Saudi Journal of Biological Sciences 30 (2023) 103663

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Saudi Journal of Biological Sciences

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Original article

Chemical composition, antioxidant and antibacterial activity of *Piper chaba* stem extracts with preservative effects on storage of raw beef patties

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ARTICLE INFO

Article history:
Received 10 January 2023
Revised 2 April 2023
Accepted 21 April 2023
Available online 29 April 2023

Keywords:
Piper chaba
Phytochemicals
HPLC
Preservative
Oxidative stability

ABSTRACT

Piper chaba, a traditional South-east Asian medicinal herb and well-known curry spice, was studied to evaluate its suitability as a source of natural preservatives for beef products. Plant extracts that are high in phenolics and have high antimicrobial and antioxidant activities are likely to be useful as a natural preservative. Therefore, the phytochemical composition and the bioactivities of both ethanolic and methanolic extracts of *P. chaba* stem were examined first. The study revealed a significant antioxidant activities and potential antibacterial activity of *P. chaba* extracts. Next we investigated the preservation characteristics of *P. chaba* by using beef patties as a model system. Beef patties were produced and treated with 0.2 % ethanolic extract (mentioned as PEE) of *P. chaba* and 0.1 % commercial preservative (mentioned as PCP). They were then assessed for various storage quality parameters under refrigerated (4° C ± 1° C) conditions, including free fatty acid, antioxidant contents, and oxidative stability at 0, 6th, 16th, and 33rd days. No significant variations were observed across the products with regard to proximate composition study such as protein, ash and fat contents. In comparison to both PEE and PCP, the control product had higher free fatty acid values throughout the storage period. This indicates that the fat content of the PEE and PCP degraded at a slower rate than the control over the 33-day storage period. Our study also showed that both PCP and PEE had increased antioxidant capacity, implying that lipid oxidation is minimized. In contrast to the control, the oxidative stability of the *P. chaba* treated products was also higher. Altogether this study revealed that *P. chaba* could be utilized commercially, particularly in the food industry to preserve muscle foods.

Practical Applications: Natural preservatives are becoming more popular as a result of the different carcinogenic and toxic side effects of conventional preservatives. *P. chaba,* an exquisite culinary herb in Bangladesh, has long been used as a traditional medicine, because of its antimicrobial and antioxidant properties. This study revealed that *P. chaba* can be utilized as a food preservative, which opens up new possibilities for its development and use in functional foods.

1. Introduction

Muscle-based food products deteriorate quickly during storage, due to reactive oxygen species generated by bacteria or the oxidation process. Textural degradation, off-flavor development, rancidity, discoloration, and a change in the nutritional value of meat products are all caused by lipid oxidation (Purriños et al., 2011). Antioxidants, on the other hand, have the ability to slow down the progression of this process (Lorenzo et al., 2018). Hence, artificial preservatives or antioxidants, for example butylated hydroxy-

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2023.103663
1319-562X/

toluene, sodium benzoate and butylated hydroxyanisole are frequently utilized to boost the oxidative stability of foods and prevent spoiling (Yang et al., 2018). Antioxidants thus help enhancing the quality of meat products by extending their shelf-life (Sun and Holley, 2012). However, as people become more concerned about the possible carcinogenicity and toxicity of these man-made preservatives, the demand for natural plant-based preservatives is growing (Sun and Holley, 2012; Saito et al., 2003; Clayson et al., 1986; Gutiérrez-Del-río et al., 2021).

Many plant extracts containing high phenolic compounds, such as those found in vegetables, herbs, spices, and fruits, are discovered that exhibit potent antioxidant and antimicrobial activities, leading to their use as effective and safer alternatives to artificial preservatives in the food industry (Bañón et al., 2007; Yogesh et al., 2012). *P. chaba* Hunter from the 'Piperaceae' family, locally known as Chui Jhal, is an economically important edible flowering vine that can be found throughout the warmer regions of Asia, including Bangladesh (Islam et al., December 2019; Taufiq-Ur-Rahman et al., 2005; Salehi et al., 2019; Jadid et al., 2018; Islam et al., 2015). It is generally exploited as an exquisite culinary herb here but also possesses a variety of pharmacological and biological properties, including immunomodulatory and anticancer actions (Islam et al., December 2019; Islam et al., 2015). Most members of the genus '*Piper*' have found widespread usage in traditional medicine to treat a variety of disorders, with the plant *P. chaba* being traditionally used for diarrhea, rheumatic pains, gastralgia, dyspepsia, piles, asthma, and other ailments (Taufiq-Ur-Rahman et al., 2005; Jadid et al., 2018; Islam et al., 2015; Islam et al., 2020; Panphut et al., 2020; 2020.). In addition, several species of the '*Piper*' genus have significant antioxidant properties when compared to synthetic antioxidants and antibacterial activities against many foodborne pathogens, suggesting that they could be used as readily available natural preservatives for food preservation (Salehi et al., 2019; Gülçin, 2005; Naz et al., 2012). However, though several types of extracts prepared, as well as compounds isolated from different parts of the *P. chaba* plant, were found to exhibit notable antimicrobial activity against both Gram + ve and –ve classes, the similar activity and also the antioxidant activity of stem extracts has yet to be investigated.

