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A NOVEL SYNTHESIS CHEMICAL CHARACTERIZATION AND BIOLOGICAL ACTIVITIES OF METAL-LEATHER PROTEIN HYDROLYSATE CHELATES

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ABSTRACT

Leather industries covers a wide chain of production and indirectly contributes to the economic flow. The different stages used in leather processing led to produce huge solid waste volumes. Because of the great effectiveness of amino acids as naturally chelates for minerals, the present study was carried out to recycling leather waste into its protein hydrolysate by CaO hydrolysis. The Leather protein hydrolysates (LPHs) was used to prepare metal-leather protein hydrolysate chelates (Cu^{2+}-, Zn^{2+}-& Fe^{2+}-LPHCs) and some of their physical properties (i.e. λ-max, FTIR spectra, color, melting point) and biochemical properties as its antibacterial activity, as well as using as micronutrient elements for plant were evaluated. Results showed that the Cu^{2+}-LPHC gave the highest value of melting point and λ-max than other chelates. All chelates shifted the vibration bands toward a higher frequency than LPH/CaO. Metal-leather protein hydrolysate (M-LPHCs) had antibacterial activities against E. coli, B. cereus and Micrococcus spp. mostly with Zn-LPHC and Fe-LPHC. These complexes also increased the growth characteristics and mineral absorption of spinach plants in hydroponic nutrient solution than that of mineral salts ($CuSO_4$, $ZnSO_4$ and $FeSO_4$). Finally, the study concluded that M-LPHCs can be used as antimicrobial agent, micronutrients for plant and support the minerals bioavailability in animals.

KEYWORDS:
Leather protein hydrolysate, metal-protein hydrolysate chelates, antibacterial, plant, micronutrients, bioavailability

INTRODUCTION

As ancient and traditional industry, leather processing not only meets the social development demand, but also subsidizes to the global economy all over the world [1]. The solid wastes generated from leather processing such as keratin wastes, skin trimmings, chrome shaving waste, fleshing wastes and buffing wastes categorized leather industry to become on a head of polluting industries [2].

Leather manufacturing process are carried out in three sequencing phases; beam house operations, tannery process and finishing process [3]. It starts with the animal skin handling with series of chemical treatments named tanning process which causing in release of harmful wastes to the environment [4,5]. Tanning operation phase is energetic because of at this stage, the raw material from hide or skin is converted into resistant and durable leather, also it generates effluents and solid wastes. Every year, global production from solid wastes generated by leather industries was recorded as 6 million tons [6]. These solid wastes are classified into four types: tanned collagen, untanned collagen, non-collagenous protein waste, non-proteinious waste [7]. Tanned collagen is hydrolyzed to a mixture of peptides by different ways of hydrolysis such as alkali, acidic or enzymatic process [8,9]. The alkaline treatment is softer than the acid one, and is more appropriate to obtain hydrolysates of industrial application. The enzymatic hydrolysis is highly appreciated because it obtains products with various functional properties [10].

Regarding to leather protein hydrolysates production with alkalis, many of previous studies focused on the recycle of the hydrolyzing chromium-containing leather waste (CCLW) to produce of peptides and amino acids for fertilizers and feeds applications. Different methods of treatment of CCLW have been developed based on hydrolysis process used such as using $Ca(OH)_2$ with steam or NaOH/KOH at elevated temperature and/or pressure, CaO or MgO at moderate temperatures [11, 12]. Recently, in our previous study, we used of alkaline hydrolysis (CaO or KOH) and enzymatic hydrolysis (protease or trypsin) to produce leather protein hydrolysates (LPHs) from chrome-containing leather waste (CCLW) and evaluated their antioxidant and metal chelating activities [13].

The amino acids and peptides are considered as natural-chelating agents, they have the ability to coordinate metal ions as Zn via their carboxyl and

amino groups [14]. Therefore, the present study was focused on develop a feasible method for the pollutant eliminating and recycling of the leather solid wastes by synthesis of metal-ion complexes (Cu, Zn and Fe) from leather protein hydrolysate obtained by CaO hydrolysis, as well as, estimate its physical properties and evaluate its biological activities for using as antimicrobial, plant micronutrient and mineral bioavailability supporting in animals.

MATERIALS AND METHODS

Materials. Chrome-containing leather waste (CCLW), as animal waste, was obtained from a commercial leather tannery, Al Basatin, Al Maadi, Cairo. Leather was kept at 5°C in refrigerator for further analysis. Spinach (Spinacia oleracea) seeds were obtained from local market, Cairo, Egypt. Escherichia coli 0157, Micrococcus spp. and Bacillus cereus were obtained from Microbiology Laboratory, Faculty of Agriculture, Cairo University, Giza, Egypt. All chemicals used were of analytical reagent grade.

Preparation of Leather Protein Hydrolysate. Alkaline hydrolysis by CaO was applied on leather proteins to produce leather protein hydrolysate (LPH).

Alkaline Hydrolysis. Alkaline hydrolysis of CCLW was carried out according to the method described by Mu et al. [15] as follows: Fifty grams of CCLW were shaken in 500 ml of distilled water and 5.0 g of CaO were added into digestion flask. The hydrolysis was done at 98°C for 24 h. After cooling at room temperature, clear hydrolysate was obtained by filtration. The filtered solution of protein hydrolysate was completed to 500 ml with distilled water and stored at 4°C for analysis and further studies.

