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Effect of *Emblica officinalis* fruit extracts on the storage quality of pork meatballs under refrigerated storage

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Abstract

The present work aimed to evaluate the effects of the supplementation of *E. officinalis* fruit extracts on the biochemical properties, antioxidant capacities, antimicrobial activities, and sensory attributes of pork meatballs under refrigerated storage at 4°C for 18 days. The meatball samples were divided into eight formulations including control, 0.02% butylated hydroxytoluene (BHT), 0.2% distilled water and ethanolic extracts, 0.4% distilled water and ethanolic extracts, and 0.8% distilled water and ethanolic extracts. Aqueous fruit extract exhibited higher antioxidant activity ranging from 34.30 to 75.59%, with IC_{50} 9.74 µg/mL as compared to the other extracts. These findings were in accordance with the highest total phenolic (1,550.22 mg GAE/g extract) and flavonoid (19.35 mg CE/g extract) contents of distilled water crude extract, followed by methanolic extract, ethanolic extract, and acetonic extract, respectively. The meatballs supplemented with both ethanolic and aqueous extracts showed higher antioxidant activity than control and BHT samples, particularly at the highest concentration of 0.8%. Similarly, the lowest TBARS values were observed in the samples with 0.8% ethanolic extracts ranging from 0.08 - 0.45 mg MDA/kg of sample. In contrast, the meatballs supplemented with aqueous extracts yielded the lowest microbial counts of 1.94 - 4.90 log CFU/g in comparison with the samples supplemented with ethanolic extracts. This was in agreement with the lowest MIC and MBC values of aqueous crude extracts (3.98 mg/mL) against all the tested foodborne pathogens. Based on sensory analysis, supplementing the pork meatballs with either ethanolic or aqueous extracts resulted in decreased sensory attributes in a concentration-dependent manner. *E. officinalis* fruit extracts could have an impact on unpleasant sensory characteristics in the meatballs with increasing levels of supplementation.

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Introduction

Emblica officinalis (synonym *Phyllanthus emblica*; Indian gooseberry) is known as *amla* or *makhampom* in Thailand. This plant is widely grown in tropical and subtropical regions particularly Bangladesh, Sri Lanka, China, India, Malaysia, and Thailand. *E. officinalis* fruit has been reported to possess a number of bioactive compounds including emblicanin, gallic acid, ellagic acid, chebulinic acid, quercetin, chebulagic acid, corilagin, phyllantine, and phyllantidine. These bioactive compounds have been confirmed to exert diverse pharmacological activities such as anti-inflammatory, antioxidant, antimicrobial, anticancer, hepatoprotective, hypoglycaemic, and immunomodulatory properties (Hasan *et al*., 2016). Consequently, *E. officinalis* fruit has become a major constituent in nutritional and pharmaceutical products. It has also been reported that *E. officinalis* fruit contains quantifiable amounts of minerals, proteins, amino acids, phenolic compounds, and hydrolysable tannins which are believed to be the bioactive ingredient in traditional medicines for various disorders (Bhandari and Kamdod, 2012). Although there have been plentiful research focusing on the medicinal aspects of *E.*

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officinalis fruit over the years, it is imperative to note that there have been limited studies about its nutritional advantages in food products.

At present, natural plant-based food preservatives are of interest within the food industry, particularly regarding meat products. In general, oxidative reactions are known to play an important role in enhancing innate biochemical and physiological changes, thus resulting in meat spoilage (Tang *et al*., 2001). Synthetic antioxidants, like butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT), have been widely used as chemical food preservatives in the food industry to inhibit microbial growth and enzymatic degradation. However, BHA and BHT have been associated with toxic effects and allergies in some cases of prolonged exposure. Previous studies have also associated BHA and BHT with carcinogenesis (Silva and Lidon, 2016). Therefore, natural plant-based preservatives may be a promising choice to avoid harmful effects among food consumers.

To date, there have been limited studies about using *E. officinalis* fruit extracts in meat products. It has been reported that the pulp and seed coat powder of *E. officinalis* extended the shelf life of chicken meat nuggets while improving their physicochemical and sensory properties during cold storage as compared to controls (Manigiri *et al*., 2019). Furthermore, it has been demonstrated that restructured buffalo meat steaks supplemented with *E. officinalis* fruit powder can be stored in aerobically packaged pouches at refrigerated temperatures without any significant changes in their physicochemical, microbiological, and sensory attributes (Giriprasad *et al*., 2015). Therefore, the present work aimed to evaluate whether *E. officinalis* fruit extracts can be used as a natural preservative ingredient to improve the shelf life of pork meatballs under cold storage. Physicochemical, antimicrobial, antioxidant and sensory properties were subsequently determined in order to verify the effect of *E. officinalis* fruit extracts on the quality of pork meatballs.

Materials and methods

Preparation of the E. officinalis fruit extracts

E. officinalis fruits were purchased from local markets in Prachinburi, Thailand. Following cleaning, the fruits were cut into small pieces, and dried in hot air ovens at 50°C for 24 h. Thereafter, the dried samples were ground into powder with an electric grinder, and stored in polyethylene bags at 4°C. The fruit powder were macerated at room temperature in distilled water, 95% ethanol, 99.8% methanol, and acetone (QREC, Auckland, New Zealand) at a ratio of 1:8 (w/v) for 24 h in an orbital shaker, and then filtered through Whatman No.1 filter paper. Following filtration, the remaining solvents were eliminated using a rotary evaporator (Eyela, Tokyo, Japan) at 50°C to obtain the extracts. The extracts were freeze-dried in lyophiliser (Thermo Electron Corporation, Massachusetts, USA) to obtain the crude extract, and stored at -20°C until subsequent analyses.

