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Research Article
Antioxidant and Biological Activities of Proteinaceous Extract from Algerian *Glycine max* Plant

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Abstract

Objective: *Glycine max* is commonly used in Algeria for treatment of anemia deficiency and osteoporosis, it ranks first in terms of vegetal proteins. The experiment was aimed at characterizing the proteinaceous *Glycine max* extract and evaluating its antioxidant, biological and hematological potential. **Methodology:** Extraction of proteinaceous materials from *Glycine max* plant was undertaken using water and n-hexane as extracting media. The isolation of proteins from the crude materials was done, providing the use of ammonium sulfate. The *Glycine max* proteins were characterized by UV-visible and FT-IR spectroscopy and analyzed by SEM micrograph and x-ray diffraction (XRD). Rheological parameters G′ and G″ were assessed. The isolated proteins were tested for their antioxidant, antimicrobial and hemagglutination activities. **Results:** There was a gelling effect of the protein extract which can be used as an alternative in principally made vaccines with its microbiological and antifungal activities. **Conclusion:** The proteinaceous extract from Algerian *Glycine max* would have a potential use in biomedical application.

Key words: Antioxidant activity, biological activity, hemagglutination activity, *Glycine max*, protein

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Proteins are ubiquitous in the tissues of several plants. They exist in an extent range of 7-40% in common cereal grains, oil-seeds and legumes[1]. Of these, soybean ranks first as far as protein content is of a great importance (35-40%). They can also be extracted from roots, leaves, flowers and stems. On the other hand, leaves from tropical plants also contain an extent of protein up to about 34%, depending on the country where the plant grows. In many cases, the vegetal proteins constitute the basic ingredients for dietary recipes, lowering cholesterol levels, blood pressure and body weight index[2]. Besides those that play an indispensable role in the animal growth, there are vegetal proteins that possess antimicrobial activity[3]. For example, purothionin, a protein extracted from wheat endosperm is active against several bacteria including *P. solanacearum*, *X. phaseoli*, *X. campestris*, *E. amylovora*, *C. flaccumfaciens*, *C. michiganense*, *C. poinsettiae*, *C. sepedonicum* and *C. fascians*[4]. In addition, vegetal proteins are reputed for their antioxidant properties[5].

Herein, we present results of the extraction work-up of proteins from Algerian *Glycine max*, grown in the West-South Algerian region (Timimoun, state of Adrar). *Glycine max* or yellow soya, sown in August and harvested in October is known in this region of the country under the name "Tedleghit". The *Glycine max* beans are small and are akin to kidney beans. In this region of the country, the soya bean by virtue of its high nutritive value and calorie intake is taken as a dietary supplement in malnutrition events. In addition and due to its iron and calcium content, its uptake is highly recommended against anemia deficiency and osteoporosis, particularly after childbirth[6]. Recently, its dermatological and cosmeceutical benefits were reported[7].

MATERIALS AND METHODS

The *Glycine max* (Tedleghit) was sown at Timimoun (state of Adrar, South-West of Algeria) in July, 2014 and harvested in October, 2014. The yellow soyabean was dried over a period of 5 months before use. Chemicals were purchased from Sigma-Aldrich (Netherlands). Specific chemicals are coomassie brilliant blue G-250 dye (Bradford reagent), 2,2-diphenyl-1 -picrylhydrazyl (DPPH) and biuret. The extract was diluted in PBS solution (pH 7.4). The latter buffer solution was made by mixing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.76 mM KH_2PO_4 in 1 L of distilled water and the pH was adjusted to 7.4 by addition of 1 M HCl. The different bacteria and yeasts were provided by CRD of Saidal (Algiers (Hydra), Algeria) and the fungi were from LBSM (Ecole Normale Supérieure, Kouba, Algiers, Algeria). Molecular weight of the extracted protein was measured by light scattering method using Zetasiser type Horiba Scientific.

