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Research Article

Selection of a Natural Extract Rich in Antioxidants to Reduce Lipid and Protein Oxidation in Rabbit Meatballs During Refrigerated Storage

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Although consumption of rabbit meat is limited worldwide, it is highly nutritious and offers significant health benefits. Lipid and protein oxidation affect the quality and acceptability of rabbit meat products by consumers. These processes can be suppressed using nonharmful antioxidant agents, such as polyphenol-rich extracts obtained from natural sources. This paper compared the antioxidant properties of extracts obtained from *Euphorbia antisyphilitica* (Ea), *Momordica charantia*, *Acrocomia aculeata*, and *Dactylopius opuntiae* to select the best antioxidant extract. This extract was used in ready-made rabbit meatballs to evaluate its preservative actions during refrigerated storage. Among all extracts, the Ea extract exhibited the highest polyphenol content and antioxidant activity compared with the other extracts (Ea semisolid extract contained 337.45 ± 11.53 mg GAE/g and showed half-maximal inhibitory concentrations [IC_{50}'s] of 66.32 ± 11.47 and 755.60 ± 132.4 μg/mL against DPPH and ABTS radicals, respectively). As the selected semisolid extract could not be added to meat, a drying step was applied to obtain a powdered extract, which exhibited higher polyphenol amount (485.97 ± 29.05 mg GAE/g) and antioxidant activity (IC_{50}'s of 47.36 ± 2.55 and 549.6 ± 36.81 μg/mL against DPPH and ABTS radicals, respectively) than its semisolid form. To obtain the powdered form, a simple method was employed (samples were heated at 200°C for 2 min and pulverized using a mortar and pestle). Compared with preservative-free meatballs, meatballs fortified with the powdered Ea extract showed reduced cooking loss (reduction of 33.8% from the comparative value), enhanced color and antioxidant ability (antioxidant increase of up to 101.7% from the comparative value), and suppressed lipid and protein oxidation (reduction of up to 71.4% and increase of 81.3% from comparative values for lipid deterioration and protein preservation, respectively). Therefore, this natural powder has the potential to be used as a preservative in the meat industry after additional tests, such as sensory evaluations, among others.

Keywords: antioxidant preservative; candelilla; gallic acid; malondialdehyde; rabbit meatball; sulfhydryl group content

1. Introduction

Rabbit meat is considered a functional food because it contains low levels of fat and cholesterol and high levels of protein, polyunsaturated fatty acids, essential amino acids, and B vitamins [1]. In 2020, data showed that rabbit meat production played a minor role compared to meat produced from other farm animals. It has increased by 134% in China (from 370,00 to 865,477 t/year), 8% in Mexico (from 4160 to 4483 t/year), and 2% in Italy (from 42,174 to 43,109 t/year). Although China is the largest producer of rabbit meat worldwide, it has the highest proportion of nonrabbit consumers. On the contrary, most of the population in Italy, France, Brazil, and Mexico consumes rabbit meat [2].

A global consumer study of rabbit meat showed that one-third of consumers preferred to purchase the whole carcass. Among the cut-up products, the thighs were given the highest preference, followed by the loin, whereas the foreleg was not a preferred product for purchase. At present, the meat processing industry is more inclined toward the production of modern products, such as ready meals and ready-to-cook items, mainly for young individuals who want to spend a shorter time preparing food [2]. Consequently, ready-made meatballs are becoming increasingly popular among consumers because of their excellent, juicy flavor and high nutritional value [3]. However, ready-made rabbit meat products remain underrepresented in the market owing to thermally induced lipid oxidation of their high unsaturated fatty acid content, which generates undesirable odor compounds during processing [1].

Lipid and protein oxidation are two crucial processes that occur during refrigerated storage of meat. Commonly, a period of refrigeration of 10 days is required to distribute meat products to retail outlets. To prevent these oxidation processes, compounds with antioxidant properties are used to preserve the products, including premade rabbit meatballs [1, 3, 4]. For this purpose, both synthetic and natural antioxidants are available. Synthetic preservatives, such as butylated hydroxytoluene (BHT), have been associated with harmful health effects; therefore, their replacement with antioxidant compounds obtained from natural sources is highly desirable [4, 5].

Plant polyphenols are well-known natural compounds with strong antioxidant properties [6]. These compounds can be found in the stems, leaves, and fruits of plants, such as *Euphorbia antisyphilitica* (Ea) stems, *Momordica charantia* leaves, and *Acrocomia aculeata* fruits [7–9]. Ea, commonly known as candelilla plant, is one of the 10 nontimber forest products with a significant economic impact in desert and semidesert regions of Mexico since it is a source of phytochemicals, wax, and fiber, which are used by the food, cosmetic, and pharmaceutical industries [9]. *M. charantia* is a climber growing which is commonly known as bitter melon. This plant is cultivated for its use as a vegetable or popular folk medicine in regions of Africa, such as Benin. It is reported that *M. charantia* has important hypoglycemic, antidiabetic, antioxidant, antiviral, antimalarial, and antimicrobial activities [7]. *A. aculeata*, commonly known as macaúba or bocaiuva, is a plant that reaches up to 20 m in height and produces spherical fruits that are edible. This plant is found in Central, North, and South America. The pulp of the fruit is consumed directly or used in products for human consumption, such as jelly, flour, and cakes, among others. The oil obtained from the mesocarp and seed has an important economic impact since it is used in food, pharmaceuticals, cosmetic products, and biodiesel production. Furthermore, the oil extracted from the fruit pulp is a rich source of monounsaturated fatty acids and bioactive compounds. These compounds have several biological activities, such as anti-inflammatory, antimicrobial, and antioxidant actions [8]. In addition, polyphenols can be found in insects mainly owing to their consumption of plants [10], whereas some insects produce other types of compounds with antioxidant properties. An example is carminic acid, which has antioxidant activity and is the main metabolite found in the red dye produced by insects from the genus *Dactylopius* [4].

To our knowledge, extracts obtained from Ea stems, *M. charantia* leaves, *A. aculeata* fruits, or *D. opuntiae* have not been evaluated for their antioxidant properties and preservative ability in rabbit meatballs during refrigerated storage. Therefore, this study is aimed at comparing the antioxidant properties of these extracts and identifying the extract with the best antioxidant activity for its use in the preservation of ready-made rabbit meatballs during cold storage. The analysis of extracts allows us to identify the Ea extract as the best antioxidant, and we confirm its usefulness as a novel preservative for the meat industry.

