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ORIGINAL ARTICLE

The Effect of Roselle Calyx Extract and Nano-ZnO Biofilm on Walnut's Chemical and Sensory Properties

Neda Sadat Aghayan[1], Maede Sadat Abbasi[2], Anna Etemadi Razlighi[*3], Atousa Kokabian[*4], Neda Fallah[*5,6], Grace Morine[7]

[1]*Department of Food Science and Technology, Shahrood Branch, Islamic Azad University, Shahrood, Iran*

[2]*Department of Food Science and Technology, Qom Branch, Islamic Azad University, Qom, Iran*

[3] *Department of Food Science & Egineering, Mahalat Branch, Islamic Azad University, Mahalat, Iran*

[4]*Quality Control Manager of Zarvar Company, Hamedan, Iran*

[5]*Production and Recycling of Materials and Energy Research Center, Qom Branch, Islamic Azad University, Qom, Iran*

[6]*Department of Food Science and Technology, Najafabad Branch, Islamic Azad University, Najafabad, Iran*

[7]*Veterinary Mycology Group, Facultat de Veterinària, Universitat Autònoma de Barcelona, 08193 Bellaterra, Catalunya, Spain*

K E Y W O R D S

Bionanocomposite film;
Biopackaging;
Free radicals;
Fungal growth;
Oxidation reaction

A B S T R A C T

Bionanocomposite active films made from tapioca starch and bovine gelatin, with the addition of roselle calyx extract (RCE) and zinc oxide nanorod (ZnO-N), were created as packaging material to protect walnuts against mold, yeast contamination, and lipid oxidation. Three types of packaging were produced: tapioca starch and bovine gelatin (control sample), tapioca starch, bovine gelatin, and RCE, and tapioca starch, bovine gelatin, ZnO-N, and RCE. Approximately 30 grams of walnuts were packed using each type of packaging and evaluated for acidity value, mold and yeast count, peroxide value, and sensory tests. After 90 days, the mold and yeast count of walnuts packed with RCE/ZnO-N and RCE was 4.49 and 4.65 log cfu/g respectively, compared to 4.95 log cfu/g in the control sample. At the end of the conservation period, the aroma score was 3.59 for walnuts packed with RCE/ZnO-N, compared to 2.5 for those packed with tapioca starch and bovine gelatin. The acidity value indicated that walnuts packed with RCE and RCE/ZnO-N had a positive effect on acidity, with the lowest value found in walnuts packed with RCE/ZnO-N. The study showed that bionanocomposite packaging films containing RCE and ZnO-N are effective in protecting walnuts against fungal contamination and oxidation.

Introduction

Walnut is an economically and nutritionally valuable product that is widely consumed. Not only nuts but other components of walnuts are used (Hassankhah *et al*., 2017; Akca and Sahin, 2022). The walnut kernel is an important section of the human diet and its regular consumption shows positive effects, especially concerning cardiovascular diseases (Jahanbani *et al*., 2018; Sarikhani *et al*., 2021). Active compounds including polyphenols, ellagic acid, melatonin, and tocopherols have been detected in walnuts (Kavuncuoglu *et al*., 2021).

The walnut kernel is a perishable product under

[*]**Corresponding author**: Email address: anna.etemadi@yahoo.com, akokabiyan@yahoo.com, neda.fallah.tie@gmail.com
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natural conservation conditions (Habibi *et al.*, 2022). High concentrations of unsaturated fatty acids and fat in the walnut kernel make them susceptible to oxidative rancidity (Chatrabnous *et al.*, 2018). Oxygen concentration, relative humidity (RH), temperature, processing technique, natural antioxidants, and light are the parameters that influence the oxidation capacity of the walnut kernel (Adiletta *et al*., 2020) . Walnut kernels, at harvest period, indicate high water activity which facilitates the increase of mold population and the activity enzymes of lipase and polyphenol oxidase involved in fatty acid hydrolysis and browning reactions, respectively (Habibi *et al.*, 2022). The reduced economic walnut is due to the oxidation rancidity, which produces unfavorable rancid taste, and browning reactions (Ozturk *et al*., 2016).

Additionally, oxidation rancidity can decrease because of the use of bionanoconposite packaging and improve quality of product (Davoodi & Naji, 2018).