The UV-visible analyses were performed using UV-vis spectrophotometer Shimadzu. The FTIR spectra were recorded with Shimadzu FTIR 9800, using KBr pellets. Zeta potentials and the hydrodynamic diameter of proteinaceous particles were measured with nanopartical SZ-100 (Horiba Scientific). Microscopic characterizations were made with JEOL-JSM 6360 DLX (for SEM) and optika microscope (Italy) (for optical microscopy). The XRD analysis was made using Bruker D8 Advance diffractometer operated at a voltage of 40 kV and a current of 40 mA using Cu Kα radiation ($\lambda = 1.5406$ Å) in the range of 0-100° at a scanning speed of 0.05° sec^{-1}. Rheological parameters of the protein were measured with a modular compact rheometer MCR 302 (Anton-Paar) having parallel plate geometry (diameter = 25 mm), the frequency range was 0.1-100 rad sec^{-1}. The employed lyophilizer was christ alpha 2-4 LSC plus.

Crude extract: The first step was to collect the healthy grains (among the infected ones) from the crude material (crop) (a rough size of the grain was about 1-2 mm). An amount of 10 g of healthy grains was ground with an electrical mill and immersed into a 50 mL of physiological water (pH = 7). By doing so, both cell bursting and protein denaturation could be prevented. The whole system was then gently stirred for 8 h. An extraction process was executed by stirring the system at 20°C for 8 h and the system was afterwards cooled down to room temperature, in the aim to prevent screening effect of nucleic acid cells for the proteins in case of cell bursting during the extraction, 3-4 g of EDTA (ethylenediamine tetra acetic acid) as a chelating agent were added prior to the extraction operation. The crude extract was isolated and filtered, the filtrate was then kept at 4°C for 1 day. In another extraction process, n-hexane was employed instead of water and the same work-up was applied. The crude extracts with water and n-hexane are herein designated as CE_W and CE_H, respectively. Crude protein extract is illustrated in Fig. 1.

Tests for the presence of proteins

Bradford test: Kruger[8] protein assay was applied to confirm the presence of proteins in the crude extracts. The test was positive as the solution of coomassie brilliant blue G-250 dye turned blue in the presence of protein (the solution was red in the absence of protein) and its UV-visible absorption shifted from 465 to 595 nm.

Fig. 1: Crude extract from *Glycine max* plant

Biuret test: Two milliliters of vegetal extract of the crude sediment charged were added in a test tube of 2 mL of a 3 M NaOH solution. After mixing the two solutions, 1-2 drops of 0.1% aqueous solution of copper sulfate were added. Shortly, a pink color appeared, indicating the presence of proteins.

Coagulation test: A precipitate was formed upon either heating a 2 mL of vegetal extract in water-bath or mixing 1 mL of it with 2 mL of ethanol. The formation of precipitate indicated the presence of proteins.

Proteins isolation: Purification of protein was performed through a salting-out process by adding ammonium sulfate at a concentration of 75% to the obtained filtrate, followed by stirring for 1 h at room temperature. Afterwards, the mixture was vigorously agitated and centrifuged (10000 rpm) during 20 min. The sediment part containing the crude proteins was dialyzed through semi-permeable cellulose-based membrane. The ammonium sulfate-contaminated solution which contained the crude proteins, was then charged into a dialysis bag and stirred at 4°C for 6 h. The dialysate was subsequently diluted with PBS solution (pH = 7.4). The proteinaceous isolates from the crude extracts CE_W and CE_H are herein designated $Prot_w$ and $Prot_h$, respectively.

Lyophilization: The isolated proteins underwent freeze-drying under reduced pressure of 0.1 mbar, comprising a freezing for about 30 min, a primary drying (02 days, 2 h and 10 min) and a secondary drying (4 h and 10 min).