Preparation of Metal Complexes Chelating with LPH as Ligands. After hydrolyzation of leather waste with CaO, 100 ml of LPH were mixed with 300 µl of concentrated phosphoric acid (85%, w/w) then centrifuged at 1968 g for 5-10 min, to remove calcium ion. Clear solution was used to prepare metal-chelate complexes with some individual metal ions (Cu^{2+}, Zn^{2+} and Fe^{2+}) using the method described by Jie et al. [16] as follows. In a clean conical flask, 2 mole of appropriate metal salt ($CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$ or $FeSO_4 \cdot 7H_2O$) was separately added to one mole of total free amino acids (the most appropriate ratio). The mixture was shaken for one minute and kept at room temperature for 30 minutes. Before mixing in iron chelate complex, the iron salt and 1% of ascorbic acid were added. The obtained product was concentrated by evaporation then washed with ethanol (95%). The washed products were dried in air for 12 h.

Determination of Physical Properties of Metal-ion Complexes. General Appearance and Color. The metal-ion complexes obtained from LPH were examined for their appearance and color and noted down.

Determination of Moisture, Ion Percentage Chelated, Metal Ion Percentage and Yield Percentage of M-LPHCs. Moisture was determined according to the AOAC [17]. Ion % chelated and M-ion % were determined using of Inductively Coupled Plasma (ICP) under Argon Gas (ICP/MS, NEXION 300X Series). The yield of each complex percentage was calculated according to the following equation: dry weight of M-LPHC/salt weight × 100

Determination of Melting Point. Melting point of each metal-ion complex was measured using a differential thermal analyzer under nitrogen gas (DTA-50, Shimadzu, Japan).

Ultraviolet Visible Absorption Measurements. The Ultraviolet visible absorption of saturated solution of each metal-ion complex was manually recorded at room temperature using a T80 PG UV/VIS Spectrophotometer. For data collection, absorption was begun at 190 nm and stepped at 10 nm intervals to 700 nm. If absorption was over scale, data were recorded at 1 nm interval of wavelength.

IR Spectra. A 0.2 g sample of solid Zn^{2+}-, Fe^{2+}- or Cu^{2+}--chelate was mixed with potassium bromide and pressed into a disc. The structural characteristics of the chelate were determined using infrared spectroscopy (JASCO FTIR 400-4000, Japan).

Assay of Antibacterial Properties. Antibacterial activity of LPH metal-chelate complexes against three different bacteria strains has been tested by disc diffusion method with some modifications as described by Stanila et al. [18] as follows: The culture medium was composed (%) from peptone (0.5), beef (0.3), yeast extract, agar (1.5) and sodium chloride (0.5). The pH of medium was adjusted to 6.8 with HCl or NaOH solution at 25°C. Three strains of bacteria (E. coli 0157, Micrococcus spp. and Bacillus cereus) were tested. Antibacterial tests were carried using of suspension containing 107 CFU/ml of bacteria, inoculated in nutrient agar. The metal-chelate complexes were sterilized by filtration through 0.45 µm Millipore filters. Sterilized filter paper discs (6 mm) were placed on the inoculated agar. Sterilized filter paper discs were individually saturated with 20, 40 or 60 µl/disc of individually saturated solution of Cu^{2+}-LPHC, Zn^{2+}- LPHC or Fe^{2+}-LPHC with 30, 60 or 90 µl/disc. Sterilized distilled water was used as negative control. Flumequine (30 µg/disc) was used as positive reference standard to determine the sensitivity of each bacterial species tested. The plates were inoculated at 37ºC for 48 h. At the end of the

period, antibacterial activity was evaluated by measuring the inhibition zones in millimetres carefully around the paper disc against the test organisms. After farther incubation at 28±2ºC for 30 h, inhibition zones were measured. All inhibitory tests were performed in triplicate. An average of two independent readings for each compound was recorded.

Hydroponic-Culture Experiment. The method of Ghasemia et al. [19] was employed to evaluate the efficiency of metal-ion complexes, instead of free ions, on spinach seeds grown in hydroponic nutrient solution. The basic nutrient solution consisted of 1.0 mM KNO_3, 1.0 mM $NH_4H_2PO_4$,1.0 mM $Ca(NO_3)_2$, 25.0 µM H_3BO_3, 1.0 mM $MgSO_4$, 2.0 µM $ZnSO_4$, 50.0 µM KCl, 2.0 µM $MnSO_4$, 01.0 µM $NiSO_4$, 5 µM $CuSO_4$ and 0.02 µM $H_2Mo_7O_4$ in deionized water. The Fe level ($FeSO_4$) in the nutrient solution was 100 µM. The pH of solution was adjusted to 6 with NaOH or HCl solution as a buffer. Tested nutrient solutions consisted of basic nutrient solution and LPH metal-chelate complexes of copper, zinc or ferrous instead of $CuSO_4$, $ZnSO_4$ and $FeSO_4$ (the most common sources used in nutrient solutions). Seeds of spinach were sterilized with H_2O_2 solution (1.0 Volume) for 15 min then rinsed five times with distilled water and kept at 25ºC on wet filter paper for germination. Only seedlings with uniform size were transferred to jars under specific conditions of 10 h light period 25/20ºC day/night temperature. All pots were covered with black polyethylene to prevent passing of light from reaching to solution and roots. Two plants were planted in each pot. All solutions were regularly daily renewed. The lengths, numbers and fresh weights of shoots and roots for each plant were recorded every day. At the experiment of (18 days after seeding), plants were harvested and divided into shoot and roots. Shoot and root dry matter yields were determined for each pot treatment. Plant parts (shoots and roots) were dried and stored for further analysis, determination of metal concentrations. Analyses of Zn^{2+}, Fe^{2+} and Cu^{2+} were carried out with an inductively coupled plasma atomic emission spectrometry (ICP-AES, ultima 2).

