Physicochemical and antimicrobial properties of citral and quercetin incorporated kafirin-based bioactive films

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Abstract: The aim of this study was to determine the physicochemical and antimicrobial properties of kafirin-based bioactive films incorporating the plant essential oil citral and the polyphenol quercetin. The addition of quercetin and citral both imparted a yellowish color to the films. The tensile strength of films significantly decreased and elongation at break increased when citral was incorporated, whereas addition of quercetin did not alter these two film parameters. The rate of water vapor transmission of the films decreased with citral incorporation but the water vapor permeability was not affected by either citral or quercetin incorporation. Furthermore, incorporation of citral and quercetin significantly lowered the oxygen permeability of the films. Film made of kafirin alone had antimicrobial activity against *Listeria monocytogenes*, however films incorporating citral exhibited the highest antimicrobial activity against *Campylobacter jejuni*, *Listeria monocytogenes*, and *Pseudomonas fluorescens*. These results suggest that kafirin-based films incorporating citral and quercetin have potential as bioactive packaging to improve food safety and quality.

Keywords: Kafirin, citral, quercetin, bioactive film, antimicrobial activity

1 Introduction

During the past decade, there has been an increasing interest in the development and use of bio-based active films with demonstrated antimicrobial activities to improve food safety and reduce the use of chemical preservatives (Aider, 2010). These bio-based active films may also possess the abilities to avoid moisture loss or water absorption by the food matrix, oxygen penetration to the food material, aromas loss and solute transport (Dutta, Tripathi, Mehrotra, & Dutta, 2009), therefore providing further potential to maintain food quality during product transportation and storage. These films can be made from food grade biopolymers including proteins, lipids, and carbohydrates, which are edible and biodegradable (Coma, 2008). However, as these biopolymers can be more expensive than current plastic films and the total cost of a food product is highly related to the packaging material cost. Therefore the search for more economical packaging materials is a very important subject in the food industry (Aider, 2010). For instance, it is recommended that the use of biopolymers from underutilised materials or from agro-food by-products and waste would significantly reduce the cost of bioactive films (Campos, Gerschenson, & Flores, 2011).

Kafirin is a prolamin protein found in the grain of the cereal crop sorghum and accounts for about 60-80% of the total grain protein (Da Silva & Taylor, 2005). Because kafirin is highly hydrophobic, it has low solubility in water, and not easily enzymatically digestible after wet heat processing, it is an ideal biopolymer for coating and film manufacture, due to its potential to provide water and gas barrier properties (Taylor, Taylor, Dutton, & de Kock, 2005a). In addition, unlike some other predominant cereal crops such as rice and wheat, sorghum is tolerant to poor soil conditions, drought, and a variety of crop diseases, and is well-adapted to high temperatures; highlighting its potential as a sustainable crop for future agro-food security in the predicted challenge of climate change (Stefaniak & Rooney, 2013).

To enhance the bioactivity (e.g. antimicrobial and antioxidant activity) of bio-based films, plant essential oils and natural antioxidants (polyphenols) can be incorporated into the film. For example, the essential oils from oregano, rosemary and garlic have been incorporated in whey protein films (Seydim & Sarikus, 2006) and cinnamon essential oils in chitosan based films to improve the antimicrobial and physicochemical properties (Ojagh, Rezaei, Razavi, & Hosseini, 2010). The potential of cellulose-based packaging films containing cinnamaldehyde and eugenol for lowering the growth rate of *Pseudomonas aeruginosa* has also been documented (Sanla-Ead, Jangchud, Chonhenchob, & Suppakul, 2012). Current natural polymer-based packaging materials often have poor mechanical properties and low water resistance (Rhim & Ng, 2007), therefore the improved mechanical property, oxygen/water barrier properties, and antimicrobial activities reported for kafirin films incorporating polyphenols (tannins) (Emmambux, Stading, & Taylor, 2004), indicate the promise of kafirin-based films as active packaging for foods.

The incorporation of plant essential oil and polyphenols into bioactive films might be a feasible approach for improving their industrial applications. It has been reported that the essential oil citral is effective in inhibiting the growth of a wide range of microorganisms (Seow, Yeo, L., & Yuk, 2014) and the polyphenol quercetin is widely used as a potent antioxidant in breakfast cereals, dairy products, processed fruits, fats and oils (Harwood, Danielewska-Nikiel, Borzelleca, Flamm, Williams, & Lines, 2007). The purpose of this study was to develop a bio-based active film using kafirin with the incorporation of citral and quercetin as natural antimicrobial and antioxidant agents. The potential of the films for food quality preservation was investigated by evaluating their antimicrobial properties against selected spoilage and pathogenic bacteria and their physicochemical strength and barrier properties.

