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Comparative Evaluation of Antimicrobial Activity and Functional Properties of Native and Esterified Legume Proteins

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Abstract

Tow legume proteins (cowpea and common bean proteins) were isolated and esterified with methanol in the presence of hydrochloric acid for 10 h at 4°C to give esterification extent 82% and 79%; respectively. Esterification raised the pIs (iso-electric points) of legume proteins from pH 4 for the native legume proteins, to pH 6 in the case of cowpea protein and pH 8 in the case of common bean. Applying methylated proteins at four different concentrations (0.5, 0.75,1 and 2 mg/ml) to Petri dishes containing nutrient agar infected with two pathogenic Gram- negative *(Enterobacter cloacae* and *Serratia marcescens*) and Gram- positive bacteria (*Listeria monocytogenes*) gave rise to concentration-dependent inhibition zones.

Keywords: *protein esterification -legume proteins - antibacterial activity -functional properties*

Introduction

Plants are considered a worthy source of natural products for infections control and maintaining human health because, microbial infections cause harm of health, and plants are a good source of antimicrobial agents (Ashish *et al.,* 2013 and Ram *et al.,* 2015)

In the past legumes have been utilized mainly as whole seeds but now, interest has grown in the utilization of legumes in many other forms (e.g. flour, concentrate and isolate) (Doxastakis, 2000).

Proteins of legumes are characterized with a high content in polar amino acids with opposite charge (glutamic and aspartic acid on one side and lysine and arginine on the other one) (Duke, 1982). Thus, it is reasonable to intend that these amino acids are involved in the association-dissociation phenomena of protein sub-units that are at the basis of the solubility properties of legume oligomeric proteins. The bean proteins of high content of lysine/arginine and aspartic/glutamic acids would explain the condition for this mechanism of interaction to be effectively (Marletta *et al.,* 1992). In this respect such a mechanism might be common with legume proteins, as all of these are characterized by a very high content of these acid and basic amino acids (Duke, 1982). Mosses (1990) stated that cereal grains and legumes or oil seeds remain by far the predominant source of protein used for human food and for animal feed. Functionality of food protein has been defined as those chemical and physical characteristics which impact the behavior of proteins in food systems during

processing, storage, preparation and consumption (Fennema, 1996).

Recently resistance to antimicrobial agents by pathogenic bacteria has emerged, representing a great health problem (Nanda and Saravanan 2009), so the identification of new antimicrobial agents with different mechanisms of action is highly required. Cationic antimicrobial peptides or proteins (AMPs) that stop mechanism is due to the interaction with the cytoplasmic membrane are promising candidates (Friedrich*, et al.*, 2000). Intensive work is currently devoted to study the effects of AMPs on intact cells using electron microscopy techniques to reveal the damage caused by these molecules on the bacterial morphology and membranes (Giuliani, *et al.,* 2010).

Another approach to get new cationic proteins is through the intentionally tailored chemical modifications of native proteins. Chemical modification of native proteins was one of the first methods employed to test structure-function relationships. Esterification is an important and easy tool for the proteins modification. Esterification blocks free carboxyl groups thus raising the net positive charge and rendering more basic the modified protein (Halpin and Richardson, 1985 and Sitohy *et al.*, 2001b). Generally, increasing the positive charge on the protein and peptide molecules enhances their antibacterial effects. Amidation, which also increases the positive charges on the modified protein molecules, was found to improve the impacts of bovine lactoferrin (LF) against a range of Gram-positive and Gram-negative bacteria (Pan *et al*., 2005). The negatively charged carboxyl groups of the aspartyl and glutamyl residues on protein molecules can be neutralized by esterification transforming their net charge into positive. The obtained positively charged proteins were proved antimicrobially active (Sitohy and Osman 2010). The antimicrobial activity of some proteins may be occured from their ability to interact with carbohydrates on the cell surface of microbes. Antibacterial protein can interact with bacterial cell wall components such as N -acetylglucosamine, N -acetylmuramic Acid (MurNAc), tetrapeptides linked to MurNAc, and lipopolysaccharides (Gomes *et al.,* 2013).