The based-on solution mentioned, nano-biopackaging is recently the most important issue in food packaging. The purpose of the application of nano-biopackaging in the food industry is the shelf life extension of products by preventing chemical reactions, oxygen and water vapor molecules penetration, and microbial contaminations (Bumbudsanpharoke & Ko, 2015).

Some nanofillers including ZnO, Ag, TiO_2, and Cuo have antifungal activity and can also improve the biofilm properties to prevent gas molecule transfer which creates negative conditions for fungal and microbial activity (Javidi *et al*., 2022).

In our previous work, we studied the usage of active films from nano-ZnO/RCE based on tapioca starch/bovine gelatin, and the findings indicated that the inhibitory effect of filler on wvp of biodegradable film was 3.36×10^{-7} gr/mPah (Sadeghi *et al*., 2023). Tavakoli *et al*. (2017) studied the antimicrobial effect of polyethylene containing silver nanoparticles on walnuts. It was observed that bionanocomposite film containing nanofiller has higher antimicrobial properties than the neat sample. The effect of the coating with nano-silver/montmorillonite on the microbiological quality of carrot slices was studied by Costa *et al*. (2011). The obtained results indicated that the use of nanoadditives causes preservation of the product up to 70 days. Vera et *al*. (2018) reported that laminates with nano-selenium prevent the oxidation rancidity of hazelnuts and increase the shelf life of nuts. Walnuts packaged with synthetic packaging based on low-density polyethylene under vacuum or nitrogen conditions heated at 70°C for 2 min significantly increased the oxidation stability of walnuts (Ziaolhagh *et al*., 2020). The application limitations of petroleum such as the environmental concern and the high price of oil, attracted the scientific thoughts of scientists to develop biopolymers. Based on current works on bionanocomposite films illustrated that the application of nanofillers in biodegradable film such as starch (universally available, low cost) can improve the barrier, mechanical, and antibacterial properties of biopackaging and improve the oxidative stability of the product (Marvizadeh *et al*., 2021; Nobari *et al*., 2022).

Pigments or colorants are additives applied to improve the price, color, and barrier properties. Natural pigments can be used in many applications due to their inherent positive characteristics including; light barrier activity, antibacterial, antioxidant activity, and plasticizing ability. Different parts of plants for example the bark, flowers, leaves, fruits, heartwoods, and calyx can be chosen and extracted to obtain colorant. In general, natural colorants are used to packaging materials using a casting technique (NANSU *et al*., 2021).

Talebi *et al*. (2019) investigated the impact of using various levels of chitosan/thyme essential oil, on the free fatty acid of walnuts during 4 months of storage. They illustrated that the coated sample has high levels of free fatty acids (FFAs) compared to the neat specimens. It can be attributed to the moisture content of walnuts. On the other hand, The use of biofilms containing roselle calyx extract improve the barrier properties of film, decrease water vapor traveling, and enhances the shelf life of food production (Sadeghi *et al*., 2023).

Hibiscus sabdariffa L. pigments possess flavonoid compounds as the major group of compounds in roselle calyx and sappan heartwood (Vinha *et al*., 2018). Roselle is a herbal that is well-known around the world and is widely grown throughout Southeast Asia countries including Thailand. The anthocyanin pigment is the major compound in roselle calyx extract and is also pH sensitive. The red color of *Hibiscus sabdariffa* results from the anthocyanin pigment changing its chemical to a flavylium cation (Etemadi Razlighi *et al*., 2023). Scientific work states that monosaccharides of roselle can lead to long-term performance benefits in sports. This is associated with the impact of polysaccharide molecules on skeletal muscle metabolism, which can change performance (Sadeghi *et al*., 2022).

To the best of our knowledge, no research has been conducted on the chemical, sensory, and microbial properties of walnuts packaged with tapioca starch, bovine gelatin, and roselle calyx extract (TB/RCE) or the combination of TB/RCE with ZnO-N. Therefore, this study aims to investigate the effects of ZnO-N and roselle calyx extract on the quality of walnut kernels.

Materials and Methods

Materials

Bovine gelatin, tapioca starch, and plasticizer including liquid sorbitol and glycerol were supplied from Sigma Chemical Co (Burlington, USA). ZnO nanoparticles were purchased from US-NANO (Oklahoma, USA). The walnut kernel that is newly harvested was provided from a local producer in Damghan, Iran. Also, other laboratory materials were provided from Merck Co. (Germany).