Determination of total phenolic content

To determine the total phenolic content (TPC) of the crude extracts, the Folin-Ciocalteu colorimetric method (Baba and Malik, 2015) was used. Briefly, the crude extracts were prepared by dissolving them with 10% dimethyl sulfoxide (DMSO) to obtain a concentration of 1 mg/mL. Thereafter, the crude extract solutions (1 mg/mL) were mixed with Folin-Ciocalteu reagent (Merck, Darmstadt, Germany) at a ratio of 2:1 (v/v), and incubated for 5 min in the dark. Subsequently, 2 mL of 10% Na_2CO_3 (Sigma Aldrich, Saint Louis, USA) and 1 mL of distilled water were added into the mixture. The absorbance was measured at 765 nm using a spectrophotometer (Biochrom, Cambridge, United Kingdom) following incubation in the dark for 30 min at room temperature. Gallic acid (Sigma Aldrich, Saint Louis, USA) was used as the standard for interpreting the TPC of the crude extracts. The results were expressed in mg of gallic acid equivalent per g of extract (mg GAE/g extract).

Determination of total flavonoid content

The aluminium chloride colorimetric method (Baba and Malik, 2015) was used to analyse the total flavonoid content (TFC) of the crude extracts. Briefly, the crude extracts (1 mg/mL) were mixed with 5% $NaNO_2$ (Sigma Aldrich, Saint Louis, USA) at a ratio of 2:1 (v/v), and incubated for 5 min in the dark. Thereafter, 0.3 mL of 10% $AlCl_3$ (Sigma Aldrich, Saint Louis, USA) solution was added into the mixtures, and the mixture was incubated for 6 min. Subsequently, 2 mL of 1 M NaOH solution (Sigma Aldrich, Saint Louis, USA) and 4 mL of distilled water were added in the final step. The final mixture's absorbance was measured at 510 nm using

a spectrophotometer (Biochrom, Cambridge, United Kingdom). Catechin (Sigma Aldrich, Saint Louis, USA) was used as the standard for interpreting the TFC in the crude extracts. The results were expressed as mg of catechin equivalent per g of extract (mg CE/g extract).

Determination of DPPH radical scavenging activity

1,1-diphenyl-2-picryl-hydrazyl, DPPH (Sigma Aldrich, Saint Louis, USA), was used to evaluate the antioxidant activity in the crude extracts following the method in a previous study (Ajila *et al*., 2010). Briefly, the crude extracts (25 - 400 µg/mL) were mixed in 0.1 mM DPPH solution (Sigma Aldrich, Saint Louis, USA) at a ratio of 1:2 (v/v), and incubated for 30 min in the dark. Following incubation, the absorbance was measured at 517 nm using a spectrophotometer (Biochrom, Cambridge, United Kingdom). In order to determine the antioxidant activity in the pork meatballs, the following extraction procedure was employed. Briefly, 8 g of meat was homogenised with 20 mL of distilled water for 10 min. The mixture was centrifuged at 3,000 *g* (Labnet International, New York, USA) for 5 min. Subsequently, 1 mL of the supernatant was used following the same protocol. The DPPH radical scavenging activity was calculated using Eq. 1:

$$\text{DPPH radical scavenging activity (\%)} = [(\text{Control OD} - \text{Sample OD}) / \text{Control OD}] \times 100 \quad \text{(Eq. 1)}$$

where, control OD = distilled water's absorbance, and sample OD = extract's absorbance.

The inhibitory concentration at 50% (IC_{50}) value was determined by using a linear graph of percentage of DPPH radical scavenging activity and the various concentrations of the extracts (µg/mL).

Bacterial strains preparation

Staphylococcus aureus (TISTR 1466), *Bacillus cereus* (TISTR 678), *Escherichia coli* (TISTR 780), and *Salmonella* Typhimurium (ATCC 13311) were purchased from the Thailand Institute Scientific and Technological Research (TISTR) Culture Collection, in Pathum Thani, Thailand. These strains were sub-cultured overnight at 37°C on nutrient agar (Difco, BD, New Jersey, USA). The cultures were the grown on nutrient broth (Difco, BD, New Jersey, USA), and adjusted to a microbial count of about 10^8 CFU/mL.

Antimicrobial susceptibility testing by disc diffusion assay

The crude extracts were prepared by dissolving them in 10% DMSO to obtain a concentration of 500 mg/mL. The sterilised Mueller-Hinton agar (Difco, BD, New Jersey, USA) was poured into Petri dishes. Following solidification, a sterile cotton swab was dipped into the adjusted bacterial suspensions of about 1.5×10^8 CFU/mL (0.5 McFarland Standard) and spread on the agar surface. Sterile paper disks (6 mm diameter) were soaked in a final concentration of 16 mg/disc of each crude extracts, and placed on the inoculated Mueller-Hinton agar plates. Amoxicillin (30 µg/disc) was used as the positive control, while 10% DMSO was used as the negative control. Plates were incubated for 18 - 24 h at 37°C. Antimicrobial susceptibility was interpreted by measurement of the inhibition zones using Vernier callipers (mm). All experiments were performed in triplicate.

Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC)

Determination of MIC was performed using the broth microdilution method following the Clinical and Laboratory Standards Institute (CLSI) protocols. Serial 2-fold dilutions of the crude extracts were prepared in Mueller-Hinton broth (Difco, BD, New Jersey, USA) for a final concentration of 0.97 - 500 mg/mL. The bacterial cultures were prepared in Mueller-Hinton broth, and adjusted to a 0.5 McFarland turbidity standard. Next, 10 µL of the cultures were added to each well of 96-well microplates to prepare 1.5×10^6 CFU/mL viable cells, and incubated at 37°C for 18 - 24 h. Thereafter, 30 µL of 0.01% resazurin (Sigma Aldrich, Saint Louis, USA) was added to each well, and then incubated for 4 h. Resazurin dye was used as the indicator of cell viability. The MIC values were interpreted as the lowest concentration of the crude extracts which showed no visible bacterial growth. Amoxicilin (1.46 – 3,000 µg/mL) was used as the positive control, while 10% DMSO was used as the negative control. MBC was performed by aliquoting the suspension from each well used for the MIC values onto Mueller-Hinton agar plates. The lowest concentration of the crude extract which showed no visible bacterial growth after 18 - 24 h of incubation was regarded as the MBC. All experiments were performed in triplicate.

Preparation of the pork meatballs

In order to determine the effect of the crude extracts on pork meatballs, aqueous and ethanolic extracts of *E. officinalis* fruits at three concentrations were supplemented into the pork meatballs. The supplementation of 0.2, 0.4, and 0.8% of the crude extracts were defined as test groups, with positive control (0.02% BHT) and negative control (no addition of BHT/the crude extracts) groups. The meatballs were formulated with 83.95% lean ground pork, 0.25% sodium phosphate, 1.65% salt, 1.65% corn starch, and 12.5% ice. The meatballs were prepared following our previous study (Prasajak *et al*., 2021). Polyethylene bags were used for aerobic packaging and storage at 4°C for 18 days. Random sampling was used to analyse the physicochemical properties of the meatballs every three days during cold storage. All experiments were performed in triplicate.

Determination of pH

The pH value was measured with a pH meter (WTW, inoLab, Weilheim, Germany). Briefly, 8 g of pork meatball was homogenised with 20 mL of distilled water. Thereafter, the filtrate was obtained using Whatman No.1 filter paper, and the pH value determined. All experiments were performed in triplicate.

Determination of bacterial growth

The total viable count (TVC) of the meatball samples was carried out throughout the storage period. Briefly, 25 g of sample was homogenised with 225 mL of sterile peptone water following homogenisation in stomacher bag at 250 rpm for 1 min. Serial ten-fold dilutions were plated on plate count agar (Difco, BD, New Jersey, USA) by pour plate technique, and incubated at 37°C for 48 h. The bacterial growth was expressed as the log CFU/g of the sample. All experiments were performed in triplicate.

Determination of TBARs values

The levels of lipid oxidation in the meatballs were determined by 2-thiobarbituric acid reactive substances (TBARs) assay. Briefly, 8 g of sample was homogenised in 20 mL of deionised distilled water to obtain the homogenate. Subsequently, the homogenate was mixed with 75 µL of 7% BHT followed by 3 mL of 0.02 M 2-thiobarbituric acid (TBA) in 15% trichloroacetic acid (TCA) (Sigma Aldrich, Saint Louis, USA) solution. The mixture was heated in a water bath at 90°C for 30 min, then cooled to room temperature before being centrifuged at 4,000 *g* for 10 min. A spectrophotometer was used to measure the concentration of malonaldehyde (MDA) in the samples at 532 nm. The TBARs value was expressed in milligrams of MDA per kilogram of meatballs in the samples (mg MDA/kg sample) from a standard curve of 1, 1, 3, 3-tertramethoxypropane (precursor of MDA). All experiments were performed in triplicate.

Sensory evaluation

The sensory attributes of the meatball samples were evaluated by 50 untrained panellists between 20 and 40 years of age. A 9-point hedonic scale was used for sensory analysis which included colour, texture, flavour, odour, and overall acceptability. The quality of each attribute was scored as follows: 1 = extremely dislike, 5 = neither like nor dislike, and 9 = extremely like.

Statistical analysis

Statistical analysis was conducted using One-way ANOVA on SPSS version 21.0 software followed by means comparisons with Duncan's multiple range tests (DMRT) at a significance level of $p \leq 0.05$.

Results and discussion

Total phenolic content, total flavonoid content, and antioxidant activity of E. officinalis fruit crude extracts

Based on the free radical scavenging activity determined by DPPH assay, results (Table 1) showed that aqueous extract exhibited higher antioxidant activity, ranging from 34.30 to 75.59% with IC_{50} 9.74 µg/mL, as compared to the other extracts. These findings were in accordance with the highest TPC (1,550.22 mg GAE/g extract) and TFC (19.35 mg CE/g extract) of the aqueous extracts, followed by the methanolic extracts, ethanolic extracts, and acetonic extracts.

In accordance with previous study, biological compounds of the fruits extracted from various solvents demonstrated free radical scavenging activity, similar to that observed in the present work. The highest TPC and antioxidant activities were obtained by aqueous extraction, followed by ethanolic, methanolic, and acetonic at absolute

concentration (Verma *et al*., 2018). For quantitative compounds obtained from the aqueous extraction, fruit extracts exhibit a wide range of TPC ranging from 59.18 - 62.50 mg GAE/g extract (Liu *et al*., 2008). Charoenteeraboon *et al*. (2010) demonstrated that aqueous extract of *E. officinalis* fruits had TPC of 34.22 g gallic acid/100 g extract, and IC_{50} 51.30 µg/mL. In comparison with these studies, our results showed higher TPC and lower IC_{50} values. The inconsistency may be explained by the effects of the boiling extraction and spray-drying method during the processing of the crude extracts in prior studies, whereas maceration and freeze-drying techniques were used herein for preparing the dried extracts in order to preserve their bioactive molecules.

