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Antifungal activities of combined treatments of irradiation and essential oils (EOs) encapsulated chitosan nanocomposite films in *in vitro* and *in situ* conditions

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ABSTRACT

Cellulose nanocrystals (CNCs) reinforced chitosan based antifungal films were prepared by encapsulating essential oils (EOs) nanoemulsion. Vapor phase assays of the chitosan-based nanocomposite films loaded with thyme-oregano, thyme-tea tree and thyme-peppermint EO mixtures showed significant antifungal activity against *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus parasiticus*, and *Penicillium chrysogenum*, reducing their growth by 51–77%. Combining the bioactive chitosan films loaded with thyme and oregano EOs produced ~2 log reduction in fungal growth in inoculated rice during 8 weeks of storage at 28 °C. The bioactive films showed a slow release (26%) of volatile components over 12 weeks of storage. Sensorial evaluation of rice samples packed with the bioactive films showed no significant change in odor, taste, color and general appreciation compared with untreated rice. Incorporation of cellulose nanocrystals (CNCs) with the chitosan matrix played an important role in stabilizing the physicochemical and release properties of the nanocomposite films. In addition, combining the bioactive chitosan films with a dose of 750 Gy of ionizing radiation showed significantly higher antifungal and mechanical properties than treatment with the bioactive film or irradiation alone.

1. Introduction

Protection of food crops against storage pathogens is a major concern for the food industry, farmers, public health organizations, and environmental agencies. In 2010, about 133 billion pounds of food, representing 31% of the global food available, was wasted at the retail and consumer level in the United States due to spoilage caused by storage food pests (Buzby et al., 2014). One of the main foods affected by pest infestation are cereal crops, which is commonly contaminated by mycotoxins (25%) produced by storage fungi (Smith et al., 2016; Tarazona et al., 2018). Aflatoxins, produced as secondary metabolites by fungal species namely *Aspergillus flavus* and *Aspergillus parasiticus*, are considered the most dangerous group of mycotoxins, as they deteriorate liver and kidney functions and increase the risk of cancer. High concentrations of aflatoxin can lead to aflatoxicosis, a condition causing severe illness, and in extreme cases, death (Hossain et al., 2016; Mateo et al., 2017).

Bio-nanocomposite based packaging containing plant-derived essential oils (EOs) is currently playing an important role to reduce fungal contamination and proliferation in processed food (Hossain et al., 2017). EOs are more efficiently used in foods when encapsulated in appropriate delivery systems to overcome dosage limitations and increase the biological stability of active compounds (Van Long et al., 2016). When encapsulation is at the nanoscale, the bioactivity of EOs can be enhanced through the activation of passive mechanisms of cell absorption or tissue infusion, thereby enabling the reduction of the EOs doses required to ensure antimicrobial activity (Bilia et al., 2014). Such low doses minimize the impact of the bioactive compounds on the natural aroma, flavor and taste of the food (Lu et al., 2016).

Among biopolymers, chitosan is the second most abundant naturally occurring polysaccharide after cellulose, and is commonly found in chitinous exoskeletons of crustaceans. Chitosan can also be obtained from insect cuticle and the cell walls of some fungi (Elsabee and Abdou, 2013; Khan et al., 2014). In addition chitosan has been approved by the

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Food and Drug Administration (FDA) as a food ingredient (Ma et al., 2016). Cellulose nanocrystals (CNCs), which can be extracted from lignocellulosic materials, bacteria or algae, have reinforcing properties that make them potential nanofillers. Reinforcement with CNCs can also improve the thermal, mechanical and barrier properties of polymers, as well as their surface wettability, and controlled release of active compounds/drugs (Salmieri et al., 2014a, b).

Food irradiation or "cold pasteurization" is a disinfection treatment option, and involves exposing food to ionizing radiation to reduce the microbial load, particularly for control of food borne pathogens (Maherani et al., 2016). Combining irradiation with other treatments such as active films can increase microbial radiosensitivity and reduce the dose of the individual treatments while maintaining the quality and safety of the food product (Lacroix and Follett, 2015).

The aim of this current study was to develop chitosan-based nanocomposite films with enhanced antifungal properties, alone and in combination with gamma irradiation. The objectives of the research were to (i) characterize EO nanoemulsions for three EO combinations, (ii) evaluate the antifungal activity of the chitosan based bioactive films in combination with gamma radiation in rice, (iii) evaluate the effect of CNC on the physico-chemical and release properties, and (iv) evaluate the organoleptic properties of cooked rice stored with developed film.

2. Materials and methods

2.1. Essential oils

Essential oil of oregano (*Origanum compactum*; Moroccan oregano), thyme (*Thymus vulgaris*), tea tree (*Melaleuca alternifolia*), and peppermint (*Mentha piperita*) were obtained from Robert & Fils (Ghislenghien, Belgium), and stored at 4 °C prior to use. Chromatograms of the EOs provided by the manufacturer showed that the oregano oil contained 46.37% carvacrol, 13.70% thymol, 13.33% *p*-cymene and 12.32% ɣ-terpinene. Thyme oil contained 26.04% thymol, 26.36% *p*-Cymene, and 16.69% γ-terpinene. Tea tree contained 38.4% terpinene-4-ol, 22.6% γ terpinene, 8.1% α terpinene and peppermint oil contained 33.38% menthol, 34.31% menthone, and 6.34% 1,8-cineole.

