ACTIVITY OF NANO ENCAPSULED ANNATTO SEEDS AND BEETROOT EXTRACTS TO EXTENDING THE SHELF LIFE OF BEEF BURGER

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ABSTRACT

The present study highlighted the effectiveness of natural extracts derived from annatto and beetroot in prolonging the shelf life of refrigerated beef burgers for up to 12 days. The experimental design included five treatment groups: T1 (control, no additives), T2 (185.6 µg/ml nano-encapsulated annatto), T3 (187.6 µg/ml nano-encapsulated beetroot), T4 (a combination of nano-encapsulated annatto and beetroot at the same concentrations), and T5 (0.01% BHA). Samples were stored under refrigeration for 1, 3, 6, 9, and 12 days, after which they were subjected to a series of physical, chemical, microbiological, and sensory evaluations. The data revealed that the nano-encapsulation of annatto and beetroot extracts led to statistically significant increases ($P < 0.01$) in protein, fat, and ash content, as well as elevated levels of myoglobin pigment and thiobarbituric acid (TBA) values. In contrast, moisture content and water-holding capacity (WHC) showed significant reductions ($P < 0.01$). These findings suggest that incorporating nano-formulated annatto and beetroot extracts into beef burgers contributes to enhanced antioxidant activity during chilled storage, as evidenced by lower oxidative indicators and reduced total bacterial counts.

Keywords:norbixin,capsule,bixin,anti-oxidation,betalains, food safety

فعالية مستخلصات بذور الأناتو والشمندر المغلفة بكبسولات نانوية في إطالة العمر الخزني لبركر اللحم البقري

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المستخلص

سلطت هذه الدراسة الضوء على فعالية المستخلصات الطبيعية المستخرجة من بذور الأناناتو والشمندر في إطالة العمر التخزيني لبرغر اللحم البقري المحفوظ بالتبريد لمدة تصل إلى 12 يومًا. وقد شمل التصميم التجريبي خمس معاملات كما يلي: T1 السيطرة، دون إضافات(،T2 (185.6 ميكروغرام/مل من مستخلص الأناناتو المغلف نانوياً(،T3 (187.6 ميكروغرام/مل من مستخلص الشمندر المغلف نانوياً T4 (مزيج من الأناناتو والشمندر المغلفين نانوياً بنفس التراكيز)، و T5 (0.01% BHA). تم حفظ العينات في التبريد لفترات 1، 3، 6، 9، و12 يومًا، ثم خضعت لسلسلة من التقييمات الفيزيائية والكيميائية و الميكروبيولوجية والحسية. أظهرت البيانات أن تغليف مستخلصي الأناناتو والشمندر بتقنية النانو أدى إلى زيادات معنوية إحصائيًا ($P < 0.01$) في محتوى البروتين والدهن والرماد، بالإضافة إلى ارتفاع مستويات صبغة المايوكلوبين وقيم حمض الثايوباربتيوريك (TBA). في المقابل، انخفضت نسبة الرطوبة وقدرة حفظ الماء (WHC) بشكل معنوي ($P < 0.01$). تشير هذه النتائج إلى أن إدخال مستخلصات الأناناتو والشمندر المغلفة نانوياً في برغر اللحم يساهم في تعزيز النشاط المضاد للأكسدة أثناء التخزين المبرد، كما يتضح من انخفاض مؤشرات الأكسدة وتراجع أعداد البكتيريا الكلية.

الكلمات المفتاحية: نوربيكسين، الكبسلة، بيكسين، مضادات الاكسدة، بيتالين، سلامة الغذاء