In Vitro Absorption Experiment. The experiment of everted gut sacs of broilers intestinal segments as described by Ji et al. [20] was used as follows:

Mineral Free Tris-Krebs Buffer Solution. Tris-Krebs buffer solution (free of minerals) with the following composition (mmol/l, pH 7.0) was prepared: 15.5 Tris, 5.6 KCl, 120.7 NaCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$ and 11.5 glucose.

Everted Gut Sacs Procedure. One end of the sac was tied with cotton and 2.0 ml of mineral free Tris-Krebs buffer solution were put into the gut sac and the other end tied. Sacs were incubated for 80 min at 40°C (body temperature of broilers) in 20.0 ml of the same buffer containing individual different source of Cu at a constant concentration of 57,344 ppb, $CuSO_4$ (control) or Cu^{2+}-LPH complex. Ileal sacs were incubated in Tris-Krebs media of pH 7.0. All media were saturated with 95% O_2, 5% CO_2 before incubation. Three replicates of ileal sacs were used. At the end of experiment, the concentration of Cu was determined with an inductively coupled plasma under argon gas (ICP/MS, NEXION 300X series).

Statistical Analysis. Values are presented the means ± standard deviations for 6 replicates. Statistical analysis was performed using the Statically Analysis System for Windows (SAS 2008). One-way analysis of variance was performed to evaluate significant differences between sample means, with a significance level of $p < 0.05$. Means were compared by Duncan's test.

RESULTS AND DISCUSSION

Metal-Leather Protein Hydrolysate Chelates. Because leather protein hydrolysate obtained by CaO hydrolysis consists mainly of free amino acids [13]. It has been used in order to prepare metal-leather protein hydrolysate chelates (M-LPHCs). 2 mole of appropriate metal salt ($CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$ or $FeSO_4 \cdot 7H_2O$) was separately added to one mole of total free amino acids (the most appropriate ratio). One mole of total free amino acids to 2 moles of metal were chosen because this ratio was preferred (the most appropriate ratio). In this respect, a mixing of 10.0 ml of LPH with each of 1.46 g of ($CuSO_4 \cdot 5H_2O$), 1.72 g of ($ZnSO_4 \cdot 7H_2O$) or 0.912 g of ($FeSO_4 \cdot 7H_2O$) yielded 0.86 g of Cu^{2+}-LPHC, 0.70 g of Zn^{2+}-LPHC or 0.90 g of Fe^{2+}-LPHC, respectively. In this part of the present study, three different M-LPHCs were used: copper (II)-leather protein hydrolysate chelates (Cu^{2+}-LPHC), zinc-leather protein hydrolysate chelates (Zn^{2+}-LPHC) and iron (II)-leather protein hydrolysate chelates (Fe^{2+}-LPHC). The characterization and biological activities of these M-LPHCs were investigated.

Characterization of Metal-Leather Protein Hydrolysate Chelates. Analytical Data and Physical Properties. Some of analytical data, including moisture% of M-LPHCs, ion% chelated with LPHs as a ligand, M-ion% in M-LPHCs and yield% of M-LPHCs, and some physical properties, including the color of M-LPHCs (whether in solid state or solution), melting point (M.P, °C) and UV-vis λ-max (nm) are summarized and presented in Table 1. Regarding to the analytical data of M-LPHCs and the yield of each chelate it can be said that moisture% of M-LPHCs was ranged between about 19 and 26%,

about 1/5 or ¼ of M-LPHC. Concerning the reaction of metal salts with ligands (LPH) in order to prepare M-LPHCs, data also revealed that 23.68% of total Cu ions reacted with ligands (LPH) and formed Cu^{2+}-LPHC, 17.59% of total Zn ions reacted with LPH and formed Zn^{2+}-LPHC, and 22.91% of total Fe ions reacted with LPH and formed Fe^{2+}-LPHC. In other word, 23.68%, 17.59% and 22.91% of total Cu, Zn and Fe ions were incorporated in complex ions (Cu^{2+}-, Zn^{2+} and Fe^{2+}-LPHC, respectively).

The chemical analysis of M-LPHCs revealed that the solid form of M-LPHCs contained a different percentage of metal ion. The percentages of metal ions in the complexes reached 10.3% in Cu^{2+}-LPHC, 9.8% in Zn^{2+}-LPHC and 8.4% in Fe^{2+}-LPHC. The yield of complexation reaction percentage (dry weight of M-LPHC/salt weight × 100) was calculated and presented in Table 1. They recorded 57.72% for Cu^{2+}-LPHC, 40.70% for Zn^{2+}-LPHC and 98.68% for Fe^{2+}-LPHC. The obtained results showed that each M-LPHC has a particular color distinct for the chelate. The blue and Pale-yellow colors of Cu^{2+}-LPHC and Fe^{2+}-LPHC of course are attributed to the ions of copper (II) and ferrous or iron (II), respectively. It is not expected that the Zn^{2+}-LPHC have orange color because zinc ion is colorless. The orange color of Zn^{2+}-LPHC, whether in the form of solid state or solution, is probably due to the presence of chromium (VI) ion, where LPH contained a considerable amount of chromium ion. Chromium compounds in oxidation state (VI) has a beautifully orange color [21]. The melting points of M-LPHCs were almost close to each other and higher than 130°C (138.95, 135.50 and 130.44°C for Cu^{2+}-LPHC, Zn^{2+}-LPHC and Fe^{2+}-LPHC, respectively). This may be due to that the ligands in all chelates were the same (LPH).

TABLE 1
Some of analytical data and physical properties of metal-leather protein hydrolysate chelates (M-LPHCs) prepared from LPH obtained by CaO hydrolysis (LPH/CaO).