2 Materials and methods

2.1 Materials

***Sorghum flour and chemicals*:** Sorghum *(Sorghum bicolor)* grain variety Liberty (tannin free, grain with un-pigmented pericarp/testa) was obtained from Queensland Department of Agriculture, Fisheries and Forestry, Brisbane, Australia. The sorghum wholegrain was milled to flour with a ZM 200 Retch Mill (Retsch Gmbh & Co, Haan, Germany) using a 500 micron screen. All chemicals including citral and quercetin were purchased from Sigma-Aldrich (Sydney, Australia).

***Microbiological test cultures and media**:* A common food spoilage organism *Pseudomonas fluorescens* A150 and 2 pathogenic bacteria strains, *Campylobacter jejuni* A625, *Listeria monocytogenes* A360 were provided by the School of Biomedical Sciences, Curtin University. Viability and purity of the cultures were maintained by sub-culturing single colonies fortnightly on appropriate media until the end of the experiments. *C. jejuni* was maintained at 42 ℃ under microaerophilic conditions on campylobacter blood agar base No.2 (Skirrow selective supplement, SA Analytical Lab Services, Adelaide, Australia). *P. fluorescens* was maintained aerobically at 22 ℃ on brain heart infusion agar (SA Analytical Lab Services, Adelaide, Australia) and *L. monocytogenes* aerobically at 37 ℃ on tryptic soy agar (Becton, Dickinson and Company, Sydney, Australia).

2.2 Kafirin extraction

Kafirin was extracted using the method described by Taylor, Taylor, Dutton, and de Kock (2005b). Briefly, the sorghum flour (250g) was extracted for 1 h with 900 mL aqueous ethanol (70% v/v) containing 4.38g (≈ 0.5% w/w) sodium hydroxide and 6.25g (≈ 0.4% w/w) sodium metabisulphite at 70 ℃ with continuous stirring. The kafirin containing supernatant was separated by centrifugation at 3,000 rpm at 23° C for 5 min. Kafirin precipitate was

obtained through neutralization by adjusting the supernatant to pH 5.0 and was collected by vacuum filtration. The kafirin filter-cake was then freeze-dried (Christ Alpha 1-2 / LD plus, John Morris Scientific, Sydney, Australia). The freeze-dried kafirin was then ground using a domestic coffee grinder and defatted three times with hexane at a protein to solvent ratio of 1:10 (w/w) and air-dried at ambient temperature. The isolated kafirin had 82.12 ± 1.68% protein (dry basis) as determined by the Kjeldahl method of AOAC 925.10 (AOAC, 2005) using a nitrogen to protein conversion factor of N × 6.25.

2.3 Film preparation

Kafirin film forming solution was prepared by dissolving the kafirin in ethanol as described by Buffo, Weller, & Gennadios (1997) with some modifications. Briefly, 30.88g of kafirin was dissolved in 180.67g of 96% ethanol containing 12.64 g of plasticizer (1:1:1 w/w polyethylene glycol 400: lactic acid: glycerol). The mixture was heated for 10 min at 80 ± 2 °C with rapid and continuous stirring. Four formulations were made from the film forming solution with or without the addition of citral and/or quercetin (Table 1).

Consideration for the 2.5% and 1.25% citral was based on a preliminary antimicrobial efficiency study carried out in this work. Two concentrations of quercetin (2% and 1%) were considered based on the study by Fujisawa and Kadoma (2006). All formulations were homogenized for 2 minutes at 6,000 rpm at 23 °C using a homogenizer disperser mixer AD500S-P (IKA Works Inc. Wilmington, NC, USA).

Casting was carried out on glass sheets fabricated to form 320 × 290× 3 mm (L× W× D) glass trays. The film forming solutions were placed on glass trays and evenly distributed using a spreading rod. The films were dried for 24 h using forced draft in an oven (Panasonic Biomedical, Leicestershire, UK) at 28 ± 2° C and 33 ± 2% RH. After peeling from the casting wells the resulting free-standing films were conditioned in a desiccator at room temperature

125 (23 ± 2 °C) and 52 ± 3% relative humidity (RH). Conditioned films were used for analysis of
126 their physical and antimicrobial properties. The process of film-making and analysis was
127 performed in triplicate.