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INTRODUCTION

In the last years, enormous growth in the demand for processed meat products has been observed that can respect both health and safety measures as well as satisfy the taste preferences of the consumers (1, 40). This has led to a revolutionary change wherein the paradigm of the meat processing industry has shifted greatly toward fulfilling the expectations of the consumers by developing products that are functional and safe for long-term consumption (3, 41). In the past, nitrite salts were popular as meat preservatives since they inhibited the microbial growth in the meat, thus enhancing flavor and contributing to the characteristic cured meat color (5). However, information has for long been building up, inclusive of assessments by WHO, in terms of the implication of nitrites and nitrates intake concerning health, most notably as carried out under conditions and high levels (11, 12, 21, 37). These have brought the required proper attention to the public and, therefore, directed the industry to move towards exploring new, naturally synthesized alternatives. As a result, the use of natural colorants and preservatives of plant origin has been high. These compounds give a dual benefit by enhancing the visual and organoleptic features of meat products along with antioxidant and antimicrobial properties. Some commonly available vegetables, such as annatto, beetroot, celery, spinach, radish, and lettuce, have strong resources toward nitrates which can act as analogs for some of the preservative actions of synthetic nitrites (15). Among them, beetroot is very promising due to its abundant betalains and phenolic compounds which can give not only color as natural pigments but also as strong antioxidants that can fight reactive oxygen species (30). Such active compounds in beetroot are responsible for providing enhanced oxidative stability, better texture, and color retention in processed meats as well as significant chemo preventive properties (6, 39). Likewise, annatto seeds obtained from Bixa orellana are very rich in flavonoids and phenolic acids responsible for their strong coloration and bioactivity. These compounds give annatto a broad range of functional properties including activities toward being an antioxidant, anti-inflammatory, and antimicrobial; therefore, it may be an ideal candidate to use as a multifunctional additive in meat formulations (34). At the same time, nanotechnology has come about, which means new ways that are changing the food making and wrapping parts. Because they are very small, nanomaterials have more surface area for their size and show better special qualities which is very useful when it comes to food (13). When used in wraps, nanocomposites have better solidness against heat, a barrier against moisture and gas, and a barrier against microbes. Also, new ideas for putting Nano sensors together make it possible to watch how fresh the food is, how spoiled it is, and how much bacteria are on it, therefore making the product safer and having a longer shelf life. (24, 25, 42). Driven by environmental concerns, there is also an increasing interest in replacing synthetic packaging materials with biodegradable ones. Since the biodegradable polymers are of natural origin also having intrinsic biological activities, cellulose, carrageenan, and chitosan have been increasingly used and adopted (37). Indeed, such materials greatly lower the footprint for the packaging of foods and, in addition, further aid in the preservative action via means such as antimicrobial and antifungal effects. This, therefore, complies with the very recent development in the production of food in a sustainable manner (21). In view of the convergence of these developments, this study sets up to undertake a systematic appraisal of the promise that nano-encapsulated beetroot and annatto extracts offer as additives sourced naturally in the formulation of beef burgers. The work centers on their assay for supporting prolonged shelf life under refrigerated storage, concomitant with an appraisal of their functionality as antioxidants and antibacterial agents. This work adopts an approach that synthesizes bioactive compounds with nanotechnology to address current drawbacks in the preservation of meat by offering a safer and more sustainable alternative to synthetic additives.

MATERIALS AND METHODS

This experiment took place at the Meat Science and Technology Laboratory of the Department of Animal Production, College of

Agricultural Engineering Sciences, University of Baghdad, in collaboration with the laboratories of the Scientific Research Authority under the Ministry of Higher Education. 8 kilograms of beef were collected from the rump section of a calf carcass, and 2 kilograms of kidney and pelvic fat were taken from the same animal. All samples were collected just after slaughtering and were kept in refrigeration for over 12 hours so that the rigor mortis condition could be relaxed. Following this initial cooling phase, the meat was manually sliced using a sterile knife into small chunks measuring approximately 3–4 cm, to facilitate uniform mincing. The meat and fat were first ground using an electric meat grinder equipped with a 6 mm plate. To enhance sample uniformity, a second grinding step was performed using a 3 mm plate, resulting in a finer and more homogenous meat-fat blend. The homogenized mixture was then divided into five equal portions, each weighing 2 kg. To each portion, 1 gram of burger seasoning (Malouya spice blend) was added to standardize flavor. Each portion was subsequently treated with a specific additive: nano-encapsulated beetroot extract, nano-encapsulated annatto seed extract, a combination of both nano-encapsulated extracts, or synthetic antioxidant BHA (butylated hydroxyanisole). The treatment groups were defined as follows:

T1: Control (no additive)
T2: 185.6 µg/ml nano-annatto
T3: 187.6 µg/ml nano-beetroot
T4: 187.6 µg/ml nano-beetroot + 185.6 µg/ml nano-annatto
T5: 0.01% BHA

Each sample was uniformly mixed with its respective treatment to ensure consistent distribution of the additives. The treated burger mixtures were then packed into sterile polyethylene bags, sealed, and stored in a freezer at standard conditions. Storage intervals were set at 0, 3, 6, 9, and 12 days, after which samples were withdrawn for analytical evaluations. Several physicochemical and microbiological analyses were conducted to assess the impact of the applied treatments on the quality and shelf life of beef burgers. Moisture, protein, fat, and ash contents were determined following the procedures outlined in (9). Lipid oxidation was assessed by measuring thiobarbituric acid (TBA) reactive substances, while myoglobin concentration was quantified. Total bacterial counts were estimated using standard microbiological techniques, and water-holding capacity (WHC) was evaluated. The experimental design followed a 5×5 factorial scheme based on a completely randomized design **(CRD)** to examine the effects of both treatment type and storage duration on the measured variables. Statistical comparisons of means were performed using a **multinomial test** (16). All statistical analyses were conducted using specialized software tools, as referenced in (32), to ensure data accuracy and the validity of the findings.

