

# Preserving apple (*Malus domestica* var. *Anna*) fruit bioactive substances using olive wastes extract-chitosan film coating



Ibrahim Khalifa<sup>a,\*</sup>, Hassan Barakat<sup>a,b</sup>, Hamdy A. El-Mansy<sup>a</sup>, Soliman A. Soliman<sup>a</sup>

<sup>a</sup> Food Technology Department, Faculty of Agriculture, Benha University, Moshtohor, 13736 Qaliubia, Egypt

<sup>b</sup> Food Science and Human Nutrition Department, College of Agriculture and Veterinary Medicine, Qassim University, Buraidah, Saudi Arabia

## ARTICLE INFO

### Article history:

Received 26 June 2016

Received in revised form

25 October 2016

Accepted 10 November 2016

Available online 17 November 2016

### Chemical compounds studied in this article:

DPPH\* (PubChem CID: 2735032)

Trolox (PubChem CID: 40634)

Gallic acid (PubChem CID: 370)

Quercetin (PubChem CID: 5280343)

Chitosan (PubChem CID: 3086191)

Thiabendazole (PubChem CID: 5430)

### Keywords:

Olive wastes extracts

Chitosan

Coating

Nutritional quality

Microstructure analysis

## ABSTRACT

Apple fruits have components of therapeutic nature. Such components, to a great extent, decline or decompose during post-harvest that negatively affect fruit shelf-life. Chitosan fruit-filming has proved useful in maintaining these compounds. This study aims, therefore, at enhancing Chitosan coating-film performance by mixing with some olive wastes extracts of leaf and pomace extracts. Apple fruits were sprayed with six different coating formulas including chitosan-water wax coating, in addition to the uncoated fruits. Then, the total phenolic, flavonoids, antioxidants, pigments, weight loss, decay area, and microstructure were assayed. The bioactive substances drastically changed in uncoated rather than coated fruits. Conversely, weight loss and decay area significantly increased in uncoated fruits. Amazingly, the addition of olive leaf extract to chitosan coating films meaningfully reduced the gradual decline in total phenolic, flavonoids and antioxidants. Olive pomace extract recorded the lowest influencing on anthocyanins during storage at  $4 \pm 1$  °C for 35 d. Also, both olive leaf and pomace extracts enhanced the coating distribution, due to no pores were observed in the fruits' surfaces. Decidedly, incorporation of olive leaf extracts with 2% into chitosan coating solution was the best formula comparable with the others. Thus, olive wastes extracts, incorporated into chitosan fruit coatings; relatively improve the nutritional quality of apple fruits during post-harvest.

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## 1. Introduction

Apple (*Malus domestica* var. *Anna*) fruits greatly vary in the bioactive substances like polyphenols, flavonoids, vitamins, pigments and others [1,2] which provide high nutritional values. Unfortunately, these components dramatically decline during post-harvest times [35]. This might refer to the activity

\* Corresponding author. Fax: +20 132467786.

E-mail addresses: [ibrahim.khalifa@fagr.bu.edu.eg](mailto:ibrahim.khalifa@fagr.bu.edu.eg) (I. Khalifa), [hassan.barakat@fagr.bu.edu.eg](mailto:hassan.barakat@fagr.bu.edu.eg) (H. Barakat), [Hamdy.elmansy@fagr.bu.edu.eg](mailto:Hamdy.elmansy@fagr.bu.edu.eg) (H.A. El-Mansy), [Soliman.soliman@fagr.bu.edu.eg](mailto:Soliman.soliman@fagr.bu.edu.eg) (S.A. Soliman).

Peer review under responsibility of China Agricultural University.

<http://dx.doi.org/10.1016/j.inpa.2016.11.001>

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of some microorganisms which grow on the fruit surfaces' during post-harvest time. They produce mycotoxins and degrade phytochemicals [6]. Additionally, other factors may responsible for phytochemicals degradation includes cultivar type, environmental and agronomic conditions, harvest and food processing operations, and storage factors [7].

Commonly, these challenges might be fixed using coating fruits with commercial waxes such as water wax incorporated with artificial additives like thiabendazole (WW-TBZ). But, such materials might cause some dangerous side effects related to bladder cancer [8]. Switching to using some natural polymers, such as chitosan (CH) (poly B-(1,4) N-acetyl-D-glucosamine) incorporated with natural additives like food processing wastes, has recently been applied to fruit coating techniques [9–11]. The CH, following cellulose, is the second most abundant polysaccharide found in nature [12]. In addition to being environmentally safe, it has good film-forming properties, antimicrobial activity, and has been recommended as GRAS food additive [13–16].

Olive (*Olea europaea* var. *Kronakii*) plant extracts has been one of these natural additives which play a functional role in fruit film-coating components. Olive oil processing wastes (OOW) contain considerable amounts of bioactive substances, although it causes some economic losses and some environmental problems [17–20]. However, OOW are promoting by-products for functional food and/or nutraceuticals. They can be used as antioxidants, antifungal and antibacterial agents [20,21]. Thus, OOW has been valued before in edible-film approach as in the cases of polyesters and polylactic films [22,23]. Incorporating OOW extracts into chitosan coating materials may be one safe approach to maintaining quality of cold-stored apple fruits. The objective of this study was to infer how both olive wastes extracts, when incorporated into Chitosan film coating, enhance apple fruits shelf-life during post-harvest cold storage time by maintaining fruit nutritional and keeping quality.

## 2. Materials and methods

### 2.1. Reagents and solutions

1,1-Diphenyl-2-picrylhydrazyl radical (DPPH), 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one (Quercetin) and 6-hydroxy-2,5,7,8-tetramethylchroman carboxylic acid (Trolox) were obtained from Sigma Aldrich, Co., Germany. Chitosan 95% deacetylation, high molecular weight (viscosity 500–2000 cps) was procured from Oxford Co., India. Folin-Ciocalteu reagent was purchased from Fluka Biochemical, Co., Switzerland. Gallic acid Serva was obtained from Biochemical, Co., New York. Thiabendazole and water wax® WW-TBZ were obtained from Fomesa Fruitech, Co., Spain. All reagents and indicators are pure and analytical grade.