128 ***2.4 Film thickness***

129 The film thickness, taken as the average of eight random locations, was obtained using a
130 digital caliper micrometer (Thorlabs, NJ, USA) according to the method of Mei & Zhao
131 (2003). The film thickness was used in the calculations for the tensile and water vapor
132 permeability (WVP) properties of the films.

133 ***2.5 Moisture content of the film***

134 The moisture content of the films was determined according to AOAC 925.10 (AOAC,
135 2005). Samples of 30 – 40 cm^2 (300 - 400 mg) were placed on pre-dried aluminum dishes in
136 triplicate followed by drying to a constant mass in a forced draft oven at 105 ± 2° C for 24 h.

137 ***2.6 Film solubility in water***

138 The solubility of the films in water was determined using a modification of the method of
139 Kavoosi, Dadfar, and Purfard (2013). The initial dry matter of the films was obtained after
140 oven drying 28 - 32 cm^2 samples for 24 h at 105 ± 2°C. Dried samples were weighed into 50
141 mL centrifuge tubes and 40 mL deionized water was added. The tubes were sealed and
142 agitated in a shaking water bath at 30 °C for 24 h. The films were separated from the water
143 by vacuum filtration using a pre-dried, desiccator-equilibrated and pre-weighed filter paper
144 Whatman #2 (Thermo Fisher Scientific, Scoresby, Australia]. The final dry weight of the
145 filter paper and film residue were obtained after oven drying at 105 ± 2°C for 24 h. The tests
146 were performed in triplicate for each film and the solubility was calculated as equation (1):

$$Solubility(\%) = \left(\frac{Film\ initial\ dry\ weight\ (g) - Film\ final\ dry\ weight(g)}{Film\ initial\ dry\ weight\ (g)}\right) \times 100$$

(1)

2.7 Film tensile properties

Tensile properties of the film were evaluated according to ASTM standard method D882 – 12 (ASTM, 2013a) with some modifications. Rectangular strips of 60 × 10 mm were cut from the pre-conditioned film samples. The ends of the strips were mounted between the tensile grips (40mm apart) on a TA-XT2 Texture Analyzer (Stable Micro Systems, Goldalming, UK). Tensile grips were coated with an abrasive paper, orbital sanding sheet (P180) obtained from a local retail store (Bunnings, Perth, Australia). Tension was applied at a test speed of 0.6 mm/s, pre-test speed 1.0 mm/s, post-test speed 8.0 mm/s, distance 80.0 mm using a load cell of 5 kg, at a temperature of 23 ± 2 °C and 53 ± 3% RH. The maximum force and distance at break were obtained automatically from the computer.

The tensile strength and elongation at break (ELB) were obtained using equations (2) and (3) respectively.

$$Tensile\ strength\ (N/mm^2) = F\ /A \quad (2)$$

$$EAB\ (\%) = [Increase\ in\ length\ at\ breaking\ point\ (\Delta l)/original\ length] \times 100 \quad (3)$$

where, F (N) is the maximum force at break, and A (mm^2) is the initial cross-sectional area (thickness (mm) × width (mm)). EAB is elongation at break, and (Δl) is the difference between the original distance between grips holding the specimen before and distance after the break of the sample.

2.8 Water vapor permeability

Water vapor permeability was determined gravimetrically according to Taylor, Taylor, Dutton, and de Kock (2005a). Circles of the films (43 mm diameter) were mounted on the mouth of 100 mL Schott bottles containing 90 mL deionized water. A watertight seal was maintained by holding a 2 mm ledge of the film in place with a parafilm (Hach Pacific, Sydney, Australia) reinforced by silicon tape. The test assembly was weighed to ± 0.001 g and placed in an oven at 30 ± 3 °C at 32 ± 2 % RH. The assembly was weighed 7 times at 4 h intervals and the changes in their weight over time were plotted in a scatter plot ($R^2 > 0.95$). The rate of water vapor transmission (WVTR) was calculated using equation (4) and water vapor permeability (WVP) was obtained from equation (5). Each film formulation was evaluated in triplicate.

$$WVTR\ (g/h \cdot m^2) = (G/t)/A \quad (4)$$

$$WVP(g \cdot mm/m^2 \cdot h \cdot kPa) = G.X/A.\Delta P \quad (5)$$

Where, G is the weight change from the straight line (g), t is the time during which G occurred (h), G/t is the slope of the straight line (g/h), A is the test area (cup mouth area, m^2). X is the film thickness (mm), ΔP is the differential water vapor partial pressure across the film (Eck Van, 2004), and ΔP = (Po (kPa) × (RH1-RH2)/100: Po at 30 °C = 4.76 kPa (Aider, 2010). RH1 is the relative humidity inside the bottle and RH2 is the relative humidity outside the bottle. An assumption was made that the relative humidity inside the bottle (RH1) was 100% (ASTM, 2013b). The relative humidity outside the bottle (RH2) was measured using a Tiny Tag Ultra2 data logger for temperature and humidity (Omni instruments, Parkes, Australia).