Phenolic acids and flavonoids have been reported to serve as potential phytochemicals derived from fruits and vegetables which are responsible for their antioxidant capacity. Kumar *et al*. (2006) has identified gallic and tannic acids as the major antioxidant components in phenolic fractions of *E. officinalis* fruits. Moreover, free and bound phenolic compounds of *E. officinalis* fruits have been demonstrated to have potent antioxidant capacity *in vitro*, which, based on evidence, are related to acute and chronic anti-inflammatory activities in rat models (Muthuraman *et al*., 2011; Middha *et al*., 2015). Altogether, *E. officinalis* fruits are rich sources of phenolic acids and flavonoids which are associated with antioxidant capacity.

Table 1. Total phenolic contents, total flavonoid contents, and IC_{50} values of *E. officinalis* fruit crude extracts from various solvents.

Solvent	*E. officinalis* fruits crude extract		
	Total phenolic (mg GAE/g)	**DPPH assay IC_{50} (µg/ml)**	**Total flavonoid (mg CE/g)**
Distilled water extract	1,550.22 ± 123.71^{a}	9.74 ± 0.48^{c}	19.35 ± 2.06^{a}
Methanolic extract	1,456.06 ± 101.74^{ab}	10.52 ± 0.41^{c}	16.29 ± 1.50^{b}
Ethanolic extract	1,421.86 ± 91.80^{b}	14.27 ± 0.28^{b}	16.93 ± 1.02^{b}
Acetonic extract	592.42 ± 29.23^{c}	20.14 ± 0.56^{a}	7.04 ± 1.34^{c}

Values are mean ± SD of three independent experiments (n = 3). Means followed by different lowercase superscripts in the same column represent significant difference ($p \leq 0.05$).

Antibacterial activity of E. officinalis fruit crude extracts

The antibacterial activity of *E. officinalis* fruit extracts against foodborne pathogens was assessed by agar disc diffusion assay, MIC, and MBC values (Table 2). Among the extracts obtained with various solvents, methanolic extract yielded the highest antibacterial activity against Gram-positive bacterial strains (14.50 - 15.67 mm inhibition zones), *S. aureus*, and *B. cereus*, followed by acetonic, ethanolic, and aqueous. Against Gram-negative bacteria, none of the extracts showed a zone of inhibition. This might be explained by the nature of Gram-negative bacteria which have an additional lipopolysaccharide barrier in the outer cell membrane, thus rendering them less sensitive than Gram-positive bacteria.

For MIC and MBC, aqueous extracts of *E. officinalis* fruits yielded the lowest MIC and MBC values, 3.98 mg/mL, against all tested strains as compared to the other extracts. Similarly, it has been reported that the antibacterial activities of *E. officinalis* fruits were different in different solvent extracts. Ethanolic and acetonic extracts of *E. officinalis* fruits have showed moderate activity against *S. aureus*, *B. subtilis*, *P. aeruginosa*, *S. dysenteriae*, and *E. coli*, which provided a 12.7 mm maximum zone of inhibition (Hossain *et al*., 2012). Aqueous extracts of *E. officinalis* fruits exhibited antibacterial action against *S. aureus*, *B. subtilis*, and *E. coli* (Jadon and Dixit, 2014). In addition, methanolic extracts of *E. officinalis* fruits have produced MIC values ranging from 0.26 - 0.34 mg/mL for *S. aureus* and *K. pneumoniae* (Raghu and Ravindra, 2010). Several studies have reported that tannins, saponins, flavanoids, terpenoids, and phenols were identified in the extracts derived from aqueous and methanolic extracts (Kumar *et al*., 2011; Badoni *et al*., 2016). Therefore, these phytochemical compounds may play an important role in producing the observed antibacterial properties.

Table 2. Inhibition zones, MIC, and MBC values of *E. officinalis* fruit crude extracts from various solvents against different foodborne bacteria.

Foodborne bacterium	Crude extract	MIC (mg/mL)	MBC (mg/mL)	Inhibition zone (mm)
Staphylococcus aureus	Distilled water extract	3.98	3.98	14.17 ± 0.41[c]
	Methanolic extract	3.98	3.98	15.67 ± 0.52[b]
	Ethanolic extract	3.98	3.98	15.83 ± 0.41[b]
	Acetonic extract	7.81	7.81	15.83 ± 0.41[b]
	Amoxicillin	0.001	0.001	41.33 ± 0.82[a]
Bacillus cereus	Distilled water extract	3.98	3.98	13.83 ± 0.41[c]
	Methanolic extract	3.98	15.62	14.50 ± 0.55[bc]
	Ethanolic extract	3.98	7.81	12.33 ± 0.52[d]
	Acetonic extract	7.81	62.50	14.00 ± 0.63[ab]
	Amoxicillin	0.02	0.02	14.67 ± 0.52[a]
Salmonella Typhimurium	Distilled water extract	3.98	3.98	ND
	Methanolic extract	3.98	7.81	ND
	Ethanolic extract	3.98	7.81	ND
	Acetonic extract	7.81	15.62	ND
	Amoxicillin	0.001	0.001	25.88 ± 1.36
Escherichia coli	Distilled water extract	1.95	3.98	ND
	Methanolic extract	3.98	3.98	ND
	Ethanolic extract	3.98	15.62	ND
	Acetonic extract	3.98	7.81	ND
	Amoxicillin	0.001	0.001	23.63 ± 0.96

Values are mean ± SD of three independent experiments (n = 3). Means followed by different lowercase superscripts in the same column represent significant difference ($p \leq 0.05$). ND: not detected.