Hence, the main purpose of the current investigation was to specify and characterize the extent and mode of action of these antimicrobial cationic proteins against these main pathogens (*Enterobacter Cloacae*, *Serratia Marcescens* and *Listeria Monocytogenes*) using standard media while identifying the main constituents responsible for this action.

Materials and Methods

Plant materials

Cowpea (*Vigna unguiculata*.) and common bean (*Phaseolus vulgaris*) seeds were obtained from local market, Zagazig city, Sharkia Governorate, Egypt.

Microorganisms

(*Enterobacter Cloacae*, *Serratia Marcescens* and *Listeria Monocytogenes*) were obtained from Fundamental Medicine and Biology Institute, Kazan (Volga region) Federal University, Russia.

Methods

Sample preparation

Cowpea and common bean seeds were manually cleaned and ground for 3 min using a Moulinex mixer (Type 716, France) at the maximum speed setting and the meals were ground to pass through a 1 mm2 sieve. The powder was then defatted using chloroform: methanol (3:1v/v) for 8 h. Solvent was evaporated by rotary-evaporator and dried-defatted meal was stored at 4°C for analysis.

Extraction of protein isolates

Dispersions of 5% (w/v) defatted Cowpea and common bean flour in water were adjusted to pH 9 with 0.1 N NaOH at room temperature, shaken for 1 h and centrifuged for 15 min at 2000 rpm. The extraction and centrifugation procedures were repeated on the residue to increase the yield. The extracts were combined and the pH was adjusted to 4.5 with 1 N HCl to precipitate the protein. The proteins were recovered by centrifugation at 2000 rpm for 15 min followed by removal of the supernatant by decantation. Crude protein was washed with distilled water, then dispersed in a limited volume of distilled water at pH 7.5, dialysed overnight and lyophilised. (Johnson and Brekke, 1983).

Protein esterification

The procedure outline by Sitohy, *et al.,* (2000a, 2000b) was used. Different protein isolates (Cowpea and common bean protein) were esterified by dispersing appropriate amounts in concentrated (>99.5%) methanol (5% w/v). Amounts of hydrochloric acid were added drop-wise at the start of the reaction to induce the protonation of carboxylates. All the reaction mixtures were kept at 4°C under continuous stirring. At the end of the reaction duration (10 h), the samples were centrifuged at 10 kg for 10 min. The resulting supernatant was discarded and the residue was dispersed in a volume of alcohol equal to that of the discarded supernatant and mixed well before re-centrifuging under the same conditions. The washing step was repeated three times. The final precipitate was dissolved in an appropriate amount of distilled water at pH 7.5, dialysed for 3 days at 4°C against distilled water then lyophilized. The lyophilized samples were kept at 20°C for analysis.

Esterification extent

The extent of esterification of proteins was quantified by the color reaction with hydroxylamine hydrochloride as developed by Halpin and Richardson (1985) and modified by Bertrand *et al.,* (1991).

Iso-electric point (protein pH-solubility curves)

The iso-electric points were deduced from the protein pH-solubility curves as the pH at which the protein is less soluble. Protein pH-solubility curves were assayed in the pH range from 2–10 according to the procedure outlined by Chobert *et al.,* (1991).

Emulsifying activity (EA)

Emulsifying activity was determined using the method of Neto *et al.,* (2001). Five milliliters of protein solution (2%w/v) were homogenized with 5 ml corn oil. The emulsions were centrifuged at 1100 g for 5min. The height of emulsified layer and that of the total contents in the tube was measured.

The emulsifying activity (EA) was calculated as:

$$EA\ (\%) = \frac{\text{Height of emulsified layer in the tube x 100}}{\text{Height of the total contents in the tube}}$$

Influence of pH was investigated by preparing protein solutions of various pH ranging from 2 to 10.