2.2. Preparation and size and antifungal activity characterization of combined EO emulsions

Three EO mixtures, thyme and oregano (formulation 1), thyme and tea tree (formulation 2), and thyme and peppermint (formulation 3), were selected based on previous antifungal tests (Hossain et al., 2016). A coarse emulsion was prepared by blending 5% (w/v) mixed oil phase (1:1 ratio) with an aqueous phase (water) containing 1.25% lecithin and 3.75% tween 80 as an emulsifier. The mixture was homogenized using an Ultra-Turrax homogenizer at 15,000 rpm for 2 min. Based on some preliminary tests the resulting coarse emulsion was passed through a Microfluidizer (Microfluidics Inc., Newton, MA, USA) at 15,000 psi pressure using 3 cycles to produce EOs nanoemulsion. The average particle size, polydispersity index (PDI) and ζ-potential of the prepared emulsions were determined by dynamic light scattering using photon correlation spectroscopy (Malvern Zetasizer Nano-ZS, Model ZEN3600) with a scattering angle of 173° and at a temperature of 25 °C. The antifungal activity of nanoemulsion was carried out against *A. niger* following vapor contact assays described by Hossain et al. (2016).

2.3. Preparation of chitosan-based bioactive composite films containing EO emulsion

Chitosan (CH) based composite films containing cellulose nanocrystals (CNCs) were developed following Khan et al. (2012). In brief, a quantity of 2% (w/v) chitosan was added to the 0.1% (w/w) CNCs (FPInnovations, Pointe-Claire, QC, Canada) suspension followed by 2% (v/v) acetic acid and 0.5% (w/v) ethylene glycol. The chitosan solution was then homogenized with an IKA RW-20 mechanical homogenizer at 1500 rpm. Combination of oregano: thyme EO emulsions at two concentrations (0.13 and 0.19% w/w), were mixed and the resulting suspensions were introduced in the microfluidizer (Microfluidics Inc., Newton, USA) at pressures of 5000 psi and 3 numbers of cycles to obtain a homogenous film forming dispersion (FFD). Composite films were cast with by applying 12 mL of the film-forming suspension onto Petri dishes (95 × 15 mm; Fisher Scientific, Canada) and allowing them to dry at room temperature for 24 h. Chitosan (CH)-based films containing no antimicrobial were used as a control.

2.4. Fungicidal activity of biopolymeric films

2.4.1. Fungal inocula and assay media

Four types of fungal species were used for the bioassays. They were *Aspergillus niger* (ATCC 1015), *Aspergillus flavus* (ATCC 9643), *Aspergillus parasiticus* (ATCC 16869), and *Penicillium chrysogenum* (ATCC 10106). Fungi conidia were prepared and adjusted to 1×10^8 conidia/mL under a microscope for subsequent *in vitro* and *in situ* assays following Hossain et al. (2016).

2.4.2. In situ antifungal activity of bioactive films combined with gamma radiation

An inoculation bath was prepared with peptone water containing 10^5 conidia/mL of *A. niger*, *A. flavus*, *A. parasiticus* and *P. chrysogenum*. A quantity of 500 g of rice grain was added to the inoculation bath and stirred gently for 30 s. The rice grains were dried on a sheet of sterile aluminum foil for 2 h under sterile condition. A quantity of 30 g of the inoculated rice was packaged in a plastic bag. The most efficient bioactive films were selected from the *in vitro* tests at two concentrations (0.13 and 0. 19% w/w) (CH-film A and CH film B) and were placed in each rice bag (1 g/cm^2). The rice samples were grouped into two subsets with one receiving irradiation treatment at 750 Gy based on preliminary tests at the Canadian Irradiation Center and one without irradiation. Irradiationn treatment was done in a UC-15A irradiator equipped with a ^{60}cobalt source (Nordion Int. Inc., Kanata, Ont., Canada) and having a dose rate of 10.46 kGy/h. The samples were incubated at 28 °C for 8 weeks and microbiological analyses were carried out on a weekly basis.

2.4.3. Microbiological analyses

Microbiological analyses were performed by adding 60 mL of sterile peptone water (0.1%, w/v) into 30 g of rice and homogenized for 1 min at 260 rpm using a Lab-blender 400 stomacher (Laboratory Equipment, London, UK). The resulting homogenate was serially diluted using sterile peptone water. An aliquot of 0.1 mL of each dilution was inoculated evenly in triplicate onto the Potato Dextrose Agar (PDA). The plates were incubated at 28 °C for 2–4 days.

2.5. Physicochemical characteristics of irradiated and non-irradiated composite films

The mechanical and water vapor barrier properties were evaluated for four types of irradiated and non-irradiated films to evaluate the effect of CNCs on prepared film. These were i) CH film (Control) ii) CH-bioactive film containing 0.19 (w/w%) of thyme and oregano nanoemulsion iii) CH-CNCs film (control film) and iv) CH- CNCs-bioactive film containing 0.19 (w/w%) of thyme and oregano nanoemulsion.

2.5.1. Mechanical properties of biopolymeric films

Film thickness was measured using a Mitutoyo Digimatic Indicator (Mitutoyo MFG Co. Ltd., Japan), at five random positions. Films widths were measured using a Traceable® Carbon Fiber Digital Caliper (Fisher Scientific). The mechanical properties such as tensile strength (TS), tensile modulus (TM) and elongation at break (Eb) of the composite films were measured using a Universal Testing Machine (UTM) (Tinius

Table 1
Average droplet diameter, polydispersity index (PDI), zeta-potential and antifungal activity of coarse and nanoemulsions.