### 2.2. Microbial strain and media

*Penicillium expansum* ATCC 7861 was obtained from Cairo Microbiological Resource Center (MIRCEN), Faculty of Agriculture, Ain Shams University, Cairo, Egypt. Sabouraud agar No. 402005 was obtained from Biolife, Co., Italy.

### 2.3. Raw materials

- Olive (*Olea europaea* var. *Kronakii*) wastes including olive leaves and olive pomace were obtained from Cairo for Oil Industry, Co., Industrial Zone, 6th October City, Egypt.
- Apple fruits (*Malus domestica* var. *Anna*) eatable maturity form was obtained from the Alexandria Agriculture Farm, Co., Egypt.

### 2.4. Analytical techniques

**2.4.1. Olive oil processing wastes preparation and extraction**  
Olive leaves and olive pomace were oven-dried (Tit Axon S.R.L via Canova, Italy) at 40–50 °C gradually till the weight constant (4.37% and 7.60% moisture, respectively). Subsequently, they were milled by grinder (Severin, type 3871, Germany). They were passed through a 60 mesh sieve to obtain a fine homogeneous powder, then packed in dark glass jars then kept at  $-20 \pm 1$  °C until use. Both olive leaves and pomace were mixed with ethanol 80% (1:20, w/v) in dark bottles, and shaken at 120 rpm for 86400 s (Centrifuge (MLM Zentrifugenbau.TS21, Germany). The mixtures were filtered through filter paper Whatman No. 1. The filtrates were collected, then solvents were removed by rotary evaporator (NE-1-Rikakikai Co., LTD, Japan) at 40 °C according to Özge et al. [24]. The residue was collected and kept at  $-18 \pm 1$  °C.

#### 2.4.2. Film forming solution

The incorporated CH solutions with both ethanolic olive leaf and olive pomace extracts were prepared according to Lafka et al. [25] with some modifications. Chitosan 2% was dispersed in an aqueous solution of glacial acetic acid (0.5%, v/v) at 40 °C. The solution was heated and agitated constantly for 43200 s. Then the pH was adjusted to 5.6 with 1 M NaOH. Subsequently, glycerol 1.6% was added as a plasticizer [26]. The solution was stirred overnight at room temperature. Finally, olive leaves and olive pomace extracts at 1 and 2% were added and mixed to achieve the complete dispersion.

#### 2.4.3. Apple fruits coating applying

Apple fruits were sorted for uniform size, full color,  $\frac{3}{4}$  maturities, and for being free of visible defect and of decay. Then, they were sanitized by inundation on sodium hypochlorite solution 250 ppm for 2 min and washed with distilled water to eliminate chlorine traces. Subsequently, cross-shaped wounds (2, 0.1 and 0.5 mm for length, width and depth, respectively) were made to the fruits using sterilized Spatula (Dynalon 1212W16CS 391905) and inoculated by 10  $\mu$ L *P. expansum* spore suspension ( $10^5$  CFU/mL). The coating solutions (as described above Section 2.4.2) were sprayed twice on the whole fruit surface using a multi-function hand 2L pressure sprayer (Ningbo Synkemi Co., type SK-2B, China) and allowed to be air-dried at ambient temperature for 7200 s. Seven groups of samples were prepared in total: uncoated (control), CH (2% w/v), Chitosan-Olive leaves extracts CH-OLE (1 and 2% w/v), Chitosan-Olive pomace extracts CH-OPE (1 and 2% w/v), and WW-TBZ 0.1% coated fruits as a positive control,

according to Sánchez-González et al. [27]. Fruits were packed in boxes (~3 fruit per box) as a three replicate and wrapped with polyethylene sheets, then stored at  $4 \pm 1^\circ\text{C}$  for 35 d. The nutritional characteristics, keeping parameters and weight loss of fruits were evaluated at the beginning of the experiment (i.e., 0 d) then at a 7 d interval up to 35 d.

#### 2.4.4. Bioactive substances of coated apple fruits

**2.4.4.1. Anthocyanins content.** The anthocyanins content of apple fruits was determined according to Zhang et al. [28]. A 0.005 kg apple samples were extracted with 45 mL of acidified ethanol (95% ethanol: HCl 1.5 N 85:15) for 7200 s at room temperature in the dark, filtered and measured at 535 nm using spectrophotometer (CE599- Automatic Scanning Spectrophotometer, GECIL, England).

**2.4.4.2. Carotenoids and chlorophylls content.** A 0.01 kg apple sample was mixed with 50 mL acetone 85% in dark bottle and left to stand for 54,000 s at room temperature. The mixture was filtered through glass wool into a 100 mL volumetric flask and made up to appropriate volume by the same solvent. The chlorophyll *a*, *b* and carotenoids were immediately measured at 440, 644 and 662 nm using the same spectrophotometer according to Fuleki and Francis [29].

**2.4.4.3. Total phenolic compounds content.** The total phenolic compounds (TPC) for acetone extracts of apple were determined according to Raghuramulu et al. [30]. In brief, 200  $\mu\text{L}$  of each sample was mixed with 1 mL of 10-fold diluted Folin-Ciocalteu reagent; the reaction was terminated after 300 s by 1 mL of  $\text{Na}_2\text{CO}_3$  7.5%, then 1.5 mL distilled water was added. The mixtures were incubated in dark for 3600 s then the absorbance at 760 nm was measured using the same spectrophotometer. The TPC was expressed as gallic acid equivalents ( $\text{mg GAE } 100 \text{ g}^{-1}$ ) using the following straight-line linear regression equation based on the calibration curve:

$$Y = 0.0201x + 0.0538 \quad (R^2 = 0.99) \quad (1)$$

where *Y* is the concentration and *X* is the absorbance.