2.9 Film oxygen permeability

Oxygen permeability was determined according to the method of Ayranci and Tunc (2003), however each type of edible film was used to seal between two plastic cups with 5.7 cm diameter, rather than the diameter of 4 cm previously reported. The oxygen permeability equipment was assembled to contain two identical chambers each with an inlet and an outlet channel.

Oxygen was allowed to enter and leave one of the cups on one side of the film at a controlled flow rate. Constant pressure in the unit was maintained at 10 ± 0.5 Kpa using a differential pressure manometer HD750 (± 5 psi range) (Extech Instruments, New Hampshire, USA). The cup on the other side of the film was purged by a stream of nitrogen which functioned as a carrier of permeated oxygen to a wet analysis system containing manganese (II) sulphate and alkaline iodide azide solution for 1 hour.

The solution of manganese (II) sulphate and alkaline iodide azide was analyzed for oxygen concentration (mg/L) using the stoichiometric titration described by Vogel (1989) and following Equation (6). Any pre-dissolved oxygen in the wet system was determined using a blank titration. Finally, oxygen permeability was calculated using equation (7).

$$Dissolved\ oxygen\ mg/L = (Vol\ of\ thiosulphate\ (mL) \times M\ of\ thiosulphate \times 8000)\ /\ (vol\ of\ sample\ titrated(ml)\ \times [(ml\ of\ original\ solution - 8)\ /\ (ml\ of\ original\ solution)] \quad (6)$$

where: “8000” is a factor related to 8 mg oxygen / miliequivalents mEq, “8” was the volume in mL of reagents added.

$$Oxygen\ permeability = \quad m.d/A.t.\Delta P \quad (7)$$

Where: m is the mass of oxygen (g) diffusing through the film; d is the thickness (m) of the film; A (m^2) is the area through which permeation takes place; t (h) is the time interval under which permeation occurs; ΔP (Pa) is the pressure difference of oxygen between the two sides of the film (oxygen pressure on the nitrogen side of the film was assumed to be zero since all diffused oxygen was continuously carried away by nitrogen).

2.10 Color measurement

The colour of the films was determined using a Minolta Hunter LAB colorimeter (CM-508i, Konica Minolta, Tokyo, Japan) using instrumental color readings *L* a* b**. The instrument was calibrated using a standard white tile. Three replicate readings from random locations were taken for each sample and the average was recorded.

2.11 Antimicrobial activity of films

The antimicrobial property of the films was tested qualitatively against *C. jejuni, L. monocytogenes* and *P. fluorescens* using the agar disc diffusion method according to Rojas-Graü, Avena-Bustillos, Friedman, Henika, Martín-Belloso, and McHugh (2006). The selection of the three microorganisms was based on their frequent occurrence in food, especially in poultry meat and meat products (Ravishankar, Zhu, Olsen, McHugh, & Friedman, 2009). An active culture of each bacterium was obtained by inoculating single colonies into tryptic soy broth with 0.6% yeast extract (TSBYE) for 12 hours. Subsequent dilutions of the stock cultures were made in buffered peptone water (BPW) to obtain approximately 10^8 CFU/ml with reference to the 0.5 McFarland standard turbidity test (Kavoosi, Dadfar, & Purfard, 2013).

Lawns of bacteria cultures were introduced by flooding 1,000 µL of the 10^8 cells/ml inoculum onto their respective media on sterile petri dish and excess cells were drawn using a sterile micropipette. Plates were air dried in a laminar flow cabinet for 10 minutes. Films

were aseptically cut into 8 mm diameter discs and six discs were arranged in each plate following an alternating manner determined by the levels of citral concentration. All plates were incubated in an inverted position for 24 h under the specific conditions for each microorganism as described above.

The size (mm) of the inhibition zone (annular radius) around the film disk (colony-free perimeter) was measured and the growth of the bacteria at the contact area of edible film with the agar surface was visually examined (Chen, Yeh, & Chiang, 1996). An average of three measurements of the radius taken, 90 degrees apart were used for antimicrobial sensitivity analysis.