2. Materials and Methods

2.1. Collection and Preparation of Plant, Fruit, and Insect Materials. E. antisyphilitica Zucc. stem samples, which were collected in November 2022, were donated by Multiceras S.A. de C.V. (Municipio de García, Nuevo León, Mexico). The stem samples were air-dried at room temperature ($23.6°C \pm 0.8°C$) without exposure to sunlight for 30 days. The dried stems were cut into 2 cm lengths and ground in both a blender (BLSTBPST series Oster, Newell Brands Inc., Atlanta, Georgia, United States) and a grinder (GT2035MX series T-Fal, Groupe SEB Mexico, Mexico City, Mexico) for 15 s in each device. The powdered sample was transferred into a glass column for percolation and maceration extraction with the lower end closed. Then, hexane was added until the entire sample was covered and was maintained in this way for 24 h at room temperature ($22.4°C \pm 0.5°C$). After this time, the lower part of the glass column was opened, and the hexane was allowed to drip slowly. Subsequently, the sample was covered with dichloromethane and methanol in the glass column, as was performed with hexane. In this case, the methanolic fraction was collected. *M. charantia* leaf samples and *A. aculeata* fruit samples were obtained from Santa María Tonameca, Oaxaca de Juárez, Mexico, in November 2019. These samples were authenticated by Eleazar Carranza González, who is affiliated with the Herbarium Isidro Palacios of the Instituto de Investigación de Zonas Desérticas of the Universidad Autónoma de San Luis Potosí, with voucher specimens deposited in the herbarium. Dried leaf samples were ground

and macerated in an ethanol:water ratio of 96:4 (*v*/*v*) at room temperature for 12 days. Similarly, fruit pulp samples were cut into 2-cm pieces, ground, and macerated in the 96:4 *v*/*v* ethanol:water solution at room temperature for 12 days. After maceration and percolation procedures, all solutions were filtered, and the solvent was removed under reduced pressure at 40°C to obtain semisolid extracts. To obtain a *D. opuntiae* aqueous extract, we used the same methods for identification, breeding, and citric acid–free cochineal extraction as described previously [11]. The insects were collected in November 2022. To prepare standards for the *D. opuntiae* and plant extracts, deionized water and ethanol were used as solvents, respectively.

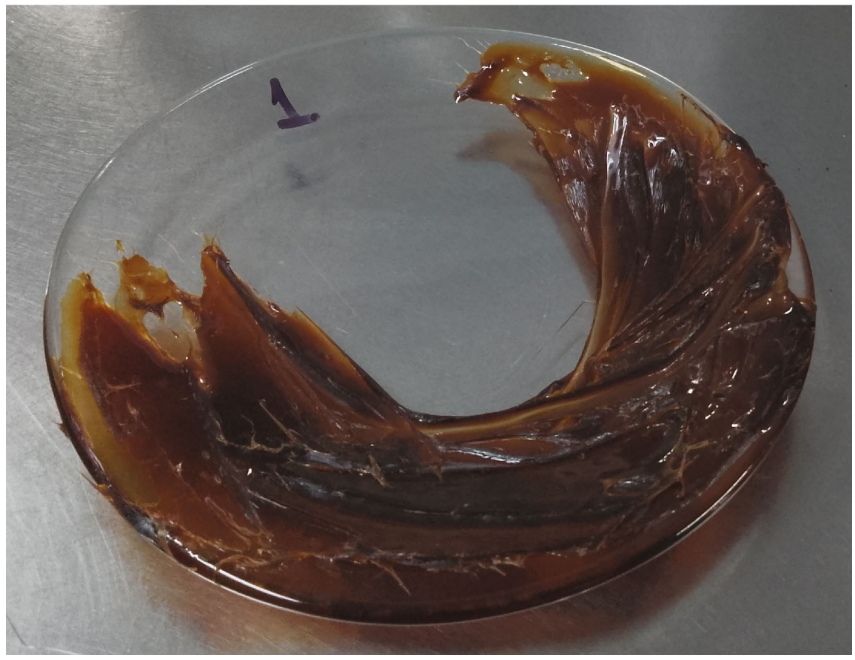
2.2. Content of Polyphenols and Flavonoids in Samples. Total polyphenol and flavonoid contents in semisolid and liquid extracts were determined using methods described previously [12]. For polyphenol quantification, each sample (30 μL) was mixed with 150 μL of Folin–Ciocalteu reagent diluted to a ratio of 1:10 with deionized water. After 5 min of incubation at room temperature, 120 μL of a 10% (*w*/*v*) sodium carbonate solution was added to the mixture, followed by incubation at room temperature for 30 min. Subsequently, the mixture was centrifuged at 3000 g for 5 min at 10°C, and the supernatant (200 μL) was added to one well of a 96-well plate (Costar 3595, Corning Incorporated, New York, United States). Absorbance was measured against a blank (ethanol) at 765 nm on a Cytation 3 microplate reader controlled by the Gen5 software (Biotek Instruments Inc., Vermont, United States). The concentration of polyphenols was obtained by interpolating the absorbance value of the tested sample into a gallic acid calibration curve. Based on the data of the volume and grams of the extract used, each result was expressed as milligrams of gallic acid equivalent (GAE) per gram of extract. For these experiments, quercetin and citric acid were used as the positive and negative controls, respectively. Quercetin is a flavonoid, whereas citric acid is a polyprotic α-hydroxy acid that does not belong to polyphenols or flavonoids [5, 13]. For flavonoid quantification, each sample (40 μL) was mixed with 40 μL of a 10% (*w*/*v*) aluminum chloride solution, 40 μL of a 1 M potassium acetate solution, and 360 μL of deionized water. After 30 min of incubation at room temperature, the mixture was centrifuged at 3000 g for 5 min at 10°C, and 200 μL of the supernatant was added to one well of a 96-well plate. Absorbance was measured against a blank (ethanol) at 415 nm using the same microplate reader and software mentioned above. Based on the data of interpolation into a rutin calibration curve and the volume and grams of the extract used, the flavonoid concentration was expressed as milligrams of rutin equivalent (RE) per gram of extract. The positive and negative controls used in this experiment were the same as those used for polyphenol quantification.

2.3. Evaluation of Inhibitory Effects Against Synthetic Radicals. The antioxidant activities of the extracts were evaluated using 2,2′-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical–scavenging assays as described previously [4]. For the DPPH radical–scavenging assay, each sample (50 μL) was mixed with 200 μL of 202 μmol/L DPPH solution. This mixture was incubated at 30°C for 20 min. Subsequently, 150 μL of the mixture was added to one well of a 96-well plate, and absorbance was measured at 517 nm using the Cytation 3 microplate reader controlled by the Gen5 software. During these measurements, water and ethanol were used as blanks for *D. opuntiae* and plant extracts, respectively. The absorbance values of blanks and test samples were used to calculate the percentage of inhibition against DPPH radicals. For each extract, the number of samples and the range of concentrations were prepared in accordance with the guidelines for an accurate estimation of the half-maximal inhibitory concentration (IC_{50}) [14]. The concentration–inhibition curves and IC_{50} values were obtained using the GraphPad Prism software Version 5.01 (San Diego, California, United States). Quercetin and citric acid were used as the positive and negative controls, respectively. Quercetin possesses antioxidant properties [5], whereas citric acid does not have antioxidant activity [11]. For the ABTS radical–scavenging assay, the radical was produced through the reaction of a 7.0 mmol/L ABTS diammonium salt solution with a 2.4 mmol/L potassium persulfate solution for 16 h in the dark. After the reaction, the solution (1.14 mL) was diluted with deionized water (48.86 mL) to obtain an ABTS radical concentration of 95.7 μmol/L. Subsequently, 300 μL of this diluted solution was mixed with 3 μL of each test sample. After incubation at 30°C for 4 min, 150 μL of the mixture was added to each well of a 96-well plate, and absorbance was measured at 730 nm using the same microplate reader and software mentioned above. The blanks (water and ethanol) and positive and negative controls used in this experiment were the same as those used in the DPPH radical–scavenging assay. In addition, the percentage of inhibition, concentration–inhibition curves, and IC_{50} values were obtained using the same methods as in the DPPH radical–scavenging assay.