RESULTS AND DISCUSSION

Moisture percentage: Table (1) presents the interaction effect between different treatment groups and refrigeration storage durations on the moisture percentage of beef burger samples. The statistical analysis revealed a highly significant difference ($P < 0.01$) in moisture content across both variables treatment and storage time. Notably, the treatment labeled T4, which combined 187.6 µg/ml nano-beetroot and 185.6 µg/ml nano-annatto, recorded the highest moisture percentage (69.73%) after just one day of refrigerated storage. In contrast, the control group T1 (untreated) exhibited the lowest moisture content (47.27%) after 12 days of refrigeration. These findings underscore the potential of nano-encapsulated natural extracts in preserving moisture content during early storage. The interaction between treatment and storage duration produced varying results, suggesting that both factors contribute significantly to moisture retention. Comparable results have been documented in previous studies. For instance, (26) reported that incorporating annatto seed extract into refrigerated beef sausages significantly improved moisture retention. Similarly, (39) observed enhanced moisture levels in sausages treated with beetroot powder and stored at 4°C, further supporting the current findings. Furthermore, the storage duration itself exerted a significant effect ($P < 0.01$) on moisture content. Across all treatments, moisture percentage was highest at day 1, then exhibited a progressive decline with increasing storage time, reaching the lowest point by day 12. This trend is consistent with the natural dehydration that occurs during prolonged refrigeration, which results in increased dry matter content, including protein, fat, and ash. These patterns align with earlier studies reporting that the addition of medicinal plant extracts or their bioactive components can enhance water retention in meat matrices during early storage stages (4, 39). The moisture-retaining properties of these natural additives may be attributed to their polyphenolic and hydrophilic constituents, which could help maintain cellular integrity and reduce drip loss.

Table 1. Effect of the interaction between different treatments and cold storage periods on the moisture percentage (%) ± standard error (SE) of beef burgers treated with nano-encapsulated plant extracts

Transactions	% Moisture					Average
	1 day	3 day	6day	9day	12 day	
T1	[illegible]±0.16^{d}	67.92±0.19^{i}	62.89±0.15^{m}	55.69±0.22^{q}	47.27±0.15^{u}	61.27±2.39^{C}
T2	[illegible]±0.13^{b}	69.72±0.15^{f}	64.84±0.18^{j}	57.75±0.15^{no}	50.27±0.18^{s}	63.30±2.26^{B}
T3	[illegible]±0.19^{c}	69.17±0.16^{g}	64.27±0.15^{k}	57.47±0.12^{o}	50.17±0.16^{s}	62.89±2.21^{C}
T4	[illegible]±0.09^{a}	70.31±0.15^{e}	65.25±0.11^{j}	58.09±0.09^{n}	51.12±0.11^{r}	63.88±2.25^{A}
T5	[illegible]±0.12^{c}	68.59±0.20^{h}	63.80±0.13^{l}	56.98±0.15^{p}	48.19±0.13^{t}	62.13±2.34^{D}
Average	[illegible]±0.19^{A}	69.14±0.23^{B}	64.21±0.22^{C}	57.19±0.23^{D}	49.40±0.38^{E}	

Means with different letters are significantly different ($P < 0.01$). Capital letters indicate the main effect of treatments and storage durations; lowercase letters indicate the interaction effect between treatments and storage durations. Treatments are defined as follows: T1 = no addition (control), T2 = 185.6 µg/ml Nano Annatto, T3 = 187.6 µg/ml Nano Beetroot, T4 = 187.6 µg/ml Nano Beetroot + 185.6 µg/ml Nano Annatto, T5 = 0.01% BHA

Protein percentage: Table (2) demonstrates the effects of various treatments under different durations of refrigerated storage on protein content in beef burger samples. The data showed a highly significant ($P < 0.01$) difference between the type of treatment and storage period. Between all groups tested, treatment T4, which is 187.6 µg/ml nano-beetroot plus 185.6 µg/ml nano-annatto, gave the maximum percentage of protein content, which is 30.98%, on the 12th day of cold storage. The control (T1) with no addition has the least percentage of protein content, which is 15.52%, in the first day of storage. The protein content in the matrix goes up with the progress of storage time due to the steady loss of moisture from the meat. As the water content reduces, the concentration of solid components, which include protein, is realized. This is the trend observed in the findings of the study of (27) and it proves the fact of the inverse relationship between moisture and protein percentages with storage. Also, the high protein levels in nano-treated samples could be due to the protective role of beetroot compounds, which are known to have antioxidant capacities. These active moieties help preserve the structure of proteins by reducing oxidative degradation and thus add to the stabilization of meat proteins over time (2). The results agree with the available literature, indicating that the use of natural additives, such as plant extracts or powders, helps maintain protein content of meat products stored under refrigeration (7). Additional statistical analysis confirmed a marked increase ($P < 0.01$) in protein percentage with the advancement in the period of storage. The lowest values were recorded on day 1 for all the treatments, protein content increasing progressively up to day 12. The gradual enrichment of protein goes hand in hand with the natural desiccation process of the refrigeration and the concomitant rise in the concentration of dry matter components such as protein, fat, and ash. This indicates not only that natural nano-extracts are able to improve shelf-life quality, but shows their potential for quality retention, especially for protein content. These changes could be attributed to the antioxidant properties of beetroot and annatto working together to build up the integrity of muscle tissues over storage periods (2, 27).

