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Rice Milk Fortification Using Calcium Hydroxyl Phosphate Nanoparticles and Hydrolyzed Peanut Protein Fractions

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THIS study aimed to develop a rice milk-based meal (Roz Bel Laban) containing the necessary nutrients for overcome malnutrition problems. The meal was prepared in three stages and its antimicrobial activities and sensory properties were evaluated. First, calcium hydroxyl phosphate nanoparticles (CaHPNPs) were synthesized. Their morphology and particle size were examined by high-resolution transmission electron microscopy, and the composition was investigated by X-ray diffraction. Second, peanut meal (PM) was hydrolyzed with different solvents using ultrasonic waves and analyzed by native and SDS electrophoresis. Third, both CaHPNPs and hydrolyzed peanut (HP) were added while cooking the rice milk. The rice milk cooked with (HP + CaHPNPs) showed greater quality and storage time than that cooked with HP only. The quality and storage time were the lowest for the control. The fortified rice milk also displayed various antimicrobial activities. The bacterial species most affected by the HP extract were Salmonella typhi (inhibition zone = 11.5 mm) and B. cereus (inhibition zone = 11.0 mm). All fortified rice milk was free of proteolytic and lipolytic bacteria, while two bacterial isolates were categorized based on their phenotypic characteristics in the control. Our findings suggest that HP and CaHPNPs can be used to improve the nutritional value and antibacterial properties of prepared foods.

Keywords: Calcium hydroxyl phosphate nanoparticles, Peanut meal, Bioactive compounds, Antioxidant, Electrophoresis, Antimicrobial, Sensory properties.

Introduction

Hunger and malnutrition in developing countries are responsible for disease outbreaks such as cholera, malaria, polio and cancer that cause morbidity and mortality besides death directly through starvation. Recently, international aid agencies have increasingly focused on what is referred to public health scientists and the World Health Organization as the "epidemiological transition" issue [1].

The aim of this study was to optimize waste of peanut by-products as a new theory to alleviate hunger/malnutrition in poor and developing countries, because Peanut is considered one of the most important sources of vegetable protein, and has been received increasing attention in food industry as an additive in dairy products and health baked food [2]. Peanuts (*Arachis hypogaea* L.) contain essential nutrients, including minerals such as iron, potassium, zinc, magnesium, copper and selenium, vitamin E, and most B vitamins, and they also display antioxidant capacity [3, 4]. According to the protein digestibility-corrected amino acid score, proteins present in peanut, soy, and other legumes are nutritionally equivalent to meat and eggs for human growth and health [5]. Dissociated oil seed protein byproducts/waste

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materials (e.g., soybean, sunflower, peanut and cottonseeds) are used as fertilizers and animal feed [6,7]. Peanut seed proteins possess good functional properties, and combine arachin and non-arachin components with several polypeptides. Arachin contains six molecular weight classes (between 15,500 and 68,000) of polypeptides with isoelectric points between 4.7 and 8.4, and non-arachin consists of nine molecular weight classes (between 16,000 and 170,000) with isoelectric points between 4.7 and 7.9 [8].

Children without awareness prefer fast food, which is poorer in quality, higher in energy, more expensive and lower in nutrients than homemade food. Yogurt, cheese, and rice milk can serve as dairy milk substitutes and provide the body with the calcium needed for bone growth in school-aged children who dislike drinking milk. Even so, rice, soy, and almond milk have emerged as attractive plant-based alternatives to products for health reasons [6,7].

Rice (*Oryza sativa* L) is one of the principle foods consumed by humans around the world especially in Asia and Egypt [9].

Commercial and homemade rice milk is often supplied with sugars and some flavoring agents. In addition to being easy and fast to cook, rice milk is rich in carbohydrates and low in fat. However, because of its low protein, Calcium and phosphates content, this type of rice milk is not as nutritionally balanced as dairy milk. The addition of small nCaP which exhibits high solubility at different concentrations, fast transport in the physiological system are of interest for many biomedical applications due to their good biocompatibility and bioactivity [10, 11, 12].

For the above reasons, this study was based on the dissociation of peanut protein byproducts. To identify the best cost-effective proteins equivalent to those in meat and eggs for human growth and health, the authors prepared high-quality and low-cost (HP) samples that differ in dissociation behavior and properties. The sample with the best dissociation was mixed with rice milk ingredients and the mixture was cooked with and without calcium hydroxyl phosphate nanoparticles (CaHPNPs). This work suggests that acid and base-(HP) with CaHPNPs as nutritional supplements in a simple food such as rice milk may serve as the basis for a solution to hunger/ malnutrition, vitamins and mineral deficiency.

Materials and Methods

All the chemicals and solvents used in this study were purchased from Sigma-Aldrich, St. Louis, MO, USA. Calcium hydroxide and orthophosphoric acid as calcium and phosphorous precursors, respectively.

Peanut (Arachis hypogea L.) was brought from the local market.

Recipe: Ingredients of rice milk: 100 g Egyptian Rice [white], 500 to 600ml water , 25 g dry milk(milk powder derived from cow's milk), 10 g hydrolyzed peanut meal with or without Nano (Ca,PNps), vanilla, granulated sugar, to taste and two tea spoons of starch if needed.

Note: The proportions of rice to water may vary depending on the type of rice used and your own preference for thick or thin rice milk (Table 1).

TABLE 1. Ingredients used in Egyptian rice milk preparation.

Ingredient Amounts (g)	Egyptian Rice Milk (control)	Egyptian Rice Milk with Hydrolyzed Peanut	Egyptian Rice Milk with Hydrolyzed Peanut& CaHPNPs
Egyptian Rice (white)	100	90	90
Hydrolyzed Peanut	0.0	10	10
Dry Milk	25	25	25
Vanillia	0.5	0.5	0.5
Peanut Protein	0.0	5.5	5.5
Total Protein Added	5.25 from milk	10.75 from milk+ (HP)	10.75 from milk+(HP)
Peanut Fat	0.0	0.0	0.0
Milk Fat	7.0	7.0	7.0

Microbial strains

Listeria monocytogenes V7 and *Yersenia enterocolitica subsp. enterocolitica* ATCC9610TM were obtained from Liofil chem S.r.l. Italy. *Bacillus cereus* (ATCC133018), *Salmonella typhimurium* 14028, *E. coli O157:H7* (ATCC 6933) and *staph. aurius* (ATCC 20231) obtained from the stock cultures of the Agricultural Research Centre in Giza., and *Aspergillus flavus (A. flavus)* ATCC 16872.

Preparation of calcium hydroxyl phosphate nanoparticles

Calcium hydroxyl phosphate nanoparticles (CaHPNPs) have been prepared according to a direct precipitation reaction between orthophosphoric acid solution and calcium hydroxide solution as described by Bianco et al. [13]. The procedure was carried out with ratio of calcium and phosphorus more than the stoichiometric one (Ca/P = 1.67).In this work, the aqueous solution of calcium hydroxide and the solution of orthophosphoric acid were prepared with deionized water (Milli-Q, Millipore, USA), respectively. The orthophosphoric acid solution was slowly added into calcium hydroxide solution at room temperature under vigorous stirring. The obtained mixture was then stirred by a magnetic stirrer for 2 hours at the speed of 1000 rpm, aged for 24 hours at room temperature and centrifuged to complete the precipitation process. The precipitate was dried at 100°C during 12 hours.