2.4. Application of the Selected Extract on Meatballs

2.4.1. Additional Drying Step. After the abovementioned experiments were completed and the results were analyzed, the utility of the Ea extract as a preservative in rabbit meatballs was assessed. However, an additional process was applied to this extract, as it had a semisolid consistency. Briefly, a thin layer (2 g) of the semisolid extract was placed on the surface of a watch glass and heated at 200°C for 2 min in an electric oven (Koblenz, Thorne Electric Co., San Antonio, Texas, United States). The dried extract was recovered from the watch glass using a spatula and weighed. The dried sample was pulverized using a mortar and pestle (Figure 1). This procedure was repeated as many times as necessary to obtain the grams of powdered extracts required for subsequent experiments. Each powdered sample (0.3 mm particle size) was stored in an opaque polypropylene bottle at room temperature. The weights recorded before and after the drying step were used to calculate recovery.

2.4.2. Evaluation of the Powdered Extract. The total polyphenol and flavonoid contents and antioxidant activities



(a)



(b)

Figure 1: Representative Ea extracts. (a) Extract in its semisolid form. (b) Extract after the additional drying step.

(against DPPH and ABTS radicals) of the powdered Ea extract were measured as described above, whereas the gallic acid concentration of the powdered and semisolid extracts was determined using a liquid chromatography (LC) method. The LC method described by Seal [15] was modified to quantify gallic acid, ascorbic acid, and other phenolic acids and flavonoids in Ea extracts. Calibrators containing ascorbic acid (1.79–114 μg/mL), p-coumaric acid (1.80–115 μg/mL), sinapic acid (1.22–78 μg/mL), caffeic acid (1.24–79 μg/mL), gallic acid (1.50–96 μg/mL), o-coumaric acid (1.14–72 μg/mL), gentisic acid (1.92–122 μg/mL), rutin (1.54–98 μg/mL), quercetin (1.09–70 μg/mL), methyl gallate (1.94–124 μg/mL), benzoic acid (1.77–113 μg/mL), myricetin (1.17–74 μg/mL), kaempferol (1.09–70 μg/mL), and apigenin (1.07–68 μg/mL) were prepared in ethanol. All these reagents were obtained from Sigma-Aldrich (St. Louis, Missouri, United States). The semisolid and powdered extracts were also prepared in ethanol. All calibrators and extracts were filtered using a 0.45-μm filter unit (Millipore Corporation, Bedford, Massachusetts, United States), and 200 μL of

each filtrate was transferred to a vial insert inside an amber glass tube for chromatographic analysis. A 1100 series Agilent LC system and Agilent ChemStation software for 3D LC systems (Rev. B.02.01-SR1) were used for measurement and data analysis (Agilent Technologies, Palo Alto, California, United States). The LC system consisted of a quaternary pump with a degasser, autosampler, thermostatted column compartment, and diode array detector. For chromatographic separation, a 250-mm-long Syncronis C18 column (Thermo Fisher Scientific, Waltham, Massachusetts, United States) was used, with a 5-μm particle size and a 4.6-mm internal diameter. The column temperature was maintained at 30°C, and the mobile phase consisted of a 28% sodium acetate solution at pH 3.0 in an acetonitrile gradient. The separation was initiated with a composition of 95% mobile phase plus 5% acetonitrile, which changed over time to reach a composition of 80% mobile phase and 20% acetonitrile at 28 min, 60% mobile phase and 40% acetonitrile at 39 min, and 10% mobile phase and 90% acetonitrile at 50 min. Finally, the composition returned to the initial condition (95% mobile phase and 5% acetonitrile) at 55 min and was maintained for 10 min. The analysis was performed using an injection volume of 20 μL of the sample at a 1 mL/min rate, with monitoring performed at 230, 270, 310, and 350 nm with a run time of 65 min and a scanning range of 190–600 nm. The compounds were identified based on their retention times and spectra, whereas their concentrations in the extracts were determined by interpolating their responses (peak areas) into corresponding calibration curves. The results were expressed as micrograms of compound per gram of extract. Additional flavanols and flavones were identified using the spectral data of their B-ring cinnamoyl and A-ring benzoyl systems, which were reflected by two major ultraviolet (UV) absorption peaks called Band I and Band II, respectively [16]. The abundances of flavanols and flavones in the extracts were calculated using the area of their peaks and all peaks in the chromatogram.

2.4.3. Preparation of Meatballs. Meatballs were prepared using a previously described method with slight modifications [3]. Fresh skinless legs of five New Zealand and three Californian rabbits of both sexes were obtained from Facultad de Agronomía y Veterinaria, Universidad Autónoma de San Luis Potosí (San Luis Potosí, S.L.P., Mexico), and transported to our laboratory (Figure 2a). Meat was separated from the bone and ground in a meat grinder fitted with a 4.5-mm grind plate (Tartare, Metaltex International, Molsheim Cedex, France). The base mince was divided into two formulations: meat without additives (Co) and meat with 100 mg/kg powdered Ea extract. The formulations were prepared according to Table 1. Each formulation was mixed at a low speed for 3 min in a mixer (Model 64650, Hamilton Beach Brands Inc., Glen Allen, Virginia, United States). Each meatball was approximately 33 mm in diameter and weighed approximately 21 g (Figure 2b). The meatballs were heated in a water bath at 80°C for 20 min, cooled to room temperature for 30 min (Figure 2c), dried slightly on the surface, wrapped with a plastic wrap, and stored at 4°C in the absence of light. The meatballs were analyzed for cooking loss, color, antioxidant capacity against the DPPH radical, pH, thiobarbituric acid reactive substances (TBARSs), and total sulfhydryl group content on Days 0, 6, and 12. The evaluation on Day 0 was performed within 40 min of meatball preparation at a temperature of 4°C. The amount of the extract added was determined according to the amount of BHT (100 mg/kg), a synthetic phenolic compound used as a preservative in meat products and approved for human consumption by US regulations [5].

2.4.4. Cooking Loss. Cooking loss was evaluated as described by Li et al. [3], with slight modifications. The meatballs were weighed on a Mettler PE3600 balance (Mettler Instrument Corporation, Hightstown, New Jersey, United States) both before and after cooking on Days 0 and 12. For frozen meatballs, the water stains on the surface were dried with an absorbent paper before weighing. The weight of each meatball was expressed in grams.

2.4.5. Color Evaluation. Color was evaluated as described by Li et al. and Aragon-Martinez et al. [3, 4]. After each meatball was cut in half, a Color Muse colorimeter (Variable Inc., Chattanooga, Tennessee, United States) was placed on the surface of the meatball in two internal and two external areas. The CIE Lab system was used for each measurement. L^*, a^*, and b^* values were obtained, which represented a measure of lightness, redness, and yellowness in samples, respectively. Before measurements, the colorimeter was calibrated using a standard white plate provided by the manufacturer. Finally, the mean value of each parameter was calculated in each sample.

2.4.6. Antioxidant Activity in Meat. The antioxidant activity of rabbit meatballs was assessed as described for beef patties [5], with some modifications. Each meatball sample (0.50 g) was placed in a tube containing 500 μL of ethanol, ground manually with a stirrer for 20 s, agitated on a vortex mixer (Labnet International, Inc., Woodbridge, New Jersey, United States) for 20 s, and centrifuged at 1500 g for 10 min at 4°C. A total of 50 μL of the resultant supernatant or an ascorbic acid calibrator (6.0–120 μg/mL) was processed using a DPPH radical solution as described earlier, with the DPPH concentration being 450 μmol/L. Subsequently, 200 μL of each mixture was added to a well of a 96-well plate. Based on the data of interpolation into an ascorbic acid calibration curve and the volume and mass of the meatball sample used, antioxidant capacity was expressed as micrograms of ascorbic acid equivalent (AAE) per gram of meatball sample.