**2.4.4.4. Total flavonoids content.** The total flavonoids content (TF), for acetone extracts of apple, was determined according to Khalifa et al. [31]. A 0.5 mL aliquot of  $\text{AlCl}_3$  2% ethanolic solution was added to 0.5 mL of extracts and mixed well. Then it was kept for 3600 s at room temperature and the absorbance at 420 nm was measured using the same spectrophotometer. The final concentration of TF was expressed as quercetin equivalent ( $\text{mg QE } \text{g}^{-1}$ ) which was calculated using the following straight line linear regression equation based on the calibration curve:

$$Y = 0.037x + 0.1363 \quad (R^2 = 0.98) \quad (2)$$

where *Y* is the concentration and *X* is the absorbance.

**2.4.4.5. Antioxidant activity.** The antioxidant activity (AOA) of apple acetonetic extracts was evaluated according to Khalifa et al. [32]. A 0.1 mL extract was added to 3.9 mL of DPPH\* methanolic solution, then the absorbance at 517 nm was measured after the solution had been allowed to stand in the dark for 600 s at 517 nm using the same spectropho-

tometer. The final results were expressed as Trolox equivalents ( $\mu\text{mol TE } \text{g}^{-1}$ ).

**2.4.4.6. Decay area determination.** Mold growth area of inoculated apple was checked by measuring in terms of the decay area every seven days using micrometric ruler according to Gniewosz et al. [33].

**2.4.4.7. Microstructure analysis.** The surface and cross-section microstructures of apple skins, which had been coated by selected formulas such as CH 2%, CH-OLE 2%, CH-OPE 2%, and uncoated fruits, were examined and scanned using an electron microscope. Tissues from different treatments were fixed in 4% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 4.1) for 14,400 s, formerly fixated later in osmium tetroxide for 7200 s. Fixed tissues were rinsed in the same buffer three times and dehydrated through a graded ethanol series 10 to 100% for 600 s up to 1800 s in final concentration. The specimens were transferred on copper slide and dehydrated using critical point dryer with liquid carbon dioxide, then coated with gold using (S150A Sputter coater-Edwards-England). The specimens were examined and photographed using scanning electron microscope with proper magnification (JXA-840A, Electron Probe Micro analyzer-JEOL, Japan).

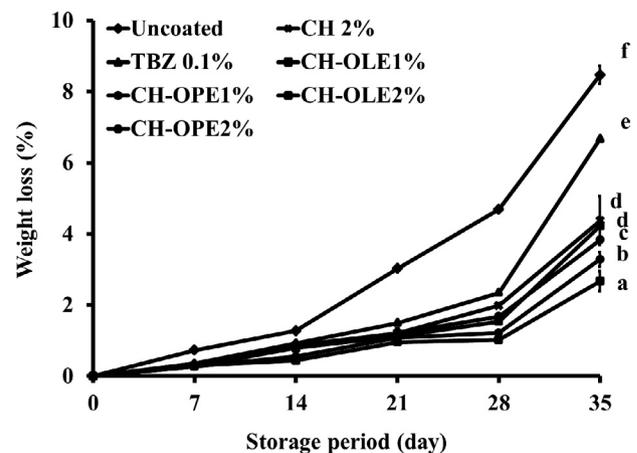
#### 2.4.5. Statistical analysis

The statistical analysis was carried out using SPSS program with multi-function utility regarding to the experimental design and multiple comparisons were carried out applying LSD according to Steel et al. [34].

## 3. Results and discussions

### 3.1. Weight loss

The effect of CH-incorporated films on weight loss of apple fruits during cold storage is shown in Fig. 1. The weight loss steadily increased during a prolonged storage period either



**Fig. 1 – Effect of CH, CH-OOW and WW-TBZ coating solution on weight loss in apple fruits during cold storage at  $4 \pm 1^\circ\text{C}$ , (Mean  $\pm$  SD, *n* = 3).**

coated or uncoated fruits. Significant difference ( $p < 0.05$ ) was observed between uncoated and coated fruits with the progression of storage period. Uncoated apples fruits showed weight loss was as high during storage period. Significant difference ( $p < 0.05$ ) was found between CH incorporated film and WW-TBZ. Statistically, uncoated apples evident the highest weight loss to be 3.03% during the whole storage period and 8.50% after 35 days. Conversely, the lowest observed loss was 2.66% using CH-OLE 2%. The formed CH film on surface of coated fruits delayed the migration of moisture. These CH-based coating strategies to reduce the weight loss during storage and its concept were used before as mentioned [10].

### 3.2. Anthocyanins content

The initial anthocyanins of coated apple with CH-OPE 2% and uncoated apple were 20.83 and 19.00 mg 100 g<sup>-1</sup>, respectively (Fig. 2). Indeed, during the preliminary stage of cold storage the uncoated and coated fruits show a significant increase in anthocyanins content. Shao et al. [35] reported that fruits become darker during storage due to releasing cell anthocyanins' after its decomposition. Following 14 d of storage, there were, generally, plodding declines of anthocyanins. At 35 d, CH-OPE 2% film had relatively higher anthocyanins content. Based on Fig. 2, the control had about 41% less anthocyanin relative to both CH-OPE 2% and CH-OLE 2% at 35 d. Applying CH-OOW films maintained the anthocyanins better than WW-TBZ films. Coating acts as a gas barrier, thus lowering internal O<sub>2</sub> levels and increasing CO<sub>2</sub>. Under these conditions, metabolism and catabolism are slowed down which contribute to preserving nutraceuticals for longer periods [36]. Also, olive pomace extracts contains a lot of AOA that prevent cell wall oxidation [37].