The morphology and particle size of calcium hydroxyl phosphate was observed using high resolution transmission electron microscope (TEM). The transmission electron microscope (TEM) images were taken on (HR-TEM, Tecnia G20, FEI, Netherlands), operating at 80 kV, and the composition was investigated by X-ray diffraction (XRD, X'pert Pro, Pan Analytical, Netherlands) in the 2θ range 0° to 80° using CuKα1 radiation (λ=1.54056 Å).

Preparation of defatted peanut meal (PM)

Peanuts were hulled manually, The seeds were hydraulic pressed(as obtained from oil Co.), and then was subjected in the laboratory to complete defatting using a soxhlet apparatus and *n*-hexane as defatting solvent, then allowed to air-dry in a fume hood to remove residual hexane. The resulting defatted meal was ground in coffee mill to obtain a finely divided material suitable for extraction studies then saved in refrigerator until used.

Preparation of Hydrolyzed Peanut (HP)

Dried PM (1g) mixed with 90ml of each solvent [Ethanol: 0.5 N HCl (70:30), Ethanol: 0.5 N NaOH (70: 30), 1 N NaOH, 0.5 N NaOH, 1 N HCl and 0.5 N HCl], leave in the ultrasonic bath for one hour, soaking with the same solvents 24 h and stirring 15 min. The hydrolyzed samples were centrifuged, and the soluble phase were stored at -20°C until testing, while the residue (insoluble phase) allowed to air-dry in a fume hood and saved in cool place.

Analysis of soluble phase (Soluble protein, phenolics, Flavonoids and Saponins) of (HP)

Determination of Soluble protein

Protein was determined by using Bradford method [14]. Hence, the assay is based on the colour change of coomassie brilliant blue dye G250 in response to protein concentrations. The absorbance of the mixture was measured after 5 minutes (and before 1 hr) at 595 nm against blank (prepared from 0.1 ml of bidistilled water and 5ml of dye reagent).

Determination of Soluble Phenolic

The content of phenolic compounds was determined according to Mc Donald et al. [15]. Absorbance of the solution was measured at 765 nm using a spectrophotometer (T80 UVvis spectrophotometers).

Determination of Soluble Flavonoids

The colorimetric determination of soluble Flavonoids was performed according to Kanatt et al., [16].

Determination of Soluble Saponins

The colorimetric determination was performed according to Hiai et al. [17].

Evaluation of antioxidant activity of all soluble phase

For each hydrolyzed sample three series of antioxidant capacity methods were applied, 2, 2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, hydrogen peroxide (H_2O_2) scavenging and total reducing-capability of the free radical-scavenging assay (Table 2).

1- DPPH radical-scavenging

The method described by De Ancose et al [18] was utilized to determine the DPPH radical-scavenging .The reduction of the DPPH radical was measured at 517 nm. Results were expressed as percentage inhibition of the DPPH using the following equation:

Inhibition of DPPH (%) = absorbance control-absorbance sample/absorbance control × 100

Where, absorbance control is the absorbance of DPPH solution without extract.

2- Hydrogen peroxide (H_2O_2) scavenging

The H_2O_2 scavenging ability of each extract was determined according to Sfahlan et al. [19]. The absorbance value of the reaction mixture was recorded at 230 nm after 10 min.

3- Estimation of total reducing capability

The reducing power of each extract was determined according to Zhao et al. [20].The absorbance was measured spectrophotometrically at 700 nm. The measurement was compared to the standard curve of prepared BHT solution. The final results were expressed as milligram of BHT equivalents per gram based on dry weigh

Electrophoresis

A- native-electrophoresis

Samples analyses: About 0.01 g of the dried (HP) insoluble fractions was mixed with 0.5 mL of sample buffer. Gel preparation and sample loading were similar as the running conditions according to the methods prescribed by Jensen &Lixue and Laemmli [21, 22]. Except that SDS and beta mercapto-ethanol were not the part of sample and running buffers. The concentrate separating gel were prepared 8%.

B-Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDSPAGE was performed according to the method of Laemmli [22]. The soluble and insoluble fractions of all hydrolyzed samples were analysed by polyacrylamide gels vertical type using a Mini-protein II Electrophoresis.. Using 12% separating gel and 5% stacking gel according to the methods prescribed by Jensen & Lixue and Laemmli [21,22].

Gel documentation and analysis: Molecular weight of different bands was compared with (FISHER BIO reagents) a mixture of standard protein markers include range in size from 10-200 kDa when analyzed by SDS-PAGE and stained with coomassie blue.

Preparation of fortified rice milk

First: Method of preparation of rice milk

The rice was rinsed under running water, drained, put in a sauce pan and covered completely with water. The mixture of rice and water was boiled and cooked under low heat for ten minutes. The boiled milk was added to the cooked rice, then the (HP) with or without Nano (Ca,HPNps), vanilla and sugar were added. The final rice milk was poured in dessert bowls and let cool then refrigerate until serving.

Second: Fortified rice milk:

Rice milk was fortified with (HP) and (CaHPNPs) as in Fig.1.

The control sample was made by the same method (Table 1) but without any additions of (HP) or (CaHPNPs). All samples were examined every week for its microbiological analysis during storage period time at 4-5 ±2 °C. (Table 5).

Antimicrobial activity of hydrolysed peanut (HP) and (HP + CaHPNPs)

Assay for antimicrobial activities: The (HP) and (HP + CaHPNPs) were examined for its antimicrobial against *L. monocytogenes V7* and *Y. enterocolitica* ATCC9610TM, *B. cereus* (ATCC133018), *Salmonella typhimurium* 9027, *E. coli O157:H7* (ATCC 6933), *staph. aureus*(ATCC 25175) and *A. flavus (A. flavus)* ATCC 16872. using disc diffusion method Assefa et al, [23]. The (HP) and (HP + CaHPNPs) were absorbed onto sterile filter paper discs (diameter: 7 mm) individually. Each agar plate had two sample discs and one control discs (with sterile H_2O) was incubated at 37°C for 24h, then inhibition zones around were measured. All experiments were repeated for three times.

Microbiological examinations of Rice milk with (HP)

The refrigerated samples of Rice Milk with HP and ca HPNPs were examined microbiologically at zero time and at time intervals of 5, 10, 15 and 21 days for their safety and quality: Rice Milk with and without HP and caHPNPs were microbiologically examined according to APHA and FDA [24, 25] for total aerobic colony count (TACC) using plate count agar, molds and yeasts counts using acidified potato dextrose agar, enumeration of *S. aureus* using Baird Parker agar medium, and Coliform group using violet red bile agar (VRBA). All media used were Oxoid Co. brand.