2.4.7. Lipid Peroxidation. TBARS content was determined in rabbit meatballs using a method described for beef patties [5], with some modifications. Each meatball sample (0.18–0.20 g) was ground in 1 mL of 50 g/L trichloroacetic acid and 6 μL of 1 g/L BHT using a stirrer for 20 s and mixed on a vortex for 20 s. This mixture was centrifuged at 1900 g for 10 min at 18°C, and 250 μL of the supernatant or a malondialdehyde (MDA) calibrator was incubated with 150 μL of 0.8% w/v thiobarbituric acid at 75°C for 30 min and stored at 4°C for 3 min. The mixture (200 μL) was added to a well of a 96-well plate, and absorbance was measured at 532 nm



(a)



(b)



(c)

Figure 2: Representative images showing the meat used to prepare the rabbit meatballs fortified with the powdered Ea extract. (a) A leg of a rabbit. (b) Crude rabbit meatballs. (c) Cooked rabbit meatballs.

using the microplate reader and software used to measure the total polyphenol content. The MDA calibrators used had a concentration ranging from 0.019 to 4.80 μg/mL and were prepared from a 240 μg/mL MDA stock solution. This stock solution was obtained by exposing 1,1,3,3-tetraethoxypropane to 0.1 N hydrochloric acid in a boiling water bath.

TABLE 1: Rabbit meatball formulations prepared with or without plant powder.

Ingredient (g)	Meat without Ea	Meat with Ea
Base mince	150.00	150.00
Powdered extract	0.0000	0.0150
Sodium chloride	2.40	2.40
Disodium phosphate	0.45	0.45
Potato starch	7.50	7.50
Ice-cold drinking water	24.00	24.00

Note: Three independent batches of meatballs were made for each formulation, with each group containing 21 meatballs.

TBARS content was calculated through interpolation into an MDA calibration curve, and the volume and mass of the sample were used. The results were expressed as micrograms of MDA equivalent per gram of meatball.

2.4.8. Total Sulfhydryl Group Content. To evaluate total sulfhydryl group content, three sequential steps were performed. In the first step, myofibrillar proteins (MPs) were extracted as described by Hemung and Chin [17], with some modifications. Briefly, each meatball sample (0.50–0.53 g) was homogenized in 2 mL of a washing buffer (0.05 M Na_2HPO_4 plus 0.1 M NaCl, pH 6.25) using the Tekmar homogenizer at 8000 rpm for 1 min. The homogenate was centrifuged at 1000 g for 15 min at 4°C. After the supernatant was discarded, the pellet was resuspended in 1 mL of the washing buffer and centrifuged. Again, resuspension and centrifugation of the sample were done. Subsequently, the pellet was resuspended in 1 mL of a 0.1 M NaCl solution (pH 6.25) and filtered using a Swinnex filter holder (Millipore Corporation, Bedford, Massachusetts, United States) with a Whatman Grade 1 filter paper (GE Healthcare Company, Little Chalfont, Buckinghamshire, United Kingdom). The filtrate was centrifuged at 1000 g for 15 min at 4°C. Finally, the supernatant was discarded, and the pellet, which contained MPs, was stored at −20°C for subsequent analysis. In the second step, the protein concentration of each MP pellet was determined using Bradford microassay as described previously [18], with slight modifications. Before the assay, each MP pellet was suspended in 400 μL of a 0.9% (*w*/*v*) NaCl solution. Bradford reagent (400 μL) was added to 40 μL of the MP suspension, and this mixture was incubated at room temperature for 10 min. Subsequently, 200 μL of the mixture was added to a well of a 96-well plate, and absorbance was measured at 595 nm using the microplate reader and software used for polyphenol quantification. Bovine serum albumin was used to construct protein calibration curves (13–416 μg/mL), and the absorbance values of MP suspensions were interpolated in these curves to determine the protein concentration (μg/mL) of the suspensions. In the third step, sulfhydryl groups in MP suspensions were quantified as described by Li et al. [3], with slight modifications. Briefly, each MP suspension (100 μL) was vigorously mixed with 800 μL of a Tris–glycine–urea solution (pH 8.0; 10.4 g of Tris, 6.9 g of glycine, 1.2 g of ethylenediaminetetraacetic acid [EDTA], and 0.48 g of urea per liter) and centrifuged at 11,000 g for 10 min at 15°C. The supernatant (450 μL) was mixed with 50 μL of 10 mmol/L Hellman reagent and incubated at room temperature for 30 min. After incubation, 200 μL of the mixture was added to a well of a 96-well plate, and absorbance was measured against a blank (water) at 412 nm using the microplate reader and software described above. The content of sulfhydryl groups in MP suspensions (μmol/L) was calculated using the Beer–Lambert law equation, with a molar extinction coefficient of 13,600 mol/L•cm. Based on the data obtained in the last two steps and the volume of MP suspension, the results were expressed as micrograms of sulfhydryl groups per gram of protein determined in the MP pellet.

2.4.9. pH Monitoring. pH was measured as described previously [3], with slight modifications. Each meatball sample (0.50 g) was weighed, chopped, and homogenized in distilled water (5 mL) using a homogenizer (Tekmar Company, Cincinnati, Ohio, United States) at a medium speed (8000 rpm) for 1 min. The homogenate was allowed to stand for 30 s, and pH was subsequently monitored using an Accumet AR15 pH meter (Fisher Scientific, Pittsburgh, Pennsylvania, United States). Before using the pH meter, it was calibrated with a phosphate buffer solution at pH 7 (J.T. Baker, Mallinckrodt Baker Inc., Mexico City, Mexico). Based on the pH equation and the volume and mass of the sample used, the results were expressed as nanomoles of H^+ per gram of sample.

2.5. Data Analysis. Data were expressed as the mean and standard deviation. For all the analyses, normality, variances, and number of groups were considered to select a parametric or nonparametric test. In this sense, data obtained from the quantification of polyphenols and flavonoids in the four extracts ($N = 6$) or the two variants of the Ea extract ($N = 6$) were analyzed using the Kruskal–Wallis test with Dunn's multiple comparison test or an unpaired *t*-test, respectively. Data obtained from the assessment of antioxidant activity in the four extracts ($N = 5$) or the two extracts of Ea ($N = 5$) were analyzed using one-way analysis of variance (ANOVA) with Tukey's post hoc test or an unpaired *t*-test with Welch's correction, respectively. LC data ($N = 3$) were evaluated using an unpaired *t*-test. Finally, the color, cooking loss, pH, antioxidant capacity, TBARS content, and sulfhydryl content ($N = 7$) of rabbit meatballs were analyzed using two-way ANOVA with the Bonferroni post hoc test.

A p value of < 0.05 was considered statistically significant. Calculation of IC_{50} values and statistical analyses were performed using the GraphPad Prism 5 software (San Diego, California, United States).

3. Results

3.1. Polyphenols and Flavonoids in Extracts. Ea extract had a higher polyphenol content than the other three extracts. Flavonoid content showed no significant differences among the Ea, *M. charantia*, and *A. aculeata* extracts, whereas flavonoids were not detected in *D. opuntiae* extract (Table 2).

TABLE 2: Polyphenol and flavonoid concentrations in the tested extracts ($N = 6$).