Hemagglutination test: In the first step, the red cells were washed as follows: A 5 mL test tube containing an amount of heparin as coagulant was charged with 3 mL of blood (Rh^+ = O) from a healthy human. The test tube was then filled up with physiological water and the system was centrifuged at 1000 rpm for 4-5 min, the supernatant part was then discarded. This studying process was quadruplicated. The sediment containing the red cells was dispersed into 9% saline solution (1 v of sediment for 20 v of saline solution) to end up with 5% diluted solution. Two methods of hemagglutination test were evaluated: Opaline plate and tube methods. The test is quantified in titers as being the reciprocal of the highest dilution for which agglutination is observed.

Opaline plate method: A mixture of one drop of 5% red cells solution and one drop of an isolated protein was cast on a circular plate (2 cm of diameter) and the system was smoothly and constantly moved back and forth. After 5 min of such a work-up, a hemagglutination of erythrocytes was observed.

Tube method: A mixture (1:1 v) of 5% red cells solution and an isolated protein was charged into a test tube containing a volume of 0.9% physiological water, forming the dilution 1/2. After the brewing time, a volume of the content of this tube was placed in a second test tube that contained the same volume of 0.9% physiological water to form the dilution 1/4 and a volume of the content of this second volume is placed in a third test tube that contained the same volume of 0.9% physiological water to form the dilution 1/8 and so on. The result is taken as the last dilution upon which no hemagglutination occurred.

Antioxidant activity assay: The antioxidant property of the protein was assessed via the anti-radicalar activity according to the method described by Banerjee *et al.*[9].

In dry test tubes were charged 100 µL of different dilutions of vegetal extract (or extracted proteins) (100, 200, 400, 600, 800 and 1000 µg mL^{-1}), followed by addition of 2.9 mL of 0.004% ethanolic solution of DPPH (1,1-diphenyl -2-picrylhydrazyl). The mixtures were incubated for 30 min in darkness at room temperature, then their absorbances were measured at λ_{max} = 517 nm. A blank test was performed in the absence of vegetal extract. The antiradicalar activity of vitamin C was determined for a comparison purpose. The results are given in terms of radical inhibition power of DPPH (I_{DPPH}) using the following Eq. 1:

$$I_{DPPH}\ (\%) = ([A_{blank}-A_{sample}]/A_{blank})\times 100 \quad (1)$$

where, A_{sample} and A_{blank} are the absorbances of the mixture in the presence and in the absence of the vegetal extract (or extracted proteins), respectively. The final results were the means of triplicate runs.

The antioxidant activity of the vegetal extract (or extracted proteins) is quantified as the inhibitory concentration of the extract for scavenging 50% of the initial DPPH concentration (IC_{50}), the inhibitory concentration IC_{50} was estimated by extrapolating the curve of the plot of I_{DPPH} (%) in function of the vegetal extract (or extracted proteins) concentration.

Antimicrobial activity assay: Agar dilution method was employed to determine the Minimum Inhibitory Concentration (MIC) of the vegetal extract or extracted proteins vis-à-vis the strains. The tested strains were bacteria (*Klebsiella pneumoniae* (-) ATCC 4352, *Pseudomonas aeruginosa* (-) ATCC 27893, *Escherichia coli* (-) ATCC 4157, *Staphylococcus aureus* (+) ATCC 6538, *Bacillus subtilis* (+) ATCC 9372), fungi (*Aspergillus flavus* NRRL 62477, *Aspergillus paraciticus* NCPT 217, *Aspergillus carbonarius* NRRL 369, *Fusarium graminearum* NRA 155) and yeasts (*Candida albican* ATCC 24433, *Saccharomyces cerevisiae* ATCC 2601). Solutions of concentrations from 0.1-5 mg mL^{-1} were prepared by sequential dilutions of the mixture of the extract and the sterilized, melted nutrient agar enriched with 10 g L^{-1} of glucose and 0.5% of tween 80. The as-made solutions were stirred and instantaneously spread over petri dishes. After drying the petri dishes, they were inoculated with 2 µL of each suspension of target microorganisms.