Compound	Moisture (%)	Ion chelated (%)	M-ion (%)	Yield (%)	Color		M.P (°C)	UV-vis λ-max (nm)
	Solid	Solid	Solid	Solid	Solid	Sol.	Solid	Sol.
LPH/CaO		-	-	-	Light-yellow	Light-yellow	-	314
Cu^{2+}-LPHC	22.03	23.68	10.3	57.72	Blue	Blue	138.95	234
Zn^{2+}-LPHC	19.05	17.59	9.8	40.70	Orange	Pale-orange	135.50	214
Fe^{2+}-LPHC	26.48	22.91	8.4	98.68	Pale-yellow	Light- green	130.44	214

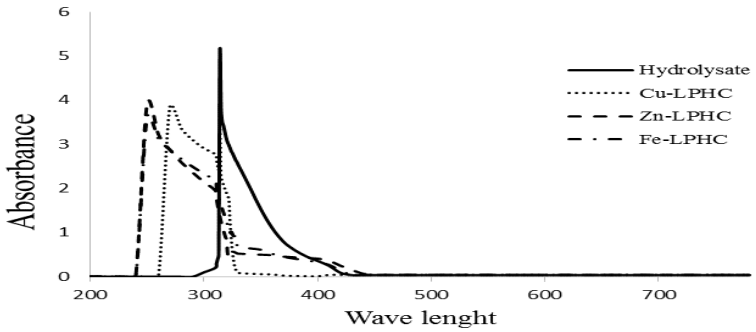

FIGURE 1
UV-Visible Spectra of metal-leather protein hydrolysate chelates.

TABLE 2
Some important IR bands (cm^{-1}) of leather protein hydrolysate chelates (LPHC) obtained by CaO hydrolysis (LPH/CaO) and its metal complexes

Compound	$\upsilon_{st}(NH_2)$	$\upsilon_{st}(C=O)$	$\upsilon_{s}(COO^{-})$	$\upsilon_{st}(O–H)$	$\upsilon_{st}(C–N)$
LPH/CaO	3367.10	1642.09	1412.60	3075.90	1111.76
Cu^{2+}-LPHC	3406.64	1623.77	1451.17	2514.72	1114.65
Zn^{2+}-LPHC	3543.56	1624.73	1447.31	3408.57	1132.01
Fe^{2+}-LPHC	3406.64	1625.70	1434.78	2514.72	1116.58

υ: vibration, st: stretching, s: symmetric.

In respect to UV-visible spectroscopy, the symmetry around the metallic ions was determined comparing the LPH and the saturated solution of metallic complexes UV-visible spectra. The electronic spectra of the complexes were recorded in water and their assignments (λ-max, wavelength of maximum absorbance in nm) were given in Table 1. One representative ligand field spectra of M-LPHCs are shown in Figure 1 and band position is presented in Table 1. The λ-max (wavelength of maximum absorbance) of M-LPHCs was in UV-region (234 nm for Cu^{2+}-LPHC, 214 nm for each Zn^{2+}-LPHC and Fe^{2+}-LPHC). As it's known, the peptide bond absorbs light in the range of 180 to 230 nm (which is called the "far-UV" range). The aromatic residues, tyrosine, tryptophan, and phenylalanine, also absorb light in this region and, in addition, show bands near 260 to 280 nm (in the "near-UV"). Therefore, the λ-max of metal-leather protein hydrolysate chelates may be due to the presence of aromatic amino acids and peptides in all-chelate preparations. Tripathi and Kamal [22] mentioned the characteristic π–π* transitions are observed in the spectrum of complexes at 257, 288, and 364 nm. The electronic spectrum also exhibits a broad band at 815 nm attributable to d-d transitions, which strongly distorted the octahedral geometry around the Cu(II) ion. The absorption bands of the complexes corresponded to the n→σ*, n→π* and π→π* transitions of -NH_2 and –COO. Shifts in these bands and the observed d-d transitions of the complexes indicated coordination. The UV-visible spectra of the complexes show absorption bands assigned to a large band around 634 nm. The presence of the later band mentions an octahedral stereochemistry for these complexes.