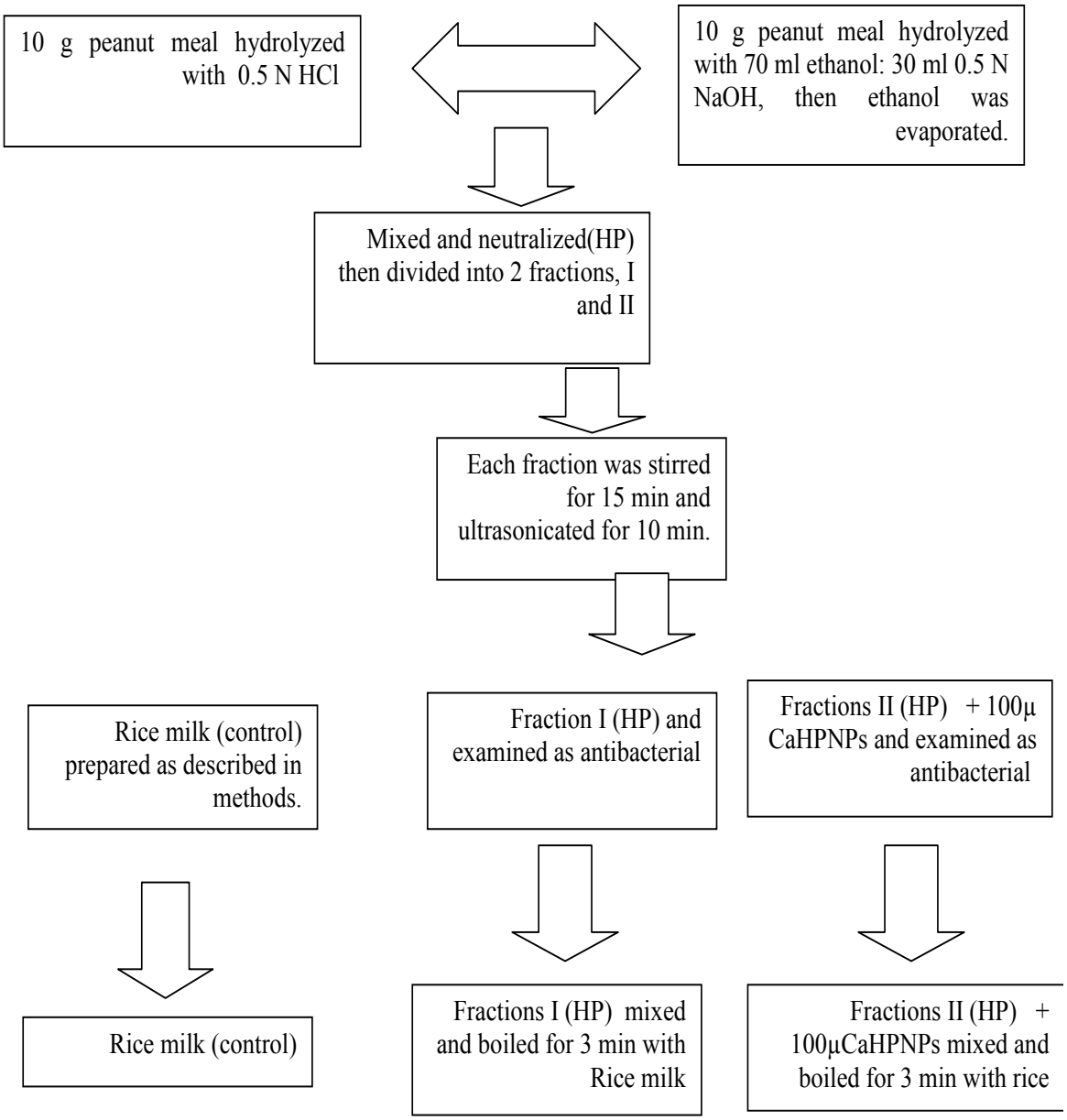


Fig. 1. A flow diagram of mixing (HP) and (CaHPNPs) with rice milk during cooking.

Proteolytic and lipolytic bacteria

Proteolytic and lipolytic bacterial isolation and identification were carried out according to APHA, Harrigan & McCance, and Tanasupawat et al. [25, 26, 27].

Calculations and statistics

All results were carried out in triplicates and values were expressed as Means ± Standard Deviation (SD). Significant statistical differences of investigated parameters were determined and analyzed using one way analysis of variance (ANOVA PC-STAT, 1985 version IA copyright, University of Georgia).

Results and Discussion

The aim of the current paper is to prepare a low-cost and easily prepared formulation of rice milk with calcium hydroxyl phosphate nanoparticles and hydrolyzed peanut protein. The preparation involves synthesizing the nanoparticles, hydrolyzing peanut meal using acidic or basic solutions, and incorporating the resulting additives in the rice milk preparation before cooking Figure (1). These fortified rice milk exhibited higher nutritional value and shelf

life. The preparation process involves three steps and starts with the synthesis of the nanoparticles the authors performed HR-TEM, to show the shape and/or size of the particles Figure (1). And X-ray powder diffraction (XRD) to improve pattern of synthesized CaHPNPs. High-resolution transmission electron microscopy (HR-TEM) analysis Figure (2) showed that the CaHPNPs adopted a nanorod-shaped morphology with diameters between 14.5 and 21.1 nm and lengths between 33 and 57.5 nm and these data indication to their large surface area. Figure (3) shows a typical X-ray powder diffraction (XRD) pattern of synthesized CaHPNPs with low crystalline shape. The pattern shows broad peaks at 2 theta values of 25.8°, 31.8°, 32.2°, 32.9°, and 49.4°, in good agreement with previous data for the calcium hydroxyl phosphate structure (JCPDS 01-074-9761. The main (h k l) indices for nanometer-sized HAP are (002), (121), (112), (300), and (213) indicating a finer crystal size and perfect structure, the above data indication of Calcium phosphates combine several features, such as their large surface area facilitates the incorporation of drugs and easy-to-transport systems with desired release profiles [11,12]. Calcium phosphate nanoparticles (CaPNPs) exhibit synergistic growth promotion [28]. Monocalcium phosphate monohydrate (MCPM) is the most acidic and water-soluble CaP phase. Marked as food additive E341, it is often added to toothpastes. However, pure MCPM is not biocompatible with bone due to its acidity [29, 30].

The aim of addition calcium phosphate nanoparticles is to provide the body with the calcium and phosphates needed for bone growth in school-aged children who dislike drinking milk.

The effects of CaHPNPs on the antimicrobial activity of hydrolyzed peanut and sensory properties of rice milk were investigated (Tables 5, 6 and 7). The fortified rice milk in this study exhibited higher antimicrobial activity and shelf life than rice milk alone. Commercial rice milk is often low in Calcium and phosphates content. These results agree with those of a previous study involving a composite coating consisting of antibacterial agents, such as lactoferrin, tetracycline, and gatifloxacin, immobilized on an ethylenevinyl alcohol copolymer and integrated into CaP. The composite exhibited activity against E. coli and S. Aureus [31]. CaHPNPs can be incorporated with either drug release systems or antifouling agents to prevent infection [28].

Hydrolyzed peanut

Peanut posses high nutritive value, an excellent source of protein, which can be supplied to people who suffering from hunger and malnutrition especially in the developing and underdeveloped countries. Peanut meal can be used for human consumption after partial hydrolysis of its protein. Such products are easily digestible and nutritious.

This work briefly describes various treatments

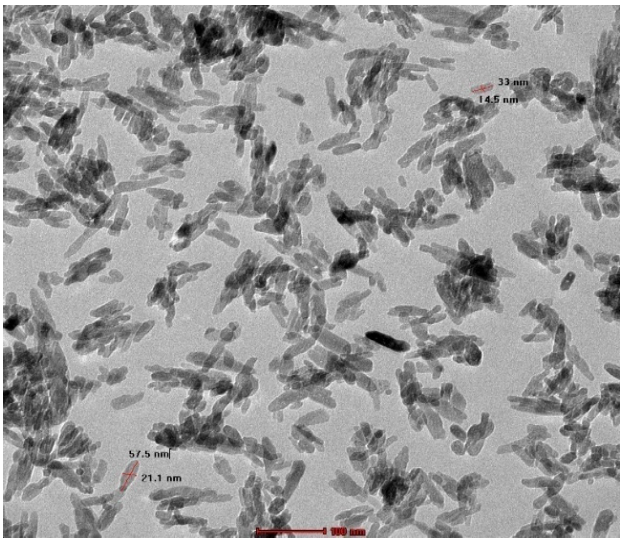


Fig. 2. HR-TEM micrograph of the CaHPNPs.