Foaming properties

The foaming capacity and stability were studied according to the method of Conffman and Garcia (1977). Weighed amount of protein isolate was dispersed in 100 ml-distilled water. The resulting solution was whipped vigorously for 2min in a Moulinex mixer (Type 716, France) at the maximum speed. Volumes were recorded before and after whipping. The percentage volume increase was calculated according to the following equation:

$$\% \text{ Volume} = (V_2 - V_1)/ (V_1) \text{ x}100$$

Where: V_2 is the volume of protein solution after whipping and V_1 the volume of protein solution before whipping.

Antibacterial assay (Disc-diffusion method)

Kirby-Bauer method was used for disc diffusion assays (Baur *et al.,* 1966) to determine the sensitivity or resistance of bacteria to plant proteins at four different concentrations (0.5, 0.75, 1.5 and 2 mg/ml). In vitro antimicrobial activity was screened using Meat Peptone Agar (MPA) obtained from the Scientific Research Center of Pharmacotherapy (SRCP), город, Saint Petersburg, Russia. The sterile filter-paper disks impregnated with different proteins (30µl/disc) were placed on the surface of the MPA in Petri plates. Sterilized phosphate buffer solution pH 7was used as a negative control. The plates were incubated at 37°C for 24 h. Inhibition zones formed around the discs were measured in millimeters. The treatments were replicated three times.

Minimum Inhibitory Concentration (MIC)

The antimicrobial activity of the tested substances at different concentration (0.05, 0.1, 0.25, 0.5, 1.0, 2.0 mg/ml) against (*Enterobacter cloacae*, *Serratia marcescens* and *Listeria monocytogenes*) was assayed by the conventional broth dilution assay (Lana, *et al.,* 2006). Negative control (sterilized phosphate buffer solution pH 7) and positive control (kanamycin) were conducted simultaneously.

Statistical analysis

All investigated treatments were performed in three replicates and results were expressed by the mean plus the standard error.

Results and Discussion

Esterification extent

The color reaction using hydroxylamine hydrochloride was used with modification to quantify the extent of esterification of proteins and the results showed that the highest level of esterification was recorded for esterified cowpea protein isolate (82%) followed by esterified common bean protein isolate (79%).

Functional properties of native and modified proteins

PH-solubility profile

The pH-solubility curves of native and esterified cowpea protein are shown in Fig.1. The solubility profile of native cowpea indicate that protein solubility reduced as the pH increased from 2 to 4, which corresponding to its isoelectric point, after which subsequent increases in pH increased

protein solubility progressively. The minimum solubility for native cowpea (10 %) was at pH 4 which corresponds to its isoelectric point (IP).The highest protein solubility (80%) was observed at pH 10. Esterification increased protein solubility in the acidic pH range from 2 to 5. Increasing pH more than 5 reduced the solubility and giving a minimum value (9 %) at pH 6. Methylated cowpea protein was more soluble in the acidic range of pH and less soluble in the alkaline range of pH as compared to unmodified protein. On the other hand The solubility profile of native common bean indicate that protein solubility reduced as the pH increased from 2 to 5, which corresponding to its isoelectric point, after which subsequent increases in pH increased protein solubility progressively. Esterification increased protein solubility in the acidic pH range from 2 to 7. Increasing pH more than 7 reduced the solubility and gave a minimum value (12 %) at pH 8. These data are in good agreement with Sitohy *et al.,* (2001b) who recorded that solubility of β-lacto globulin esters depends on the degree of esterification as well as on the nature of the grafted ester groups. The conformation of the esterified protein might play an important role on the solubility of the protein. Hence; it is not only the nature of the grafted ester group that determines the solubility of the modified protein but also the nature of the protein itself and the pH degree.