Parameter	Sample	Content
Total polyphenols (mg GAE/g of sample)	Ea leaf extract	337.45 ± 11.53[a]
	Mc leaf extract	21.36 ± 7.07
	Aa pulp extract	26.18 ± 5.49
	Do insect extract	23.57 ± 0.58
	Positive control (quercetin)	1460.40 ± 32.59
	Negative control (citric acid)	Not detected
Total flavonoids (mg RE/g of sample)	Ea leaf extract	17.32 ± 1.24
	Mc leaf extract	43.02 ± 2.39
	Aa pulp extract	11.54 ± 6.06
	Do insect extract	Not detected
	Positive control (quercetin)	1589.95 ± 138.11
	Negative control (citric acid)	Not detected

Abbreviations: Aa, *A. aculeata*; Do, *D. opuntiae*; Ea, *E. antisyphilitica*; GAE, gallic acid equivalent; Mc, *M. charantia*; RE, rutin equivalent.
[a]p value of < 0.05 versus Mc, Do, and Aa extracts.

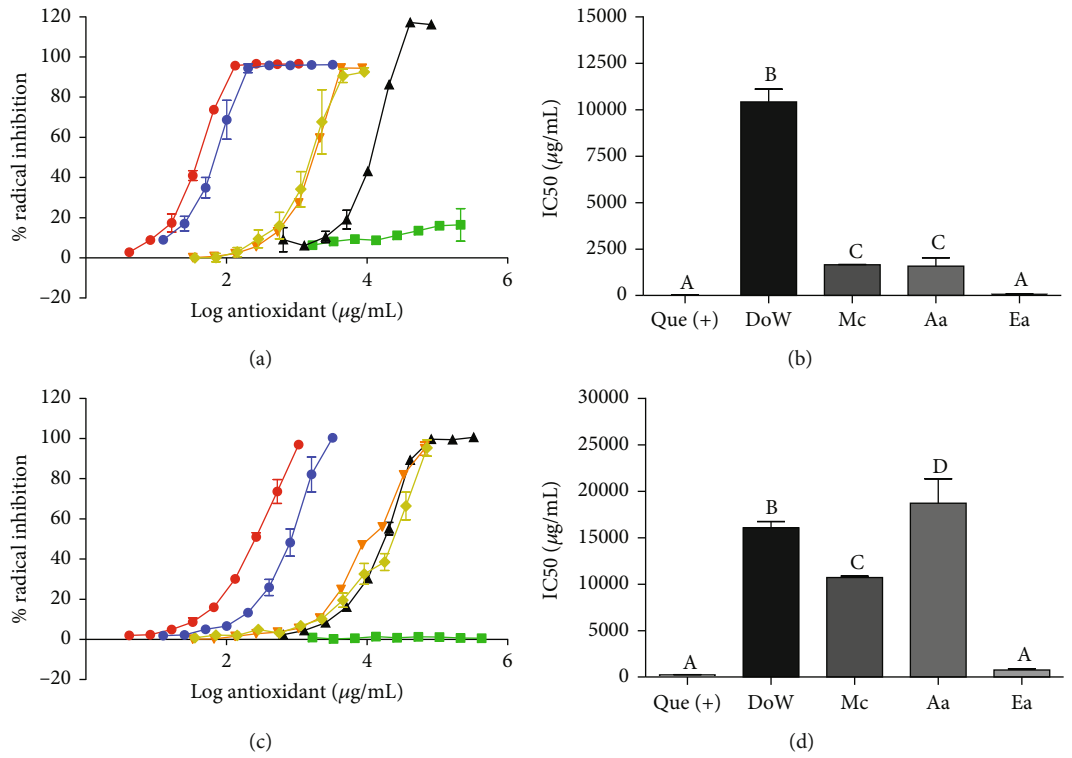


FIGURE 3: Antioxidant activities of extracts against synthetic radicals. (a, b) Graph and IC_{50} values obtained from DPPH radical–scavenging assay. (c, d) Graph and IC_{50} values obtained from ABTS radical–scavenging assay. In each curve, 8–12 points were plotted, with $N = 5$ for each point. In this sense, when one column is different from another column, this statistically significant difference was represented by a different letter above it, and the p value was < 0.0001. The red, blue, olive, orange, black, and green lines represent positive control (Que +), Ea extract, *A. aculeata* (Aa) extract, *M. charantia* (Mc) extract, *D. opuntiae* (DoW) extract, and negative control, respectively.

3.2. Antioxidant Activity in Extracts. Among the four extracts, Ea extract exhibited the highest antioxidant activity against DPPH and ABTS radicals as evidenced by its lower IC_{50} values than those of the other three extracts. The antioxidant activity of Ea was similar to that of pure quercetin, which was used as a positive control (Figure 3).

3.3. Powdered Ea Extract. The powdered Ea extract exhibited a recovery of 78.8% ± 1.4% ($N = 5$). The drying process increased polyphenol and flavonoid contents and reduced I C_{50} values against DPPH and ABTS radicals, indicating an increase in antioxidant activity in the powdered extract compared with the semisolid Ea extract. Consistently, the

TABLE 3: Antioxidant activity and composition of Ea extracts.

Parameter	Semisolid extract	Powdered extract
Polyphenols (mg GAE/g of sample, $N = 6$)	Shown in Table 1	485.97 ± 29.05[a]
Flavonoids (mg RE/g of sample, $N = 6$)	Shown in Table 1	20.25 ± 1.79[b]
IC_{50} value, DPPH radical (μg/mL, $N = 5$)	66.32 ± 11.47	47.36 ± 2.55[c]
IC_{50} value, ABTS radical (μg/mL, $N = 5$)	755.6 ± 132.4	549.6 ± 36.81[d]
Gallic acid (μg/g of sample, $N = 3$)	5006.4 ± 1657.9	16,390.8 ± 3946.0[e]
Methyl gallate (μg/g of sample, $N = 3$)	12,213.0 ± 1576.1	13,559.3 ± 806.5
Rutin (μg/g of sample, $N = 3$)	8810.3 ± 385.6	11,771.8 ± 177.9[f]
Myricetin (μg/g of sample, $N = 3$)	4547.4 ± 1246.8	3821.0 ± 352.6
Quercetin (μg/g of sample, $N = 3$)	810.9 ± 74.3	1051.2 ± 59.7[g]
Apigenin (μg/g of sample, $N = 3$)	261.5 ± 36.3	306.8 ± 13.9
Ascorbic acid (μg/g of sample, $N = 3$)	Not detected	Not detected
p-Coumaric acid (μg/g of sample, $N = 3$)	Not detected	Not detected
Sinapic acid (μg/g of sample, $N = 3$)	Not detected	Not detected
Caffeic acid (μg/g of sample, $N = 3$)	Not detected	Not detected
o-Coumaric acid (μg/g of sample, $N = 3$)	Not detected	Not detected
Gentisic acid (μg/g of sample, $N = 3$)	Not detected	Not detected
Benzoic acid (μg/g of sample, $N = 3$)	Not detected	Not detected
Kaempferol (μg/g of sample, $N = 3$)	Not detected	Not detected
Other flavanols and flavones (%, $N = 3$)	89.1 ± 1.2	86.8 ± 0.2[h]

Note: Superscripted letters (a–h) represent p values of < 0.0001, 0.0082, 0.0226, 0.0285, 0.0100, 0.0003, 0.0120, and 0.0339 for the comparison between the values found in the powdered extract versus those found in the semisolid extract for the same parameter analyzed in both samples.

amounts of gallic acid, rutin, and quercetin were higher in the powdered extract than in the semisolid extract. The additional flavanol and flavone fractions were reduced in the powdered extract compared with the semisolid extract (Table 3).