### 3.3. Carotenoids contents

Over the 35 d period, a decremental rate has been observed during cold storage in either uncoated or coated fruits (Fig. 3). Between both CH-OLE 2% and CH-OPE 2% on one side

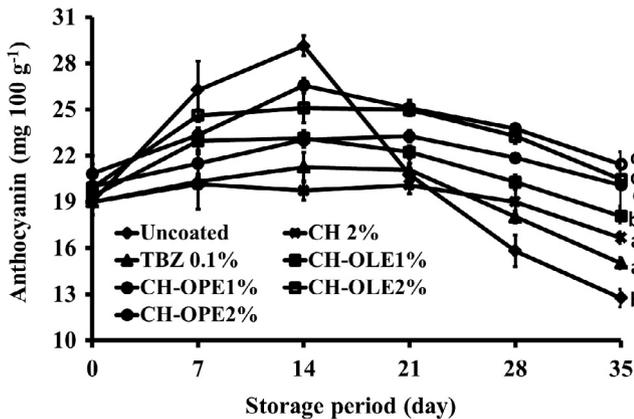


Fig. 2 – Monitoring of anthocyanins for coated and uncoated apple fruits with different chitosan based coating formulas during cold storage at 4 ± 1 °C, (Mean ± SD, n = 3).

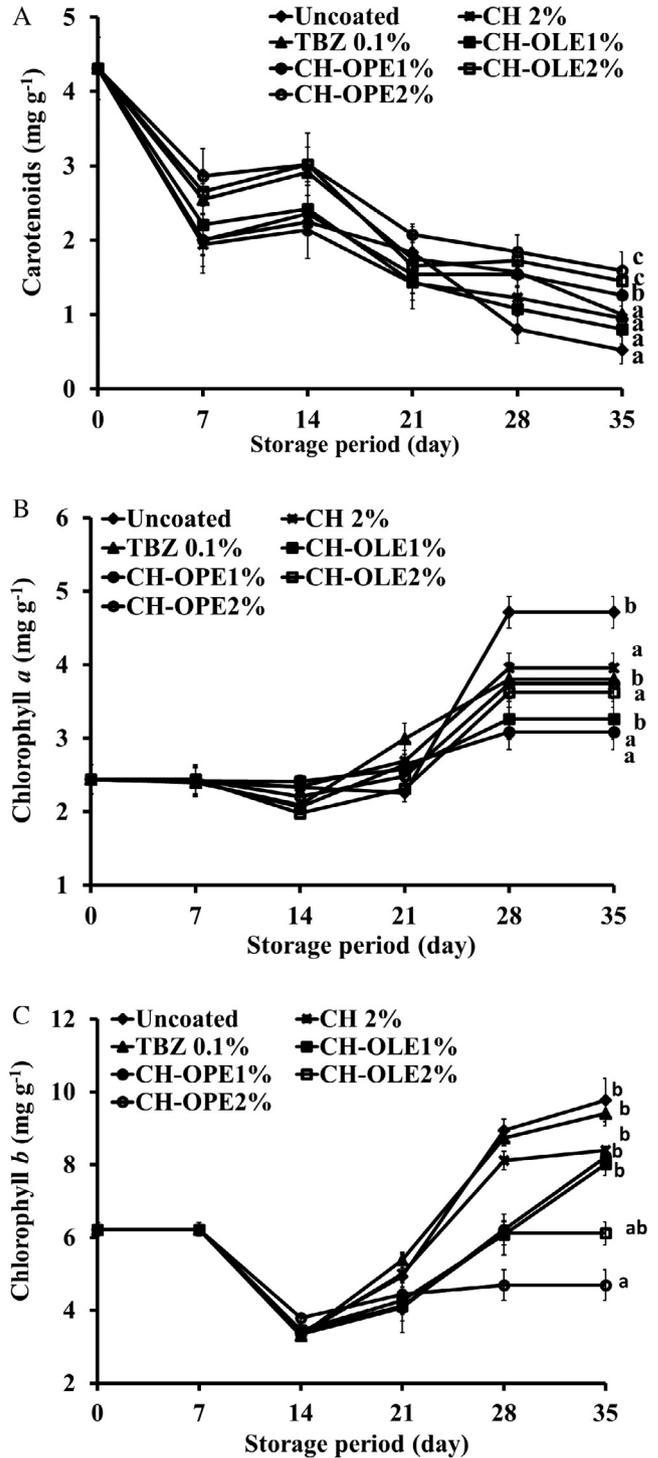


Fig. 3 – Changing of carotenoids in coated and uncoated apple fruits with different chitosan based coating formulas during cold storage at 4 ± 1 °C, (Mean ± SD, n = 3).

and the rest of the other films, there were marginal differences ( $p < 0.05$ ). The coated apple with CH-OPE 2% was the lowest decreases in carotenoids contents (1.59 mg g<sup>-1</sup>) compared with uncoated apple (0.52 mg g<sup>-1</sup>) at the end of storage period. So far, there are no studies on the effect of the coating

materials on the content of carotenoids in fruits. However, Anjum et al. [38] found that cold storage was decreased the carotenoids in some vegetable and fruits.