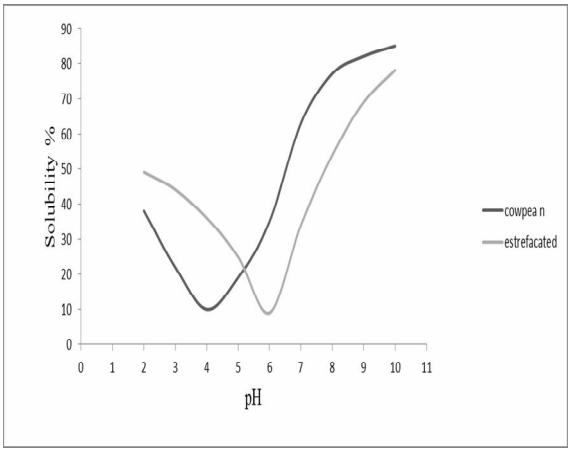

Fig.1. Effect of pH on in cowpea protein solubility.

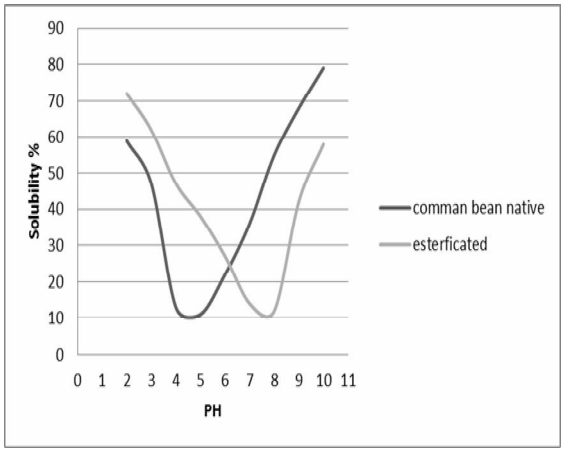

Fig.2. Effect of pH on common bean protein solubility.

Emulsifying properties

Effect of pH on emulsifying activity is a reflection of the influence of pH on protein solubility. Effect of pH on emulsifying activity of native and modified proteins is presented in Fig.3, 4. Emulsifying activity decreased with decrease in pH until it reached minimum value at iso electric point. Esterification increased emulsifying activity in the acidic pH. Increase in the emulsifying activity after modification is an indication of increased solubility. As the protein became more soluble, it formed a layer around the fat droplets to facilitate their association with the aqueous phase (Ponnampalam *et al.,* 1990).

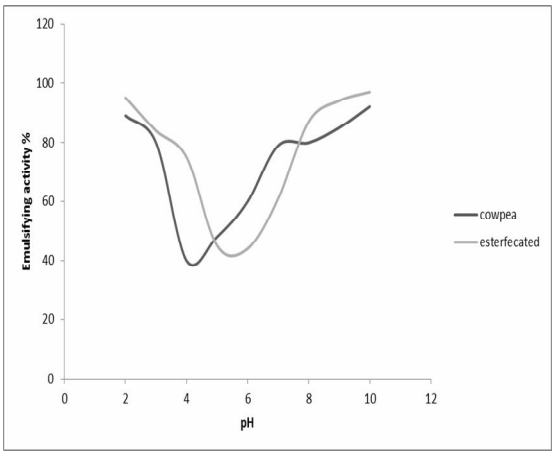

Fig.3. Effect of pH on in cowpea protein emulsifying.

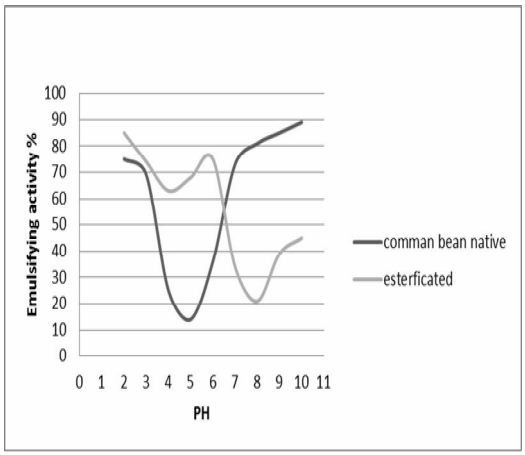

Fig.4. Effect of pH on common bean protein emulsifying.