3.4. Effects of the Powdered Ea Extract on Rabbit Meatballs. As shown in Table 4, the use of the powdered Ea extract in meatballs prevented weight reduction caused by refrigerated storage on Day 12, which was observed in meatballs without a preservative. For this analysis, only cooking was identified as the source of variation ($p = 0.0320$).

In frozen meatballs free of any preservatives (control group), lightness increased by Day 6 and remained elevated until Day 12. The addition of Ea powder to meatballs reduced lightness from the beginning to the last day of evaluation compared to that in the case of the control group (Figure 4a). The sources of variation in lightness were time ($p < 0.0001$) and the use of the powdered Ea extract ($p < 0.0001$). In the control group, the redness of meatballs diminished over time, reaching statistical significance on Day 12. However, the use of the powdered Ea extract prevented the reduction in redness throughout the storage period (Figure 4b). For redness analysis, only time was identified as a source of variation ($p = 0.0299$). The use of the powdered Ea extract decreased the yellowness value of frozen meatballs at all time points when compared with the control group (Figure 4c). For yellowness analysis, the use of the powdered Ea extract was identified as a source of variation ($p < 0.0001$). Figure 4d shows the external and internal surfaces of rabbit meatballs that were monitored using a colorimeter.

TABLE 4: Effects of cooking on the mass (g) of the meatballs tested ($N = 7$).

Group	Uncooked meat	Cooked meat, Day 0	Cooked meat, Day 12
Co	20.69 ± 1.11	19.14 ± 1.56	18.62 ± 1.03[a]
Ea	20.69 ± 1.69	19.49 ± 2.56	19.32 ± 2.05

Abbreviations: Co, meat without preservatives; Ea, meat fortified with powdered Ea extract.
[a]p value of < 0.05 versus the mass of uncooked meatballs from the same group.

The antioxidant activity of preservative-free meatballs against the DPPH radical decreased during refrigerated storage; however, the use of the powdered Ea extract in meatballs increased antioxidant activity, with the activity remaining high at all time points evaluated (Figure 5a). The sources of variation in antioxidant activity were time ($p = 0.0070$) and the use of the powdered extract ($p < 0.0001$).

The MDA content of preservative-free meatballs increased during refrigerated storage. However, the use of Ea powder in meatballs decreased MDA content at all time points evaluated. The MDA content of meatball samples with Ea powder increased on Day 12; however, it was substantially lower than that of preservative-free meatballs (Figure 5b). For MDA analysis, time ($p < 0.0001$), the use of the powdered extract ($p < 0.0001$), and interactions

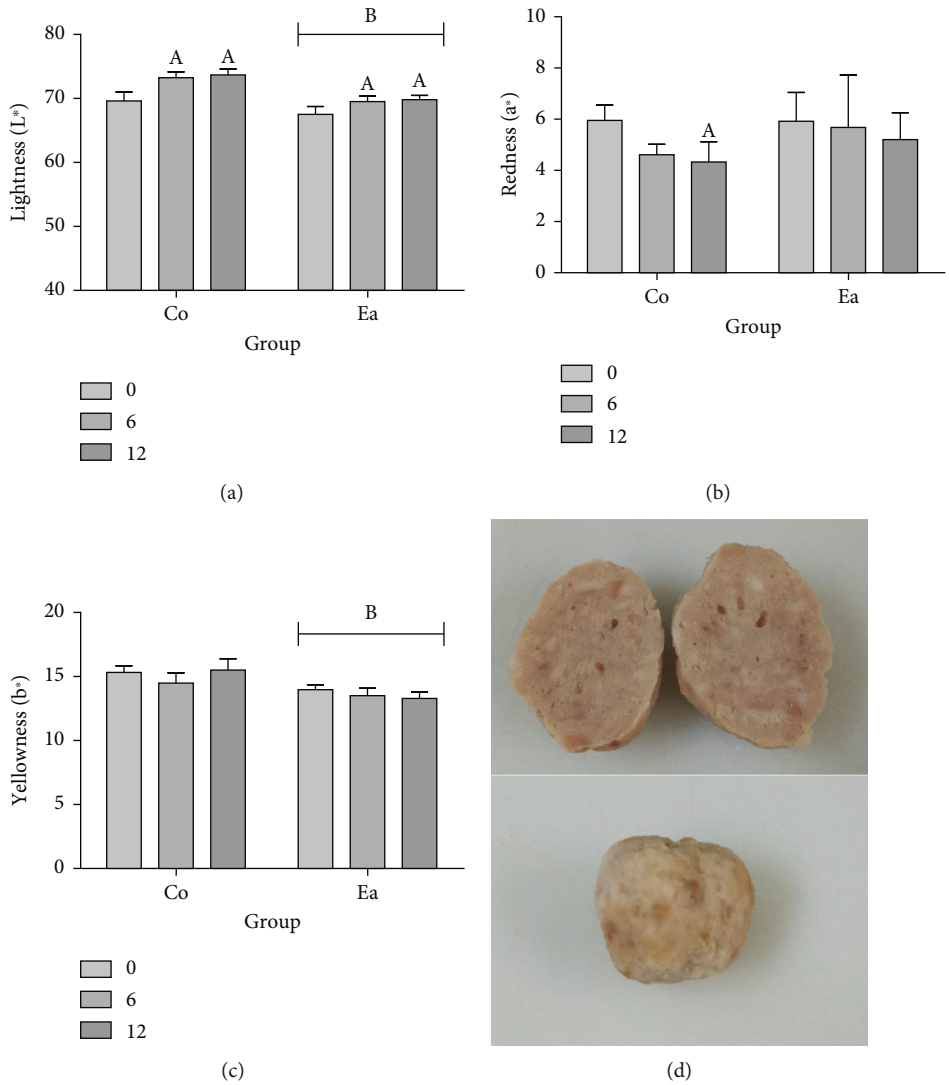


Figure 4: Color parameters obtained from the groups tested ($N = 7$). (a) Lightness in meatball samples on Days 0, 6, and 12 of cold storage. (b) Redness of meatballs during cold storage. (c) Yellowness of meatballs during cold storage. (d) Representative images of rabbit meatballs, one whole and the other cut in half, fortified with the powdered Ea extract at Day 0. For lightness: ^{a}p value of < 0.01 versus Day 0 in the same group and ^{b}p value of < 0.01 versus the Co group. For redness: ^{a}p value of < 0.05 versus Day 0 in the same group. For yellowness: ^{b}p value of < 0.05 versus the control group. Co, meat without preservatives; Ea, meat fortified with powdered Ea extract.

between these two factors ($p < 0.0001$) were identified as the sources of variation.

The content of sulfhydryl groups in preservative-free meatballs showed no significant changes during cold storage. The addition of Ea powder increased sulfhydryl content on Day 0, with no further changes observed over time (Figure 5c). Only time was identified as the source of variation ($p = 0.0175$). The pH values did not show statistically significant differences between the control and extract groups at any time point (Figure 5d).