Roselle calyx extract

The roselle extract was carried out according to the modified technique suggested by Giusti *et al*.'s [13]. About 1 g of roselle calyx powder was combined with approximately 15 mL of ethanol (80%); the pH of the mixture was subsequently adjusted to pH 2 using HCl. After being heated for 60 min at 50°C to obtain the final extract, the specimens were centrifuged for 5 min at 3000 rpm. The solvent was separated at 50°C using a rotary evaporator model Hei-VAP (Heidolph, GER).

Film preparation

The casting technique was used to form treatment biofilms containing ZnO-N and RCE and neat film (tapioca starch/bovine gelatin film without ZnO-N and RCE). Nanofiller was added to 100 mL of distilled water at a concentration of %2 (w/w, based on tapioca), and the combination was magnetically agitated at 25°C for 60 min before being exposed to ultrasound wavelength. About 0.4 g of gelatin, 4g of starch, (Marvizadeh *et al*., 2016; Marvizadeh *et al*., 2014), and plasticizer (3.2g) (combination of glycerol/sorbitol) (Mosavian *et al*., 2017) were added to biosolution. Each of the solutions was heated for 1h at 85°C. About 2g of roselle calyx extract for TP/RCE and 1.5g of roselle calyx extract for TP/RCE/ZnO-N were added to the dispersion and homogenized for 1h while the biodegradable solution was cooling (40°C). Finally, glass plates (17×17 cm^2) were used to decant the solutions before they were allowed to dry at room temperature to create biodegradable films.

Walnut kernel packaging

The walnut kernel samples were carefully separated from the walnut shell. About 30g of walnut kernels were put between biodegradable sheets, using a heat sealer (Iran), and stored at refrigerator temperature for 90 days. The control and treatment biopackages size was 12 × 16 cm^2.

Peroxide value

About 5 g of walnut kernel oil and 30 ml of acetic acid-chloroform mix in a baker to dissolve the kernel oil. A 0.5 ml KI solution was added to the mentioned mixture. The final solution was agitated to dissolve the walnut kernel oil and taken for 10 min in the dark conditions at 25°C. Then, distilled water (30 ml) was added to the solution. After adding 0.5 ml of starch solution (indicator) at concentration 1% w/v, the dispersion was titrated with sodium thiosulfate (0.01N) until the blue color was removed (Atarés, Pérez-Masiá, & Chiralt, 2011).

Acidity measurement

The acid value was determined by the standard method. To estimate the acidity index of walnuts, 2 ml of alcohol-ether was added to 5 g of the sample. After agitating flasks, phenolphthalein as an indicator was added to the mentioned mixture and then was titrated with sodium hydroxide (0.01 N) (AOAC, 2012).

Mold and yeast count

About ten grams of samples were aseptically taken sterile blender. Subsequently, they were mixed in 450 mL of peptone (0.1%) for 1 min. Serial dilutions of the homogenates in 0.1% peptone were poured on DG18 agar (0.1 mL/plate), and the samples were incubated at 25°C for 3 days. At last, colonies were calculated, and counts were stated as log cfu/g.

Sensory properties

Samples were prepared for sensory properties and placed in three-digit coded plates. A sensory quality, using panelists comprising 10 trained members from Islamic Azad University, was employed to measure flavor and aroma factors differences between control and treatment samples. Panelists evaluated the sample for sensory properties on a 5-point scale (1=very bad and 5=very good) according to Al-Bachir (2004).

Statistical analysis

One-way ANOVA and independent sample t test were applied to compare means of chemical properties and sensory factors of walnuts at $p<0.05$. Experiment data were analyzed by Minitab software (21.4.1).

Results

Peroxide value

Based on Fig. 1. the findings represent that the peroxide value of control and treatment specimens increased during storage time. TB packaging containing RCE and ZnO-N significantly affected the PVs of walnut kernels ($p < 0.05$). There was significant difference ($p < 0.05$) between the control and TB/RCE after one month of storage. According to Fig. 1. TB film containing RCE/ZnO-N showed the lowest peroxide value. The final PV of control packaged walnut kernels was about 8.96 meq O_2/1000g, while the specimen containing RCE and RCE/ZnO-N, showed the lowest values (8.01 and 6.61 meq O_2/1000g).