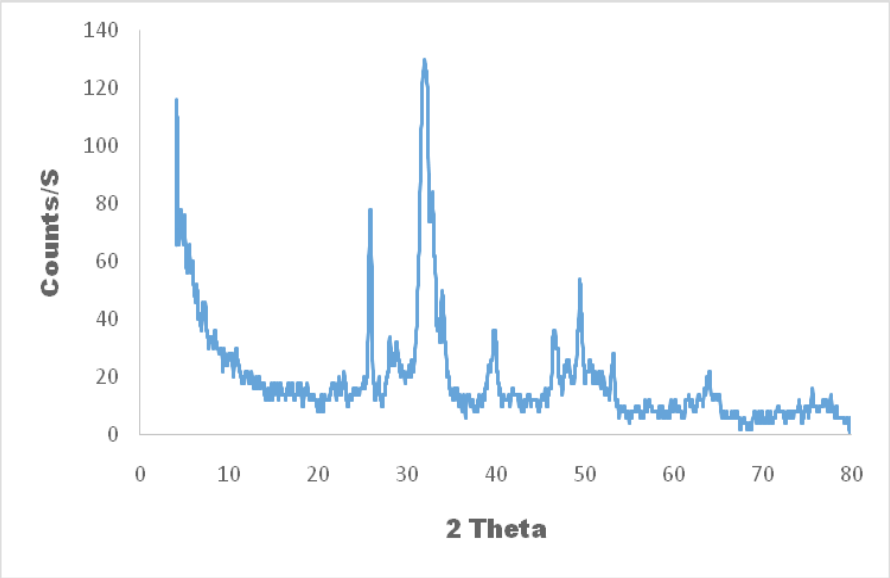


Fig. 3. XRD pattern of the prepared CaHPNPs.

of peanut by-products; it can also be incorporated in many food preparations as supplementary food like rice milk [32].

Table (2) shows the differences in hydrolysis between treatments with the effect of different medium, ultrasounds, soaking time and stirring resulted in substantial variations between dissociation products. The hydrolysis of treatment mixtures tested, noticed that the 70:30 ethanol/0.5 N NaOH mixture extracted the highest protein and saponin yields from (PM). The 1 N NaOH solution produced the highest amount of phenolic compound, followed by 0.5 N NaOH then 70: 30 ethanol/0.5 N NaOH. The flavonoids were extracted by the 70:30 ethanol/0.5 N HCl mixture. Furthermore, 1 N HCl generated the lowest amounts of protein, phenolic, saponin, and flavonoids. There is no significant difference between 1 N and 0.5 N NaOH treatments in terms of flavonoids extraction.

Soluble protein and bioactive compounds

The PM was hydrolyzed using an acid or base, with or without ethanol. Other factors, such as ultrasound waves, soaking time, and stirring, also exhibited a great effect on the hydrolysis. These treatments are fast and low in cost. Previous methods for preparing antioxidant peptides have relied on protein extraction and subsequent hydrolysis using proteases [33, 34].

The bioactive compounds consisted of aromatic rings with one or more different functional groups (OH or CH3), and their structures may range from a simple molecule to a polymer complex [35, 36, 37].

Additionally, bioactive compounds can play an important role because of their antioxidant properties and ability to adsorb free radicals by electrons or hydrogen donors to form stable diamagnetic molecules. Their chemical properties and biological activity of bioactive compounds change according to the type of hydrolysis (in the presence of acids/alkali or hydrothermolysis) and storage [38].

In addition, Special structures of (Phe, Tyr, Trp, and Pro) are present in both peanut and soya from the digestion of protein, suggesting that the aromatic amino acid peptide is a fast donor of hydrogen atoms and that scavenging free radicals contributes to its antioxidant activities [39, 40].

Table 3 shows the antioxidant activities of Hydrolyzed peanut for each assay of the different treatments design. In this investigation three different methods have been used for the determination of the extracts activities (soluble phase) : the first method is the DPPH free radical scavenging activity, second method is Hydrogen peroxide-scavenging effect and finally Total reductive capability measured by ferric reducing antioxidant power (FRAP).

In this study (PM) used with its skin because peanut skin contains antioxidant compounds such as phenolic compounds flavonoids, procyanidin. The previous antioxidant assays showed that (PM)

TABLE 1. Ingredients used in Egyptian rice milk preparation.

Ingredient Amounts (g)	Egyptian Rice Milk (control)	Egyptian Rice Milk with Hydrolyzed Peanut	Egyptian Rice Milk with Hydrolyzed Peanut& CaHPNPs
Egyptian Rice (white)	100	90	90
Hydrolyzed Peanut	0.0	10	10
Dry Milk	25	25	25
Vanillia	0.5	0.5	0.5
Peanut Protein	0.0	5.5	5.5
Total Protein Added	5.25 from milk	10.75 from milk+ (HP)	10.75 from milk+(HP)
Peanut Fat	0.0	0.0	0.0
Milk Fat	7.0	7.0	7.0

TABLE 2. Yields of soluble protein, phenolic, saponin, and flavonoid compounds produced by different treatments (hydrolysis medium) of (PM) at room temperature.

Hydrolyzed peanut	Soluble Protein mg/g ±SD	Soluble Phenolic mg/g ±SD	Saponin m/g±SD	Soluble Flavonoid µ/g ±SD
Ethanol: 0.5 N HCl 70 : 30	17±0.01^{f}	15±0.5^{d}	0.42±0.01^{c}	0.016±0.002^{a}
Ethanol: 0.5 N NaOH 70 : 30	110.6±0.5^{a}	15.8±0.4^{c}	0.61±0.02^{a}	0.0014±0.001^{c}
1 N NaOH	50.7±0.1^{c}	26.7±0.2^{a}	0.62±0.03^{a}	0.008±0.001^{b}
0.5 N NaOH	60.4±0.3^{b}	23.5±0.3^{b}	0.55±0.04^{b}	0.007±0.002^{b}
1 N HCl	27.6±0.2^{e}	8.7±0.1^{e}	0.22±0.01^{d}	0.002±0.001^{c}
0.5 N HCl	32.1±0.1^{d}	6.4±0.2^{f}	0.23±0.02^{d}	-
LSD at the 5% level	0.458475	.5579488	.042970	.0022968

The different letters in each column indicate significant differences between solvents at P<0.05 for each treatment concentration.

TABLE 3. Antioxidant activity of soluble bioactive compounds from hydrolyzed peanut meal at room temperature.

Hydrolyzed peanut	DPPH-scavenging effect (%± SD). 200 µl	Hydrogen peroxide-scavenging effect (%± SD). 200 µl	Total reductive capability (m/g) ± SD. 200 µl
Ethanol: 0.5 N HCl 70 : 30	91.81±0.5^{d}	11.8±0.1^{b}	9.2±0.2^{c}
Ethanol: 0.5 N NaOH 70 : 30	95. 5±0.4^{b}	13.5±0.2^{a}	3.1±0.1^{d}
1 N NaOH	100±0.2^{a}	13.4±0.3^{a}	11.5±0.4^{a}
0.5 N NaOH	100±0.2^{a}	11.5±0.4^{b}	10.1±0.3^{b}
1 N HCl	93.9±0.3^{c}	-	-
0.5 N HCl	81.2±0.2^{e}	6.1±0.1^{c}	0.6±0.05^{e}
LSD at the 5% level	.509567	.452919	.447456

The different letters in each column indicate significant differences between solvents at P<0.05 for each treatment concentration.

extracts exhibited good antioxidant activities [2]. It is clear from the results that all the extracts were able to scavenge the hydrogen radical but at different levels.