Table 2. Effect of the interaction between different treatments and cold storage periods on the protein percentage (%) ± standard error (SE) of beef burgers treated with nano-encapsulated plant extracts:

Transactions	Protein%					Average
	1 day	3 day	6day	9day	12 day	
T1	15.52±0.15 q	18.95±0.14 n	19.84±0.11kl	22.62±0.09 h	27.19±0.15 d	20.82±1.04 D
T2	16.79±0.13 op	19.69±0.13 l	20.59±0.12j	25.17±0.15 f	30.29±0.09 b	22.50±1.26 B
T3	16.55±0.16 p	19.50±0.15 lm	20.47±0.15j	25.13±0.14 f	30.18±0.07 b	22.36±1.27 B
T4	17.02±0.11 o	19.97±0.12 kl	21.17±0.09 i	26.10±0.18 e	30.78±0.18 a	23.00±1.30 A
T5	15.91±0.14 q	19.15±0.13 mn	20.17±0.13 jk	23.73±0.13 g	29.09±0.19 c	21.61±1.20 C
Average	16.35±0.16 E	19.45±0.11 D	20.44±0.13 C	24.55±0.33 B	29.50±0.34 A	

Means with different letters are significantly different (P < 0.01). Capital letters indicate the main effect of treatments and storage durations; lowercase letters indicate the interaction effect between treatments and storage durations. Treatments are defined as follows: T1 = no addition (control), T2 = 185.6 µg/ml Nano Annatto, T3 = 187.6 µg/ml Nano Beetroot, T4 = 187.6 µg/ml Nano Beetroot + 185.6 µg/ml Nano Annatto, T5 = 0.01% BHA.

Fat percentage

Table (3) presents the results regarding the interaction between treatment types and storage durations on the fat content of beef burger samples. The statistical analysis revealed a highly significant difference (P < 0.01) in fat percentages depending on both the applied treatment and the length of refrigerated storage. Interestingly, the highest fat percentage (20.99%) was observed in the control group T1 (without any additive) after 12 days of storage, while the lowest fat percentage (5.35%) was recorded for treatment T4, which combined 187.6 µg/ml nano-beetroot and 185.6 µg/ml nano-annatto, on the first day of storage. This variation across treatments and storage intervals can be explained by the progressive loss of moisture during refrigeration, which leads to an increase in dry matter content, including fat, protein, and ash. These results agree with the findings reported by (5), who emphasized that longer storage periods naturally concentrate non-aqueous components in meat products. Furthermore, similar patterns were observed by (33), who investigated the use of whey membrane films in packaging fish meat and noted a rise in fat percentage after extended storage, attributing it to water loss and concentration of solids. The decline in fat percentage observed in the nano-treated groups, particularly in T4, may be attributed to the antioxidant properties of the natural extracts used. Both beetroot and annatto are known for their ability to inhibit lipid oxidation by neutralizing free radicals and reactive oxygen species, thereby preserving fat molecules from degradation over time (20, 31). This antioxidant mechanism not only reduces oxidative rancidity but also contributes to maintaining the nutritional quality of the product during storage. From a temporal perspective, the data revealed that fat content was lowest at day 1 across all treatments, and then increased progressively (P < 0.01) with the extension of the storage period, reaching its peak by day 12. This trend reinforces the known relationship between moisture loss and fat concentration during refrigeration and reflects the importance of additive intervention in maintaining product quality. In summary, the results confirm that while storage time naturally elevates fat concentration due to moisture reduction, the use of nano-encapsulated plant-based additives can effectively mitigate fat oxidation and modulate fat retention, enhancing the shelf life and stability of meat products (20, 31, 33).

Table 3. Effect of the interaction between different treatments and cold storage periods on the fat percentage (%) ± standard error (SE) of beef burgers treated with nano-encapsulated plant extracts

Transactions	Fat%					Average
	1 day	3 day	6day	9day	12 day	
T1	8.20±0.32 ^{mn}	9.83±0.34 ^{l}	13.19±0.20 ^{gh}	17.38±0.16 c	20.99±0.31 ^{a}	13.92±1.27 ^{A}
T2	6.41±0.30 ^{o}	7.23±0.29 ^{no}	10.93±0.25 ^{k}	13.86±0.32 ^{fg}	15.92±0.30 ^{d}	10.87±0.99 ^{C}
T3	6.78±0.29 ^{o}	8.15±0.22 ^{mn}	11.43±0.32 ^{jk}	13.83±0.30 ^{fg}	15.80±0.28 ^{d}	11.20±0.91 ^{C}
T4	5.35±0.28 ^{p}	6.80±0.19 ^{o}	10.74±0.23 ^{kl}	12.76±0.33 ^{hi}	14.77±0.26 ^{ef}	10.08±0.95 ^{D}
T5	7.41±0.33 ^{no}	8.90±0.32 ^{m}	12.17±0.18 ^{ij}	15.45±0.32 ^{de}	18.77±0.35 ^{b}	12.54±1.12 ^{B}
Average	6.83±0.28 ^{E}	8.18±0.31 ^{D}	11.69±0.26 ^{C}	14.65±0.44 ^{B}	17.25±0.62 ^{A}	

Means with different letters are significantly different (P < 0.01). Capital letters indicate the main effect of treatments and storage durations; lowercase letters indicate the interaction effect between treatments and storage durations. Treatments are defined as follows: T1 = no addition (control), T2 = 185.6 µg/ml Nano Annatto, T3 = 187.6 µg/ml Nano Beetroot, T4 = 187.6 µg/ml Nano Beetroot + 185.6 µg/ml Nano Annatto, T5 = 0.01% BHA

