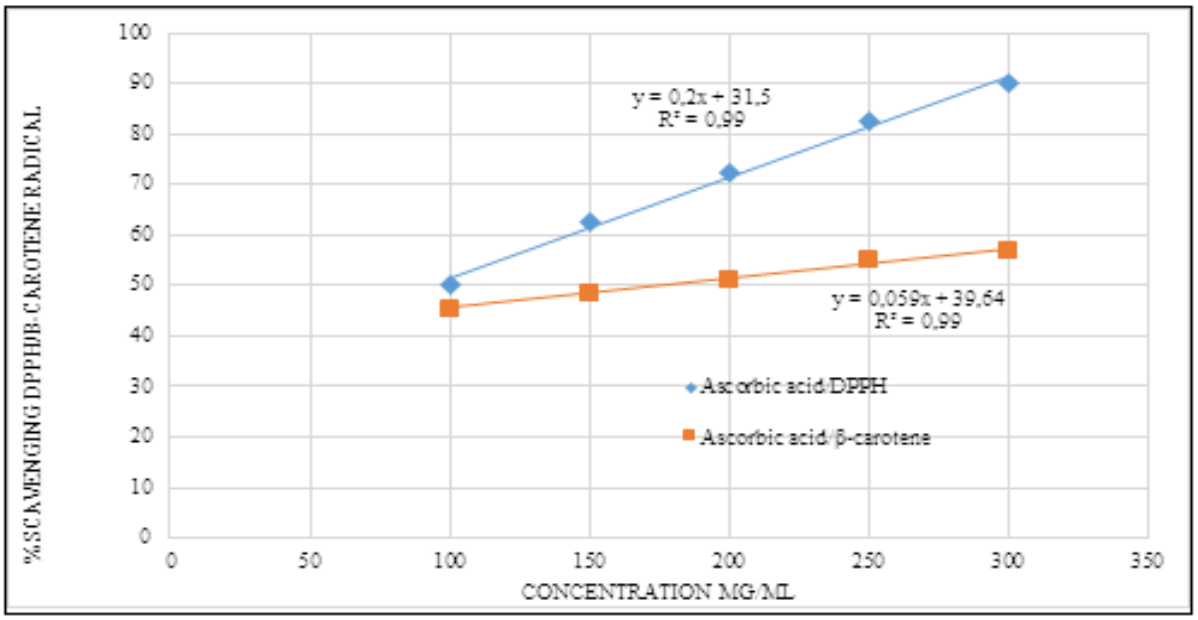

Fig. 3. Scavenging of DPPH / β-carotene radical by ascorbic acid as a function of concentration.

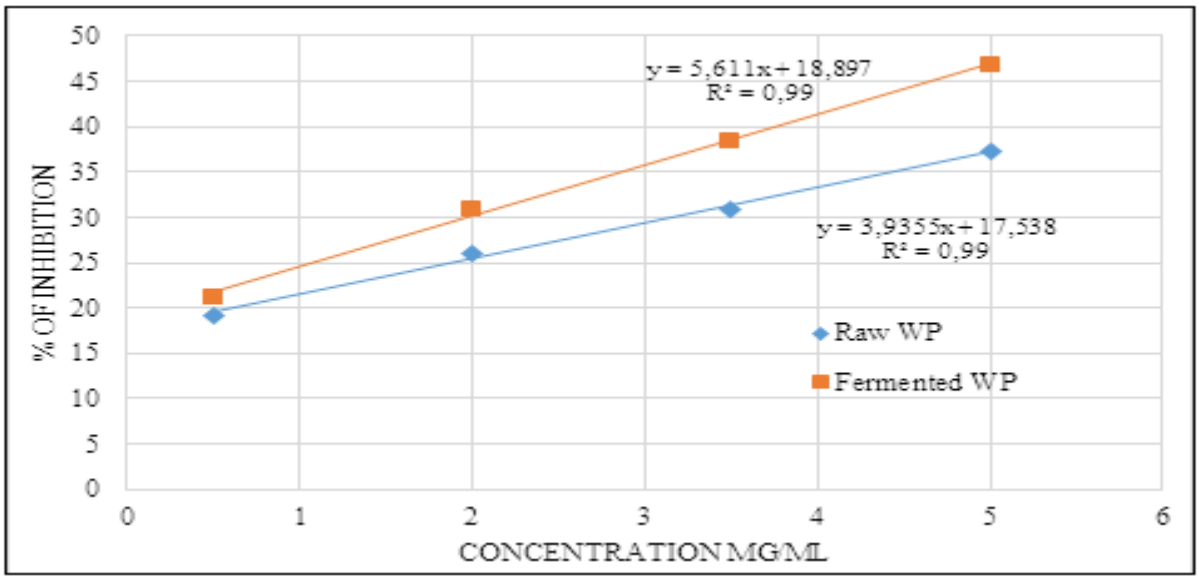

Fig. 4. Effect of camel WP on inhibition of albumin denaturation.