Combination of EOs	Type of emulsion	Size (nm)	PDI	Zeta potential (mV)	Percentage (%) inhibition of *A. niger* Vapor contact assay		
					24 h	48 h	72 h
Formulation 1: Thyme-Oregano	Coarse	219.0 ± 1.62^{d}	0.45^{e}	-56.2^{e}	39.75 ± $1.70^{ab,C}$	31.32 ± $1.70^{b,B}$	27.71 ± $1.70^{c,A}$
	Nano	76.58 ± 0.35^{c}	0.25^{b}	-51.0^{c}	83.73 ± $2.55^{d,\ C}$	71.08 ± $1.70^{e,B}$	64.45 ± $2.55^{f,A}$
Formulation 2: Thyme-tea tree	Coarse	262.4 ± 0.70^{e}	0.37^{d}	-43.7^{a}	36.71 ± $0.85^{a,C}$	22.28 ± $0.85^{a,B}$	16.26 ± $2.55^{a,A}$
	Nano	69.9 ± 1.13^{b}	0.21^{a}	-50.7^{c}	75.60 ± $1.27^{c,C}$	55.42 ± $3.40^{c,B}$	43.97 ± $4.25^{d,A}$
Formulation 3: Thyme-peppermint	Coarse	279.0 ± 0.35^{f}	0.46^{e}	-48.0^{b}	43.07 ± $0.42^{b,B}$	28.91 ± $3.40^{b,B}$	22.59 ± $2.98^{b,A}$
	Nano	57.9 ± 0.70^{a}	0.32^{c}	-53.3^{d}	87.95 ± $6.81^{c,D}$	66.26 ± $6.^{81c,D}$	51.20 ± $0.85^{e,A}$

Values are means ± standard error. Within each column means with the same lowercase letter are not significantly different (P > 0.05). Within each row means with the same uppercase letter are not significantly different ($P > 0.05$).

Olsen Testing Machine Co., Inc., USA), equipped with a 100 N-load cell and 1.5 kN-specimen grips.

2.5.2. Water vapor permeability (WVP)

WVP tests were conducted following the procedure described by Salmieri et al. (2014a, b). The films were mechanically sealed onto Vapometer cells (model 68-1; Thwing-Albert Instrument Co., USA) containing 30 g of anhydrous calcium chloride. Cells were initially weighed and placed in a Shellab 9010L controlled humidity chamber (Sheldon Manufacturing Inc., Cornelius, OR, USA) maintained at 25 °C and 60% RH for 24 h. The assemblies were weighed initially and after 24 h for all samples. The weight gain of the cell represented the amount of water vapor transferred through the film and absorbed by the desiccant (anhydrous $CaCl_2$).

WVP was calculated according to the equation below.

$$\text{WVP}\ (\text{g mm m}^{-2}\ \text{day}^{-1}\ \text{kPa}^{-1}) = \Delta w \cdot x / A \cdot \Delta P$$

where, Δw is the weight gain of the cell (g) after 24 h, x is the film thickness (mm), A is the area of exposed film ($31.67 \times 10^{-4}\ m^2$), and ΔP is the differential vapor pressure of water through the film ($\Delta P = 3.282$ kPa at 25 °C).

2.6. Release/diffusion of volatile component encapsulated in biopolymeric films during storage (in situ)

The controlled release of volatile components from essential oils was evaluated by a method described by (Tunç and Duman, 2011). Two types of films were tested: i) CH based film and ii) CH reinforced with CNCs based film containing (0.19% w/w) oregano:thyme EO nanoemulsion to evaluate the effect of CNCs on controlled release properties. Bioactive chitosan based films were kept in rice grains (1 g/cm^2) incubated (at 28 °C and 65% RH) for 12 weeks. Films were taken out from the rice grains every week and cut into (500 mg) known sizes to keep the weight of samples as constant as possible. Then, the films were placed into 10 mL of ethanol for 4 h with constant agitation for the extraction of volatile components from the film matrix. The release of volatile components were determined by spectrophotometer at 274 nm. The initial concentration of volatile components in each type of film was determined before they were put into the chamber. The decrease concentration of volatiles components in the film samples was considered as release of volatile components.

2.7. Sensory analysis

The most efficient chitosan-based bioactive film containing [0.19% of EOs emulsion (highest concentration)] was chosen for an evaluation of its effect on sensorial properties of cooked rice. A panel comprised of 12 individuals evaluated the odor, the taste, and the color independently, and provided global appreciation of the samples stored with bioactive films for two months. Rice samples were cooked for 20 min in steam rice cookers at a 1:2 (v/v) rice-to-water ratio (Meullenet et al., 1998). The rice samples were served in separate cups with closed lids and were identified by 3 random digits. The evaluation was held on a 9-point hedonic scale: 9 = Like extremely, 8 = Like very much, 7 = Like moderately, 6 = Like slightly, 5 = Neither like nor dislike, 4 = Dislike slightly, 3 = Dislike moderately, 2 = Dislike very much, 1 = Dislike extremely. Sensory evaluation of rice packed in irradiated films was also conducted; however, since it did not lead to any change in the characteristics of rice samples, we did not include these data in the manuscript.