The different microbial suspensions were prepared with bacteria, fungi (including yeasts) grown after culture times of 24 and 72 h, respectively and were adjusted to about 10^6 CFU mL^{-1}. The latter adjustment was made by spores counting with haemocytometer, a mallassez cell. The inoculated dishes were then incubated at 30°C for 24 and 48 h for bacteria and fungi (including yeasts), respectively.

RESULTS AND DISCUSSION

Extraction and characterization: The presence of proteins in the extract from the plant *Glycine max* that grows in the South-west region of Algeria, was confirmed by virtue of the Bradford protein assay[10] and the biuret and coagulation tests. In the study assay, the red color of the dye coomassie brilliant blue G-250 which absorbs at 465 nm was shifted to blue color when mixed with the extract sample, indeed, the UV-visible absorption band was displaced to 595 nm (Fig. 2), a band attributable to a complex stemmed from the binding of the dye to the protein. Such an observation is a strong indication of the presence of protein. Isolation of the proteins was undertaken by means of ammonium sulfate salting-out

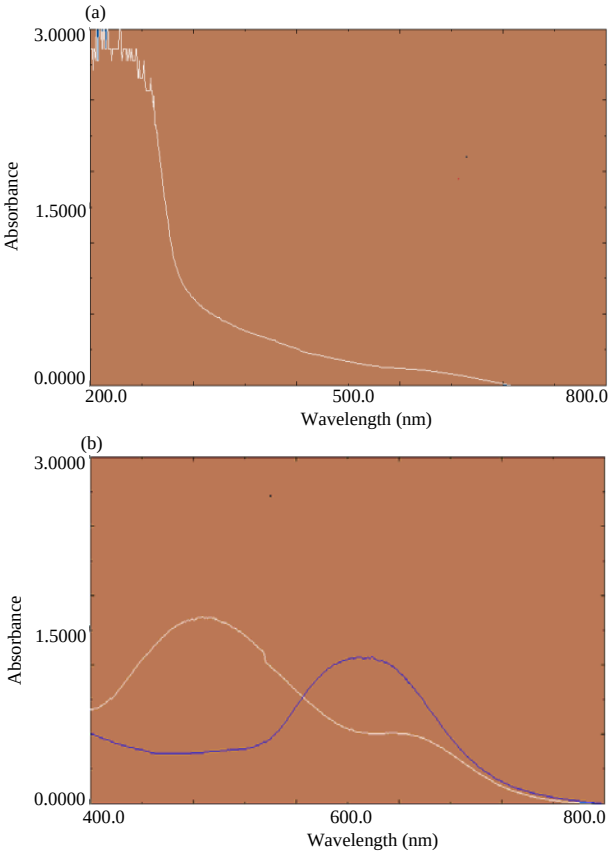

Fig. 2(a-b): UV-visible spectra, (a) Vegetal extract, (b) Coomassie blue (white curve), mixture of the vegetal extract and comassie blue (blue curve)

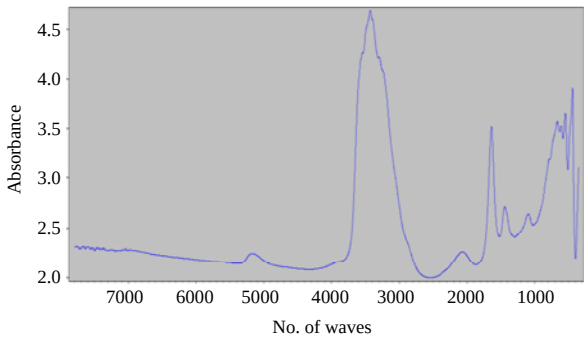

Fig. 3: FT-IR spectrum of the raw proteins

methodology[11] and their purification was done by dialysis. The yields of the crude extracts CE_W and CE_H were found to be 51.47 and 53.33%, respectively, whereas those of the proteinaceous isolates $Prot_W$ and $Prot_H$ were nearly close to each other, 17.66 against 18.88%.