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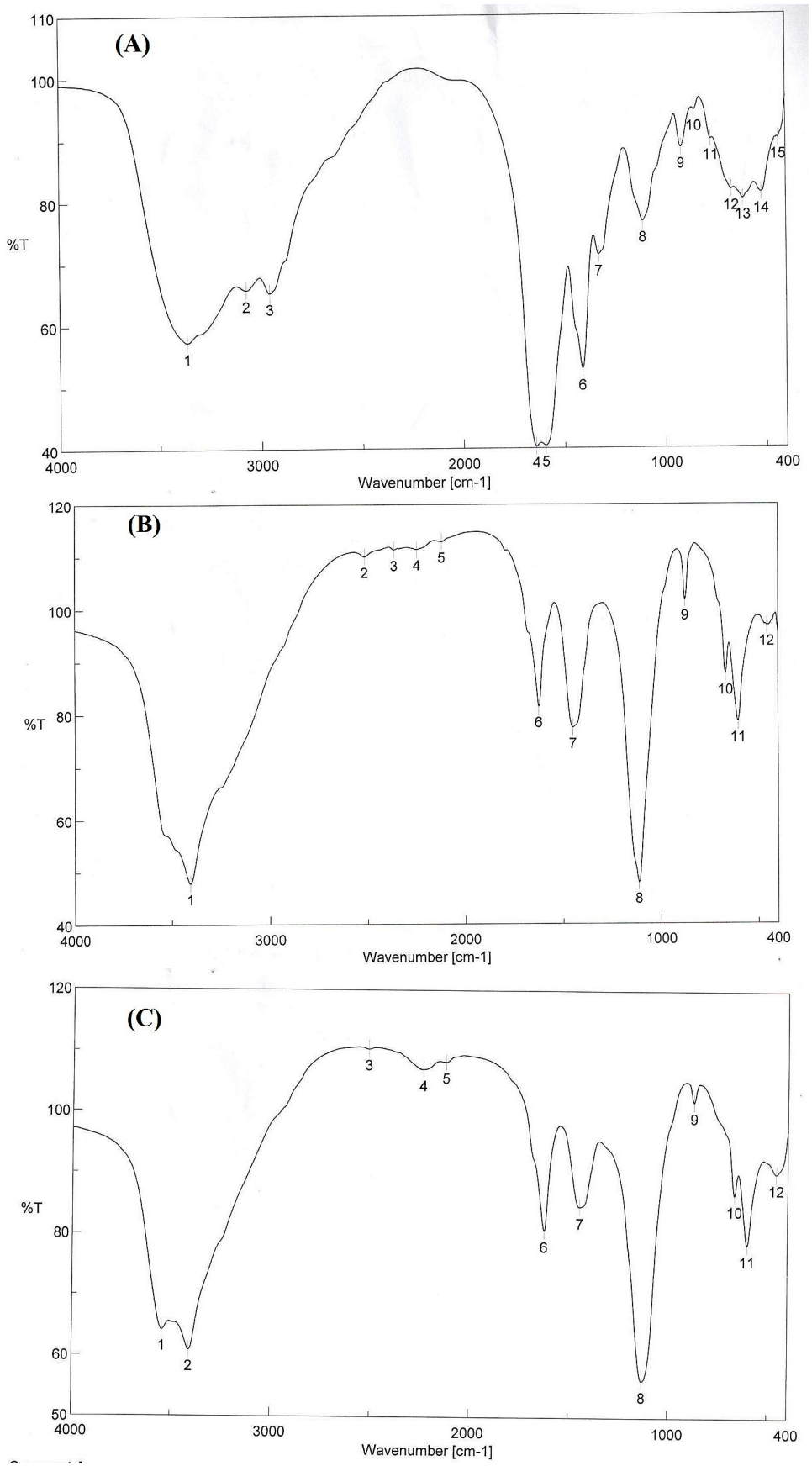
FTIR spectroscopy. The amino acids are existing as zwitterions in the crystalline state and predominant vibrations for free amino acid ligands are associated with υst(–NH_2), υst(C = O), υs(–COO^-), υst(O–H) and υst(C–N). In their complexes, the amino acids (AAs) are generally act as bidentate ligands with respect to pH, it binds with the metal by one oxygen and one nitrogen atom. Also, the noncoordinating groups (C = O) are hydrogen-bonded with the adjacent complex or with lattice water, as well as, it forms weakly bonded with the metal containing neighboring complex. Thus, υ(–COO^-) of AA complexes are affected by each of coordination and intermolecular interactions. The major factor in determining the frequency order in AA complexes is the coordination effect. Our data indicate that, the order of the metal–oxygen interaction increased because of the more asymmetrical of –COO^- group and the metal–oxygen interaction becomes stronger. The selected vibrations and assignments of LPH/CaO, Cu^{2+}-LPHC, Zn^{2+}-LPHC and Fe^{2+}-LPHC are described in Table 2.

The FTIR spectra (Figure 2) of three metal leather protein hydrolysate chelates showed an absorption pattern in the 4,000–400 cm^{-1} region which similar to region of AA. The predominant vibrations of the Cu^{2+}-LPHC, Zn^{2+}-LPHC and Fe^{2+}-LPHC are associated with υst(–NH_2), υst(C = O), υs(–COO^-), υst(O–H) and υst(C–N). The vibration bands for –OH and –NH from 3300-3500 cm_1 (peak no. 1 in Figure 2 A, B, C, D and peak no. 2 in Figure 2C) and the possibility of this vibration due to intermolecular hydrogen bond in polypeptides, histidine and arginine. The vibration band of –NH from 3130-3030 cm^{-1} and represented as peak No. 2 and 3, indicate the presence of free amino acids liberated from LPH (Figure 2A). The stretching band of –NH in amino acid from 1660-1610 cm^{-1}, peak No. 4 (Figure 2A) and peak No. 6 (Figure B, C, D). The vibration of C = O from 1550-1610 cm^{-1}, peak No. 5 (Figure 2A) related to aspartic and glutamic acid. δCH_2 from (1470-1430 cm^{-1}) in proline, peak No. 6 (Figure 2A) and No. 7 (Figure 2B, C, D). νC-OH (phenol) from 1230-1140 cm^{-1} in tyrosine (peak no. 8 in Figure 2C), and in secondary alcohol (theronine) from 1120-1100 cm^{-1}, peak no. 8 (Figure 2A, B, D). δCH in glycine from 500-1000 cm^{-1}, peaks No. 9, 10 and 11 (Figure 2A, B, C, D) and No. 12 (Figure 2A, B, C) and No. 13,14 and 15 (Figure 2A).

The observed vibrational bands of –NH2 groups around 3367.10–3543.56 cm_1 were very sensitive to the intermolecular interaction effect in the solid state and these bands sometimes appear to be wide broad. Moreover, the Cu(II)–NH_2– or Zn(II)–NH_2– or Fe(II)–NH_2–bond from the υ(–NH_2) are strong. In comparison with the free AA, the vibration of N–H bands appears like to be shifted in direction of higher frequency of the three metal-leather protein hydrolysate chelates (Cu^{2+}-LPHC, Zn^{2+}-LPHC and Fe^{2+}-LPHC), with the involvement of the amine group in the complex formation. The carboxylate ion of AA coordinates to Cu(II) or Zn(II) or Fe(II) as a unidentate mode. The C = O groups of Cu^{2+}-LPHC, Zn^{2+}-LPHC and Fe^{2+}-LPHC have approximately the same frequency around 1623.77–1642.09 cm^{-1} and the υ(CO) is metal-sensitive [23].