Antioxidant activity and lipid oxidation analysis of pork meatballs supplemented with E. officinalis fruit crude extracts

The aqueous and ethanolic crude extracts were selected to determine their effects on physicochemical properties of pork meatballs in terms of DPPH scavenging activity. The meatballs supplemented with both ethanolic and aqueous extracts showed higher antioxidant activity than those in control and BHT samples, particularly at the highest concentration of 0.8% (Table 3). Even though ethanolic and aqueous extract meatballs did not show a significant difference in antioxidant activity in a concentration-dependent manner, higher levels of the extracts have been shown to exert higher DPPH scavenging activity than lower concentrations. The 0.8% ethanolic extracts have shown excellent antioxidant capacity in pork meatballs, ranging from 82.08 - 94.10%, followed by the 0.8% aqueous extracts under storage at 4°C for 18 days. Remarkably, all samples supplemented with the different concentrations of extracts exhibited superior DPPH scavenging activity over the control and BHT groups throughout the storage period. In addition, the control and BHT meatballs showed a decrease in antioxidant activity of up to 50% on day 12 of storage, while the others were observed as having only decreased by approximately 7 - 10% on the same day. This demonstrated the sustainable antioxidant effects of the crude extracts on the pork meatballs during cold storage. Similarly, a recent study revealed that the supplementation of 2% *E. officinalis* fruit powder

Table 3. Antioxidant activity of pork meatballs supplemented with *E. officinalis* fruit crude extracts under refrigerated storage for 18 days.

Storage time (day)	% Inhibition activity (DPPH assay) in pork meatball							
	Control	0.02% BHT	% Distilled water extract addition			% Ethanolic extract addition		
			0.2%	0.4%	0.8%	0.2%	0.4%	0.8%
0	43.94 ± 4.99^{Af}	52.35 ± 9.84^{Ae}	86.21 ± 1.34^{Ad}	91.99 ± 0.39^{Abc}	92.59 ± 0.37^{Ab}	90.90 ± 1.46^{Ac}	92.64 ± 0.77^{Ab}	94.10 ± 0.78^{Aa}
3	38.61 ± 10.41^{Bf}	41.01 ± 2.55^{Be}	83.79 ± 0.38^{Bd}	90.84 ± 0.49^{Ab}	91.40 ± 0.34^{Ab}	88.32 ± 0.59^{Ac}	90.18 ± 1.25^{Ab}	92.41 ± 0.58^{Aa}
6	30.10 ± 4.28^{Cf}	35.23 ± 2.60^{Ce}	82.29 ± 0.48^{Bd}	88.86 ± 0.52^{Bb}	89.33 ± 0.38^{Bb}	86.47 ± 0.96^{Bc}	88.98 ± 0.73^{Bb}	90.51 ± 0.80^{Ba}
9	25.30 ± 5.93^{De}	30.99 ± 3.00^{Dd}	81.74 ± 0.49^{Cc}	87.81 ± 1.13^{Bab}	88.48 ± 0.81^{Ba}	85.53 ± 1.28^{Cb}	86.95 ± 0.84^{Bb}	88.71 ± 0.59^{Ca}
12	21.72 ± 3.32^{Ee}	26.86 ± 1.36^{Ed}	80.56 ± 0.65^{Dc}	83.16 ± 2.26^{Cb}	85.69 ± 0.92^{Ca}	82.35 ± 1.11^{Db}	83.03 ± 0.84^{Cb}	85.92 ± 0.81^{Da}
15	17.65 ± 1.46^{Ff}	24.43 ± 1.66^{Fe}	77.31 ± 0.57^{Ed}	80.56 ± 1.21^{Db}	80.75 ± 0.77^{Db}	78.54 ± 1.04^{Ec}	79.81 ± 0.91^{Dc}	84.09 ± 0.71^{Da}
18	12.22 ± 1.33^{Gf}	18.54 ± 3.08^{Ge}	76.28 ± 1.01^{Ec}	79.57 ± 0.93^{Db}	79.62 ± 0.65^{Db}	75.78 ± 1.41^{Ed}	77.49 ± 0.96^{Dc}	82.08 ± 0.61^{Ea}

Values are mean ± SD of three independent experiments (n = 3). Means followed by different lowercase superscripts in the same row represent significant difference among the treatments ($p \leq 0.05$). Means followed by different uppercase superscripts in the same column represent significant difference among the day of storage time ($p \leq 0.05$).

extract significantly improved the antioxidant potential of goat meat nuggets during refrigerated storage (Verma and Rajkumar, 2021). These findings indicate that *E. officinalis* fruit extract could be a potent source of antioxidants which can be used as natural food additives to improve the dietary value of pork meatballs.

Over the past ten years, there have been several assays to determine antioxidant properties of plant extracts *in vitro*. These methods work on different mechanisms including free radical scavengers, singlet oxygen quenchers, and metal ion chelators. Among the many methods, DPPH is the most rapid, simple, and economical approach for testing antioxidant activity. Depending on the free radicle scavenging mechanism, DPPH is a single electron transfer reaction-based assay. The assay determines the capacities of antioxidants to neutralise the stable DPPH free radical, thus leading to the discoloration of the purple DPPH radical which can be measured as a decrease in absorbance at 517 nm. Although this approach is simple and cost-effective, several factors have been noted to possibly interfere with its sensitivity such as the presence of hydrogen and metal ions, including some types of solvents used for DPPH testing systems (Shahidi and Zhong, 2015). This limitation makes the DPPH assay only suitable for preliminary screening of antioxidant activities.