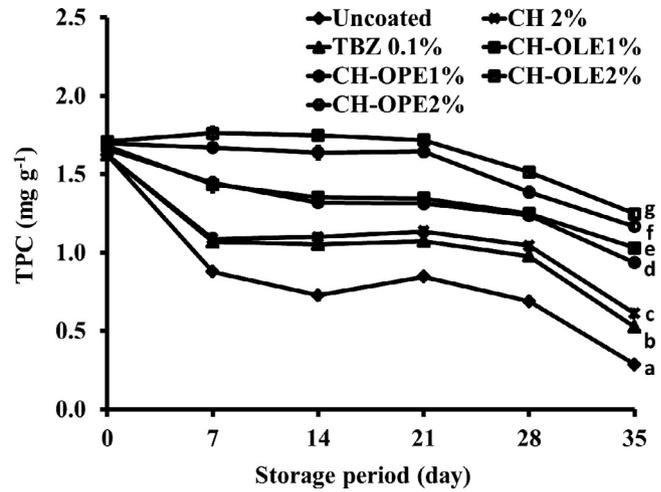
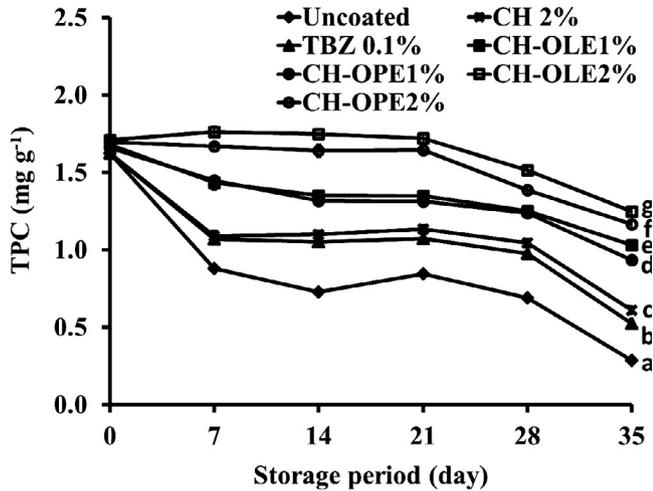


Fig. 5 - Monitoring of TPC for coated and uncoated apple fruits with different chitosan based coating formulas during cold storage at  $4 \pm 1 \text{ }^\circ\text{C}$ , (Mean  $\pm$  SD,  $n = 3$ ).

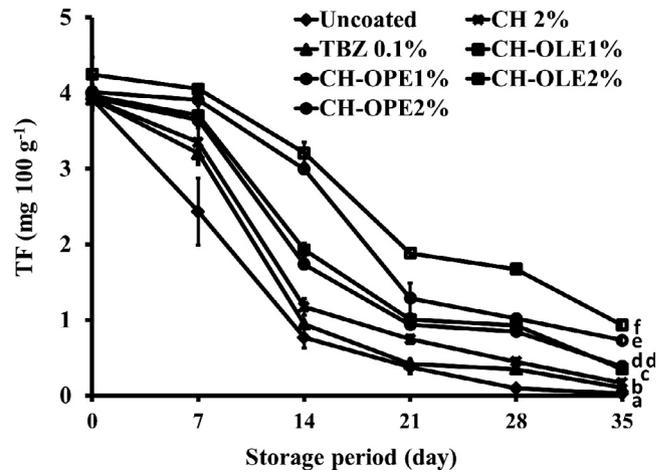
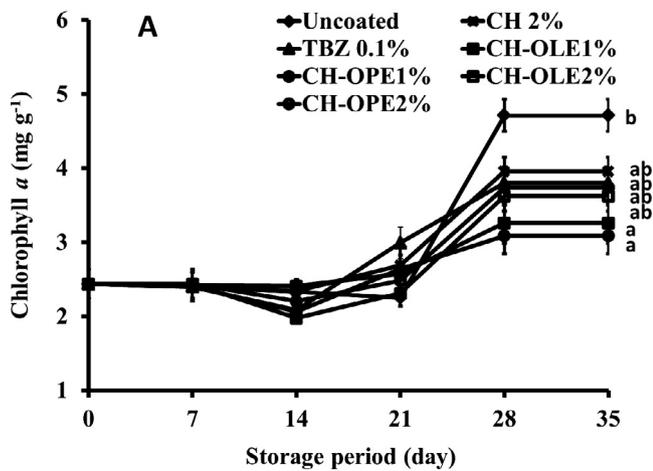


Fig. 6 - Monitoring of TF for coated and uncoated apple fruits with different chitosan based coating formulas during cold storage at  $4 \pm 1 \text{ }^\circ\text{C}$ , (Mean  $\pm$  SD,  $n = 3$ ).

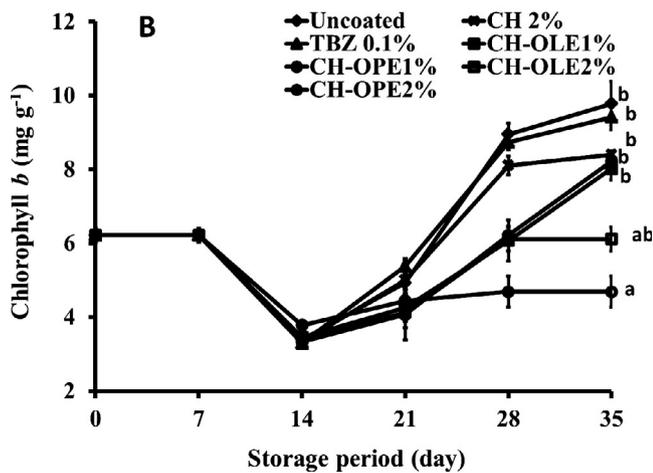


Fig. 4 - Changing of chlorophyll a (A) and chlorophyll b (B) in coated and uncoated apple fruits with different chitosan based coating formulas during cold storage at  $4 \pm 1 \text{ }^\circ\text{C}$ , (Mean  $\pm$  SD,  $n = 3$ ).

### 3.4. Chlorophylls contents

The differences among coatings were quite trivial up to 21 d; however, a sharp upward content of Chlorophyll a ( $\sim 2.5$  to  $\sim 4.5 \text{ mg g}^{-1}$ ), and for Chlorophyll b ( $\sim 4.5$  to  $\sim 9.0 \text{ mg g}^{-1}$ ) occurred towards the 28 d for the uncoated fruits (Fig. 4 A, B). However, the uncoated apple scored the highest chlorophyll a and b regardless the storage periods. Then it was followed by CH 2% for chlorophyll a and WW-TBZ for chlorophyll b. In contrast, CH-OOW led to decrease the increasing of chlorophylls releasing during storage. These changes may occur because so-called weight losses in fruits during post-harvest related to the result mentioned before (Section 3.1). There are no studies on the effect of the coating materials on apples' chlorophylls content.