Foaming properties

Effect of pH on foam capacity of native and modified proteins is presented in Fig. 5, 6. Foam capacity decreased with decrease in pH until it reached minimum value at iso electric point. Esterification increased foam capacity in the acidic pH. Increase in the foam capacity after modification is an indication of increased solubility. The functionality of proteins in foam capacity depends also upon their abilities to reduce interfacial tension structures, solubility and physical chemical conditions (Cherry and Watters, 1981; Halling, 1981 and Kinsella and Srinivasan, 1981).

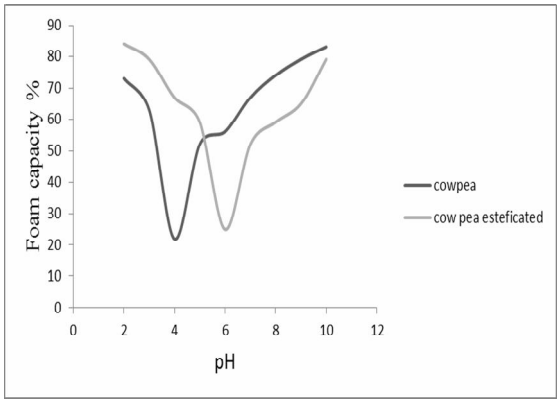

Fig.5. Effect of pH on in cowpea protein foaming.

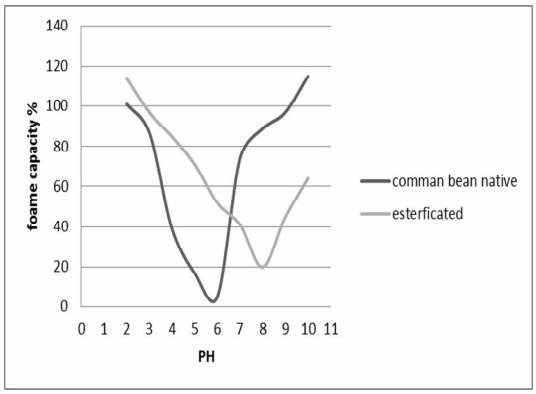

Fig.6. Effect of pH on common bean protein foaming.

Disc assay results

Two methylated legume proteins; (cowpea and common bean proteins) were investigated at four different concentrations (0.5, 0.75, 1.5 and 2 mg/ml) at Petri dishes containing nutrient agar infected with two pathogenic Gram- negative (*Enterobacter Cloacae* and *Serratia Marcescens*) and Gram- positive bacteria (*Listeria Monocytogenes*) incubated at 37°C for 24 h and the diameter of the resulting inhibition zones are recorded as shown in figs 7, 8. Native proteins were prepared at the same aforementioned concentrations. Control was in the same conditions except that protein was replaced by

distilled water. It was observed that either control or native proteins samples did not produce any inhibition zones (data not shown). It is showed that all methylated proteins gave rise to concentration dependent inhibition zones. Generally, the two types of bacteria (positive and negative Gram) were in the same level but both of two methylated legume proteins had no effect with 0,5 mg/ml conc. Similar results were obtained by (Sitohy and Osman 2010) who stated that the antibacterial activity observed with the tested proteins is mostly due to the positive charges condensed on protein molecules through the process of esterification and these positively charged modified proteins may have a direct inhibitory action on the bacterial growth. Esterification blocks free carboxyl groups rising, thus the net positive charge and rendering more basic the modified protein (Halpin and Richardson, 1985; Sitohy *et al.,* 2001a, 2001b). In general, increasing the positive charge on the protein and peptide molecules enhances their antimicrobial, and more specifically, antibacterial effects. For example, amidation improved the effectiveness of bovine lactoferrin (LF) against a range of Gram-positive and Gram-negative bacteria, including common dairy spoilage psychrotroph *Pseudomonas fluorescents* (Pan *et al.,* 2005, 2007). These effects are dependent on the interactions of the antimicrobial protein or peptides with structural elements of the bacterial cell wall and membranes (Hancock, 2004 and Mantyla *et al.,* 2005).