Biological Activities of Metal-Leather Protein Hydrolysate Chelate. Antibacterial Activity. The antibacterial activities of metal-leather protein hydrolysate chelate (Cu^{2+}-LPHC, Zn^{2+}-LPHC and Fe^{2+}-LPHC) prepared from leather protein hydrolysate obtained by CaO hydrolysis (LPH/CaO) were investigated against isolated gram-positive strains (*Bacillus cereus* and *Micrococcus* spp.) and one standard gram-negative bacteria (*Escherichia coli*). According to the results given in Table 3, all metal-leather protein hydrolysate chelates had potential of antibacterial activity against bacteria species tested.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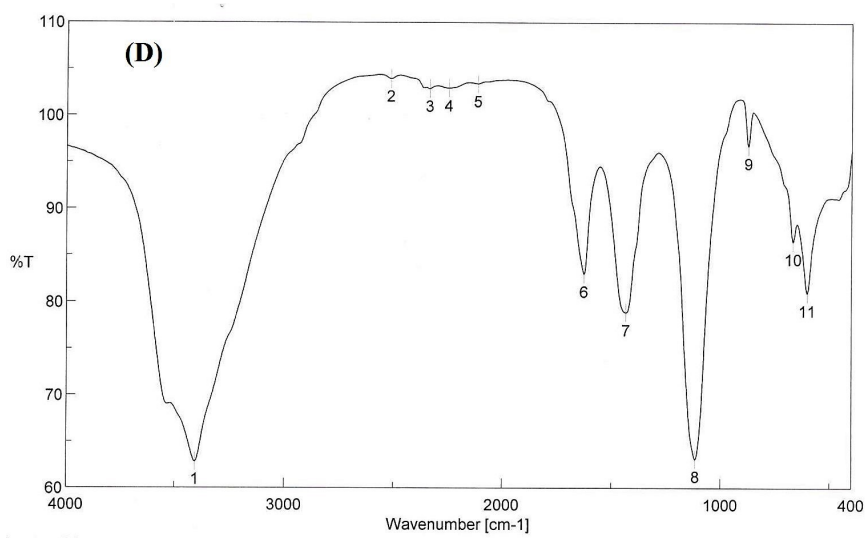

FIGURE 2
FTIR spectral analysis of LPH/CaO (A), Cu^{2+}-LPHC (B), Zn^{2+}-LPHC (C) and Fe^{2+}-LPHC (D).

The M-LPHCs were mainly active against gram-positive strains. The data indicated that the volumes of M-LPHCs tested (30, 60 and 90 µl/disc) exhibited varying levels of antibacterial activity as compared with Flumoquine (positive reference standard). Some of M-LPHCs have no antibacterial activity at low level (30 µl/disc). For example, at 30 µl/disc, Zn^{2+}- and Fe^{2+}-LPHC had no activities against *E. coli* whilst Cu^{2+}-LPHC had no activity against *Micrococcus* spp. Generally, it was clear that there was a direct correlation between the volume (concentration) of M-LPHC and its inhibitory activity against bacteria species tested. The most active chelate was Zn^{2+}-LPHC followed by Fe^{2+}-LPHC then Cu^{2+}-LPHC. The antibacterial activity of Flumoquine (positive reference standard) and metal-leather protein hydrolysate chelates at a higher volume (90 µl/disc) for *Escherichia coli* was in the following order: Flumoquine (3.54 mm) > Zn-LPHC (2.70 mm) > Fe-LPHC (1.70 mm) > Cu-LPHC (1.40 mm), while for *Bacillus cereus* the order was: Fe^{2+}-LPHC (3.75 mm) > Zn^{2+}-LPHC (3.10 mm) > Flumoquine (3.01 mm) > Cu^{2+}-LPHC (1.75 mm) and for *Micrococcus* spp. the order was: Zn^{2+}-LPHC (5.05 mm) > Cu^{2+}-LPHC (3.50 mm) > Fe^{2+}-LPHC (2.55 mm) > Flumoquine (1.46 mm).

The previous studies reported that the antibacterial activity of the amino acid complex are affected by its stability, where, the lower stability, the greater antibacterial activity. This may be due to the presence of high free ions in the solution, which enhance the interaction between the ligands and the metal ions [24,25]. Moreover, the activity of the complex as antibacterial may be referred to presence of partially sharing between the positive charge of the metal and the ligands donor atoms which increases the lipophilic properties of the metal chelate and facilitation its movement through the phospholipid bilayers of the bacterial cell membranes. In addition to,

TABLE 3
Inhibition zone (mm) of metal-leather protein hydrolysate chelates (Cu^{2+}-LPHC, Zn^{2+}-LPHC and Fe^{2+}-LPHC) prepared from leather protein hydrolysate obtained by CaO hydrolysis against test strains

Hydrolysate complex	Volume (µl/disc)	Diameter of inhibition zone (mm)		
		E. coli	*B. cereus*	*Micrococcus* spp.
Cu^{2+}-LPHC	30	1.32	1.00	0.00
	60	1.40	1.42	2.15
	90	1.60	1.75	3.50
Zn^{2+}-LPHC	30	0.00	1.47	2.65
	60	1.10	2.10	3.65
	90	2.70	3.10	5.05
Fe^{2+}-LPHC	30	0.00	0.40	0.00
	60	0.85	1.75	0.50
	90	1.70	3.75	2.55
Flumoquine (St., 30 µg/disc)	-	3.54	3.01	1.46

other factors such as conductivity, solubility and dipole moment may also be the possible reasons of the antibacterial activity increasing [26].