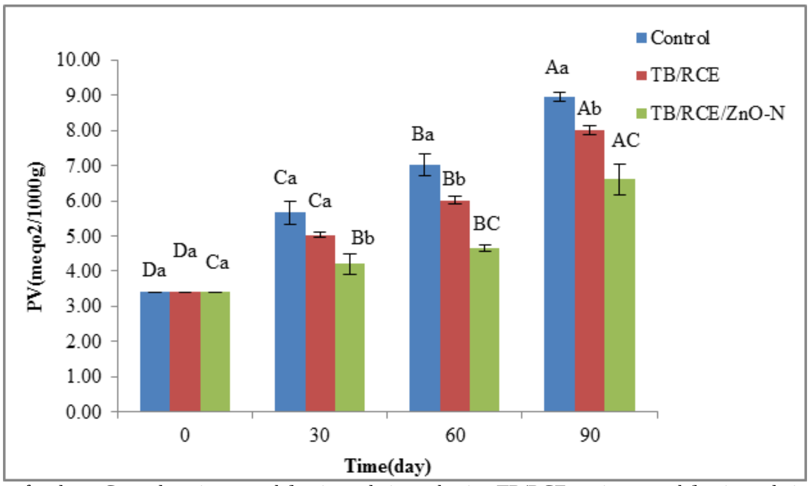

Fig.1. Peroxide value of walnuts Control: tapioca starch/bovine gelatin packaging TB/RCE: tapioca starch/bovine gelatin/roselle calyx extract TB/RCE/ZnO-N: tapioca starch/bovine gelatin /roselle calyx extract/Zinc Oxide nanorod. Different small and capital letters display significant difference between means ± SD of different amounts and one specimen during 20-day storage respectively.

Acidity value

Fig. 2. displays the effect of different packagings during 3 months of storage on walnut kernel. The use of RCE and ZnO-N had a significant impact on the acidity value of walnuts ($p<0.05$). The findings illustrate that the acidity value of walnuts had the increasing trend for 3 months; there was significant between various specimens on 90 days of storage. The acidity value of walnut packaged with TB was increased from 0.93 to 2.48 mg NaoH/g after 3 months of storage.

Regarding to Fig. 2. the increased acidity value of walnut packaged with TB/RCE/ZnO-N are lower than other samples.

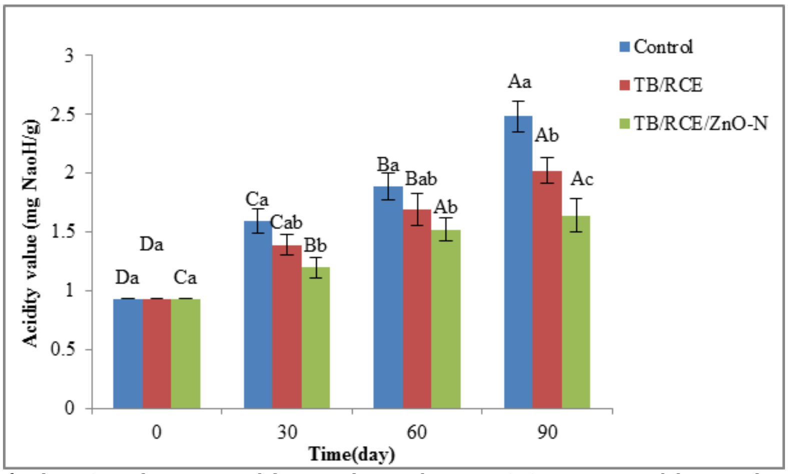

Fig.2. Acidity value of walnuts Control: tapioca starch/bovine gelatin packaging TB/RCE: tapioca starch/bovine gelatin/roselle calyx extract TB/RCE/ZnO-N: tapioca starch/bovine gelatin /roselle calyx extract/Zinc Oxide nanorod. Different small and capital letters display significant difference between means±SD of different amounts and one specimen during 90-day storage respectively.

Mold and yeast count

The number of mold and yeast of walnuts during 90-day storage is indicated in Fig. 3. Regarding to findings, the mold and yeast population of walnuts had an increment trend during conservation. The using of RCE and ZnO-N had positive effect on reducing the mold and yeast population of samples. Also, findings represented that increasing the number of mold and yeast of walnuts that have been packaged with RCE/ZnO-N active films were lower than other films. The number of mold and yeast of walnuts treated through TB packaging with RCE/ZnO-N was 4.49 log cfu/g on the 90th day of storage. The obtained findings indicated no significant difference ($p < 0.05$) between TB/RCE and TB/RCE/ZnO-N during storage time.