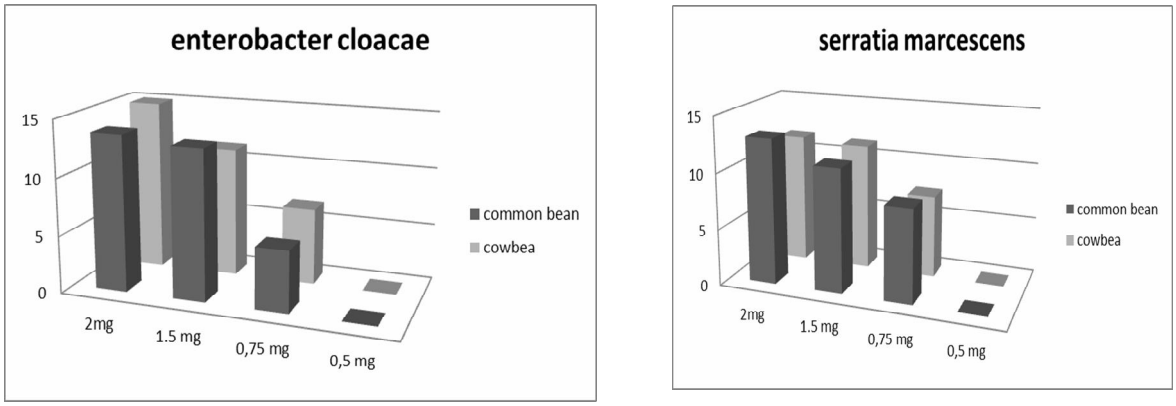

Fig.7. Antibacterial activity of modified proteins using Gram-negative bacteria

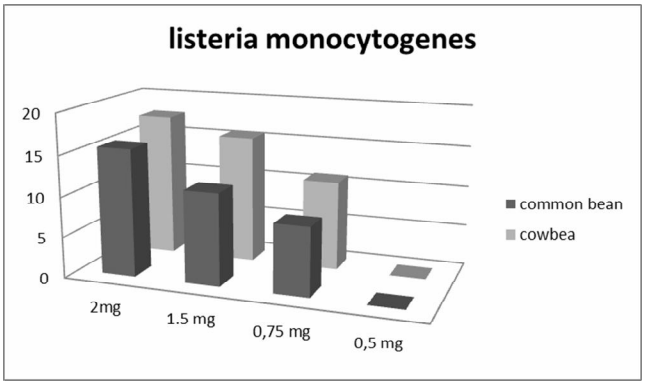

Fig.8. Antibacterial activity of modified proteins using Gram-positive bacteria

Bacterial growth curves

Two esterified proteins were applied at 100 µl of different concentration (0.05, 0.1, 0.25, 0.5, 1.0, 2.0 mg/ml) to a Test tubes containing 800 µl nutrient broth infected with 100 µl of either Gram-positive or Gram-negative bacteria and incubated at 37°C for 24h. The turbidity of the media was measured at OD600 and taken as an indicator of the bacterial growth extent. The results are illustrated in Figs. 9 and 10. The negative control was treated and applied under the same test conditions; however this did not contain protein and positive control was treated and applied under the same test conditions and replaced the protein by kanamycin. In general, data show that esterified proteins has a good inhibition as compared to positive and negative control. This result goes in line with (Sitohy and Osman 2010) who stated that the positive charge may explain the antimicrobial action exerted by the esterified legume proteins against a wide range of Gram-positive and Gram-negative bacteria.