Ash percentage: Table (4) illustrates the impact of the interaction between different treatments and refrigeration storage durations on the ash percentage in beef burger samples. The results of the statistical analysis showed a highly significant difference (P < 0.01) in ash content across treatments and storage intervals. The highest ash content (3.72%) was recorded in the control group (T1) which received no additive after 12 days of refrigerated storage. In contrast, the lowest ash value (1.43%) was observed in treatment T4, which involved the combination of 187.6 µg/ml nano-beetroot and 185.6 µg/ml nano-annatto, on the first day of storage. These results reflect the natural progression of dry matter concentration during cold storage, where the loss of moisture leads to a relative increase in mineral content, including ash. This trend is supported by findings from previous research. For example, study (38) reported an increase in ash content after incorporating beetroot extract into sausages stored under refrigeration for four months. This indicates that certain plant-based additives may influence the ash content by interacting with the meat matrix or by contributing their own mineral content. The variation in ash content across treatments could also be attributed to the differing abilities of natural antioxidants to stabilize the chemical composition of meat during storage. Although T4 initially showed the lowest ash content, this may reflect the protective effect of nano-encapsulated compounds in preventing oxidative mineral changes and loss in early storage stages.

Table 4. Effect of the interaction between different treatments and cold storage periods on ash content (%) ± standard error (SE) of beef burgers treated with nano-encapsulated plant extracts

Transactions	Ash%					Average
	1 day	3 day	6day	9day	12 day	
T1	2.31±0.2 ^{hij}	2.48±0.08 ^{ghi}	2.70±0.09 ^{fg}	3.20±0.04 ^{bc}	3.72±0.08 ^{a}	2.88±0.14 ^{A}
T2	1.86±0.03 ^{lm}	2.10±0.04 ^{jkl}	2.31±0.03 ^{hij}	2.58±0.08 ^{fgh}	2.82±0.02 ^{def}	2.33±0.09 ^{D}
T3	1.94±0.09 ^{lm}	2.23±0.02 ^{ijk}	2.49±0.06 ^{ghi}	2.74±0.03 ^{efg}	2.98±0.07 ^{cd}	2.47±0.10 ^{C}
T4	1.80±0.08 ^{m}	1.99±0.08 ^{klm}	2.21±0.05 ^{ijk}	2.40±0.08 ^{hi}	2.70±0.05 ^{fg}	2.22±0.09 ^{E}
T5	2.23±0.04 ^{ijk}	2.40±0.05 ^{hi}	2.52±0.07 ^{gh}	3.04±0.09 ^{cd}	3.32±0.08 ^{b}	2.70±0.11 ^{B}
Average	2.03±0.06 ^{E}	2.24±0.05 ^{D}	2.44±0.05 ^{C}	2.79±0.08 ^{B}	3.11±0.10 ^{A}	

Means with different letters are significantly different (P < 0.01). Capital letters indicate the main effect of treatments and storage durations; lowercase letters indicate the interaction effect between treatments and storage durations. Treatments are defined as follows: T1 = no addition (control), T2 = 185.6 µg/ml Nano Annatto, T3 = 187.6 µg/ml Nano Beetroot, T4 = 187.6 µg/ml Nano Beetroot + 185.6 µg/ml Nano Annatto, T5 = 0.01% BHA

Thiobarbituric acid concentration

Table (5) presents the interaction effects of different treatments and refrigeration storage durations on the concentration of thiobarbituric acid reactive substances (TBA) in beef burgers treated with nano-encapsulated plant extracts. The data revealed a highly significant increase ($P < 0.01$) in TBA levels, particularly in the control group (T1), which exhibited the highest malondialdehyde concentration (2.68 MDA/kg meat) after 12 days of cold storage. In contrast, the lowest TBA value (0.50 MDA/kg meat) was recorded in treatment T4, which combined 187.6 µg/ml nano-beetroot and 185.6 µg/ml nano-annatto, on the first day of storage. These results clearly demonstrate the role of nano-encapsulated plant-based antioxidants in delaying lipid oxidation in beef burgers during refrigerated storage. The variation among treatment groups and over storage time suggests that both the type of additive and duration of preservation significantly influence oxidative stability. The marked difference between T1 and T4 supports the efficacy of nano-formulated natural extracts in mitigating oxidative degradation, likely due to their free radical-scavenging activities. Natural antioxidants particularly those derived from peptides have been widely recognized for their protective roles in meat systems. Their capacity to suppress lipid peroxidation makes them valuable functional additives in processed meats (8). The observed increase in TBA concentration over time aligns with the natural progression of lipid oxidation during storage, especially in untreated samples, where antioxidant defense is absent. Statistical analysis further confirmed a significant difference ($P < 0.01$) among the different storage periods. The lowest TBA values were consistently observed on day 1, followed by a steady increase that peaked on day 12. These findings agree with prior studies, such as those by (17), who demonstrated that increasing the concentration of natural additives like Moringa oleifera and Bidens Pilosa extracts led to a reduction in TBA levels in refrigerated ground beef stored at 4°C. Furthermore, (14) observed a notable decrease in malondialdehyde formation in veal stored at 4°C for 11 days when natural antioxidants were included. Similarly, (38) reported that the application of plant-based extracts led to a significant reduction in TBA concentrations, underscoring their antioxidant effectiveness during cold storage. In summary, the results emphasize that nano-encapsulated natural extracts, particularly when combining beetroot and annatto, are effective in limiting lipid oxidation, thereby preserving meat quality during refrigeration.