Therefore, in this study, we explored the extracts prepared from the stem of *P. chaba* for phytochemical contents as well as for antioxidant and antimicrobial activities in vitro. The suitability of the produced extracts for preserving meat products was next investigated by applying the extracts in situ to some prepared beef patties and storing them in a refrigerated setting for a set period of time.

2. Materials and methods

2.1. Chemicals and reagents

Sulfuric acid, methanol, ethanol, hydrochloric acid, potassium hydroxide, peptone, sodium hydroxide, and chloroform were purchased from Merck, USA. Polyphenolic standards such as catechol (Cat), catechin hydrate (CH), vanillic acid (VA), gallic acid (GA), vanillin (Van), syringic acid (Syr), caffeic acid (CafA), chlorogenic acid (CA), benzoic acid (BA), tannic acid (TA), rutin hydrate (RH), p-coumaric acid, (-)-epicatechin, *trans*-cinnamic acid (TCA), quercetin hydrate (QH) and curcumin (Cur) as well as aluminum chloride ($AlCl_3$), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ammonium molybdate and Folin-Ciocalteu reagent were bought from Sigma-Aldrich, USA. Sodium chloride was collected from Carl Roth, Germany. Sodium carbonate and Sodium acetate were purchased from Wako, Japan. Yeast extract was obtained from Oxoid, Canada

2.2. Sample collection and processing

P. chaba stem samples were purchased from a local retail shop in Khulna, Bangladesh (22.6738° latitude, 89.3967° longitude) and brought to the lab for analysis. To remove any dirt, the stems were properly cleaned with tap water first, then distilled water. After being sliced into small pieces and dried at 60 ^{0}C in a drier (Memmert GmbH + Co. KG, Germany), the samples were turned into fine powder using a mortar and pestle.

2.3. Preparation of extracts

The powdered sample was mixed with methanol and ethanol separately in a 1:10 ratio to prepare two different extracts. The mixtures were incubated for five days at 120 rpm with continuous shaking in a shaking water bath (GFL-3005, Germany). After the stipulated time, the crude mixtures were filtered using Whatman filter paper No. 1, and then concentrated by evaporation at 60 °C in a rotary evaporator (IKA RV10 and HB10, Germany). The thickened samples were then kept in an oven at 50 °C overnight to remove any leftover solvents. Finally, the dried extracts of both the ethanol and methanol were preserved in the refrigerator at 4 °C for future application.

2.4. Quantitative determination of phytoconstituents

2.4.1. Total phenolic compounds analysis

The amount of total phenolics in the ethanolic and methanolic extracts of *P. chaba* was determined using the Folin-Ciocalteu reagent assay with slight modifications (Singleton et al., 1999). Briefly, each sample extract was diluted in three separate solvents to achieve three different concentrations (100, 200 and 1000 ppm). After that, 0.5 mL of each diluted sample was mixed with 8 mL of distilled water and 0.5 mL of Folin-Ciocalteu reagent (Merck 1090010100), respectively. The reaction was left to stand for 5 min. Then 1 mL of 35 % of Na_2CO_3 was added to the mixture. After 20 min of incubation, the absorbance was measured at 765 nm using a UV–VIS Double Beam Spectrophotometer (Thermo Fisher Scientific, Evolution 300, USA), against a blank containing only the respective solvents. Total phenolics were quantified by using a standard calibration curve of gallic acid (y = 0.0048x + 0.0153 and R^2: 0.9968). Results were expressed as mg of gallic acid equivalents (GAE)/g of fresh weight.

2.4.2. Determination of total tannin content

The tannin content was determined using a modified protocol of Folin-Ciocalteu Reagent assay (Singh et al., 2012; Marinova et al., 2005). Initially, a 35 % of Na_2CO_3 solution was prepared. Eight milliliters of distilled water were added to 0.5 mL of sample produced with the appropriate solvents, followed by 0.5 mL of Folin-Ciocalteu Reagent, and incubated at room temperature for 5 min. By adding 1 mL of 35 % of Na_2CO_3 to the mixtures, they were well mixed before being incubated for another 20 min at room temperature for color development. Finally, the absorbance was measured at 725 nm using a UV–VIS Double Beam Spectrophotometer against a blank containing only the corresponding solvent. The standard utilized here was tannic acid (y = 0.0055x – 0.0081 and R^2: 0.9973). The tannin content was expressed as mg of tannic acid equivalents (TAE)/g of fresh weight.

2.4.3. Total flavonoid assay

A previously described aluminum chloride colorimetric assay was used to estimate the flavonoid content with some modifications (Zhishen et al., 1999). In 100 mL of distilled water, 0.3325 g of $AlCl_3$ and 1 g of crystalline sodium acetate were mixed to make a reagent. Then, 2.5 mL of produced reagent and 4.8 mL of distilled

water were combined with 0.2 mL of sample generated with different solvents at varied concentrations. After that, the mixes were incubated at room temperature for 5–6 min. When the color formed, the absorbance was measured against a blank with the same solvent at 430 nm using a UV–visible spectrophotometer. Total flavonoid content for both extracts was expressed as mg quercetin equivalent (QE)/g extract ($y = 0.0047x - 0.0013$ and R^2: 0.9927).