2.12 Statistical analysis

The effect of film formulation on tensile strength and elongation at break was assessed using one-way multivariate analysis of variance. Other tests were analyzed using one-way ANOVA and non-parametric tests where applicable using SPSS version 21 (IBM Corp, NY, USA). Pair wise comparisons using Tukey (HSD) was used to separate the differences at 5% significance level. A two-way analysis of variance was used to determine the significance of the difference in the zones of inhibition of the films across the three microorganisms.

3 Results and discussion

Ethanol (96 %) was effective in solubilizing kafirin at 80 ± 2°C allowing the formation of free standing films. The films formed through this process were easy to peel from the glass surface and easy to handle. This is in agreement with the work of Buffo, Weller, and Gennadios (1997) on kafirin film preparation.

3.1 Thickness and moisture content of kafirin films

Addition of different combinations of citral and quercetin to the kafirin film forming solution caused significant ($P<0.05$) alteration of their thickness (Table 2). The thickness of the films ranged from 0.07-0.09 mm, which was in agreement with the published values of Buffo, Weller, & Gennadios (1997), and fitted the film definition of <0.25 mm (ASTM, 2013a).

The mean thickness of films without the plant extracts (P - films) was lower than that of the films where 2 % citral (C films), 1.25% citral and 1% quercetin (C+Q films) and 2 % quercetin (Q-films) were incorporated. This was possibly due to increased viscosity of film forming solutions with the addition of citral and quercetin. Taylor, Taylor, Dutton, and de Kock (2005a) reported that more viscous film forming solutions may result in thicker films. The moisture content of the various films was not significantly different, which could be attributed to the uniform storage conditions applied.

3.2 Solubility of kafirin films

Film water solubility can influence its use for protection of the packaged product from the external environment. The film may be required to be water insoluble especially when applied in high moisture foods. Kafirin has been described as a highly hydrophobic protein that is soluble only in aqueous alcohol solutions, ketones, amide solvents, esters, glycols and high concentrations of salts (Shukla & Cheryan, 2001). In the present study, the water solubility of kafirin films with the incorporation of citral and quercetin was not significantly different ($P > 0.05$) from the control (P) film (Table 2), which suggested that the kafirin film solubility was not affected by the citral and quercetin addition.

3.3 Tensile properties of kafirin films

The tensile strength (N/mm^2) and elongation at break (% EAB) were used to describe the mechanical properties of the kafirin films. Films formulation significantly affected their

tensile strength (TS) and % EAB ($P<0.05$) (Table 2). Films containing citral had lower TS and higher % EAB than the films without citral. The study showed a high standard deviation in % EAB (S.D >± 40 %) which could be attributed to the existence of a large number of variables influencing the properties of the films (Buffo, Weller, & Gennadios, 1997). Similarly high S.D. for % EAB (± 47.9 %) of kafirin film was observed by Taylor, Taylor, Dutton, and de Kock (2005a).

The mean % EAB of the control (P) film was very close to that reported by Taylor, Taylor, Dutton, and de Kock (2005a), i.e. EAB 63.5 ± 47.9% for plain film cast from 70% ethanol and 70 °C (specimen size 60 × 6 mm, tension applied = 0.4mm/s). The maximum force obtained by the authors from plain kafirin films cast was also similar to the force (4.1N) in the current study (results not shown).

Variations in specimen size, film thickness, and film conditioning have been reported to influence film mechanical properties (Buffo, Weller, & Gennadios, 1997). The present results showed that formulation affected mechanical strength of the films. The addition of citral resulted in higher elongation at break ($P<0.05$). This is of potential value for food packaging applications as these films could find application in products requiring film flexibility (Kavoosi, Dadfar, & Purfard, 2013).

3.4 Water vapor permeability test

The water vapor permeability (WVP) of kafirin films are shown in Table 3, where no significant difference was observed among the four types of film formulations ($P>0.05$). However, the overall mean water vapor transmission rate (WVTR) of plain (P) film was significantly higher than that of the films containing citral (C and C+Q) but not different from the quercetin (Q) film. Films containing citral exhibited lower water vapor transmission than plain (P) and quercetin (Q) films (Table 3). Low water transmission rate may be a useful

303 property of edible films in product packaging of food to prevent gaining or losing of moisture
304 transmission.