The infrared spectrum of the raw proteins is illustrated in Fig. 3. The band centered at 3430 cm^{-1} is assigned to NH stretching of the peptide linkage (amide A and B). The band at 1638 cm^{-1} characterized the carbonyl (C=O)

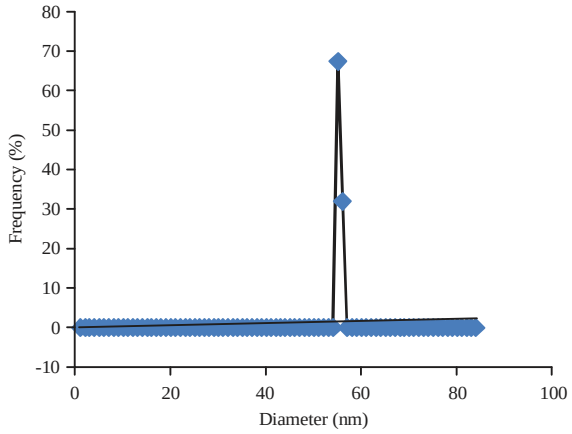

Fig. 4: Curve for diameter of the proteinaceous particles

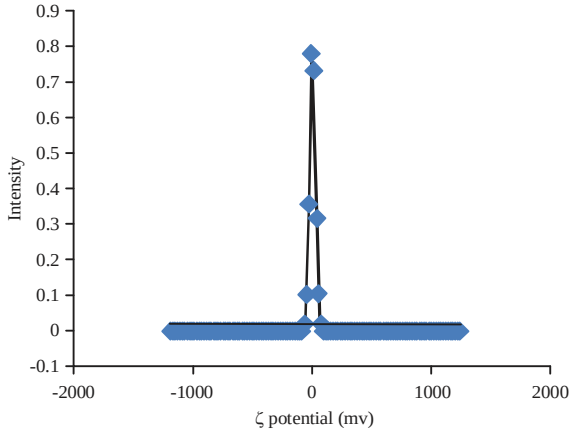

Fig. 5: Curve for ζ potential of PBS solution of the proteinaceous particles

stretching of the amide (amide I) and the band at 1444 cm^{-1} is indicative of C-N stretching and NH bending of the amide (amide II). The band at 1102 cm^{-1} is attributed to of C-N stretching and NH bending of the amide (amide III). The broad band centered at 665 cm^{-1} is the overlap of OCN bending (amide IV) and out-of-plane NH bending (amide V) and the band at 546 cm^{-1} featured the out-of-plane C=O bending (amide VI)[12]. The UV-visible analysis (Fig. 2) of the extract in PBS (pH = 7.4) revealed a broad band at 200-320 nm, hinting at the presence of an aromatic protein[13]. Hence forth, the building amino acids of the extracted proteins may be phenylalanine, tryptophan and tyrosine. The concentration of the proteins in mg mL^{-1} in the extract PBS solution was estimated according to the Warburg-Christian's method[14] as Eq. 2:

$$\text{Proteins (mg mL}^{-1}) = 1.55\ A_{280} - 0.76\ A_{260} \quad (2)$$

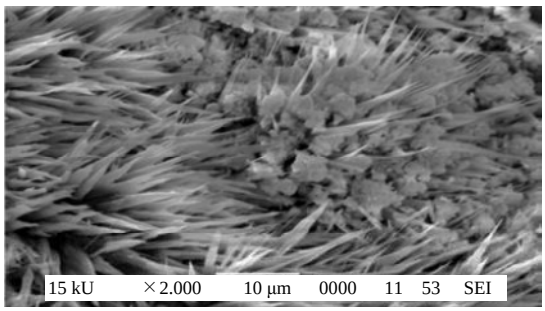

Fig. 6: SEM image of the raw proteins

where, A_{280} and A_{260} are the corresponding absorbances of the protein and nucleic acid (DNA), respectively. The DNA being the common contaminant in the protein extracts. The content of proteins in the extract was roughly 0.655 mg mL^{-1}.