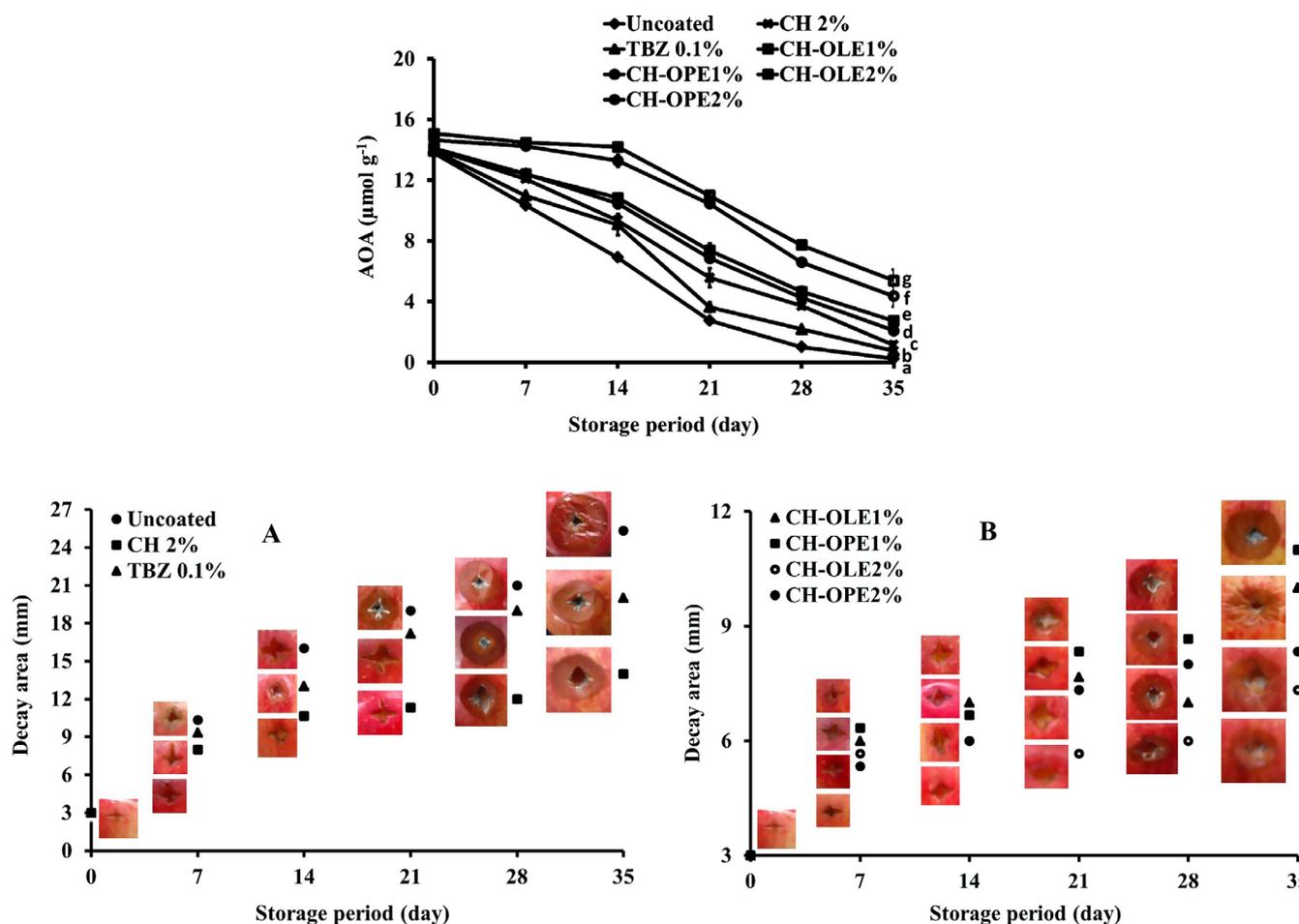


Fig. 7 – Monitoring of AOA for coated and uncoated apple fruits with different chitosan based coating formulas during cold storage at 4 ± 1 °C, (Mean ± SD, n = 3).