305 It has been proposed that the types and levels of plasticizers have influence on the migration
306 of water through thin films. According to Gillgren, Faye, and Stading (2011), addition of
307 plant extracts (e.g. citral) to film forming solutions could lower the concentration of the
308 plasticizers and then the WVTR. In the current study, plain films had higher concentration of
309 plasticizers and therefore higher WVTR than the rest. Another reason might be that citral is
310 an essential oil from citrus that has a hydrophobic nature which also reduced the water
311 transmission.

312 Taylor, Taylor, Dutton, and de Kock (2005a) reported a mean WVP of 0.43 ($g \cdot mm/m^2 hkPa$)
313 and WVTR 10.9 ($g/h/m^2$) on plain kafirin films cast using 70% ethanol at 70 °C, which were
314 lower than the results of the present study (Table 3). This difference might be related to the
315 use of a different kafirin source and/or the homogenization method used on the kafirin film-
316 forming solutions. The homogenization of the film forming solutions may generate bubbles
317 and pinholes, which have previously been shown to increase water and gas permeability of
318 polymeric films (Park & Chinnan, 1995). In the present study, we have kept the homogenized
319 solutions still for 30 min before film casting to minimize the bubble formations.

320 ***3.5 Oxygen permeability***

321 The oxygen permeability (OP) of the 4 types of films was in the range of (2.90 - 6.24) $\times 10^7$
322 $g \cdot mm/m^2.day.Pa$ (Table 3). The results showed that OP decreased significantly with addition
323 of plant extracts ($P<0.05$). Plain kafirin film exhibited the highest ($P<0.05$) oxygen
324 permeability rate whereas quercetin films had the lowest OP rates.

325 The highest values for oxygen permeability of plain kafirin films could be attributed to the
326 highly non-polar characteristic of the kafirin (Guilbert, Gontard, & Cuq, 1995). According to

Yoo and Krochta (2011), oxygen as a non-polar molecule has a tendency to dissolve more in low polarity polymers such as kafirin. As mentioned in section 3.4, plain films had relatively higher levels of plasticizers (polyethylene glycol/lactic acid/glycerol) compared to other films, which might also have increased the OP of the plain films (Gillgren, Faye, & Stading, 2011).

The protective action of flavonoids such as quercetin against movement of molecules across biological membranes has been suggested by Ionescu, Popescu, Drăguşin, Dima, Iftime, and Ganea (2007). The lowest oxygen permeability shown by quercetin films could be due to interactions between the polyphenol with the kafirin polymer through hydrogen bonding, which resulted in reduction diffusion of oxidizing molecules across the film (Ionescu et al., 2007). The authors showed that quercetin has the ability of inserting into lipid bilayers in artificial membranes as well as influencing the anion-lipid interactions in a biological system blocking the movement of oxidizing agents. The results of the present study indicated that incorporation of citral and quercetin, especially the quercetin, could significantly reduce the film oxygen permeability. Barrier to gas movement is a promising property in food packaging to prevent product oxidation, or loss of volatile aroma compounds.

3.6 Effect of film formulation on film color

Qualitative observation of the films showed that they were yellowish-brown in color (Figure 1), which was the colour of the extracted kafirin raw material. The yellowish-brown color of kafirin has previously been associated with the presence of natural pigments and polyphenols in the sorghum grain that may have been co-extracted with the kafirin (Da Silva & Taylor, 2005; Shukla & Cheryan, 2001).

The control film was the lightest L^* = 75.14 (Table 4), which was in agreement with the lightness (L*= 75.6 - 78.0) reported by Da Silva and Taylor (2005) for films of sorghum

extracted from whole grain white sorghum. In contrast, the Q and C-Q films (L^* = 61.94 and 65.44) were darker (P>0.05) than P and C films, which could be the dark yellowish coloration from quercetin (Formica & Regelson, 1995). The b^* values of films that contained citral (C and C-Q films) were higher (P<0.05) than those of P and Q films, implying that films containing citral were more yellow.

Subjective assessment showed that the films containing citral and quercetin were darker, redder and more yellow compared to the control. Similarly, Kim, Marshall, Cornell, Iii, and Wei (1995) reported that citral passed on a yellowish coloration to citral-treated fish products. Guilbert, Gontard, and Cuq (1995) reported that color, transparency, clarity, taste and odor of the films could determine its acceptability. Therefore, incorporation of citral and quercetin in kafirin film as potential bioactive packaging material could influence product color and then consumer acceptability.