The molecular weight M of the extracted protein was drawn from the plot of $\frac{KC}{R_\theta} = f(C)$ of the Raleigh equation:

$$\frac{KC}{R_\theta} = \left(\frac{1}{M} + 2A_2C\right)\frac{1}{P(\theta)} \quad (3)$$

where, K, C, R_θ, A_2 and P(θ) are the optical constant, the concentration of protein solution, the Raleigh ratio, the second virial coefficient and the shape factor, respectively. The molecular weight of the extracted protein was in the range of 100-102 kDa.

Figure 4 shows the size distribution and reveals the existence of one set of particles of a hydrodynamic diameter of around 57 nm. The zeta potential (ζ) of their PBS solution (3.32 mg L^{-1}) was about 0.9 mV (Fig. 5), a value indicating the probability of some aggregates formation[15]. The isoelectric point (pI) of the proteinaceous material was found within the range of 3.5, a pH at which this material exists in its electrically neutral state.

The SEM image of protein extract is depicted in Fig. 6 which reveals a needle-pointed morphology, stemming from aggregates of a background. Figure 7 Illustrates the XRD profile of the isolated raw proteins $Prot_w$, 2 conspicuous peaks appeared at 2θ values of 9.0 and 19.5°, revealing a high crystallinity degree at the 1st angle. The intensity of the peak at 2θ = 9.0° was about 41%, whereas that of the peak at 2θ = 9.0° was 20%. These results would hint at a higher α-helix structure over the β-sheet one in the protein morphology. This present XRD data are in disagreement with those reported for soybean protein isolated by reverse micelle and aqueous buffer extractions[16].