The variations in hydrolysis conditions considerably changed the antioxidant activity of the bioactive products (Table 3). The compounds generated in the presence of 1 N NaOH exhibited the highest antioxidant activities for all three methods, followed by those obtained using 0.5 N NaOH. This study suggested a feasible new way to produce a natural protein peptide and bioactive compounds with high antioxidant levels by acid and base-mediated hydrolysis of (PM) protein in water or ethanol/water mixtures. So, our results indicate that the soluble bioactive components of the (HP) exhibited good antioxidant properties. This is consistent with the fact that the addition of these bioactive compounds to the DPPH or hydrogen peroxide solution caused a rapid decrease in the optical density, which also indicated the scavenging capacity of all treated samples [2]. Similar results were observed by [41], who found that protein isolate hydrolysates extracted from beans have the highest antioxidant activity. Peanut protein hydrolysate treated with esperase enzyme exhibited higher antioxidant activity than those of peanut protein isolate [6]. According to Hwang et al., 2010, the solubility of peanut peptides was greater than that of peanut proteins over a wide range of pH values (2 to 12) [2]. The reducing power is regarded as an important index in the evaluation of an antioxidant. Table 2 shows the reductive capability of the soluble bioactive components of (HP). This agrees with the previous correlation between absorbance value and reductive power [42]. Further work is underway using different commercial enzymes to hydrolyse cooked peanut to prepare protein hydrolysates for more application [43], but this method cost a lot. It was concluded when take all of those results together that the current work will provide new reference data to hydrolyze peanut.

Native and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE):

Native and SDS-PAGE were evaluated to confirm the polypeptide protein in (HP), which was hydrolyzed into SDSsoluble and insoluble forms.

The 8% native PAGE of the insoluble HPPs

Native electrophoresis shows that the protein conformation changed after all hydrolysis treatments compared to that of the control and the mobility varied according to the ratio of electric charge to hydrodynamic friction [44].

Figure (4) showed that all acid and base hydrolysis treatments modified the protein surfaces [39]. Hydrolyzed samples (Lanes 1, 2, and 3, Fig. 4B) displayed higher degradation into low molecular weight compounds than the control (Fig. 4A, lane 1) also, this agree with [39] which observed that acidic proteins migrate through the gels faster than basic proteins. These findings, together with those related to antioxidant activity

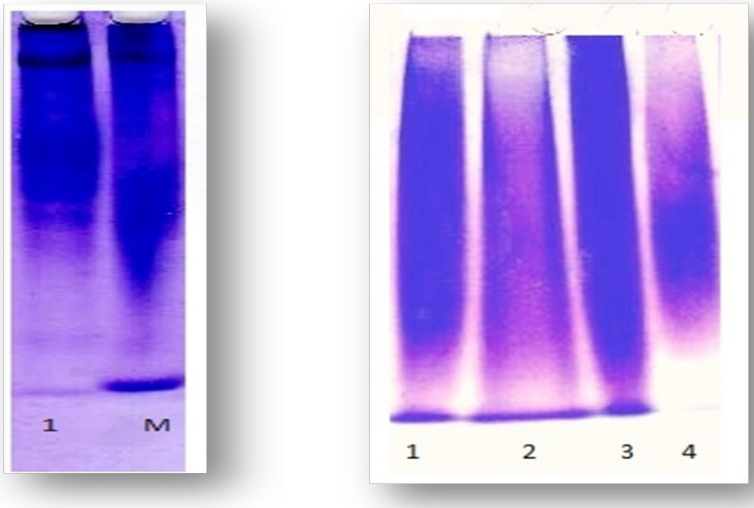


Fig: A **Fig: B**

Fig. 4. (A & B): Eight percent native polyacrylamide gel electrophoresis (PAGE) of insoluble HPPs.

A: lane 1 (peanut meal (control)), lane M (marker). B: lane 1 (0.5 N HCl), lane 2 (1 N HCl), lane 3 (70:30 ethanol/0.5 N NaOH), and lane 4 (70:30 ethanol/0.5 N HCl).

Table (3), agree with previous work showing that peptides exhibit lower molecular weights and higher antioxidant activity than large polypeptides or native proteins (control) [45, 46].

Sodium Dodecyl Sulfate (SDS)-PAGE

Data of soluble HPP are shown in Fig. 5, it is clear that the Coomassie blue staining shows extensive, intense band smearing in the upper gel regions of lanes 3–6 (Fig. 5), which should contain negatively charged proteins, even though this dye typically binds to basic amino acid side chains [39] (Figs. 5 and 6). The anionic SDS bound to lower molecular weight proteins,

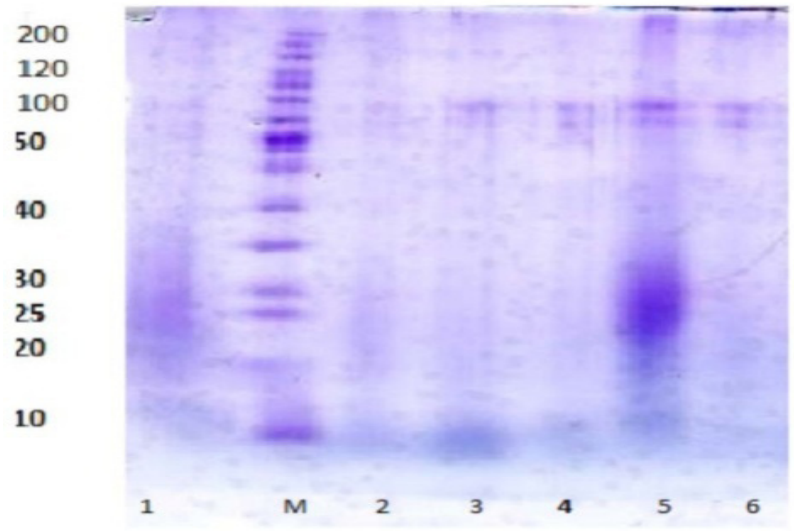
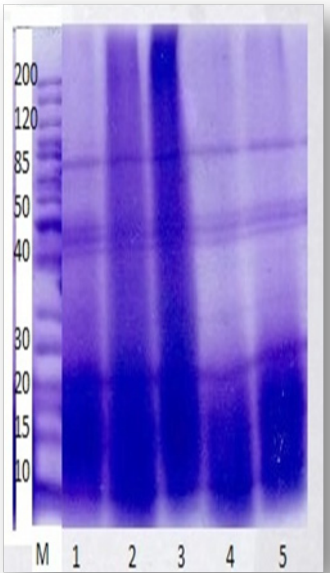


Fig. 5. SDS-PAGE of soluble HPPs.

Lane M (marker), lane 1 (0.5 N HCl), lane 2 (1 N HCl), lane 3 (0.5 N NaOH), lane 4 (1 N NaOH), lane 5 (70:30 ethanol/0.5 N NaOH), and lane 6 (70:30 ethanol/0.5 N HCl).