Table 5. Effect of the interaction between different treatments and cold storage periods on TBA concentration (mg malondialdehyde/kg meat) ± standard error (SE) in beef burgers treated with nano-encapsulated plant extracts

Transactions	Thiobarbituric acid concentration					Average
	1 day	3 days	6day	9day	12 day	
T1	0.80 ± 0.08^{ijkl}	1.33 ± 0.05^{fg}	1.81 ± 0.07^{e}	2.22 ± 0.02^{bcd}	2.68 ± 0.09^{a}	1.77 ± 0.17^{A}
T2	0.61 ± 0.06^{lm}	0.80 ± 0.02^{ijkl}	1.21 ± 0.03^{gh}	1.73 ± 0.08^{e}	1.98 ± 0.07^{ed}	1.27 ± 0.14^{D}
T3	0.65 ± 0.04^{klm}	0.89 ± 0.08^{ijk}	1.43 ± 0.03^{fg}	1.98 ± 0.07^{ed}	2.29 ± 0.04^{bc}	1.45 ± 0.16^{C}
T4	0.50 ± 0.03^{m}	0.67 ± 0.07^{klm}	1.03 ± 0.08^{hi}	1.46 ± 0.05^{f}	1.79 ± 0.03^{e}	1.09 ± 0.13^{E}
T5	0.76 ± 0.07^{jkl}	0.99 ± 0.05^{hij}	1.78 ± 0.09^{e}	2.06 ± 0.06^{cd}	4.44 ± 0.03^{b}	1.61 ± 0.17^{B}
Average	0.67 ± 0.04^{E}	0.94 ± 0.06^{D}	1.45 ± 0.08^{C}	1.89 ± 0.07^{B}	2.24 ± 0.09^{A}	

Means with different letters are significantly different ($P < 0.01$). Capital letters indicate the main effect of treatments and storage durations; lowercase letters indicate the interaction effect between treatments and storage durations. Treatments are defined as follows: T1 = no addition (control), T2 = 185.6 µg/ml Nano Annatto, T3 = 187.6 µg/ml Nano Beetroot, T4 = 187.6 µg/ml Nano Beetroot + 185.6 µg/ml Nano Annatto, T5 = 0.01% BHA

Myoglobin (mg/g meat):

Table (6) shows the influence of the interaction between treatments and cold storage durations on myoglobin concentration in beef burger samples after being treated with nano-encapsulated plant extracts. Differences between the treatments and storage periods were highly significant statistically ($P < 0.01$).

The highest myoglobin concentration level, 4.95 mg/g of meat, was in nano-beetroot 187.6 µg/ml + nano-annatto 185.6 µg/ml; this was recorded on the 1st day of cold storage and the lowest concentration, 3.22 mg/g of meat, was in the control treatment (without additives) T1 after 12 days of refrigeration. These results give important priority to beetroot and annatto as natural antioxidants in the conservation of the stability of myoglobin pigments in meat. The mode of protection seems to be related to the suppression of oxidative damage, a major factor in normal pigment degradation. Prior evidence has shown beetroot to protect mitochondrial structures and also lower free radical activities, activities which could collectively help in the coloration and stability of pigments in meat (23). This finding agrees with prior results by (26) who found that using annatto seed powder as a natural preservative and antioxidant in beef sausages stored at 4°C lowered the oxidation of myoglobin. The oxidative molecules in annatto antioxidants may hold back the conversion of bright red oxymyoglobin to dull brown metmyoglobin, which normally takes place under oxidation reactions during refrigerated storage. The statistical analysis further revealed that there was a significant ($P < 0.01$) effect of storage duration alone on myoglobin concentration. The levels were highest for all treatments on day 1 consistently, after which it started decreasing progressively with the storage period and finally attained a minimum by day 12. This decrease is most likely due to oxymyoglobin oxidation taking its own time in such factors as aerobic exposure, enzymatic activity, and light intensity, which further accelerate pigment degradation in due course (28, 26). The trend in initial pigment holding succeeded by degrading underlines the urgency of integrating effective natural antioxidants into meat formulations, more so for products intended for extended refrigerated storage. These natural compounds shall instill an attractive appearance and freshness by stabilizing color-related proteins such as myoglobin.