Application of Metal-Leather Protein Hydrolysate Chelates as Plant Growth Promoters in Hydroponic Nutrient Solution. It was suggested that the complexation of mineral ions, such as Cu, Zn and Fe, with any ligand, such as EDTA and amino acids, might improve the uptake of minerals by the plant. Accordingly, the present study was interested and designed to study the effect of metal-leather protein hydrolysate chelates (Cu^{2+}-LPHC, Zn^{2+}-LPHC and Fe^{2+}-LPHC) prepared from leather protein hydrolysate obtained by CaO hydrolysis (LPH/CaO) on the spinach grown in hydroponic nutrient solution. The salts of mineral ions ($CuSO_4$, $ZnSO_4$ and $FeSO_4$) in the hydroponic nutrient solution were individually substituted with M-LPHCs (Cu^{2+}-, Zn^{2+}- or Fe^{2+}-LPHC). The growth characteristics of the grown spinach and the content of each meaning mineral in plants (Cu, Fe and Zn) at the end of experiment (18 days) were determined.

Plant Growth Characteristics. The data on the effect of metal-leather protein hydrolysate chelates under investigation on the growth characteristics, including leaf numbers, leaf length (cm), shoot length (cm), shoot weight (g), root numbers, root length (cm) and roots weight (g), of spinach grown in hydroponic nutrient solution were summarized and illustrated in Table 4. Generally, application of M-LPHCs as sources of micronutrients (Cu, Zn and Fe) instated of mineral salts significantly increased growth characteristics of spinach plants relative to control. Application of Cu^{2+}-LPHC and Zn^{2+}-LPHC caused the greatest increase in growth characteristics of spinach plants compared with Fe^{2+}-LPHC. The Zn^{2+}-LPHC was the best to improve the most growth characteristics of spinach plants whilst the Fe^{2+}-LPHC was the lowest one to improve growth characteristics. From these results it can be say that M-LPHCs had stimulating effects on spinach plants grown in hydroponic nutrient solution. The stimulating effects of M-LPHCs on the growth of plant are depending on the plant cultivar and the type of ligand (amino acid).

Minerals Content of Plants. Table 5 shows the effect of Cu^{2+}-, Zn^{2+}- and Fe^{2+}-LPHC on the plant contents of copper, zinc and iron (ppm) of spinach grown in hydroponic nutrient solution. Our study results confirmed the greater efficacy of Cu^{2+}-LPHC, Zn^{2+}-LPHC and Fe^{2+}-LPHC in supplying Cu or Zn or Fe to spinach plants compared with that of control ($CuSO_4$, $ZnSO_4$ and $FeSO_4$). As shown in Table, copper and zinc contents of plants grown in hydroponic nutrient solution supplied with Cu^{2+}-LPHC or Zn^{2+}-LPHC reached about 200%, or more for Zn, relative to control supplied with mineral salts ($CuSO_4$ or $ZnSO_4$), i.e. the uptake of Cu or Zn by plants was doubly that of control. The same was observed with Fe^{2+}-LPHC where the ratio reached 126.07% relative to control. This means that LPHs as a ligand increase the uptake of micronutrients by plants. The results indicated that using of metal-leather protein hydrolysate chelates in the plant nutrition (nutrient solution) could improve growth characteristics of spinach plants (stimulating effect) and also supply sufficient amounts of minerals for plant uptake.

The stimulating effect of metal-leather protein hydrolysate chelates on spinach growth could be due to the amino acids role, which exist in abundance in LPH, in improving the rate of plant growth, cell division, and/or cell development [27]. Pervious study performed by Nassar et al. [28] who found that the positive effect of Arg on both of bean root and shoot growth was accompanied with elevation of certain plant growth regulators levels. Our study indicated, the growth-stimulating effect of Cu^{2+}-LPHC and Zn^{2+}-LPHC was greater than that of Fe^{2+}-LPHC, this may have revealed to the variation of mineral concentration used. Previous studies reported the effects of various amino acids on plant growth [29, 30].

TABLE 4
Effect of metal-leather protein hydrolysate chelates (Cu^{2+}-LPHC, Zn^{2+}-LPHC and Fe^{2+}-LPHC) prepared from leather protein hydrolysate obtained by CaO hydrolysis on growth characteristics of spinach grown in hydroponic nutrient solution.

Compound	Leaf No.	Leaf length (cm)	Shoot length (cm)	Shoot weight (g)	Root No.	Root length (cm)	Roots weight (g)
Mineral salts (control)	$3.0^{a}\pm0.87$	$2.75^{ab}\pm0.64$	$4.8^{a}\pm0.45$	$46.6^{b}\pm7.69$	$6.3^{c}\pm1.041$	$6.7^{a}\pm0.88$	$1.3^{b}\pm0.06$
Cu^{2+}-LPHC	$4.7^{a}\pm0.29$	$3.60^{a}\pm0.21$	$5.7^{a}\pm0.06$	$79.3^{a}\pm6.11$	$17.0^{b}\pm1.32$	$10.7^{a}\pm1.46$	$2.4^{b}\pm0.17$
Zn^{2+}-LPHC	$4.3^{a}\pm0.29$	$3.63^{a}\pm0.06$	$5.7^{a}\pm0.50$	$82.7^{a}\pm7.59$	$22.0^{a}\pm1.50$	$11.2^{a}\pm1.72$	$3.9^{a}\pm0.22$
Fe^{2+}-LPHC	$3.3^{a}\pm0.58$	$1.92^{b}\pm0.20$	$4.5^{a}\pm0.46$	$43.4^{b}\pm4.22$	$16.7^{b}\pm1.26$	$8.1^{a}\pm0.40$	$4.1^{a}\pm0.72$
L.S.D	2.105	1.324	1.544	24.6871	4.861	4.6426	1.459

-Values are means of three replicates ± SE. Numbers in the same column followed by the same letter are not significantly different at $P<0.05$.