2.8. Statistical analysis

All experiments were done in triplicate and each replicate include analysis of three samples. Data were subjected to analysis of variance and mean separations were performed using the Duncan's multiple-range test. Differences between means were considered significant when the confidence interval was smaller than 5% ($P \leq 0.05$). All analyses were performed using PASW Statistics 18 software (IBM Corporation, Somers, NY, USA).

3. Results and discussion

3.1. Characteristics of combined EO nanoemulsions

The size, polydispersity index (PDI), zeta potential and antifungal activity of three EO emulsions were evaluated. Results showed that microfluidization at a pressure of 15,000 psi and 3 cycles resulted in the formation of nanoemulsions with droplet size of < 100 nm, and exhibited a higher antifungal activity as compared to the coarse emulsions (Table 1). The size of the coarse emulsions prepared with formulation 1, 2 and 3 was 219, 262 and 279 nm respectively, while the size of the nanoemulsions prepared from the same formulations was 77, 70 and 58 nm, respectively which is significantly ($P \leq 0.05$) smaller. The nanoemulsion based on thyme and peppermint combination had smallest size (57.9 nm) as compared to the nanoemulsions 1 and 2 formulation.

The PDI value indicates the range of particle size distribution, thus, a small PDI value signifies a narrow size distribution. In present study, EOs nanoemulsions exhibited a significantly ($P \leq 0.05$) narrow range of size distribution as compared to coarse emulsion, with PDI values ranging between 0.21 and 0.32. However, the particle size distributions were significantly ($P \leq 0.05$) broader (0.37–0.46) for the coarse emulsions in comparison with the nanoemulsions. Studies have shown that microfluidization of the coarse emulsions disperses the particles with narrower particle size distributions due to the high shear stresses developed in the microchannels of the interaction chamber (Jo and Kwon, 2014). The zeta-potential of the prepared emulsions ranged between −43 to −56 mV. According to Lu and Gao (2010), low absolute zeta potential values are associated with low emulsion stability with particles showing more likelihood of aggregation and flocculation. Emulsions with zeta potential of −11 to −20 mV have been reported to be unstable and close to the threshold of agglomeration, while emulsions with zeta potential of −41 to −50 mV had good stability (Lu and Gao, 2010). In the current research, the range of zeta potentials measured (app. −43 to −56) corresponded more to emulsions displaying higher stability. The stability of emulsions may also be correlated with the composition and properties of emulsifier (Lu and Gao, 2010). In the present study lecithin and tween 80 were used as emulsifiers. Lecithin is amphiphilic in nature allowing incorporation with hydrophobic EOs and influences emulsion stability (Traynor et al., 2013). EOs also have strong interactions with the nonionic emulsifier tween 80, which adsorbs quickly at the oil-water interface and provides good stability to the dispersed system (Azarbayjani et al., 2009).

The antifungal properties were dependent on the size of the emulsions. As particle size decreased following microfluidization, the percentage inhibition of *A. niger* increased significantly ($p < 0.05$). The percent inhibition induced by the coarse and nanoemulsions was 39.7, 36.7, 43% and 83.7, 75.6 and 87.9% respectively, for the thyme and oregano, thyme and tea tree, and thyme and peppermint mixtures (after 24 h incubation period). The antifungal activity was also found to decrease significantly over time ($p \leq 0.05$). After 72 h of incubation, the percent inhibition induced by the coarse and nanoemulsions was 27.7, 16.2, 22.5% and 64.4, 43.9 and 51.2%, respectively, for thyme and oregano, thyme and tea tree, and thyme and peppermint mixtures. However, the decrement in bioactivity was significantly less ($p \leq 0.05$) for the nanoemulsions as compared to the coarse emulsions for all the EOs formulations. Smaller particles have greater surface area-to-volume ratios, which considerably increases the dissolution rate of the particles, enabling them to overcome solubility-limited bioavailability thereby exhibiting a higher biological activity. Such size-dependent antimicrobial activity has previously been observed by other authors. Jeong et al. (2014) incubated *Methylobacterium spp*. with water-soluble silver nanoparticles of 10 and 100 nm for two days, and the 10 nm nanoparticles displayed higher antimicrobial activity than the 100 nm sized particles. In accordance Floury et al. (2000) also reported that high-pressure homogenization increases the surface activity of emulsifying molecules and improve their efficiency.

3.2. In situ analysis with CNCs-reinforced chitosan (CH)

In situ analyses were performed using CNCs-reinforced chitosan (CH/CNCs) containing oregano and thyme nanoemulsions in combination with gamma irradiation. The fungal growth profiles for the control (no film) and CHs/CNC-containing the oregano-thyme nanoemulsion are shown in Fig. 1. The initial inoculation was 3 log CFU/g for each fungal species. The growth of *A. niger* in the control samples reached 6.49 log CFU/g after 8 weeks of incubation (Fig. 1a). For the samples incubated with film A (0.13% EO) and B (0.19% EO), the fungal growth was 4.94 and 4.22 log CFU/g, respectively, representing a reduction of 1.55 and 2.27 log CFU/g, respectively, as compared to the control samples. The *A. niger* growth was 3.95 log CFU/g after 8 weeks of incubation when gamma radiation at 750 Gy was applied alone, which was a reduction in the growth of *A. niger* by 2.54 log CFU/g as compared to the control sample. Combining the bioactive films with gamma irradiation at 750 Gy caused an enhanced reduction in *A. niger* growth. Irradiation of the inoculated samples and application of the bioactive films A or B caused a reduction of 4.18 and 4.82 log CFU/g respectively after 8 weeks of incubation as compared to the control samples. Fig. 2 shows photographic images of rice grains inoculated with *A. niger* treated with bioactive films A or B after 8 weeks of storage.