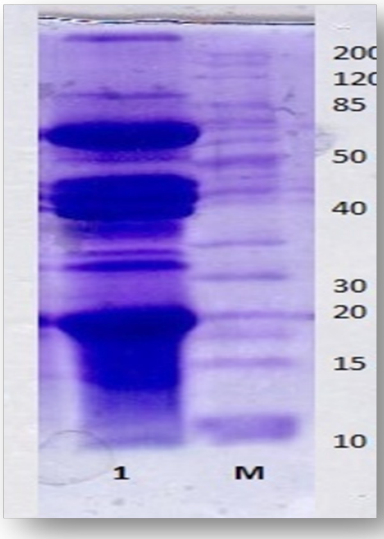


Fig. A **Fig. B**

Fig. 6. (A & B): SDS-PAGE of insoluble HPPs.

A: Lane 1 (peanut meal (control)), lane M (marker). B: Lane M (marker), lane 1 (70:30 ethanol/0.5 N HCl), lane 2 (70:30 ethanol/1 N NaOH), lane 3 (70:30 ethanol/0.5 N NaOH), lane 4 (1 N HCl), and lane 5 (0.5 N HCl).

indicating protein fragmentation (subunits), and nonspecific crosslinked peptides in the 30 to 5 kD range, generating diffuse smears. These smears were particularly visible for the SDS-soluble fraction in all lanes in the lower gel regions [39].

Variations in conditions produced peptides with different molecular weights. Greater change among the charged peptides that barely migrated into the gels than that for the meal (control).The SDS-PAGE of insoluble HPPs (Fig. 6A and B) showed strong peptide bands ranging from 85 to 40 kDa in (PM) (control, lane 1, Fig. 6A). These bands were very weak in all hydrolyzed samples (Fig. 6B), consistent with the high degradation during the treatments. All samples also displayed small peptides (less than 20 kDa, Fig. 6B), presumably resulting from high degradation or hydrolysis during acid and base treatments. These results agree with [47], which combined acid hydrolysis with heat to obtain amino acids and small peptides (less than 10 kDa). The SDS- (Fig. 6) also shows a constant strong band near 80 kDa and three constant, strong main bands ranging from 40 to 50 kDa for control and hydrolyzed samples. No bands were visible above 85 kDa for the treated samples (Fig. 6B). All the hydrolyzed samples presented a higher concentration of high-density and fused polypeptide fragments of various molecular sizes ranging from 10 to 20 kDa (Fig. 6B) than the control. This confirmed that the degraded polypeptides weighed more than 85 kDa and less than 30 kDa.). The above results agree with [48], which noticed that glutelins, prolamins, globulins, albumins, glycoprotein and lamin fractions, which are present in soluble seed proteins, are small polypeptides (MW < 14 kDa). The low molecular weight of basic albumins and globulins, especially from 5 to 15 kDa, indicate that more peptide fragmentation occurred in all treated samples than in the control (lane 1, Fig. 6B). Also, agrees with the study by [39], in which modified albumin and globulin fractions became hydrophobic, generating diffuse smears upon cross linking and moving to the SDS-soluble fraction. These diffuse smears are clearly seen in the lower region of the gels. All hydrolysis treatments produced residues containing very small peptides compared with the control, irrespective of the strength of the treatment (Fig. 6A and B), Coomassie blue staining revealed extensive intense bands that contained proteins in all gel regions [39], the same as present in the current study. Overall using native and SDS electrophoresis illustrates the effects of various conditions on hydrolysed protein.

Mixing of (HPP) and (CaHPNPs) with rice milk during cooking

The prepared meal aimed to obtain a nutritional fortified meal with the necessary components like protein, calcium and phoshate, it can be prepared with 15 percent defatted groundnut flour [32].

The addition of (HP) and (CaHPNP) to cooked rice milk enhanced antioxidant activity. Therefore, the protection of rice milk from oxidation, and the associated effect on shelf life were examined.

The principle for making rice milk at home is based on supplementing either white or brown rice with some high quality hydrolyzed proteins from treated low-cost oil seed meal. So, hydrolysed peanut (HP) was added to the rice milk mixture plus 100 ppm (CaHPNPs). The control sample was prepared by the same method but without adding (HP) or (CaHPNPs) as shown in Table (1).

The best HP was chosen and cooked with rice milk in the presence and in the absence of (CaHPNPs) Fig. (1), and then their antimicrobial activities and shelf life were examined Tables (5 and 6). According to [49], peanut peptides are natural and do not present any potential health risk or side effects. Consequently, they can be used at high concentrations to achieve good radical-scavenging effects when added to other ingredients, such as flavonoids, polyphenolics, and saponins.

Antimicrobial activity of hydrolysed peanut (HP) and (HP + CaHPNPs)

Antimicrobial activity of (HP), (CaHPNPs) and fortified rice milk

Results for (HP) extract as shown in Table 4 exhibited the strongest effect against *Salmonella typhi* (inhibition zone = 11.5 mm) and *B. cereus* (inhibition zone = 11.0 mm). In contrast, it presented moderate activity against *L. monocytogenes* and *Staph. aureus*(inhibition zones = 9.0 and 6.2 mm, respectively) and no activity against *E. coli O157:H7*, *Y. enterocolitica*, and *A. flavus.* Meanwhile, combining HP with CaHPNPs was ineffective against the *A. flavus* test species and inactive against *E. coli O157:H7* and *Y. enterocolitica*, yet displayed the highest antimicrobial activity against *Salmonella typhi, B. cereus, L. monocytogenes*, *and Staph. aureus*.

The antimicrobial activities and safe use of peanut hydrolysate and CaHPNPs, against E. coli and S. aureus, in rice milk agreed with [31, 50],

but not agreed with [28]. Mixing of (HP) and (CaHPNPs) with rice milk during cooking and the addition of (HP) and (CaHPNPs) to cooked rice milk enhanced antioxidant activity. Therefore, the protection of rice milk from oxidation was examined, which had associated effects on shelf life. The peanut protein hydrolysate showed high antibacterial activity against gram-positive and gram-negative bacteria at different concentrations, in agreement with a study by [51,52], who found that the Salmonella count decreased by approximately 3 log CFU/g during storage in peanut butter samples. However, the hydrolysate did not inhibit E. coli, Y. enterocolitica, and A. flavus. This is in contrast with findings by [52], who observed cell count reductions of 2.73 to 3.53 log CFU/g for E. coli O157:H7 strains in peanut butter. Our results show, the Gram-positive bacteria were more sensitive than the Gram-negative bacteria to the peanut extract. This change in sensitivity could be attributed to the different cellular structures. The cellular structures might influence the sensitivities of the bacteria to peanut extract. Additionally, cellular structures could be related to polysaccharide properties because the bacteria might use a portion of carbohydrates and polysaccharides [53]. The (HP) + (CaHPNPs) mixture showed variations in antimicrobial activity against gram-positive and gram-negative bacteria, similar results on antibacterial were obtained when ZnO peanut-shaped nanobunches and nanoparticles were used [54-56].