TABLE 5
Effect of copper-, zinc- and iron-leather protein hydrolysate chelates (Cu^{2+}-LPHC, Zn^{2+}-LPHC and Fe^{2+}-LPHC) prepared from leather protein hydrolysate obtained by CaO hydrolysis on copper, zinc and iron contents (ppm) of spinach plant grown in hydroponic nutrient solution

Concentration	Copper		Zinc		Iron	
	$CuSO_4$	Cu-LPHC	$ZnSO_4$	Zn-LPHC	$FeSO_4$	Fe-LPHC
ppm	0.26	0.52	0.32	0.68	26.12	32.93
%	100	200	100	212.5	100	126.07

TABLE 6
Absorption of copper-leather protein hydrolysate chelate (Cu^{2+}-LPHC) prepared from leather protein hydrolysate obtained by CaO hydrolysis and copper sulphate ($CuSO_4$) in the everted sacs of ileum after incubation time (80 min)

Compound	Cu concentration (ppb)		
	Inside the everted ileum sac	Outside the everted ileum sac	Absorption%
$CuSO_4$ (control)	7002.8	50341.3	12.21
Cu^{2+}-LPHC	20983.0	36361.1	36.59

The great ability of amino acids to forming complexes with Cu or Zn or Fe increase the bioavailability of these metals for plant uptake [31]. Furthermore, stimulated plant growth by amino acids, i.e. LPH, may result in a greater ability for Cu or Zn or Fe uptake in roots. The pervious study performed by Zhang et al. [32] reported that, the addition of amino acids to the nutrient solutions increase the uptake and translocation of Zn from root to shoot in tomato. Another study observed, the positive effect of amino acids on nutrient uptake and growth in marigold plants [33]. Regarding to our obtained results, the effect of Zn^{2+}-LPHC on shoot and root Zn accumulation varied with amino acid type. The chelators used in nutrient solutions may be transported into the plant tissue by an undeveloped casparian band at the root tip. A high concentration of chelates can remove calcium (Ca^{2+}) from the cell membrane and impairs root membrane integrity [34].

Uptake of Copper by In Vitro Everted Gut Sacs of Broilers. The effect of Cu^{2+}-LPHC prepared from leather protein hydrolysate obtained by CaO hydrolysis as a source of copper, in comparison to Cu ion ($CuSO_4$ as control) and on the absorption (bioavailability) of Cu was studied. Absorption was measured as the uptake of Cu by everted sacs. This system has been shown to be rapid and useful in predicting the trend of the absorptive response in intact animals. The absorption, in general, can take place by passive transport, involving simple diffusion, provided there is a high concentration of the nutrient outside the cell and a low concentration inside. In this system (in vitro everted gut sacs), the absorption of a nutrient (Cu) from the lumen of the intestine can take place from outside of everted gut sac with a high concentration of nutrient to inside of everted gut sac with a low nutrient concentration.

The observed Cu concentrations (ppb) inside and outside the everted ileam and absorption percentage of Cu^{2+}-LPHC relative to control ($CuSO_4$) by ileum sacs incubated for 80 min are reported in Table 6. The uptake percentage of Cu in the form of chelate by everted ileum sac was about 3 times that in the ionic form. From these results it can be say that LPH chelates (leather protein hydrolysate chelates) increase the bioavailability (absorption) of mineral ions, including Cu, by ileum.

From the data it could be concluded that LPH as a ligand was effective in facilitating Cu absorption. Organic Cu was more efficiently absorbed than inorganic Cu ($CuSO_4$) under the conditions of this study. An enhancement of Cu absorption by organic ligand has also been reported [35]. The absorption of Cu from protein chelate in the separated intestinal segments of mice was 4 times that of $CuSO_4$. These findings support the results of the current study whereas it has been proven that the uptake percentage of Cu^{2+}-LPHC was about 3 times greater than in the absence of ligand. While there are many different forms of mineral supplements available, it has been demonstrated that amino acid chelates are superior in many respects. Mineral glycine chelates, for example, have been shown to be stable and bioavailable. The unique bonding characteristics of these organic minerals set the amino acid chelate in a class of its own.

Finally, the novelty of our study is, the LPH obtained by CaO (LPH/CaO) hydrolysis is considered a source of free amino acids with low cost. LPH/CaO has potential to be used as complexing agent (ligand) with metal ions (metal-LPH chelates, i.e. Cu^{2+}, Zn^{2+} & Fe^{2+}-LPHCs). Good results are obtained with M-LPHCs as a source of metal ions in plant nutrition where the synergistic effect was evident. The obtained results in the present research strongly proved that Cu^{2+}, Zn^{2+} & Fe^{2+}-LPHCs are recommended to increase the plant growth promoters of spinach plants grown under hydroponic nutrient condition. On the other hand, we claim that the cost of 100 grams of M-LPHC crystals in average of 2.5 $ while the cost of imported one liter of metal-amino acid

chelates in average of 12.5 $. In conclusion, this research can help the leather industry in solving the difficult CCLW disposal problem and obtain economic benefits.

CONCLUSIONS

Finally, it can be concluded that metal-leather protein hydrolysate complexes have many benefits as antimicrobial and micronutrients for plant and increase the bioavailability of mineral ions by ileum. The use of M-LPHCs supports the plant with micronutrients and is a natural source of organic nitrogen, as well as support the bioavailability of minerals in animals.

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