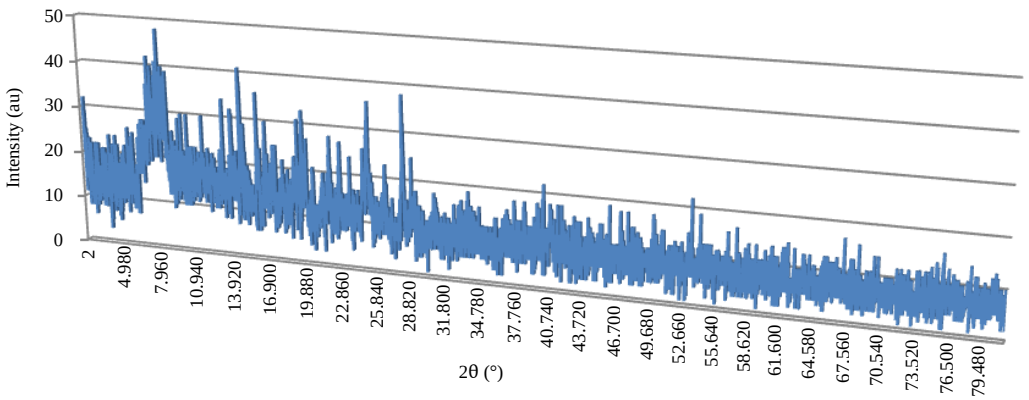

Fig. 7: XRD of the protein

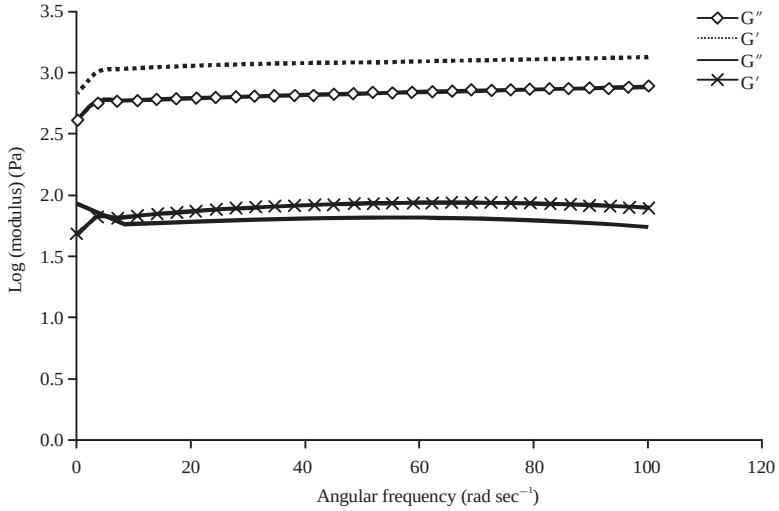

Fig. 8: Variations of G′ and G″ against angular frequency for $Prot_w$

The rheological parameters, the storage and loss moduli G′ and G″, of $Prot_w$ and $Prot_h$ in their gel state and viscous solution state (aqueous solution of 3.32 mg L^{-1}) were examined and are shown in Fig. 8 and 9, the gels were obtained by setting high concentrations of the proteins in water at room temperature until gelation onset (after about 10 days). The formation of gel could be the result either of a covalent intermolecular bonding (chemical gel) or an intermolecular entanglement or a Van der Waals bonding (physical gel)[17], yet, the 1st probability could be precluded for the $Prot_h$ as a heat-provoked denaturation could have occurred during the evaporation process[18]. As can be noticed, the storage moduli G′ are generally greater than the loss moduli G″, suggesting the viscoelastic behavior of these proteinaceous systems. The mechanical properties G′ and G″ of the protein $Prot_w$ in its gel state were 1259 and 501 Pa, respectively, while those of its viscous solution state were 79 and 56 Pa, respectively. Those of the protein $Prot_h$ were 708 and 501 Pa for its gel state and 79 and 79 Pa for its viscous solution state. The elastic modulus G′ of the protein $Prot_w$ in its gel state was greater than that of protein $Prot_h$, 1259 against 708, hinting at the difference in the phenomenon-inducing gel formation, that is chemical gel versus physical one. Overall, the magnitudes of the mechanical moduli would indicate the softness of the proteinaceous gels.

Antioxidant and antimicrobial activities: Antioxidant and antimicrobial activities of plant proteins have been surveyed[4,19,20]. In the present study, the crude vegetal extracts, CE_w and CE_h and their corresponding proteinaceous isolates, $Prot_w$ and $Prot_h$ were screened for these two properties. The results of antioxidant assays in terms of IC_{50} are compiled in Table 1. The lower the IC_{50}, the greater the antioxidant activity. As can be seen, CE_h and its isolate $Prot_h$

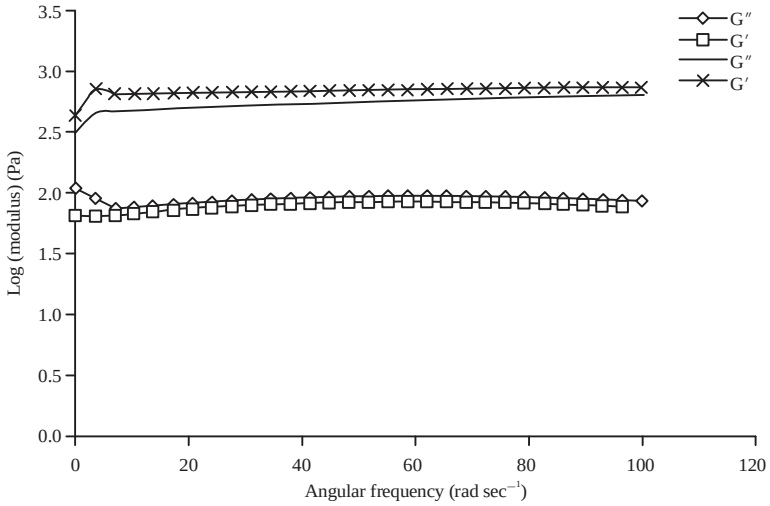

Fig. 9: Variations of G′ and G″ against angular frequency for $Prot_h$

Table 1: Results of antioxidant activity tests*

Substance	CE_w	$Prot_w$	CE_h	$Prot_h$
IC_{50} (mg mL^{-1})	0.639±0.2	0.761±0.4	0.465±0.1	0.578±0.3