Table 6. Effect of the interaction between different treatments and cold storage periods on myoglobin concentration (mg/g meat) ± standard error (SE) in beef burgers treated with nano-encapsulated plant extracts

Transactions	Myoglobin (mg/g meat)					Average
	1 day	3 days	6day	9day	12 day	
T1	4.69 ± 0.09^{abc}	4.47 ± 0.08^{cdefg}	4.30 ± 0.02^{fgh}	3.25 ± 0.09^{j}	3.22 ± 0.05^{j}	3.98 ± 0.17^{C}
T2	4.92 ± 0.03^{ab}	4.82 ± 0.09^{ab}	4.63 ± 0.05^{bcde}	4.33 ± 0.05^{efgh}	4.17 ± 0.08^{hi}	4.57 ± 0.08^{A}
T3	4.90 ± 0.05^{ab}	4.81 ± 0.07^{ab}	4.62 ± 0.01^{bcde}	4.32 ± 0.02^{fgh}	4.16 ± 0.05^{hi}	4.56 ± 0.08^{A}
T4	4.95 ± 0.02^{a}	4.83 ± 0.01^{abcd}	4.65 ± 0.08^{defgh}	4.32 ± 0.08^{ghi}	4.19 ± 0.04^{ghi}	4.59 ± 0.08^{A}
T5	4.74 ± 0.09^{abc}	4.63 ± 0.06^{bcde}	4.50 ± 0.07^{cdef}	4.13 ± 0.04^{hi}	4.00 ± 0.07^{i}	4.40 ± 0.08^{B}
Average	4.84 ± 0.04^{A}	4.71 ± 0.05^{B}	4.54 ± 0.04^{C}	4.07 ± 0.11^{D}	3.94 ± 0.10^{E}	

Means with different letters are significantly different ($P < 0.01$). Capital letters indicate the main effect of treatments and storage durations; lowercase letters indicate the interaction effect between treatments and storage durations. Treatments are defined as follows: T1 = no addition (control), T2 = 185.6 µg/ml Nano Annatto, T3 = 187.6 µg/ml Nano Beetroot, T4 = 187.6 µg/ml Nano Beetroot + 185.6 µg/ml Nano Annatto, T5 = 0.01% BHA

WHC%: Table (7) highlights the interactive effects of various treatments and storage durations on the water-holding capacity (WHC%) of beef burgers coated with nano-encapsulated plant extracts. The statistical analysis revealed a highly significant difference ($P < 0.01$) in WHC values across treatments and storage periods. The highest WHC value 61.94% was observed in treatment T4 (combination of 187.6 µg/ml nano-beetroot and 185.6 µg/ml nano-annatto) after 1 day of cold storage, while the lowest value (26.12%) was recorded in the control group (T1) without additives at the 12-day storage interval. The observed increase in WHC in T4 during early storage is likely attributed to the bioactive compounds present in beetroot and annatto extracts, which may enhance the structural integrity of muscle fibers by stabilizing cell membranes. This helps reduce protein degradation and limits water loss, allowing moisture to remain bound within the muscle matrix (26). Furthermore, the increase in pH potentially caused by the active compounds may have contributed to improving the water-retention capacity of the meat, as previously suggested in related studies (36). Regarding the effect of storage duration, the results also indicated a significant variation (P < 0.01) in WHC across different time points. WHC was maximal on day 1, but declined steadily over the course of storage, reaching its lowest value on day 12. This trend is consistent with the progressive breakdown of muscle proteins, which compromises their ability to retain moisture during extended storage (18). The overall findings reinforce the functional role of nano-encapsulated plant extracts in enhancing WHC in meat products, particularly during early stages of cold storage. By maintaining protein stability and cellular cohesion, these natural additives help improve product quality and shelf life under refrigerated conditions.

Table 7. Effect of the interaction between different treatments and cold storage periods on meat water-holding capacity (%) ± standard error (SE) in beef burgers treated with nano-encapsulated plant extracts

Transactions	WHC%					Average
	1 day	3 days	6day	9day	12 day	
T1	58.25±0.38^{de}	52.83±0.31^{g}	38.25±0.37^{l}	29.94±0.35^{o}	26.12±0.30^{q}	41.08±3.35^{E}
T2	60.84±0.32^{b}	55.97±0.39^{f}	46.55±0.34^{j}	38.55±0.38^{l}	31.01±0.39^{o}	46.59±2.92^{B}
T3	59.93±0.33^{bc}	55.53±0.35^{f}	47.87±0.32^{i}	35.86±0.32^{m}	30.47±0.33^{o}	45.93±1.14^{C}
T4	61.94±0.38^{a}	57.45±0.38^{e}	50.19±0.38^{h}	46.58±0.37^{j}	34.03±0.36^{n}	50.04±2.58^{A}
T5	59.08±0.38^{cd}	53.87±0.33^{g}	42.30±0.36^{k}	33.67±0.38^{n}	28.32±0.37^{p}	
Average	60.01±0.37^{A}	55.13±0.45^{B}	45.03±1.14^{C}	36.92±1.50^{D}	29.99±0.72^{E}	

Means with different letters are significantly different (P < 0.01). Capital letters indicate the main effect of treatments and storage durations; lowercase letters indicate the interaction effect between treatments and storage durations. Treatments are defined as follows: T1 = no addition (control), T2 = 185.6 µg/ml Nano Annatto, T3 = 187.6 µg/ml Nano Beetroot, T4 = 187.6 µg/ml Nano Beetroot + 185.6 µg/ml Nano Annatto, T5 = 0.01% BHA