For *A. flavus*, the growth of control samples reached 7.48 log CFU/g after 8 weeks of incubation. For samples treated with film Aor B, the fungal growth was 5.02 and 4.61 log CFU/g, respectively, representing a reduction of 2.46 and 2.87 log CFU/g respectively, after 8 weeks of incubation as compared to the control samples. By the application of 750 Gy of gamma irradiation, the growth of *A. flavus* was 4 log CFU/g after 8 weeks of incubation. Combining the bioactive film A or B with a treatment of gamma irradiation at 750 Gy caused a reduction of 4.35 and 4.93 respectively, after 8 weeks of incubation as compared to the control samples. Similarly, samples treated with A or B bioactive films alone reduced the growth by 1.19 and 2.08 log CFU/g for *A. parasiticus* and 1.79 and 2.39 log CFU/g for *P. chrysogenum*, respectively, as compared to the control samples. By the application of 750 Gy of gamma irradiation, the growth of *A. parasiticus* and *P. chrysogenum* was reduced by 2.77 log CFU/g and 2.79 log CFU/g respectively as compared to the control sample after 8 weeks of incubation. Combining the bioactive film A and B with a treatment of 750 Gy caused an even more pronounced reduction of these species as compared to the control samples.

The results obtained in the current study suggested that thyme and oregano EOs nanoemulsions incorporated within the CH-CNCs polymeric matrix were progressively released from the film surface to protect the rice grains over a longer period of time. The antimicrobial activity of thyme and oregano EOs is thought to cause structural and functional damage to the cytoplasmic membrane and through interaction with membrane proteins and various intracellular targets (Hyldgaard et al., 2012). CH/CNCs polymeric matrices offer great potential as a diffusion matrix for EOs nanoemulsions by extending their efficacy during storage (Deng et al., 2017). Moreover, it has been reported that CNCs stabilizes encapsulated EOs emulsions in polymers while allowing better control in their release into food (Boumail et al., 2013). As a result CH/CNCs composite matrix protected the encapsulated nanoemulsions from environmental interactions and limited their rapid loss and consumption into the surrounding surface.

Combining the encapsulated bioactive films with γ radiation exerted a more accrued inhibitory effect on the tested fungal growth. Applying a low concentration of (0.19%, w/w) oregano-thyme mixture with a low irradiation (750 Gy) dose caused an almost 70–80% reduction in the fungal growth during the 8 weeks of storage. A recent study by Severino et al. (2015) on a bioactive coating formulation involving modified chitosan-based coatings containing carvacrol nanoemulsion, gamma irradiation, modified atmosphere packaging (MAP), alone or in combinations, against *Escherichia coli* O157:H7 and *Salmonella* Typhimurium was evaluated on inoculated green bean samples during 13 days of storage period. The initial concentration of tested bacterial species was 3 log CFU/g. Their results showed the combined treatment reduced the *E. coli* population to a level not detectable after 7 days of storage. Such treatment also led to a significant reduction ($p \leq 0.05$) of 2.07 log CFU/g of *S. Typhimurium* as compared to the control after 13 days of storage, exhibiting a strong synergistic antimicrobial effect (Severino et al., 2015). The authors suggested that combined treatment of irradiation and antimicrobial agent is more effective in controlling microorganism by dual action of individual treatment. Similar observations were also made by Hossain et al. (2014) and Lacroix and Follett (2015). According to Lacroix and Follett (2015) a preliminary treatment by irradiation can induce an acute sensitivity to all the microorganism towards other preservative techniques including bioactive agents or films.

3.3. Physico-chemical properties of non-irradiated and irradiated films

The physico-chemical properties and water vapor permeability of the chitosan films without and with gamma irradiation at 750 Gy were evaluated (Table 2). For the polymeric films without irradiation, the tensile strength of CH films (thickness∼ 45 μ) was 59.67 MPa. Addition of a EOs formulation (thickness∼61 μ) reduced the tensile strength from 59.67 to 43.81 MPa ($p \leq 0.05$). Such reduction in tensile strength may be attributed to the plasticizing effect of EOs (Yanwong and Threepopnatkul, 2015). Incorporating the films with 5% (w/w) CNCs enhanced their tensile strength by 28 and 22% for CH and CH films containing EOs respectively ($p \leq 0.05$). The increase in tensile strength is an indication of strong reinforcement of the chitosan matrix by the CNCs. The high reinforcing effect of CNCs is due to a mechanical