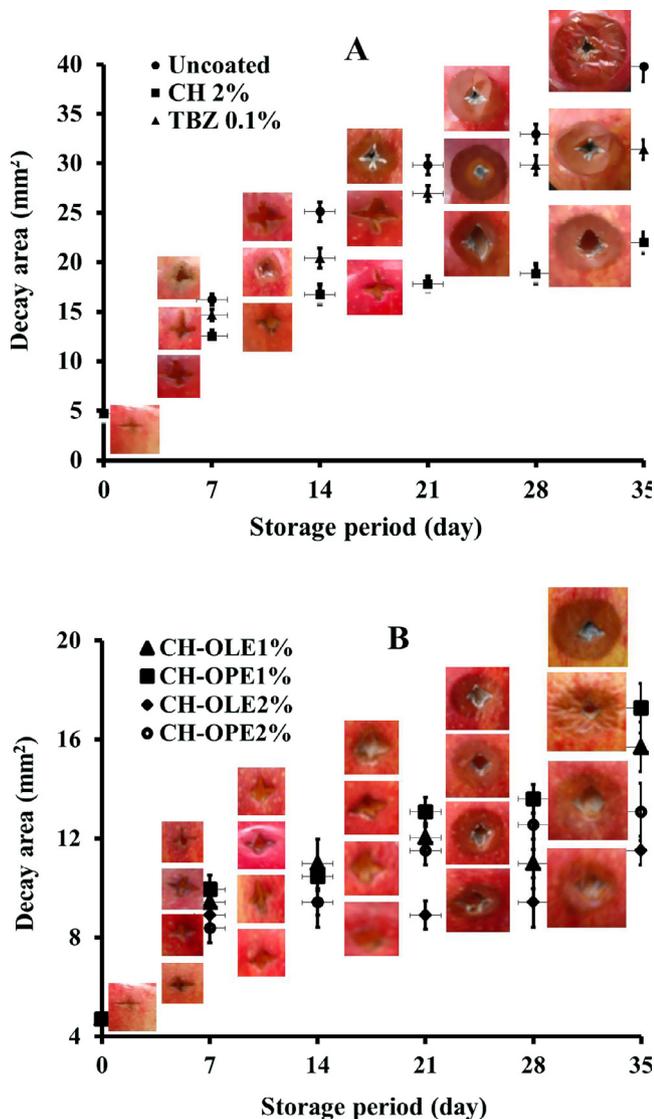
### 3.5. Total phenolic compounds

The initial TPC content was varied, for instance the mean value of TPC in uncoated and CH-OLE 2% for apple fruits were (1.62 and 1.71) mg GAE g<sup>-1</sup>, respectively. These differences owing to the phenolic were involved in the composition of OOW which used in film formation and fruits coating. However, it was 0.43 mg GAE g<sup>-1</sup> at the end of storage. Over the 35 d trial period, all coating films had varying positive impacts ( $p < 0.05$ ) on slowing down apple fruit total TPC degradation rate relative to that of the Control (Fig. 5). All four CH-OPE/OLE films caused TPC degradation rates steadily drop towards the 35 d time; yet CH 2%, TBZ 0.1%, and control, all exhibited relatively faster downward degradation rates. Both olive extracts, as basic components of CH coating thereby had potentials in maintaining fruit TPC as intact as possible in relation to CH coating impact by itself. The lowest decreases in TPC were showed in coated apple with CH-OLE 2% to be 1.24 mg GAE g<sup>-1</sup>. Otherwise the highest decreases in TPC were observed in uncoated fruits reached to 0.28 mg GAE g<sup>-1</sup> at end of storage period. Rodrigures et al. [39] explained how cell breakdown releases phenolic compounds that are exposed to enzymatic oxida-

tion. CH-OOW compounds might function as protective barriers on the fruits surface to reduce oxygen supply. This finding was similar to what Macheix and Fleuriet [40] had found in apple fruits.

### 3.6. Total flavonoids content

The variation in TF content during cold storage of uncoated and coated fruits is exhibited in Fig. 6. The TF content progressively decreased during storage period, recording a relatively greater reduction in uncoated fruits. The loss in TF in uncoated fruits was extremely rapid compared with the CH-OLE 2% coated fruits to be 0.02 vs 0.93 mg QE 100 g<sup>-1</sup>, respectively at the end of storage period. Indeed, significant difference ( $P < 0.05$ ) in TF content was noticed between all coated and uncoated fruits. Otherwise, no significant difference ( $p > 0.05$ ) was found between both CH-OLE 1% and CH-OPE 1%, or between CH-OLE 2% and CH-OPE 2%. Yet there are rarely available studies have examined the effect of coating on TF in apple fruits, whereas, Macheix and Fleuriet [40] mentioned that the TF was decreased during cold storage.



**Fig. 8 – Decay area in uncoated apple and coated with CH and WW-TBZ (A), CH-OOW (B) infected with *R. stolonifer* during cold storage at  $4 \pm 1$  °C, (Mean  $\pm$  SD,  $n = 3$ ).**

### 3.7. Antioxidant activity

Similarly, during the storage period the AOA sharply decreased especially in uncoated fruits compared to the coated ones (Fig. 7). The AOA significantly decreased ( $P < 0.05$ ) from 13.80 to  $6.90 \mu\text{mol TE g}^{-1}$  in uncoated apple after 14 d. However, low decremental rate was observed in coated fruits. Coating apple fruits with CH-OLE 2% or CH-OPE 2% exhibit the lowest decreases rather than WW-TBZ 0.1%. Carbone et al. [3] and Rodrigues et al. [39] stated valuable finding about the preventing of AOA losses in apple fruits during cold storage using edible coating.

### 3.8. Decay area

Generally, the infected area gradually increased by extending storage periods (Fig. 8 A, B). Regardless the coating treatments the initial and the final infected area was 3 and  $13.28 \text{ mm}^2$ ,