*Values are given as means of three runs

Table 2: MIC values for the proteinaceous systems

	MIC (mg mL^{-1})			
Strains	CE_w	$Prot_w$	CE_h	$Prot_h$
Bacterium				
Klebsiella pneumoniae	18	2.6	1.4	2.4
Pneudomonas aeuginosa	1.6	2.2	1.2	2.2
Escherichia coli	1.6	1.8	1.0	1.8
Staphylococus aureus	2.2	2.4	1.8	2.2
Basillus subtilis	2.0	2.4	1.6	2.0
Fungus				
Aspergillus flavus	1.8	2.4	1.4	2.0
Aspergillus parasiticus	2.0	2.2	1.6	2.2
Aspergillus carbonarius	2.2	2.4	1.8	2.4
Fusarium graminearum	1.8	2.0	1.4	2.0
Yeast				
Candida albican	3.8	>5	2.8	4.6
Saccharomyces cerevisiae	3.4	>5	2.0	>5

MIC: Minimum inhibitory concentration

proved to possess better antioxidant activities than CE_w and its isolate $Prot_w$, IC_{50}'s for the former materials were 0.465 and 0.578 mg mL^{-1}, respectively, while those for the latter ones were 0.639 and 0.761 mg mL^{-1}, respectively. Noteworthy is the greater antioxidant activity of the crude extracts compared with that of isolated proteins.

Antimicrobial activities of the as-examined materials were assessed through their Minimum Inhibitory Concentration (MIC) towards the different strains. Table 2 gathers the measured MICs against bacteria, fungi and yeasts. Also, the lower the MIC, the better the antimicrobial activity. The MIC values of CE_h towards a specific strain are lower than those of CE_w, stating higher antimicrobial activity of the crude extracted with n-hexane. This result is in a good agreement with the estimated antioxidant activity. For CE_h, the values ranged from 1-1.8 mg mL^{-1} towards bacteria, 1.4-1.8 mg mL^{-1} towards fungi and 2.0-2.8 mg mL^{-1} towards yeasts. However, the MIC values of $Prot_h$ are close to those of $Prot_w$ for all the examined strains. While the MIC values against bacteria and fungi fluctuated between 1.8 and 2.4 mg mL^{-1}, they were higher for yeasts, exceeding 5 mg mL^{-1} in most cases. Based on the MIC values, the extracts showed a good antimicrobial capacity against bacteria and fungi and were less efficient towards yeasts.

Hemagglutination activity: Hemagglutination is a practical method to detect the presence of virus in an infected cell culture. Agglutination occurs upon reaction of the proteinaceous species with red blood cells. The hemagglutination is inhibited in the presence of antibodies, preventing the adhesion of the virus to red blood cells. The titer of hemagglutination inhibition (HAI) is the highest dilution that impedes hemagglutination. The titers of HAI for $Prot_w$ and $Prot_h$ extracts were found 512 and 1024, respectively, suggesting that the extract $Prot_h$ is more efficient in hemagglutination prevention. That the latter extract showed a higher hemagglutination activity owes to the presence of higher concentration of lectins which were easily extracted with n-hexane. This finding indicated that the extract $Prot_h$ contained two-fold of sugar binding lectins over the extract $Prot_w$. Based on this hemagglutination activity, the proteinaceous extract from Algerian *Glycine max* plant would have potential use in biomedical applications.

CONCLUSION

The different properties of the extracted proteins from Algerian *Glycine max* are tightly linked to the medium used for the extraction. In general, the proteins extracted with organic medium, namely n-hexane, showed antioxidant and biological activities higher than those for the ones extracted with water.

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