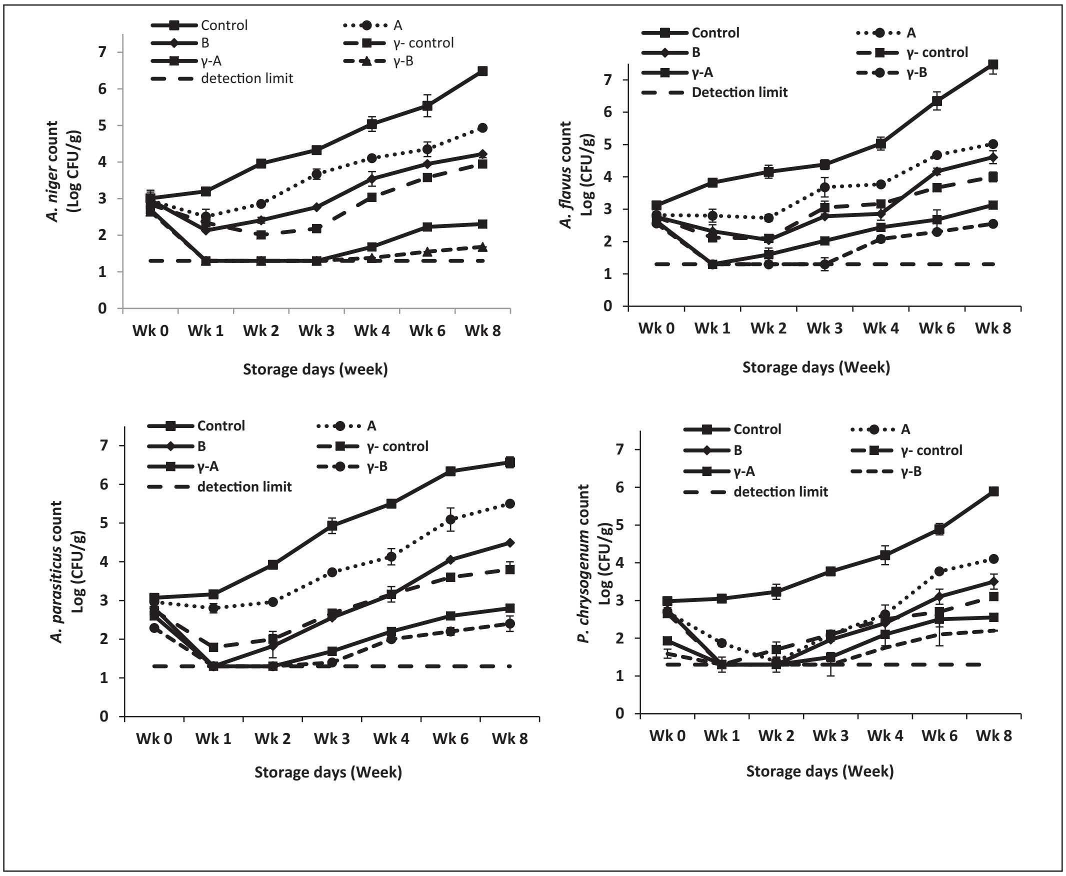

Fig. 1. Fungal growth profiles of *A. niger*, *A. flavus*, *A. parasiticus* and *P. chrysogenum* following *in situ* tests with bioactive chitosan (CH) nanocomposite based films over 8 weeks of storage. Abbreviations: Wk-week; γ-gamma irradiated sample; A-biofilm with 0.13% EO; B-biofilm containing 0.19% EO.

Fig. 2. Photographic images of rice samples inoculated with *A. niger*: i) CH-CNCs (no EOs) ii) Bioactive film A iii) Bioactive film B after 8 weeks of storage.

percolation phenomenon which forms a stiff continuous network of cellulosic nanoparticles linked through hydrogen bonding (Abou-Zeid et al., 2015).

In terms of tensile modulus (TM), the higher TM value is indicative of a more rigid material. The tensile modulus of the bioactive CH based film was less as compared to the control film indicating a less dense film matrix. Loading of 5% CNCs nanofiller caused a 10, 16% increment of the TM for bioactive and control films respectively. Bioactive CH based films displayed a higher elongation of break (Eb) corresponding to a higher film flexibility than the control. The Eb values of the film improved significantly ($p \leq 0.05$) from 29.52 to 41.65% with the incorporation of EOs. Generally, plasticizers weaken intermolecular forces between adjacent polymer chains, resulting in a decreased TS and increased film flexibility. Decreases in TS and increases in Eb are common results of essential-oil incorporation, which is consistent with the present results (Ghasemlou et al., 2013; Zivanovic et al., 2005). Addition of CNCs to the biopolymer significantly decreased the Eb between 22 and 34% for CH and bioactive CH based films. Similar observations have been reported by other authors who reinforced polymers with nanofillers (Dhar et al., 2015). Incorporation of CNCs into the polymer matrix can result in a strong interaction between the matrix and filler, restricting the motion of the matrix and hence lowering Eb (Azeredo et al., 2010).

The water vapor permeability (WVP) of biopolymeric films (Table 2) was investigated since water has a noticeable impact in deteriorative reactions as it acts as a carrier to cause texture degradation and chemical and enzymatic reactions in polymers. The WVP of the control and bioactive CH based films were not significantly different ($p > 0.05$) with respective values ranging between 9.84 and 10.66 $g\,mm\,m^{-2}\,day^{-1}\,kPa^{-1}$. However incorporating 5% CNCs decreased the WVP significantly ($p \leq 0.05$) from 9.84 to 7.61 $g\,mm\,m^{-2}\,day^{-1}\,kPa^{-1}$. Water vapor properties of bioactive films are dependent on the polymer to EOs ratio, and the homogenization technique implemented. Bonilla et al. (2011) studied the influence of homogenization conditions and basil EOs content on the physical properties of chitosan based films, and found that the WVP increased when the chitosan to oil ratio was 1:1, but did not cause any

Table 2
Mechanical properties and Water Vapor Permeabilities (WVP) of non-irradiated and irradiated films.