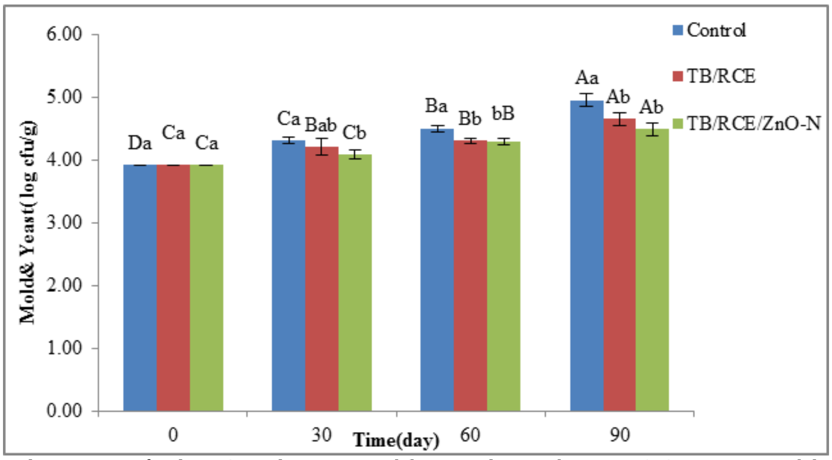

Fig.3. Number of mold and yeast count of walnuts Control: tapioca starch/bovine gelatin packaging TB/RCE: tapioca starch/bovine gelatin/roselle calyx extract TB/RCE/ZnO-N: tapioca starch/bovine gelatin /roselle calyx extract/Zinc Oxide nanorod. Different small and capital letters display significant difference between means±SD of different amounts and one specimen during 90-day storage respectively

Sensory properties

The average data of flavor and aroma of different samples of walnut kernels are displayed in Fig. 4. Walnut samples packaged with control TB biofilm indicated lower aroma and flavor scores than walnut samples packaged with RCE and RCE/ZnO-N packaging ($p < 0.05$). The results illustrated that the flavor and aroma score of all walnuts decreased during 90 days, and the score of the control specimen decreased faster than walnuts packaged with RCE and RCE/ZnO-N. According to the obtained results flavor and aroma scores of walnuts packaged with RCE and RCE/ZnO-N were higher than pure samples. The flavor and aroma result represented that significant difference was found between neat samples and RCE/ZnO-N on 90 th day of conservation.