The effect of *E. officinalis* fruit extracts on lipid oxidation in the pork meatballs was evaluated by TBARS values during refrigeration period (Table 4). The gradual increase in TBARS values was observed in all control, BHT, and test groups throughout the 18 days under refrigerated storage. Although higher levels of TBARS values were found with increased storage periods, all meatballs supplemented with the extracts still maintained values below the unacceptable level of MDA concentration of 1 mg MDA/kg of meat sample in aerobic conditions (Reitznerova *et al*., 2017). Malonaldehyde (MDA) is a secondary end-product of the lipid oxidation process which is typically used as an oxidative marker in meat products. Results showed that the crude extracts could retard lipid oxidation in the pork meatballs, even when they were used to treat precooked meat products. Interestingly, the meatballs supplemented with ethanolic crude extracts had lower TBARS values than those that were supplemented with aqueous extracts, and the values depended on the extracts' concentrations. For control meatballs, TBARS values increased dramatically at day 12 (above 1 mg MDA/kg of meat sample), and the increase progressed until the end of storage. On the other hand, the extract-supplemented meatballs maintained a lower rate of lipid oxidation, even when the samples were stored aerobically.

In accordance with the antioxidant activities, our results showed that the relation between retarding lipid oxidation and the antioxidant effects of *E. officinalis* fruit extract had a potential to protect the pork meatballs from further internal oxidation. Manigiri *et al*. (2019) reported similar results in chicken meat nuggets supplemented with pulp and seed coat powder of *E. officinalis*. They revealed that the nuggets supplemented with both powders had a better shelf life than control samples, and reduced TBARS values during refrigerated storage. Similarly, it was reported that the supplementation of *E. officinalis* fruit powder significantly reduced the concentrations of TBARS in restructured buffalo meat steaks as compared to controls (Giriprasad *et al*., 2015). Bariya *et al*. (2018) observed similar results in goat meat patties supplemented with *E. officinalis* fruit and seed coat extracts. Moreover, the shelf life of the supplemented samples was prolonged, being able to be kept up to 21 days under vacuum packed refrigerated conditions.

Antimicrobial property in pork meatballs supplemented with E. officinalis fruit crude extracts

The extracts' antimicrobial property was analysed in the pork meatballs to monitor the effects of the fruit extracts on the development of microorganisms during cold storage (Table 5). Ethanolic and aqueous extracts of *E. officinalis* fruit were supplemented in the meatballs at various concentrations ranging from 0.2 - 0.8% (w/w). Results showed lower microbial growth rates in samples supplemented with both extracts as compared to control and BHT samples. Although BHT commonly exerts antioxidant activity, some studies have revealed antimicrobial properties of synthetic phenolic antioxidants such as propyl gallate and BHA, including BHT (Ayaz *et al*., 1980; Shallangwa *et al*., 2022).

Based on the results, combined use of the extracts with synthetic phenolic antioxidant, BHT, seemed to have potential synergistic effects on both antioxidant and antimicrobial activities in the meat products. The meatballs supplemented with aqueous

Table 4. Lipid oxidation analysis of pork meatballs supplemented with *E. officinalis* fruit crude extracts under refrigerated storage for 18 days.

Storage time (day)	TBARs value (mg of MDA/kg meat) in pork meatball							
	Control	0.02% BHT	% Distilled water extract addition			% Ethanolic extract addition		
			0.2%	0.4%	0.8%	0.2%	0.4%	0.8%
0	0.42 ± 0.11^{Ga}	0.15 ± 0.10^{Gd}	0.22 ± 0.02^{Gb}	0.19 ± 0.03^{Gc}	0.12 ± 0.02^{Ge}	0.17 ± 0.02^{Gcd}	0.13 ± 0.01^{Ge}	0.08 ± 0.02^{Gf}
3	0.56 ± 0.03^{Fa}	0.30 ± 0.05^{Fb}	0.31 ± 0.03^{Fb}	0.27 ± 0.04^{Fc}	0.23 ± 0.03^{Fd}	0.24 ± 0.04^{Fcd}	0.20 ± 0.02^{Fd}	0.14 ± 0.03^{Fe}
6	0.70 ± 0.05^{Ea}	0.45 ± 0.06^{Eb}	0.36 ± 0.03^{Ec}	0.33 ± 0.03^{Ed}	0.31 ± 0.03^{Ed}	0.31 ± 0.02^{Ed}	0.24 ± 0.03^{Ee}	0.19 ± 0.03^{Eg}
9	0.82 ± 0.04^{Da}	0.69 ± 0.06^{Db}	0.48 ± 0.02^{Dc}	0.45 ± 0.05^{Dcd}	0.42 ± 0.03^{Dd}	0.38 ± 0.02^{De}	0.32 ± 0.03^{Df}	0.25 ± 0.02^{Dg}
12	1.01 ± 0.04^{Ca}	0.84 ± 0.05^{Cb}	0.55 ± 0.05^{Cc}	0.52 ± 0.05^{Cc}	0.47 ± 0.05^{Cd}	0.47 ± 0.04^{Cd}	0.40 ± 0.05^{Ce}	0.30 ± 0.02^{Cf}
15	1.10 ± 0.06^{Ba}	0.97 ± 0.04^{Bb}	0.67 ± 0.02^{Bc}	0.60 ± 0.02^{Bc}	0.53 ± 0.05^{Be}	0.59 ± 0.03^{Bd}	0.50 ± 0.04^{Be}	0.39 ± 0.03^{Bf}
18	1.42 ± 0.10^{Aa}	1.12 ± 0.05^{Ab}	0.72 ± 0.03^{Ac}	0.66 ± 0.02^{Ad}	0.61 ± 0.02^{Ae}	0.66 ± 0.03^{Ad}	0.58 ± 0.03^{Af}	0.45 ± 0.04^{Ag}

Values are mean ± SD of three independent experiments (n = 3). Means followed by different lowercase superscripts in the same row represent significant difference among the treatments ($p \leq 0.05$). Means followed by different uppercase superscripts in the same column represent significant difference among the day of storage time ($p \leq 0.05$).