Treatment	Samples	Tensile strength TS (MPa)	Tensile modulus, TM (MPa)	Elongation of break (Eb%)	Water vapor permeability g mm m^{-2} day^{-1} kPa^{-1}
Non-irradiated	CH	59.67 ± 3.35^{ef}	852.0 ± 82.72^{c}	29.52 ± 4.16^{bc}	9.84 ± 0.31^{e}
	CH + EO	43.81 ± 2.56^{a}	578.53 ± 70.70^{a}	41.65 ± 5.27^{g}	10.66 ± 0.47^{e}
	CH + CNC	77.12 ± 3.19^{h}	943.14 ± 135.6^{d}	21.91 ± 2.19^{a}	7.61 ± 0.23^{c}
	CH + CNC + EO	54.16 ± 5.17^{cd}	703.21 ± 86.87^{b}	34.04 ± 2.99^{f}	9.95 ± 0.85^{e}
Irradiated	CH	83.96 ± 5.49^{i}	994.74 ± 73.33^{d}	30.22 ± 1.98^{cd}	6.93 ± 0.16^{b}
	CH + EO	51.22 ± 4.62^{bc}	708.96 ± 59.14^{b}	36.96 ± 3.35^{f}	8.60 ± 0.37^{d}
	CH + CNC	94.26 ± 4.95^{j}	986.68 ± 65.59^{d}	26.63 ± 3.05^{b}	6.46 ± 0.29^{ab}
	CH + CNC + EO	57.02 ± 4.14^{de}	732.28 ± 51.43^{b}	35.95 ± 4.09^{ef}	7.63 ± 0.32^{c}

Values are means ± standard error. Within each column means with the same lowercase letter are not significantly different ($p > 0.05$).

improvement with the ratio of 1:0.5. The low EOs concentration used in the present study may not have significantly affected the WVP of the films. However, loading of 5% CNCs decreased the WVP by 23% as compared to the control film.

CNCs play an important role as filler for strengthening other polymeric matrices. In addition, CNCs forms a mechanical, steric barrier in the oil/water interface capable of protecting emulsion droplets against coalescence (Dickinson, 2012). Sampath et al. (2017) also found that integrating CNCs into chitosan remarkably improved the 'mechanical properties. Our results concur with previous findings and show that CNCs can effectively stabilize and strengthen chitosan polymeric matrix thereby enhancing its mechanical properties. This may be due to the percolated network of CNCs due to hydrogen bonding, thus decreasing the free volume and molecular mobility of the polymer (Pereda et al., 2012).

For chitosan based films combined with irradiation, a net improvement in the mechanical properties was observed (Table 2). The application of gamma irradiation to chitosan alone significantly ($p \leq 0.05$) improved its tensile strength and decreased water permeability. The tensile strength of irradiated CH based films increased by 40% and WVP decreased by 29.5% as compared to non-irradiated CH based films. Further addition of CNCs into CH based films positively improved their physical properties. Irradiated CH based films containing CNCs, showed a 12.2 and 6.8% improvement in tensile strength and WVP, respectively, as compared to irradiated CH based films without CNCs. Similar observations were also found with the chitosan films impregnated with EOs. The results of present studies showed that gamma irradiation has a significant positive impact on mechanical and barrier properties of the composites. Studies have shown that irradiated polymeric materials, even at low doses, undergo structural changes and produce free radicals accompanied by molecular crosslinking. Hence, it appears that gamma irradiation induced cross-linking of the chitosan based films and the effect was accentuated in the presence of CNCs. Such increased cross-linking, after irradiation may be responsible for further reinforcing the chitosan polymeric matrix. These findings are in agreement with Khan et al. (2012) and Nasreen et al. (2016).

3.4. Release/diffusion of volatile components from active CH based matrix

To obtain the release of volatile components of encapsulated EOs from the CH based film matrix, a standard curve was obtained for the oregano: thyme EOs combination at 274 nm. The extracted eluents were subjected to analysis of absorbance to obtain the release of volatile components during storage. The decrease in concentration in the film samples was assumed to be release of volatile components. Therefore the % of release was deduced from the residual availability of active components in the film. The result showed (Fig. 3) a gradual release of volatile components (oregano: thyme EO) from the chitosan based film matrix. During the 12 weeks of the experiments, the release of the EOs was found to correlate linearly with time. Similar results were also observed by other research groups as Cran et al. (2010); Patil et al. (2016) *etc.*

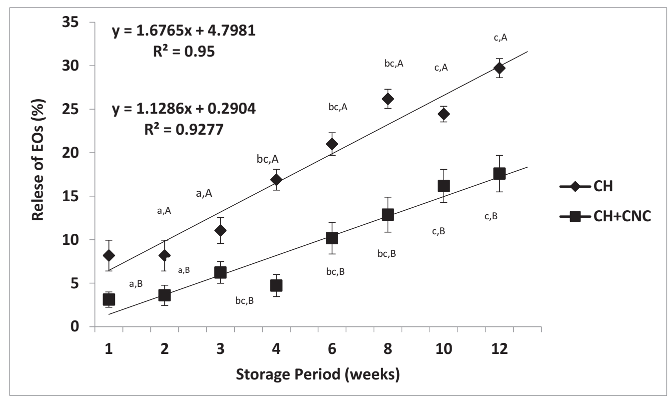

Fig. 3. Release of EOs from bioactive CH and CH + CNCs based films during storage. Means followed by the same lower case letter are not significantly different ($p > 0.05$).