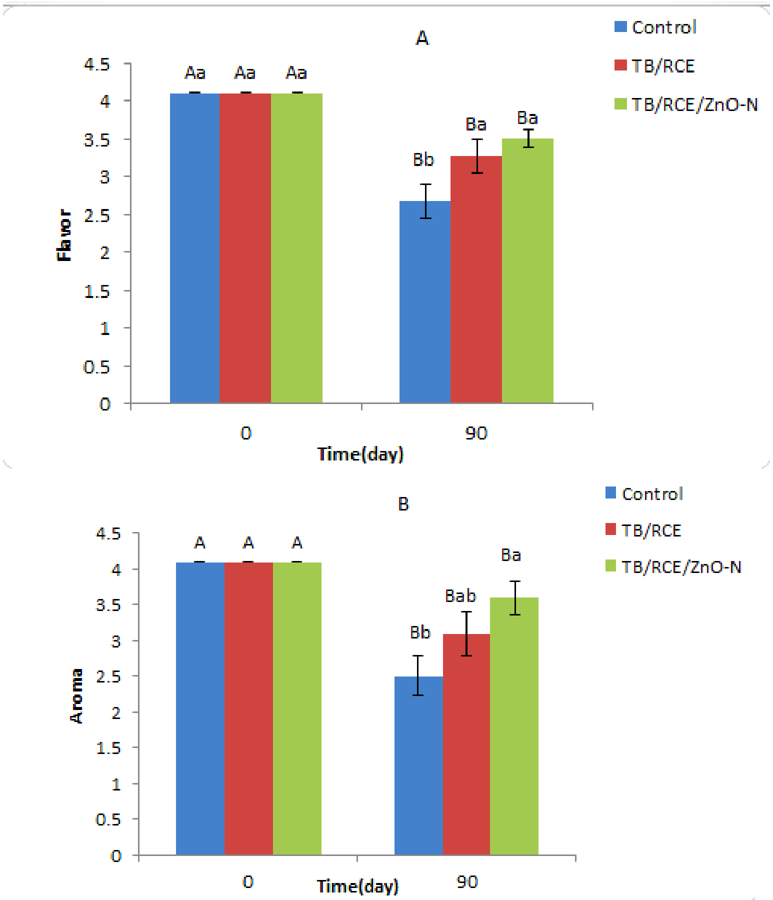

Fig.4 Flavor (A) and aroma (B) of walnuts Control: tapioca starch/bovine gelatin packaging TB/RCE: tapioca starch/bovine gelatin/roselle calyx extract TB/RCE/ZnO-N: tapioca starch/bovine gelatin /roselle calyx extract/Zinc Oxide nanorod. Different small and capital letters display significant difference between means±SD of different amounts and one specimen during 90-day storage respectively

Discussion

Peroxide value is attributed to the creation of hydroperoxides, which are an indicator of oxidative rancidity. Peroxides quickly decompose to create volatile aldehydes and ketones (Tavakoli *et al*., 2017). Changes in peroxide value are indicated in Fig. 1. The storage time and RCE, ZnO-N, significantly affected the peroxide value ($p < 0.05$). The initial peroxide value of walnuts was 3.3 meq O_2/kg. In various works, the initial peroxide value of the walnut was stated as 2.3 and 0.31 meq O_2/kg (Aydogdu *et al*., 2019; Kavuncuoglu *et al*., 2021). Mihaly Cozmuta *et al*. (2018), reported that when nano-TiO_2 and nano-Ag were applied as active agents, the oxidation rancidity level of walnut kernels during conservation was kept below 2 meq O_2/kg. These changes in the initial peroxide value may be related to differences in irrigation rate, extraction method storage conditions, and cultivar (Kavuncuoglu *et al*., 2021).

Films containing nanoadditive can act as a shield against oxygen molecules and light to the increasing oxidative rancidity quality of nuts (Fallah *et al*., 2023; Marvizadeh *et al*., 2017). The barrier properties increasing of bionanocomposite film may be related to the impacts of nanoadditive on the tortuous pathway. Hence, the mentioned result is attributed to the incorporation of nano-ZnO into biofilm and the formation of the tortuous pathway structure for oxygen molecule transfer (Fallah *et al*., 2022; Marvizadeh *et al*., 2017).

The oleic acid content is a factor in the evaluation of free fatty acids in nuts (Habashi *et al*., 2019).

Walnuts have %65 lipid content which %73 consists of polyunsaturated fatty acids. Due to having high levels of polyunsaturated fatty acids, walnut kernels are susceptible to oxidation rancidity. Thus, the prevention of oxidation reactions is an important problem to the extend shelf life of walnut kernels.

Based on Fig. 2. Tapioca starch/bovine gelatin biofilm containing RCE/ZnO-N had lower acidity value (1.64 mg NaoH/g) than other samples. The pure specimen displays the higher acidity value which is consistent with the study of Tajjedin *et al*. (2022). The author stated that carboxymethyl cellulose/polyvinyl alcohol containing nano-clay decreases the acidity value of walnuts, compared to neat samples.

In the other study, reported that the reduction in oxidation rancidity of walnuts packaged with hydroxypropyl methylcellulose/polyethylene oxide film containing 10% gallic acid loaded nanofiber was associated with nano-additives added. Also, the gallic acid compound has strong antioxidant properties and prevents oxidation rancidity of oils by showing free radical scavenger (Aydogdu *et al*., 2019).

Roselle calyx is a rich source of active compounds including, polyphenols, anthocyanin, antioxidants, and flavonoids (Peredo Pozos *et al*., 2020). In the presentation work, roselle calyx extract was successfully added into starch/protein film by casting method and they indicated antioxidant properties. Therefore, the use of RCE as an active compound was observed to be effective in reducing oxidation reactions. Also, The effect of roselle anthocyanins, on the antioxidant activity of film has been illustrated in another work (Zhang *et al*., 2019). They stated that the antioxidant activity of the film based on starch/PVA containing roselle anthocyanins was enhanced from 16% to 99.85% by the addition of roselle extract.

The mold and yeast count of walnuts stored for 90 days are represented in Fig. 3. Strach/protein film containing RCE and RCE/ZnO-N significantly affected the mold and yeast count of walnuts ($p < 0.05$). The mold and yeast count of all active films increased during conservation, and the increase in neat specimens was highest.