Total bacterial count

Table (8) presents the impact of the interaction between different treatments and refrigeration storage durations on the total bacterial count (expressed in colony-forming units per gram of meat, CFU/g) in beef burgers coated with nano-encapsulated plant extracts. The results indicated a highly significant difference (P < 0.01) across both treatment groups and storage periods. The highest bacterial load (8.17 CFU/g) was observed in treatment T1 (untreated control) after 12 days of cold storage, whereas the lowest bacterial counts were recorded in treatments T4 (combination of 187.6 µg/ml nano-beetroot and 185.6 µg/ml nano-annatto) with 3.79 CFU/g, and T3 (187.6 µg/ml nano-beetroot) with 3.97 CFU/g, both assessed on day 1 of refrigeration. These findings clearly demonstrate the antimicrobial potential of nano-encapsulated natural extracts, especially those derived from beetroot and annatto. Their effectiveness in reducing microbial growth is likely due to their rich content of bioactive antioxidant compounds, which help inhibit the proliferation of spoilage and pathogenic bacteria by scavenging free radicals and protecting cellular structures. Supporting this, earlier research by (26) reported that the incorporation of annatto seed powder in beef sausages stored at 4°C significantly contributed to reducing myoglobin oxidation and improving overall product stability. Similarly, beetroot extract has been noted for its role in preserving mitochondrial function and reducing oxidative stress in muscle tissues (23), which indirectly supports microbial stability by preserving structural integrity. From a storage perspective, the statistical analysis confirmed a significant increase (P < 0.01) in total bacterial count with advancing storage time. The lowest microbial loads were observed on day 1, and levels increased progressively over time, peaking on day 12. This trend is expected, as microbial growth is a natural consequence of storage under aerobic and refrigerated conditions, especially when antioxidant or antimicrobial protection is absent. Although this discussion references the stability of myoglobin concentrations under different treatments in earlier tables, the underlying antioxidant mechanisms such as free radical scavenging and enzymatic inhibition are equally relevant to microbial control. The findings reinforce the value of plant-based nano-additives in preserving both the microbial and physicochemical quality of beef burgers during refrigeration (28, 26).

Table 8. Effect of the interaction between different treatments and cold storage periods on total bacterial count (CFU/g) ± standard error (SE) in beef burgers treated with nano-encapsulated plant extracts

Transactions	Total bacterial count					Average
	1 day	3 days	6day	9day	12 day	
T1	4.33 ± 0.17^{mno}	5.24 ± 0.12^{ijk}	5.96 ± 0.16^{efg}	6.69 ± 0.11^{bc}	8.17 ± 0.16^{a}	6.08 ± 0.35^{A}
T2	4.08 ± 0.12^{no}	4.89 ± 0.16^{jkl}	5.46 ± 0.18^{ghij}	6.05 ± 0.17^{def}	6.76 ± 0.11^{bc}	5.45 ± 0.25^{C}
T3	3.97 ± 0.13^{o}	4.84 ± 0.10^{klm}	5.32 ± 0.12^{hijk}	5.93 ± 0.15^{efg}	6.60 ± 0.15^{bcd}	5.33 ± 0.25^{CD}
T4	3.79 ± 0.17^{o}	4.54 ± 0.15^{lmn}	5.18 ± 0.14^{jk}	5.76 ± 0.19^{fghi}	6.39 ± 0.14^{cde}	5.13 ± 0.25^{D}
T5	4.17 ± 0.11^{no}	5.08 ± 0.17^{jkl}	5.84 ± 0.13^{efgh}	6.28 ± 0.14^{cdef}	7.10 ± 0.16^{b}	5.69 ± 0.27^{B}
Average	4.07 ± 0.08^{E}	4.92 ± 0.09^{D}	5.55 ± 0.10^{C}	6.14 ± 0.10^{B}	7.00 ± 0.18^{A}	

Means with different letters are significantly different ($P < 0.01$). Capital letters indicate the main effect of treatments and storage durations; lowercase letters indicate the interaction effect between treatments and storage durations. Treatments are defined as follows: T1 = no addition (control), T2 = 185.6 µg/ml Nano Annatto, T3 = 187.6 µg/ml Nano Beetroot, T4 = 187.6 µg/ml Nano Beetroot + 185.6 µg/ml Nano Annatto, T5 = 0.01% BHA.

CONCLUSION

Based on the findings of this study, it can be concluded that applying chitosan nanoparticles loaded with annatto seed and beetroot extracts as a coating for refrigerated beef burgers effectively preserves their chemical composition, physical integrity, and sensory quality throughout a 12-day cold storage period. This nano-coating system demonstrated strong potential in delaying lipid oxidation, maintaining pigment stability, enhancing moisture retention, and reducing microbial load, thereby improving the overall shelf life and product quality without inducing any undesirable changes. Considering these promising results, it is recommended that chitosan-based nano-encapsulation techniques incorporating natural extracts such as annatto and beetroot be extended to other meat products, including poultry and processed meat formulations, as a natural, safe, and effective alternative to synthetic preservatives. Further research is encouraged to explore their application on an industrial scale and to evaluate their impact on consumer acceptance and commercial viability.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DECLARATION OF FUND

The authors declare that they have not received a fund.

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