FWP exhibited a membrane stabilizing effect by inhibiting hypotonicity induced lysis of erythrocyte membranes. There is a structural analogy between the erythrocyte membrane and the lysosome membrane. Stabilization of the lysosomal membrane is important in limiting the inflammatory response that causes

additional tissue damage. This limitation prevents the release of lysosomal constituents such as bactericidal enzymes and proteases of activated neutrophils (Kar *et al.*, 2013). Consequently, the fermented camel WP could also stabilize lysosomal membranes and present a protective effect against tissue lysis.

Several authors have worked on the *in vivo* anti-inflammatory effect of raw camel milk. Al-Hashem (2009); Darwish *et al.*, (2012) and Al-Asmari et al (2014) tested the effect of camel milk on the protection against hepatic inflammation. On the other hand, Arab *et al.*, (2014) and Hu *et al.*, (2017) used camel milk as a means of protection against gastritis. As well, Mona *et al.*, (2010) showed *in vivo* the importance of the administration effect of fermented camel milk on the cessation of diarrhea in rats compared to raw camel milk.

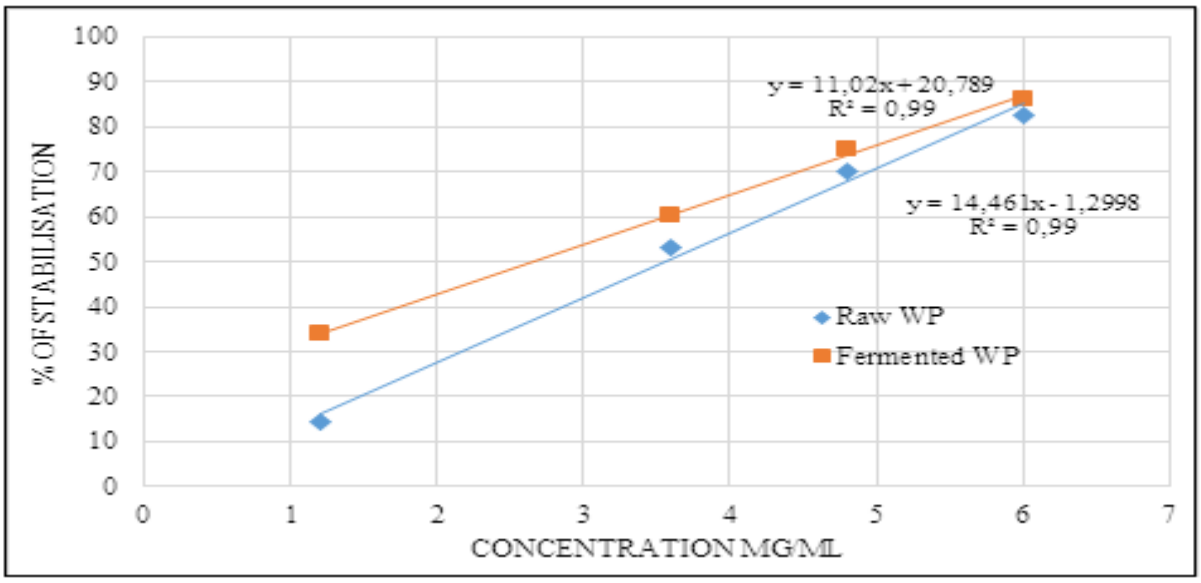

Fig. 5. Effect of camel WP on stabilization of erythrocyte membrane.

Sharma *et al.*, (2011) isolated the peptidoglycan recognition proteins from camel milk. Following its addition in the cell culture of monocytes, this protein gave an inhibition of expression of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) by use of flow cytometric techniques. In the same way, Nielsen *et al.*, (2015) used bovine casein hydrolysates in cell culture of macrophages and rat intestinal epithelial cells, leading to the lowering of the transcription of transforming growth factor-β1 (TGF-β1), cyclooxygenase-2 (COX-2) and nuclear factor κB (NFκB) (nuclear factor kappa-light-chain-enhancer of activated B cells) as inflammatory factors.

Conclusions

In conclusion, lactic acid bacteria involve actively in the fermentation process through the proteolysis of milk proteins either casein or whey proteins. This proteolysis leads to the release of peptides and bioactive amino acids in the whey of milk, which improves their bioactivities. These bioactivities corresponds to the neutralization of free radicals as harmful agents either on lysosomal membranes or on macromolecules such as proteins and nucleic acids in general. All these harmful effects are at the origin of several inflammatory and cancerous diseases.

Therefore, further works should be done to isolate and identify the bioactive peptides in fermented camel WP that are responsible for the antioxidant and the anti-inflammatory activities.

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