Table 5. Total viable count of pork meatballs supplemented with *E. officinalis* fruit crude extracts under refrigerated storage for 18 days.

Storage time (day)	Total viable count (log CFU/g) in pork meatball							
	Control	0.02% BHT	% Distilled water extract addition			% Ethanolic extract addition		
			0.2%	0.4%	0.8%	0.2%	0.4%	0.8%
0	2.18 ± 0.05^{G}	ND	ND	ND	ND	ND	ND	ND
3	3.23 ± 0.07^{Fa}	3.12 ± 0.02^{Fb}	2.66 ± 0.02^{Fd}	2.51 ± 0.05^{Ee}	1.94 ± 0.05^{Ff}	2.86 ± 0.03^{Fc}	2.80 ± 0.05^{Fc}	2.70 ± 0.02^{Fd}
6	4.47 ± 0.03^{Ea}	3.62 ± 0.07^{Eb}	3.23 ± 0.07^{Ec}	2.90 ± 0.02^{De}	2.11 ± 0.05^{Ef}	3.15 ± 0.01^{Ec}	3.00 ± 0.06^{Ed}	2.91 ± 0.01^{Ee}
9	5.95 ± 0.05^{Da}	3.81 ± 0.04^{Db}	3.61 ± 0.05^{Dc}	3.17 ± 0.01^{Cf}	2.94 ± 0.06^{Dg}	3.59 ± 0.07^{Dc}	3.42 ± 0.04^{Dd}	3.27 ± 0.07^{De}
12	6.82 ± 0.02^{Ca}	4.53 ± 0.05^{Cb}	4.39 ± 0.04^{Cc}	4.30 ± 0.02^{Bd}	3.87 ± 0.07^{Ce}	4.40 ± 0.05^{Cc}	4.25 ± 0.05^{Cd}	3.92 ± 0.02^{Ce}
15	7.72 ± 0.06^{Ba}	5.21 ± 0.03^{Bb}	4.72 ± 0.02^{Bd}	4.61 ± 0.07^{Ae}	4.59 ± 0.02^{Be}	4.90 ± 0.07^{Bc}	4.88 ± 0.03^{Bc}	4.75 ± 0.05^{Bd}
18	7.95 ± 0.04^{Aa}	5.80 ± 0.05^{Ab}	4.90 ± 0.05^{Ad}	4.74 ± 0.06^{Ae}	4.70 ± 0.03^{Ae}	5.12 ± 0.02^{Ac}	5.10 ± 0.04^{Ac}	4.98 ± 0.03^{Ad}

Values are mean ± SD of three independent experiments. Mean values followed by different lowercase letters in same row represent significant difference among the treatments ($p \leq 0.05$). Mean values followed by different uppercase letters in same column represent significant difference among the day of storage time ($p \leq 0.05$). ND refers to not detected.

extracts provided the lowest microbial counts, 1.94 - 4.90 log CFU/g, in comparison with the samples supplemented with ethanolic extracts. On the contrary, the value of total viable counts was 2.18 - 7.95 log CFU/g in the control samples, which surpassed the limit of 5 log CFU/g prescribed by the Thai FDA regulations, on day 9 of the storage period.

For supplemented samples, our results revealed that the pork meatballs with 0.2 - 0.8% concentrations of both ethanolic and aqueous extracts prolonged the shelf life up to 18 days under aerobic packed refrigerated conditions. Although there were no significant differences in antimicrobial activity with increased extracts concentrations, antimicrobial efficacy tended to be affected by the supplementation level as compared to the other treatments. Based on pH measurements, the initial pH values ranged from 5.90 - 6.70, while the final levels had dropped to 5.70 - 6.50. The pH values slightly decreased in all supplemented meatballs including the control and BHT samples, throughout storage. In addition, we observed that supplementation levels of the extracts decreased the pH values of the meatballs. Our findings were similar to Mahajan *et al*. (2017) who observed a decrease in pH value following an increase in the supplementation level of *E. officinalis* fruit juice powder in spent hen meat nuggets. This may be the result of the high content of ascorbic acids in *E. officinalis* fruit, which tends to reduce pH levels in meat products. Moreover, the decreasing pH values may be attributed to the increase in acidic substances which could be produced from lactic acid bacterial strains under refrigerated conditions. Our results indicated that *E. officinalis* fruit extracts could be highly effective against food spoilage microorganisms in the meatballs for a minimum of two weeks under cold storage. Similar findings were observed by Manigiri *et al*. (2019) who reported lower microbial growth (less than 5 log CFU/g) in chicken meat nuggets supplemented with pulp and seed coat powders of *E. officinalis*, even after 20 days of cold storage. It has also been reported that the fruit extract had antioxidation and antimicrobial effects in refrigerated raw meat batter during 12 days of storage (Kumar and Langoo, 2016). In addition, Giriprasad *et al*. (2015) also reported the antimicrobial effectiveness of *E. officinalis* supplemented in restructured buffalo meat steaks.