3.7 Antimicrobial activity of the film

The three microorganisms showed varying degrees of sensitivity against the four types of films. The mean annular radii (mm) of the inhibition zones given as the average of nine are summarized in Table 5. The control (P) films showed some inhibitory power against *L. monocytogenes* (radius = 0.17 mm) but no inhibition of *C. jejuni* and *P. fluorescens.* However, visual observation of the contact area showed that cells of all the three bacteria strains did not grow beneath (at the contact area with the agar surface) the plain film. The antibacterial activity shown by the P film may have resulted from the natural active compounds, for example, flavonoids, and phenolic acids in the kafirin extract from the sorghum grain (Afify, El-Beltagi, El-Salam, & Omran, 2012). The antimicrobial activity of grain phenolic compounds has been reported in other studies. For example, the antibacterial activity of phenolic compounds in grains against microorganisms *Bacillus cereus* and

375 *Aspergillus flavus* has been reported by Viswanath, Urooj, and Malleshi (2009). In another
376 study, Kil et al. (2009) showed that methanolic crude extracts of sorghum (*S. bicolor*)
377 inhibited the growth of *Staphylococcus aureus.*

378 Films containing citral showed a dose-related inhibition against the three microorganisms.
379 Overall, C – film (2.5% citral) was the most potent inhibitor, followed by C-Q (1.25% citral)
380 films as demonstrated by larger zones of inhibition for all three strains (Table 5).
381 Comparative antibacterial activity of citral against two other strains of foodborne pathogens
382 *Salmonella typhimurium* and its rifampicin-resistant strain has previously been demonstrated
383 by Kim, Marshall, Cornell, Iii, and Wei (1995).

384 Quercetin (Q) and plain (P) films showed similar antimicrobial reaction towards all three
385 strains. The contact area of the Q-films on the agar surface also showed that the three
386 organisms did not grow beneath the films. The potential of quercetin and other flavonoids as
387 antimicrobial agents was reviewed by Cushnie and Lamb (2005). These authors attributed the
388 antimicrobial activity of quercetin to possible reactions leading to inhibition of DNA gyrase.
389 However, in the current study, quercetin films showed similar activity to the plain film
390 probably because the plain kafirin films already contained some phenolic compounds and the
391 added quercetin concentration (2%) was not high enough to inhibit the selected
392 microorganisms. The results suggested that citral was the main contributor of the
393 antimicrobial capacity of the tested kafirin films.

394 **4 Conclusion remarks**

395 This study successfully formulated bioactive films from sorghum protein incorporating the
396 phytochemicals of citral and quercetin. The addition of quercetin and citral both imparted a
397 yellowish color on the films, and significantly lowered the film oxygen permeability. The
398 tensile strength and rate of water vapor transmission were decreased, and elongation at break

was increased when citral was incorporated into the films. In addition, the films incorporated with citral showed strong antimicrobial activity against *C. jejuni*, *L. monocytogenes* and *P. fluorescens*. The results suggested that kafirin-based films incorporating citral and quercetin have the potential to be used as bioactive packaging material to improve food safety and quality. Further research on the evaluation of the physical suitability and antimicrobial properties of these films in fresh food systems is under investigation.

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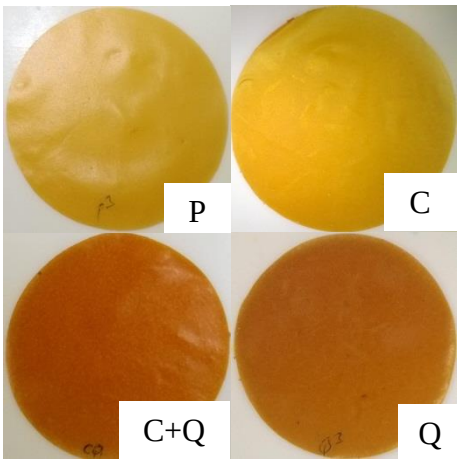

Figure 1. Visual observation of kafirin based films: P (plain film), C (P + 2.5% citral film), C+Q (P + 1.5% citral + 1% quercetin film), Q (P + 2% quercetin film).