4. Discussion

For the first time, this study showed that Ea stem extract was a better source of phenolic compounds than *M. charantia* leaf, *A. aculeata* fruit, and *D. opuntiae* extracts (Table 2). Ea is recognized as a good source of polyphenolic compounds [9]. Also, *M. charantia* leaves and *A. aculeata* fruit pulp have been shown to contain a high polyphenol content [7, 8]. In addition, we report for the first time that *D. opuntiae*, an insect, contains polyphenolic compounds. Polyphenolic compounds have been identified in various other insect species, such as *Acheta domesticus*. The presence of phenolic compounds in insects is associated with their diet and ability to synthesize these compounds through sclerotization [10]. It is important to note that the solvent used for each sample extraction was selected to achieve the greatest recovery of metabolites with antioxidant properties present in the sample, which was based on previously published information about the sample. For the extraction applied to *M. charantia* and *A. aculeata*, a mixture of alcohol–water solution was chosen to perform a complete extraction of polyphenols [19]. For the extraction used in Ea, methanol was employed as it is more efficient in extracting low

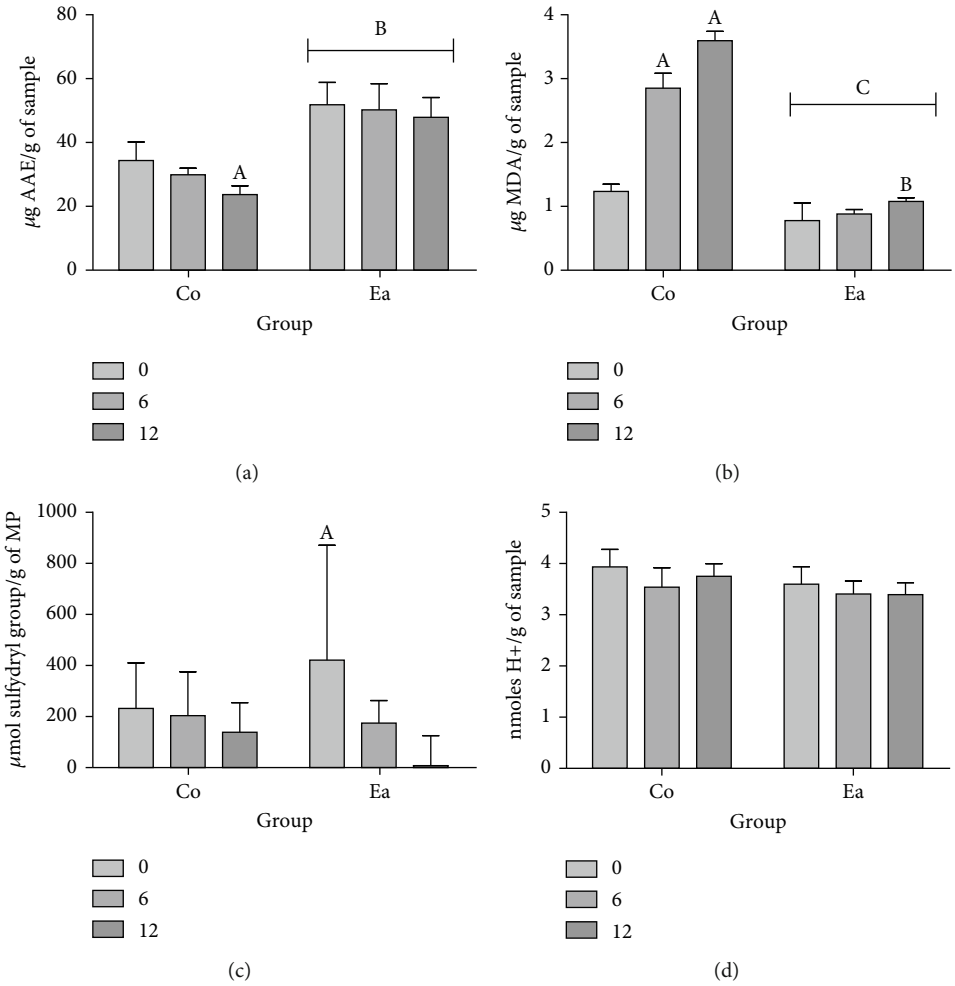


Figure 5: Antioxidant and oxidative parameters evaluated in rabbit meatballs ($N = 7$). (a) Antioxidant activity of meatballs against the DPPH radical on Days 0, 6, and 12 of cold storage. (b) MDA values found in meatballs at the time points tested. (c) Content of sulfhydryl groups recorded in stored meatballs. In addition, the pH records are shown ($N = 7$). (d) pH values obtained from the meatball samples tested. For antioxidant assay: ^{a}p value of < 0.01 versus Day 0 in the same group and ^{b}p value of < 0.001 versus the Co group. For MDA quantification: ^{a}p value of < 0.001 versus other days in the same group, ^{b}p value of < 0.01 versus Day 0 in the same group, and ^{c}p value of < 0.001 versus the Co group. For sulfhydryl group quantification: ^{a}p value of < 0.01 versus Day 12 in the same group. Co, meat without preservatives; Ea, meat fortified with powdered Ea extract.

molecular weight polyphenols, such as gallic acid, which was expected to be abundant in this sample according to previous information [9, 19]. Meanwhile, for the extraction applied to *D. opuntiae*, water was selected as it demonstrated a superior carminic acid extraction compared with a citric acid solution used as solvent. Although carminic acid is not a polyphenolic compound, its extraction was sought because it is an abundant compound with antioxidant capacity found in the insect [11]. Owing to its high polyphenol content, Ea extract exhibited the strongest antioxidant activity of all extracts (Figure 3). Polyphenols are well-known bioactive components with strong antioxidant properties [20]. Only for *D. opuntiae* extract, carminic acid, a glucosidal tetrahydroxyanthraquinone, together with the detected polyphenols, contributes to its observed antioxidant activity [11].

Because the Ea extract had the highest polyphenol content and antioxidant activity, it was selected to be used as a preservative in rabbit meatballs. The antioxidant activity of compounds has a strong relationship with their preservative ability in meat products [4]. As the Ea extract had a semisolid consistency, it could not be homogenized when added to ground meat. Consequently, an additional drying step was applied to the extract to obtain its powdered form (Figure 1). The powdered extract was readily homogenized with rabbit meat. To confirm that the powdered Ea extract did not undergo negative changes in its properties, the total polyphenol and flavonoid contents, antioxidant activity, and gallic acid concentration of the powdered extract were determined and compared with those of the semisolid extract. Despite the limited information on the chemical characterization of the candelilla plant in the literature, gallic acid concentration was monitored because it has been identified as one of the main phytochemicals in the whole Ea plant [9]. Additionally, catechin, ellagic acid, candelitannin, flavonoids with unspecified identity, tannins, saponins, and

quinones are found in the candelilla plant [9]. In our study, gallic acid concentration was three times higher in the powdered extract than in the semisolid extract. Since the semisolid Ea extract can contain tannins [9], the high temperature used in the drying step can hydrolyze these tannins, which in turn produces additional gallic acid in the powdered Ea sample [21]. For the first time, we report the presence of methyl gallate, myricetin, apigenin, rutin, and quercetin in Ea stems (Table 3). Similarly, the concentrations of rutin and quercetin were higher in the powdered extract compared with the semisolid extract. Rutin and quercetin are recognized as the main compounds found in biologically relevant extracts obtained from natural sources [12]. Since the literature does not report additional precursors or sources for rutin and quercetin in Ea plant, the increase of these two flavonoids in the powdered form was probably caused by the loss of mass of the sample after the drying process. Consequently, the increased levels of gallic acid, rutin, and quercetin produced high polyphenol content and antioxidant ability in the powdered samples relative to the semisolid samples. On the other hand, the differences observed between our and previous chemical compositions of the plant can be due to factors such as weather conditions, season, and genetic variability, as well as the methodology of extraction used [9]. It is important to mention that the maceration of our Ea samples involved the use of methanol, which is recognized as a toxic solvent. The presence of residual methanol was expected in the semisolid Ea extract, but the application of the drying step to the semisolid extract allowed the complete evaporation of methanol, and then, the powdered Ea extract was free of methanol. This situation is expected since the temperature used in the drying step was higher than the boiling point of methanol (64.7°C). Moreover, the temperature of the drying step avoided the thermal formation of byproducts from methanol due to it being lower than the temperature for pyrolysis of methanol (≥ 500°C) [22–24].