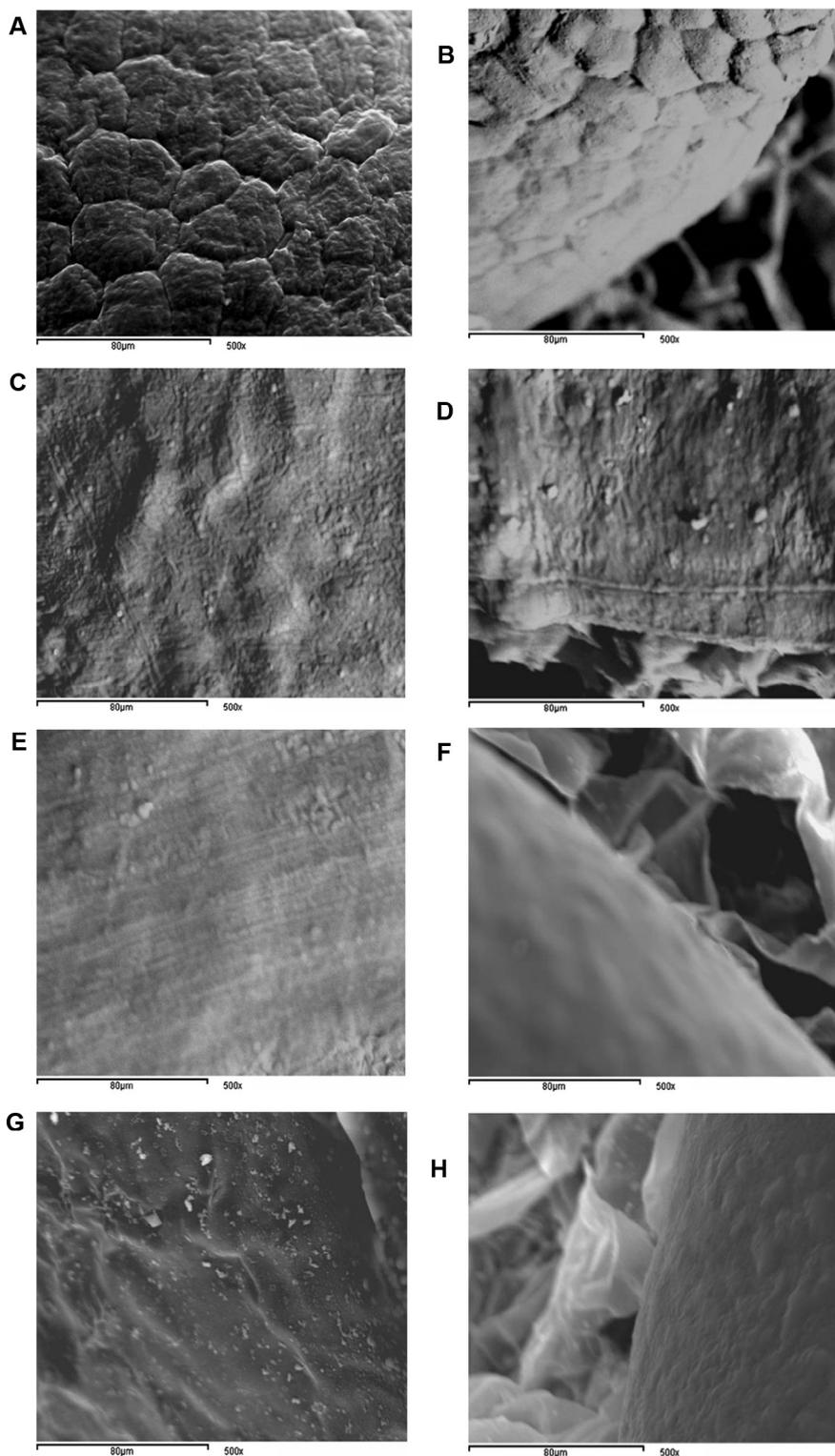
respectively. The decayed area of coated apple was smaller significantly reduced compared to the uncoated fruits. For example the early signs of mold development in uncoated apple appeared after 7 d of storage and it was delayed in the others fruits. CH-OLE 2% had a relatively low decay area compared to each of CH, WW-TBZ, and uncoated fruits. Mean decayed area on the control vs CH-OLE2% fruits was about 3-folds as much. Obviously, the highest observed areas were  $25.33 \text{ mm}^2$  in uncoated apple at the end of storage. While, the lowest observed areas were  $7.33 \text{ mm}^2$  in coated apple with CH-OLE 2%. Both CH and CH-OOW were more effective than the commercial coating material of WW-TBZ regarding growth of fungal strains Fig. 8. This is due to its antifungal activity [41]. El-Ghaouth et al. [42] suggested that CH induces chitinase, as defense enzyme catalyzes the hydrolysis of chitin, a common component of fungal cell walls, preventing or delayed the growth of fungi on the fruit. Also, these results are complimentary to those of Park et al. [43] who reported that significant antimycotic activity of methylcellulose and CH composite films incorporated with 4% of sodium benzoate or potassium sorbate.

### 3.9. Microstructure examination

The homogeneity of both CH and CH-OOW coatings, micrographs of both the surface and the cross-section areas are shown in Fig. 9. Coated apples, especially with CH-OOW, showed uniform coating distribution, and pores were not observed compared with uncoated fruits Fig. 9A and B. The higher percentage of covered surface relates to the higher water vapor resistance which slowed respiration process and water loss as observed in coated apple with CH-OLE 2% and CH-OPE 2% Fig. 9E-H. In addition, both CH Fig. 9C and D and CH-OOW coatings covered all irregularities in the fruits skin. Khalifa et al. [44] argued that the extensibility of the liquid dispersion on the covered fruit surface plays an important role in limiting water migration from the fruits.

## 4. Conclusion

The results of the present study asserted that the incorporation of OOW into CH improved the nutritional quality for cold-stored apple fruits. Also, the coatings of CH or CH-OOW have beneficial impacts on the quality retention of cold storage apple fruits especially CH-OLE 2%. The use of OOW also maintained lower weight loss. Likewise, CH-OOW resulted in effectively delaying anthocyanins, total phenolic, flavonoids, carotenoids, chlorophylls and antioxidants activities. Moreover, CH-OOW fully covered the whole surface of apple fruits in term of skin irregularities and/or pores. Hence, coatings of apple fruit with CH-OOW may be relatively more useful for improving apple post-harvest quality and shelf-life stability compared to both only CH and WW-TBZ coatings. These motivated results may encourage the food handlers to replace the chemical coating materials with the presented coatings formulas of the current study. Moreover, the applicability of such formulas on different fruits and vegetables surfaces' has to further reviewing.



**Fig. 9 – Scanning electron micrographs of surface and cross-section of uncoated; (A, B) and coated apple fruits with CH; (C, D), CH-OLE 2%; (E, F) and CH-OLP 2%; (G, H) formulas, (n = 1).**

	Surface	Cross section
Uncoated	A	B
CH 2%	C	D
CH-OLE 2%	E	F
CH-OLP 2%	G	H

## Conflict of interest

Authors have declared that there are no conflict of interest.

## Acknowledgments

The authors gratefully acknowledge the financial support from the Benha University project [Utilization of some agricultural wastes in food and feed processing – A/1/2] 2013-2015.

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