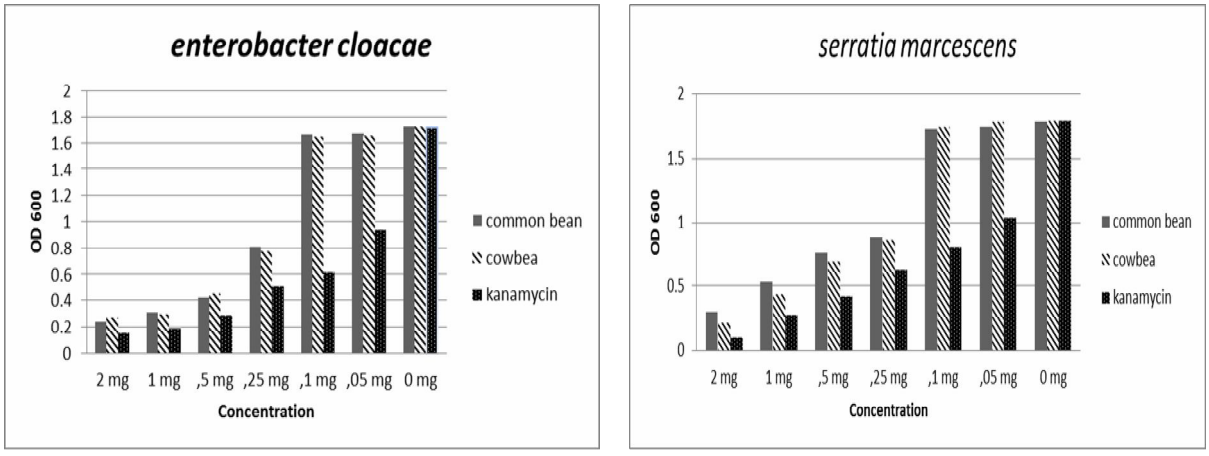

Fig.9. Broth tube dilution assay of the antimicrobial activity of modified proteins as compared to kanamycin against Gram-negative bacteria.

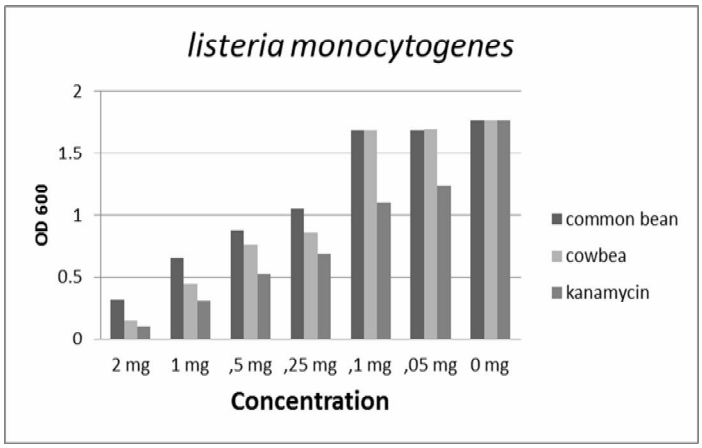

Fig.10. Broth tube dilution assay of the antimicrobial activity of modified proteins as compared to kanamycin against Gram-positive bacteria.

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تقيم التاثير المضاد للبكتريا والخصائص الوظيفية لبعض البروتينات البقولية الطبيعية والمعدلة بالاسترة

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الملخص

تم عزل نوعين من بروتينات البقوليات من بذور كل من اللوبيا والفاصوليا وتم اجراء عملية استرة بواسطة كحول الميثيل في وجود حمض الهيدروكلوريك لمدة عشر ساعات علي درجة حرارة ٤ مئوية حقق معزول البروتين للوبيا درجة استرة بنسبة ٨٢% بينما حقق معزول الفاصوليا ٧٩%. ادت عملية الاسترة الي زيادة نقطة التعادل الكهربي من درجة حموضة ٤ الي ٦ في حالة اللوبيا و٨ في حالة الفاصوليا. كما تم اختبار البروتينات المعدلة بطريقة الاسترة بعدة تركيزات (0.5,0.75,1 and 2 mg/ml) مع نوعين من الميكروبات السالبة لجرام هي (*Enterobacter cloacae* و *Serratia marcescens*) واخري موجبة لجرام (*Listeria monocytogenes*) وادي ذلك لظهور تثبيط للبكتريا معتمدة علي التركيز.