Since there are no studies of any extract obtained from the Ea plant applied to meatballs of any type of meat, our discussion does not contain comparisons with previous studies. The yield and quality of rabbit meatballs improved after the addition of Ea powder, as cooking loss was lower in these samples than in preservative-free meatballs (Table 4). Cooking loss is a crucial factor affecting the yield and quality of processed meat products [25]. Cooking loss is influenced by the type and functional properties of muscle proteins. In this case, the polyphenols from the powdered extract interacted with the proteins found in meatballs, producing a three-dimensional network structure that enhanced water retention [25, 26]. This kind of interaction between pure polyphenols and proteins was reported in studies using pork meatballs [26].

The use of Ea powder can positively influence consumer choices regarding meat products. The powdered extract improved the color-related characteristics of rabbit meatballs (Figure 4). The color of meat products is a crucial factor affecting consumer choices [3]. Herein, the use of the powdered Ea extract prevented the undesirable increase in lightness in rabbit meatballs during cold storage. Low lightness can result from reduced light reflection, which in turn is caused by a low moisture content on the surface of meatballs [3]. In addition, the powdered extract increased redness and reduced yellowness in meatballs during cold storage. High redness values and low yellowness values indicate suppression of myoglobin oxidation during cold storage [3]. Thus, Ea powder produced beneficial actions on color in rabbit meatballs in a similar fashion to other types of antioxidants, such as 6-gingerol, on mutton meatballs [3]. To our knowledge, the individual or mixture of some of the phenolic compounds identified in the present Ea extract has not been evaluated in any type of meatballs.

Meatballs supplemented with Ea powder can be considered a new functional food owing to their high antioxidant capacity (Figure 5a). The consumption of a rich source of plant antioxidants, such as phenolic acids and flavonoids, can provide health benefits to consumers, as these compounds play a crucial role in preventing the development of chronic diseases, such as cardiovascular diseases, diabetes, inflammation, and cancer [27]. On the other hand, the presence of large amounts of antioxidants leads to the suppression of lipid oxidation in fats in meat products, such as in beef patties, during cold storage [5]. Antioxidants, such as the polyphenols found in Ea extract, can prevent or delay lipid oxidation by neutralizing reactive oxygen species and free radicals through their hydrogen-donating potential. This potential is recognized as the primary mechanism against lipid oxidation in meat products. Besides, a multitude of other mechanisms of polyphenols can provide synergism that enhances their antioxidant effectiveness in muscle food. Recent research evidence shows that polyphenols inhibit the heme protein–mediated lipid oxidation, primarily in myoglobin and hemoglobin proteins, through polyphenol–protein interactions of both covalent and noncovalent natures, which in turn produce heme proteins less effective or unable to participate in the lipid oxidation process. Moreover, the partition of polyphenols into the cellular membranes of muscle generates a physical barrier that decreases the access of heme proteins to membrane phospholipids. Other mechanisms described for polyphenols to disrupt lipid oxidation include the mitigation of deleterious effects of reactive carbonyl species, regeneration of alpha-tocopherol, chelation of free iron, and decomposition of lipid hydroperoxides [28].

We found that MDA levels were lower in rabbit meatballs supplemented with Ea powder than in preservative-free ones (Figure 5b). We do not identify a cutoff MDA value that describes spoiled rabbit meat when the value is above it. Nevertheless, it is well known that the TBARS value is a parameter that determines the extent of fat oxidation during cold storage and the freshness of meatballs [3]. In a study using mutton meatballs, values of 0.202–0.664, 0.664–1.000, and >1.000 mg MDA/kg were described for fresh, second-fresh, and spoiled meat, respectively [3]. For our rabbit meatballs, all the values of the Co group and only the latest day of evaluation of the Ea group were above 1.000 mg MDA/kg. However, it is important to consider the type of meat tested because meat with a high unsaturated fatty acid content is always more prone to lipid oxidation

and deterioration, which has already been described for rabbit meat [1].

Furthermore, meatballs supplemented with Ea powder had a higher sulfhydryl group content on Day 0, which indicated that the extent of protein oxidation was slightly reduced owing to the presence of plant antioxidants in meatballs fortified with Ea powder (Figure 5c). As reported for experiments using apple polyphenols and frozen lamb meat, a high content of sulfhydryl groups reflects suppressed protein oxidation, as the amount of sulfhydryl groups in proteins decreases when converted to disulfide groups after oxidation [29]. Although the formation of alkaline byproducts that can increase the pH value was expected during the tested time, no significant differences were observed between the pH values of rabbit meatballs with and without Ea powder (Figure 5d). Alkaline products generated through the oxidation or metabolism of certain microorganisms growing during cold storage have been identified in preservative-free mutton meatballs. These compounds should result in an increased pH value [3].

Although we evaluated important meat quality parameters for the food use of the Ea extract, including key consumer acceptability predictors such as cooking loss and color [3, 25], further investigations are necessary. Among the additional assessments necessary, we can mention the cytotoxicity assay for the Ea extract as well as the monitoring of other effects on meat by the Ea supplementation, such as microorganism growth, shelf-life of the product, and sensory evaluations. Although prior experimental evidence suggests the food safety of the present product, another limitation observed in the present work is that methanol and its thermal byproducts were not quantified in the powdered extract. Despite all the limitations mentioned, this study reports the first use of an Ea powdered extract in preserving ready-made meat products such as rabbit meatballs.

5. Conclusion

Ea extract was the major source of antioxidant compounds, compared with the extracts of *M. charantia*, *A. aculeata*, and *D. opuntiae*. Moreover, the drying of the Ea extract to powder enhanced its polyphenol content and antioxidant ability in comparison with the semisolid form of the extract. The use of the powdered Ea extract in rabbit meatballs improved several quality parameters, such as the cooking loss and color, as well as changing the meat food into a rich source of natural polyphenols. Likewise, two crucial deleterious processes during meat cold storage, such as lipid and protein oxidation, were reduced by the Ea supplementation in rabbit meatballs. This plant powder exhibited some of the desired properties for its use as a natural preservative in precooked meat products; however, further evaluations are needed for its application in food products for human consumption.

Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors on request.

Ethics Statement

The animal study protocol was approved by the Institutional Animal Care and Use Committee of Universidad Autónoma de San Luis Potosí (protocol code: 0083072).

Disclosure

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization, M.M.G.-C. and O.H.A.-M.; methodology, O.H.A.-M., O.G.G.-C., B.A.-A., and J.F.L.-R.; formal analysis, O.G.G.-C., O.H.A.-M., B.A.-A., and J.F.L.-R.; investigation, O.H.A.-M. and M.M.G.-C.; data curation, O.G.G.-C., M.A.I.-E., F.M.-M., and M.M.G.-C.; writing—original draft preparation, O.H.A.-M. and M.A.I.-E.; writing—review and editing, M.M.G.-C., O.G.G.-C., M.A.I.-E., B.A.-A., J.F.L.-R., and F.M.-M.; visualization, M.M.G.-C. and O.H.A.-M.; project administration, O.H.A.-M. and M.M.G.-C.; funding acquisition, O.H.A.-M.

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