Sensory analysis in pork meatballs supplemented with E. officinalis fruit crude extracts

The effect of *E. officinalis* fruit extracts on the sensory characteristics of the meatballs was evaluated at day 0 of storage (Table 6). Results showed an increase in all sensory scores in control and BHT samples as compared to the supplemented samples. Moreover, higher supplementation with both ethanolic and aqueous extracts was linked to negative effects on the sensory attributes of the meatballs in a concentration dependent manner. The range of the overall sensory scores were as follows: appearance score, 5.10 - 6.38; colour score, 5.36 - 6.81; odour score, 4.74 - 5.67; flavour score, 4.88 - 5.90; texture score, 4.67 - 5.74; and overall acceptability score, 4.88 - 6.10.

Our findings disagreed with previous studies which reported higher sensory scores, ranging from 7.08 - 7.13, in restructured buffalo meat steaks supplemented with 0.5% *E. officinalis* fruit powder (Giriprasad *et al*., 2015), and 7.20 - 7.35 in chicken meat nuggets supplemented with 0.5% *E. officinalis* pulp powder (Manigiri *et al*., 2019). This discrepancy be related to the fact that there were no flavour enhancers or spices in the meatball ingredients. Therefore, this could possibly lead to the decreased sensory attributes in comparison to other formulations.

Among the supplemented samples, it was observed that 0.2% aqueous extract samples yielded the highest scores for all sensory attributes, while 0.8% aqueous extracts samples yielded the lowest scores for flavour, texture, and overall acceptability. These findings might have been due to the effect of polar substitutes derived from the distilled water extraction such as phenolic compounds and their esters which have been shown to be slightly bitter (Kiritsakis, 1998). Moreover, the meatballs supplemented with 0.2% aqueous extract showed no significant differences in any of the sensory scores as compared to control. *E. officinalis* fruit extracts could have an impact on unpleasant sensory characteristics in the meatballs with increasing levels of supplementation. Therefore, the use of *E. officinalis* fruit extracts in the meatballs should be carefully formulated to avoid a deterioration of the sensory quality of the products.

Table 6. Sensory analysis of pork meatballs supplemented with *E. officinalis* fruit crude extracts.

Treatments	Sensory scores (9-point hedonic scale) in pork meatball					
	Appearance	**Colour**	**Odour**	**Flavour**	**Texture**	**Overall acceptability**
Control	6.02 ± 1.39^{b}	6.48 ± 1.25^{ab}	5.36 ± 1.50^{ab}	5.40 ± 1.77^{b}	4.88 ± 1.66^{e}	5.79 ± 1.49^{ab}
0.02% BHT	6.36 ± 1.28^{a}	6.81 ± 1.13^{a}	5.67 ± 1.62^{a}	5.90 ± 1.54^{a}	5.57 ± 1.52^{ab}	6.10 ± 1.64^{a}
0.2% Distilled water extract	6.38 ± 1.53^{a}	6.33 ± 1.41^{ab}	5.45 ± 1.52^{ab}	5.79 ± 1.85^{ab}	5.64 ± 1.54^{ab}	5.52 ± 1.35^{b}
0.4% Distilled water extract	6.12 ± 1.27^{ab}	6.26 ± 1.21^{ab}	5.38 ± 1.43^{ab}	5.40 ± 1.48^{b}	5.21 ± 1.55^{c}	5.36 ± 1.27^{c}
0.8% Distilled water extract	5.95 ± 1.45^{b}	6.02 ± 1.32^{b}	5.38 ± 1.53^{ab}	4.88 ± 1.66^{d}	4.67 ± 1.58^{f}	4.88 ± 1.47^{d}
0.2% Ethanolic extract	5.62 ± 1.64^{c}	5.95 ± 1.39^{b}	5.57 ± 1.40^{a}	5.74 ± 1.48^{ab}	5.74 ± 1.38^{a}	5.88 ± 1.17^{ab}
0.4% Ethanolic extract	5.48 ± 1.49^{c}	5.60 ± 1.60^{c}	5.17 ± 1.38^{b}	5.50 ± 1.45^{b}	5.33 ± 1.36^{b}	5.57 ± 1.43^{b}
0.8% Ethanolic extract	5.10 ± 1.39^{d}	5.36 ± 1.51^{d}	4.74 ± 1.43^{c}	5.10 ± 1.45^{c}	5.02 ± 1.44^{d}	5.24 ± 1.48^{c}

Values are mean ± SD of three independent experiments (n = 3). Means followed by different lowercase superscripts in the same column represent significant difference among the treatments ($p \leq 0.05$).

Conclusion

The present work demonstrated the beneficial values of applying *E. officinalis* fruit extracts as natural-based food additives in pork meatballs. Results showed that the crude extracts significantly extended the shelf life of the meatballs under refrigerated storage for up to 18 days while still maintaining their antioxidant and antimicrobial activities that protected the product throughout the storage period. In addition, the crude extracts also generated a positive effect with regard to retarding lipid oxidation in the meatballs, even after storage in aerobic conditions. Based on the sensory analysis, however, fortifying the pork meatballs with the crude extracts resulted in a decrease in the sensory attributes with increasing amount of supplementation. Of all samples, 0.2% ethanolic extracts had the highest potential for use as a natural preservative in the meatballs based on its promising antioxidant properties, as well as retaining adequate overall acceptability attributes as compared to the controls. Although the crude extracts could extend the shelf life while maintaining the product quality of the meatballs, it should be pointed out that further studies are required to develop an appropriate formula for improving issues related to the unpleasant sensory characteristics related to the crude extracts.

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