The release was 8% and 29% with the chitosan based film matrix at week 1 and 12 respectively. The release was fast (8%) in the first week and then it became constant over week 2 and 3 (11%). Between weeks 4 and 12 the release of EOs was 16–29%. The release was significantly slower ($p \leq 0.05$) in the film containing cellulose nanocrystal (CNC) as a reinforcing agent. It is clearly seen from the figure that CNCs exhibits a significant effect in the sustained release of active components in the film. The release of volatile components from CNC reinforced chitosan based film was 3 and 17% at week 1 and week 12 respectively. The release of volatile components in CNCs reinforced CH based nanocomposite film was 41.4% slower as compared to CH based nanocomposite film. Hence the addition of CNCs in CH matrix greatly helped to preserve the EOs volatility properties, while preserving the biological activity of the EOs during storage. CNCs have found important applications in the food sector especially as food additives and in packaging films by enhancing structural and chemical properties and the efficacy of controlled delivery in active packaging systems (Fortunati et al., 2013). Comparative studies showed that CNCs application with chitosan-based films have shown to accrue delay in fruit ripening by Deng et al. (2017). Gelatin coatings containing CNCs were found to extend the shelf-life of strawberry fruit (*Fragaria ananassa*) over 8 days (Fakhouri et al., 2014). The strawberry samples covered with GEL/CNCs displayed significant extension in their shelf-life by retaining ascorbic acid (AA) (vitamin C) in the strawberries, as compared to the control samples which experienced a fast decay in AA content. Similarly, Wang et al. (2017) found chitosan-cellulose nanocrystals microencapsulation improved the encapsulation efficiency and stability of entrapped fruit anthocyanins. Results showed that CNCs incorporation into chitosan functioned as macro-ion crosslinking agent and as fillers for chitosan matrix. The chitosan-CNCs matrices generate more rigid and stable microcapsules. Hence, in present study the integration of CNCs in to CH based nanocomposite films enabled a better protection and retention of the encapsulated EOs molecules over storage period. These results were further confirmed by the antifungal effectiveness of

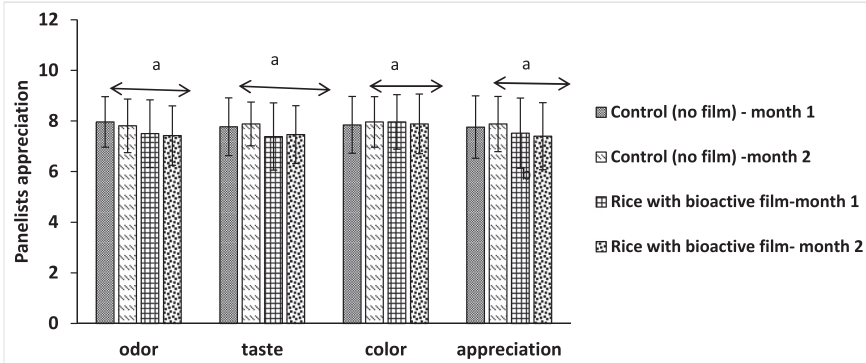

Fig. 4. Sensorial evaluation of cooked rice from packaging containing chitosan based bioactive nanocomposite films during 2 months of storage. Means followed by the same lower case letter are not significantly different at the 5% level.

the films during 8 weeks of storage period.

3.5. Nanocomposite films in cooked rice

A sensorial evaluation of rice samples incubated with 0.19% (w/w) of thyme-oregano EOs nanoemulsion loaded chitosan-based films was performed to determine if the EO volatiles were noticable to a consumer of rice after cooking. The results showed (Fig. 4) that all the control and treated samples were acceptable, and received high scores by the panelists. The scores obtained ranged between 7.4 and 8.0 for odor, taste, color and general appreciation. No negative effect ("Dislike a little" or "Lower appreciation") was reported to the organoleptic properties of any samples. Fragrance related to the presence of the EOs was expected to induce a high aromatic intensity over a prolonged period. However, incorporation of EOs to edible films requires very low concentrations as compared to direct applications (Badr et al., 2013). Further, nano encapsulating systems represent a viable and efficient approach to increase the physical stability of EOs, protection from evaporation, and providing gradual release with enhanced bioactivity. In the present study, rice exposed to a low concentration as 0.19% (w/w) of oregano-thyme nanoemulsions incorporated into the chitosan matrix remained highly acceptable after cooking until the end of the two-month storage period and did not induce any alteration to the organoleptic properties of the food. This aspect is extremely important for consumer acceptance in the global market.

4. Conclusion

Chitosan-based nanocomposite films loaded with EOs were designed and tested in the current study. Vapor phase assays showed that the chitosan films we developed were very efficient at inhibiting the growth of fungi and resulted in fungal control to a varying extent depending on the particular EO combination. Application of gamma irradiation significantly improved the tensile strength and modulus of the films. Incorporation of CNCs into the chitosan biopolymeric matrix reinforced its structure significantly in terms of tensile strength and elongation break, and extended the controlled release of active components. In addition, a higher fungal inhibitory effect was observed by combining the chitosan bioactive polymeric films with irradiation (750 Gy). Sensorial evaluation of rice samples containing CH-CNCs based films loaded with EOs nanoemulsions showed no significant ($p > 0.05$) alteration in odor, taste, color and overall appreciation. Such bioactive films exhibiting enhanced structure and antifungal properties hold significant potential for controlling fungal growth during storage of food items.

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