The antimicrobial properties of nano-ZnO can be related to various mechanisms, such as the release of antibacterial ions, the interaction of nanoadditives with microorganisms, decomposing the integrity of cell, and the formation of reactive oxygen species by the impact of light radiation (Marvizadeh *et al*., 2014). Since nano-ZnO has been thought to be abrasive due to surface flaws like edges and corners, another study claims that the antibacterial action of nano-ZnO may also be the consequence of physical damage to the cell membrane induced by the abrasive surface of nanoadditives (Javidi *et al*., 2022). The mold count of walnuts increased with increased conservation time, as illustrated by (Tavakoli *et al*., 2017). They stated that the initial mold count of walnut is 2 log CFU/g in packaging containing nano-silver and the packaging with nanoadditive shows the higher antifungal impact on mold compared with the pure sample.

Also, one of the antimicrobial impacts of herbal extracts is the penetration of the antibacterial agent into cell membrane, which causes an enhancement in cell membrane permeability. Portillo-Torres *et al*. (2019) stated hibiscus acid is one of the main antimicrobial compounds in roselle calyces. They demonstrated that the mentioned compound decomposed the membrane integrity and enhanced the permeability of cells membrane resulting in an antimicrobial impact against microorganisms. Aydin and Zorlu (2022) evaluated the antibacterial properties of alginate biofilms incorporated with extract of roselle against pathogenic bacteria. They stated higher antibacterial properties depending on increasing roselle level.

Walnut kernels packaged with neat TB film indicated lower sensory quality than walnuts packaged with RCE and RCE/ZnO-N packaging ($p < 0.05$). The results displayed that the flavor and aroma of all walnuts decreased during 90 days, and the value of neat specimens decreased faster than walnut kernels packaged with RCE and RCE/ZnO-N.

The obtained results of the sensory quality represented that, the use of active film containing

RCE and RCE/ZnO-N by barrier activity against the transfer of oxygen gas into the walnut prevented oxidation rancidity and improved aroma and the flavor of the final product (Chavoshi *et al*., 2023).

The aroma score of the sensory assay of walnuts decreased with increased conservation time, as Vera *et al*. (2018). They showed that the aroma score (last day) of walnuts are 4 and 3 in laminate containing nano-Se and control laminate, respectively, and the laminate with nanofiller indicates higher sensory quality compared with the pure sample. In another work, sensory assay represented significant difference between pure specimens and walnuts packaged with encapsulated *Cinnamomum zelanicum* essential oil (Erfani *et al*., 2022).

Since nanoparticles may migrate into packaged food during processing and preservation, the safety of their use in food packaging has led to concerns among the public. Nanoparticle migration may decrease food's sensory quality. Additionally, as some nanoparticles have safe characteristics, health issues may result if the migration rate exceeds the permitted values and limiting rate (Jafarzadeh *et al*., 2021). According to a current EFSA investigation into the safety of nano-ZnO for usage in food contact compounds, the Commission should enforce a daily Zn upper limit of 25 mg. Additionally, it was revealed that the substance does not migrate at the nanoscale, hence the safety test should be conducted using the migration of soluble Zn^{2+} (Souza *et al*., 2020).

Conclusions

In the presentation work, fungal and yeast spoilage, chemical, and sensory assays of packaged walnuts were evaluated during 90 days of conservation. The findings represented that the tapioca starch/bovine gelatin/ roselle calyx extract and tapioca starch/bovine gelatin/ roselle calyx extract/ZnO-N had a positive effect on mold and yeast count. The obtained findings indicated that the biodegradable film containing RCE and RCE/ZnO-N decreased the acidity and peroxide value of walnuts compared with the pure sample. The results of current work prove that the usage of active biofilm to walnuts can improve sensory indices, including flavor, and aroma.

Due to the lowest values in acidity and peroxide indices, the least mold and yeast count and the high quality of sensory assay during 90 days of conservation, were observed in the active film packaged with RCE/ZnO-N, therefore, the mentioned specimen was chosen as the most suitable active biopackaging. Hence, these findings state the antimicrobial effect on walnut and the consequent shelf life extension of the wrapped walnuts, since quality of chemicals and microbial of the nut remain during conservation time.

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Conflict of interests

The authors declare that there is no conflict of interest.

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