Microbiological analysis of fortified rice milk during refrigerated storage

Results as shown in Table 5 revealed the changes in microbial counts (log cfu/g) in untreated rice milk dishes (control) and those treated with (HP) and (HP +100 µ CaHPNPs) at the same concentration during refrigerated storage. The total aerobic colony counts (TC) presented no significant change ($P > 0.05$) in the control until the 15th day. Treated samples displayed numerically higher counts than the control and, especially, those mixed with (HP + CaHPNP) recorded the highest activation of bacterial growth during storage. Rice dishes typically show undesirable levels of yeast, mold, *Staph. Aureus*, and coliform. However, yeast and molds were not present in any rice milk samples in the present study (>2 log cfu/g). Moreover, there was no significant difference ($p>0.05$) in yeast and mold growth between control and treated rice milk samples after the 15th day. None of the samples contained any coliform bacteria because of the good hygienic practices adopted during their manufacturing. Additionally, it was identified two bacterial isolates from the control sample at the end of 15 days of refrigerated storage – the lipolytic *Bacillus cereus* and the proteolytic *Enterococcus faecalis* (Table 5). In contrast, treated samples were devoid of lipolytic and proteolytic bacterial strains. The microbiological assessment of rice milk revealed that the product was safe and free from most pathogenic gram positive and gram-negative bacteria during the cold storage period. This finding results from the good hygienic practices during rice milk preparation and storage. Rice milk fortified with(HP) and with(HP + CaHPNPs) displayed increases in total microbial counts compared with the control sample, which may be due to high levels of nutrients and bioactive compounds and the antimicrobial properties in the fortified rice milk [57, 58]. Proteolytic and lipolytic psychrotrophic bacteria, such as *Enterococcus faecalis* and *Bacillus cereus*, were observed in the control but not in rice milk fortified with (HP) and (CaHPNPs).. This may result from the release of proteinases and lipases by psychrotrophic bacteria [59]. Proteolytic psychrotrophs alter milk by degrading casein using heat-resistant proteolytic enzymes, which may also be made by Enterococcus faecalis in rice milk [60]. Other genera of bacteria are responsible for milk spoilage [61]. In particular, lipolytic psychrotrophs, such as Bacillus cereus, are able to grow under refrigeration temperatures and alter milk by producing heat-resistant lipolytic enzymes.

Finally, the absence of proteolytic and lipolytic psychrotrophs, such as *Enterococcus faecalis* and *Bacillus cereus* in rice milk fortified with (HP) and (CaHPNPs) and the antioxidant and antimicrobial activities of (HP) and (CaHPNPs) add nutritive value to rice milk and increased the product quality, and add value to meet a reasonable number of hygienic and nutritional requirements [51].

Sensory evaluation of rice milk:

Sensory evaluation of Egyptian rice milk products as presented in Table 6 revealed no significant differences between control and treated samples. However, the fortified rice milk showed better results than the unsupported rice milk as it was soft, tasty, and sweet. The sensory evaluation of various samples showed no significant differences in taste, odor, mouth feel, and overall acceptability between control and rice milk

TABLE 4. Antimicrobial activities (Inhibition zones, mm) of peanut on food borne pathogenic microorganisms.

Sample Extract	*B. cereus*	*E. coli O157:H7*	*Salmonella typhy*	*Y. enterocolitica*	*Staph. aureus*	*L. monocytogenes*	*A. flavus*
HP	11.0^{Bb}	0.0^{Ea}	11.5^{Ab}	0.0^{Ea}	6.2^{Da}	9.0^{Cb}	0.0^{Ea}
HP + ca HPNPs	12.0^{Ba}	0.0^{Ea}	13.30^{Aa}	0.0^{Ea}	7.0^{Da}	10.50^{Ca}	0.0^{Ea}

The means with the different capital (A, B, C…)superscript letters within the same row are significantly (P≤0.05) different between treatments during storage period. Means with the different small (a, b, c,)superscript letters within the same column indicate significant (P≤0.05) differences between treatments.

TABLE 5. Microbiological analysis of Rice-milk during storage period time at 4-5 ±2 Co.

Storage time	Sample types	Total count bacteria Log Cfu/g	Moulds & Yeasts Log Cfu/g	Coliform bacteria Log Cfu/g	Staph. aureus Log Cfu/g	Lipolytic strains Log Cfu/g	Proteolytic strains Log Cfu/g
Zero	control	$4.9^{\pm}\ 0.05^{C}$	0.0^{B}	0.0^{A}	0.0^{A}	ND	ND
	HP	$4.95\pm\ 0.2^{C}$	0.0^{B}	0.0^{A}	0.0^{A}	ND	ND
	HP + ca HPNPs	$5.08^{\pm}0.1^{C}$	0.0^{B}	0.0^{A}	0.0^{A}	ND	ND
5D	control	4.95 ± 0.01^{C}	0.0^{B}	0.0^{A}	0.0^{A}	ND	ND
	HP	5.146 ± 0.03^{B}	0.0^{B}	0.0^{A}	0.0^{A}	ND	ND
	HP + ca HPNPs	5.26 ± 0.9^{B}	0.0^{B}	0.0^{A}	0.0^{A}	ND	ND
10D	control	5.0 ± 0.81^{C}	0.0^{B}	0.0^{A}	0.0^{A}	ND	ND
	HP	5.3 ± 0.004^{B}	0.0^{B}	0.0^{A}	0.0^{A}	ND	ND
	HP + ca HPNPs	$5.29\ \pm0.05^{B}$	0.0^{B}	0.0^{A}	0.0^{A}	ND	ND
15D	control	0.23^{C}	$1.33\pm^{A}$	0.0^{A}	0.0^{A}	One strain (Bacillus cereus)	1(Enterococcus faecalis)
	HP	5.29 ± 0.005^{B}	0.0^{B}	0.0^{A}	0.0^{A}	ND	ND
	HP + ca HPNPs	5.51 ± 0.21^{A}	0.0^{B}	0.0^{A}	0.0^{A}	ND	ND
21D	control	5.0 ± 0.01^{B}	1.3 ± 0.03^{A}	0.0^{A}	0.0^{A}	1.477 (Bacillus cereus)	1.477 (Enterococcus faecalis)
	HP	$5.1^{B}\pm\ 0.12$	0.0^{B}	0.0^{A}	0.0^{A}	ND	ND
	HP + ca HPNPs	$5.21^{B}\pm0.31$	0.0^{B}	0.0^{A}	0.0^{A}	ND	ND

ND = Not detected.

TABLE 6. Sensory properties of Egyptian Rice- milk during storage period at 5 ±2 Co for 15 days.