520 **Table 1- Compositions of kafirin-based active films**

Treatment	Formulation (Percent of ingredient in formula)
Plain kafirin film (P)	Kafirin: 13.8%; Plasticizer: 5.6% (1:1:1 w/w mixture of glycerol: polyethylene glycol 400:lactic acid); 96% ethanol: 80.6%
Citral kafirin film (C)	P + 2.5% citral
Quercetin kafirin film (Q)	P + 2% quercetin
Citral quercetin kafirin film (C+Q)	P + 1.25% citral + 1% quercetin

521

522 **Table 2 Physical and mechanical properties of kafirin films**

Film	Thickness (mm)	% Moisture content	% Solubility	Tensile Strength (Nmm^{-2})	Elongation at break (%)
P	0.07 ± 0.01^{a}	14.0 ± 3.94^{a}	29.1 ± 5.59^{a}	3.48 ± 1.11^{b}	79.7 ± 41.4^{a}
C	0.09 ± 0.02^{b}	13.5 ± 4.80^{a}	24.7 ± 4.89^{a}	1.89 ± 0.55^{a}	141.0 ± 40.7^{b}
C+Q	0.09 ± 0.02^{b}	14.0 ± 2.52^{a}	24.5 ± 2.99^{a}	1.76 ± 0.50^{a}	147.0 ± 48.8^{b}
Q	0.08 ± 0.02^{ab}	11.3 ± 3.31^{a}	24.4 ± 3.53^{a}	3.25 ± 0.23^{b}	46.7 ± 44.5^{a}

523 P (plain film), C (P + 2.5% citral film), C+Q (P + 1.5% citral + 1% quercetin film), Q (P +
524 2% quercetin film).

525 Same superscript letters in the same column are not significantly different ($p > 0.05$).

526 **Table 3 Water and oxygen barrier properties of kafirin films with different levels of**
527 **citral and quercetin**

Films	WVP (g.mm/m^2.h.kPa)	WVTR (g/h.m^2)	OP × 10^7 (g/m.day.Pa)
P	0.66 ± 0.08^{a}	31.5 ± 3.74^{b}	6.24 ± 0.45^{c}
C	0.69 ± 0.10^{a}	25.6 ± 3.86^{a}	4.86 ± 1.11^{b}
C+Q	0.65 ± 0.12^{a}	23.7 ± 4.38^{a}	4.33 ± 0.34^{b}
Q	0.74 ± 0.18^{a}	28.9 ± 6.96^{ab}	2.90 ± 0.46^{a}

528 P (plain film), C (P + 2.5% citral film), C+Q (P + 1.5% citral + 1% quercetin film), Q (P +
529 2% quercetin film).
530 Same superscript letters in the same column are not significantly different at ($P > 0.05$).
531 WVTR – Water vapor transmission rate.
532 WVP – Water vapor permeability of the films.
533 OP – Oxygen permeability of the films.

534 **Table 4 CIE L* a* b* values of kafirin films**

Film type	*L**	*a**	*b**
P	75.14 ± 2.02^{c}	13.52 ± 2.18^{a}	41.78 ± 3.93^{a}
C	69.48 ± 3.87^{b}	16.01 ± 3.88^{ab}	54.62 ± 3.50^{b}
C+Q	65.44 ± 4.24^{ab}	21.84± 5.10^{c}	57.70 ± 2.75^{b}
Q	61.94 ± 4.50^{a}	19.03 ± 2.34^{bc}	43.19 ± 4.90^{a}

535 ** Mean ± S.D (n=9); L*= Lightness; a* = Redness; *b** = yellowness

536 P (plain film), C (P + 2.5% citral film), C+Q (P + 1.5% citral + 1% quercetin film), Q (P +

537 2% quercetin film).

538 Same superscript letters in the same column are not significantly different at (P > 0.05).

Table - 5 Antimicrobial activity of kafirin-based film on three bacteria strains (mean ± S.D n=9)

Microbe	***Campylobacter jejuni***		***Listeria monocytogenes***		***Pseudomonas fluorescens***	
Films	Inhibition zone (mm)	Contact area	Inhibition zone (mm)	Contact area	Inhibition zone (mm)	Contact area
P	0.00^{a}	+/-	0.17 ± 0.24^{a}	+/-	0.00^{a}	+/-
C	15.3 ± 4.70^{c}	++	2.54 ± 0.60^{c}	+	1.74 ± 0.78^{c}	+
C+Q	6.37 ± 4.20^{b}	+	0.52 ± 0.10^{b}	+	0.50 ± 0.01^{b}	+
Q	0.00^{a}	+/-	0.16 ± 0.16^{a}	+/-	0.04 ± 0.01^{a}	+/-

P (plain film), C (P + 2.5% citral film), C+Q (P + 1.5% citral + 1% quercetin film), Q (P + 2% quercetin film)

^{abcd} Mean ± S.D in the same column bearing the same superscript letters are not statistically significantly different ($p > 0.05$)

+/- .weak cells retraction at the contact area; +. clear inhibition zone present.

Same superscript letters in the same column are not significantly different at ($P > 0.05$).