No. of Person	Sample types	Appearance 40%				Body & texture 40%				smell and taste 20%			
		zero day	5 day	10 day	15 day	zero day	5 day	10 day	15 day	zero day	5 day	10 day	15 day
1	control	39.^{Aa}	38.5^{Aa}	37.^{Ab}	37^{Bb}	39^{Aa}	39^{Aa}	39^{Aa}	38.5^{Aa}	19.^{Aa}	19.0^{Aa}	18.5^{Aa}	17.8^{Ab}
	HP	38.5^{Ba}	38.1^{Ca}	37.9^{Aa}	37.2^{Ab}	39^{Aa}	38.8^{Aa}	38.9^{Aa}	38.0^{Ab}	19^{Aa}	18.5^{Ba}	18.5^{Aa}	17.8^{Ab}
	HP + ca HPNPs	39.0^{Aa}	38.7^{Aa}	37.8^{Ab}	37.9^{Ab}	39.1^{Aa}	38.7^{Aa}	39.0^{Aa}	38.0^{Aa}	18.6^{A}	19^{A}	18.2^{A}	17.5^{A}
2	control	38^{Ca}	38.0^{Ca}	37.5^{Ab}	37.5^{Ab}	38.5^{Aa}	38.5^{Aa}	38.0^{Ba}	38.0^{Aa}	19.5^{Ba}	18.5^{Ab}	18.0^{Ab}	17.0^{Ac}
	HP	38.2^{Ca}	38.0^{Ca}	37.2^{Bb}	37.4^{Ab}	38.4^{Ba}	38.5^{Aa}	37.5^{Bb}	38.0^{Aa}	19.5^{Ba}	18.2^{Bb}	18.1^{Ab}	17.0^{Ac}
	HP + ca HPNPs	38.1^{Ca}	38.0^{Ca}	37.3^{Bb}	37.3^{Ab}	39^{Aa}	38.5^{Aa}	37.4^{Bb}	38.0^{Ab}	19.0^{Aa}	18.2^{Ba}	18.3^{Aa}	17.0^{Ab}
3	control	38.5^{Ba}	38.0^{Ca}	37.0^{Bb}	36.5^{Bb}	38.0^{Ba}	38.0^{Ba}	38.0^{Ba}	37.5^{Ba}	19.0^{Aa}	18.5^{Aa}	18.0^{Ab}	16.5^{Bc}
	HP	38.5^{Ba}	38.1^{Ca}	37.5^{Ab}	36.2^{Bb}	38.1^{Ba}	38.4^{Ba}	37.9^{Ba}	37.4^{Bb}	18.7^{Aa}	18.0^{Ba}	17.8^{Ba}	17.^{Ab}
	HP + ca HPNPs	38.3^{Ca}	38.2^{Ba}	37^{Bb}	36.1^{Bb}	38.5^{Aa}	38.6^{Aa}	37.8^{Ba}	37.3^{Bb}	18.7^{Aa}	18.0^{Ba}	17.9^{Ba}	17.0^{Ab}
4	control	39^{Aa}	38.5^{Aa}	38^{Ab}	37^{Bb}	39^{Aa}	38.5^{Aa}	38^{Bb}	38^{Ab}	18.5^{Ba}	18.5^{Aa}	18.0^{Ab}	17.5^{Ab}
	HP	39^{Aa}	38.4^{Aa}	38.0^{Ab}	37.^{Bb}	38.8^{Aa}	38.5^{Aa}	37.8^{Bb}	38.0^{Ab}	18.0^{Ba}	17.7^{Ba}	17.5^{Bb}	17.4^{Ab}
	HP + ca HPNPs	39$^{A\,Ba}$	38.5$^{A\,Ba}$	38.0^{Aa}	37.^{Bb}	39^{Aa}	38.6^{Aa}	37.9^{Bb}	38.0^{Ab}	18.0$^{B\,a}$	17.8^{Ba}	17.4^{Bb}	17.1^{Ab}
5	control	39^{Aa}	38.5^{Aa}	38^{Aa}	37^{Bb}	39^{Aa}	38.5^{Aa}	38^{Bb}	38^{Ab}	18.5^{Ba}	18.5^{Aa}	18.0^{Ab}	17.5^{Ab}
	HP	38.5^{Ba}	38.1^{Ca}	37.8^{Aa}	37^{Bb}	38.8^{Aa}	38.5^{Aa}	37.8^{Bb}	37.5^{Bb}	18.4^{Ba}	18.3^{Ba}	18.0^{Aa}	17.3^{Ab}
	HP + ca HPNPs	38.6^{Ba}	38.3^{Ba}	38^{Aa}	37.1^{Bb}	38.8^{Aa}	38.6^{Aa}	37.6^{Bb}	37.5^{Bb}	18.1^{Ba}	18.0^{Ba}	18.0^{Aa}	17.5^{Aa}

The different capital (A, B, C…,) superscript letters within the same column are significantly (P≤0.05) different between treatments during storage period. Means with the different small (a, b, c...,) superscript letters within the same raw indicate significant (P≤0.05) differences between treatments

supplemented with soluble bioactive compounds from (HP) or both supplemented with (HP) and (CaHPNPs) were in agreement with [58].

This study found that (HP) and (CaHPNPs) can be used to improve the nutritional value and antibacterial properties of traditional rice milk and here upon could be used in preparing other functional foods. Also, the fortified rice milk exhibits synergistic effects of these ingredients to increase the potential nourishment and health quality.

Conclusion

The findings of this study will help researchers uncover new ways of adding seed protein byproducts/waste containing 50% to 60% high-quality protein and polyphenolics as functional compounds that can be mixed with foods to serve as functional ingredients after some treatments. In addition, (CaHPNPs) were added as high-value ingredients. Thus, the current study aimed to develop a new method to compensate for food deficiency that presents several advantages, such as fast preparation, low cost, good flavor, low fat, high energy, and high-protein content. This method prevents oxidation in food products, increase their shelf life, and provide children with good food choices.

Most byproducts of oil seed meal, such as peanut meal generated from the oil industry, have been used as animal fodder. This study proposes an alternative use of peanut meal as part of an affordable and nutritious meal, that provides natural antioxidants and (HP) derived by acid and base hydrolysis. This ingredient is added to rice milk along with (CaHPNPs), the fortified rice milk exhibits synergistic growth promotion and can supply the human body with the necessary nutrients to solve malnutrition problems. The current work will provide new reference data for the use of peanuts as potential nourishment and health commodities in the food industry.

Abbreviation

Peanut meal (PM), Calcium Hydroxyl Phosphate Nanoparticles (CaHPNPs), Transmission Electron Microscope (TEM), X-ray Diffraction (XRD), Hydrolyzed Peanut Protein (HPP), Hydrolyzed Peanut (HP), MCPM (Monocalcium phosphate monohydrate)

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الأرز باللبن المعزز باستخدام نانو جسيمات فوسفات هيدروكسيل الكالسيوم وجزيئات بروتين الفول السوداني المحلل

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تهدف هذه الدراسة إلى تطوير وجبة تعتمد على الأرز واللبن وتحتوي على العناصر الغذائية اللازمة للتغلب علي مشاكل سوء التغذية. تم تحضير الوجبة على ثلاث مراحل وتم تقييم أنشطتها المضادة للميكروبات وخصائصها الحسية. أولاً ، تم تصنيع نانو جسميات فوسفات هيدروكسيل الكالسيوم (CaHPNPs). تم فحص شكل وحجم الجسيمات عن طريق الميكروسكوب الإلكتروني النافذ عالي الدقة ، وتم التأكد من تركيبه بواسطة حيود الأشعة السينية. ثانياً ، تم تحلل كسب الفول السوداني (PM) بمذيبات مختلفة باستخدام الموجات فوق الصوتية وتحليلها بواسطة الالكتروفوريسس (رحلان كهربائي). ثالثًا ، تمت إضافة كل من CaHPNPs والفول السوداني المحلل (HP) أثناء طهي الأرز باللبن. أظهر الأرز باللبن المطهو مع (HP + CaHPNPs) جودة ووقت تخزين أكبر من ذلك المطهو مع HP فقط. كانت جودة ووقت التخزين أدنى لعينة المرجع الكنترول .HP+CaHPNPs لديه أيضًا العديد من الأنشطة المضادة للميكروبات. وكانت الأنواع البكتيرية الأكثر تأثرًا بمستخلص HP هي بكتيريا السالمونيلا التيفية (مساحة تثبيط = ١١,٥ مم) وبكتيرياالباسيلس سيريس (١١,٠ مم). كان جميع الأرز باللبن المدعم خاليًا من البكتيريا المحللة للبروتين والشحوم ، في حين تم تصنيف عزلتين بكتيرية على أساس خصائصهما الظاهرية في عينة المرجع. تشير النتائج إلى أنه يمكن استخدام HP و CaHPNPs لتحسين القيمة الغذائية والخصائص المضادة للبكتيريا للأطعمة الجاهزه.