2.5. Estimation of total antioxidant content

The phosphomolybdenum assay was used with some modifications to determine the total antioxidant content in the extracts (Prieto et al., 1999). A reagent solution was made by mixing 4 mM ammonium molybdate, 28 mM sodium phosphate, and 0.6 M sulfuric acid in a 4:2:4 ratio. Then, 3 mL of the produced reagent was added and vortexed after 0.5 mL of sample solution was mixed with 0.5 mL of distilled water. The mixtures were allowed to sit at 95° C for 90 min for color development before being measured in a UV–visible spectrophotometer at 695 nm against a blank. Here, ascorbic acid was used as a standard ($y = 0.0024x - 0.0137$ and R^2: 0.9879). Total antioxidant content was expressed in mg ascorbic acid equivalent (AAE)/g extract.

2.6. HPLC-DAD quantification of polyphenols

2.6.1. Preparation of sample and standard

Before HPLC (high performance liquid chromatography) analysis, 50,000 ppm of both ethanolic and methanolic extract solutions were prepared, then filtered using a 0.45 μm nylon syringe filter.

The standard stock solution (50,000 ppm) for each phenolic compound was prepared by weighing approximately 0.05 g of the analytes into 10 mL of methanol. By combining the standard solutions in a certain order, the mixed standard solution was made.

2.6.2. HPLC system

The chromatographic study was conducted using a Dionex UltiMate 3000 Rapid Separation LC (RSLC) system (Thermo Fisher Scientific Inc., MA, USA). The system was coupled to a rapid separation diode array detector (DAD-3000RS), Ultimate 3000RS autosampler (WPS-3000), and quaternary rapid separation pump (LPG-3400RS). Polyphenols were separated at 30 °C on an Acclaim® C18 (4.6 × 250 mm; 5 μm; 120 A°) column (Dionix, USA). Data acquisition, peak integration, and calibrations were performed using Dionix Chromeleon software (Version 6.80 RS 10).

2.6.3. Chromatographic conditions

A method previously reported by Liaudanskas, was used to determine the phenolic compositions of *P. chaba* stem extracts (Liaudanskas et al., 2014; 2014.). The mobile phase consisted of acetic acid solution pH 3.0, acetonitrile and methanol. The injection volume used was 20 μL and the flow rate was kept constant at 1 mL/min. The detection and quantification were carried out at 280 nm.

2.6.4. Peak characterization and quantification

The polyphenols were identified by comparing them to the standards given in Fig. 2. The retention time and the absorbance spectrum profile were used to identify each component. Calibration curves for each standard were used to accomplish quantification.

2.7. Bioactivity assay

2.7.1. DPPH scavenging assay

The popular DPPH assay was followed as described previously (Chang et al., 2001) for evaluating the antioxidant activity of *P. chaba* extracts with few modifications. In brief, 0.1 mM DPPH solutions were produced independently in methanol and ethanol, with ascorbic acid serving as a standard. One milliliter of this freshly prepared DPPH solution was then mixed with three milliliters of various concentrations (0–1000 ppm) of the sample extracts and standards. Finally, absorbance was read in a UV–visible spectrophotometer at 517 nm after incubating at room temperature for 30 min in the absence of light. The formula given below was used to calculate the % inhibition or DPPH scavenging activity.

$$\% \text{ of inhibition} = [(\text{Absorbance of control} - - \text{Absorbance of test sample})/\text{Absorbance of control}] \times 100.$$

IC50 value (i.e., the amount of plant extract required to prevent the formation of free radicals by 50%) was calculated from the regression line obtained. The control sample was made with methanol/ethanol and DPPH. The blanks were ethanol and methanol.

2.7.2. Antibacterial activity determination

The antimicrobial property of *P. chaba* extracts was studied in vitro using the disc diffusion method (Lai et al., 2010). Pure cultures of both Gram-positive (e.g., *Bacillus cereus* and *Staphylococcus aureus*) and Gram-negative (e.g., *Escherichia coli* and *Salmonella typhi*) bacteria were obtained from the Department of Microbiology at Jahangirnagar University. Both groups of bacteria were tested against three differently concentrated solutions (e.g., 0.02, 0.05, and 0.1 g/mL) of impregnated discs (Whatman filter paper No. 1.) of crude extracts. The positive control was a standard antibiotic disc (Oxoid Ltd., UK) soaked in 20 μL of 0.1 g/mL ampicillin solution, while the negative control was a blank disc soaked in solvent only. To analyze the antibacterial properties of the samples, the diameter of inhibition zones was measured on a millimeter scale.

2.8. Application of P. Chaba ethanolic extract to beef patties preservation

2.8.1. Preparation of beef patties

Beef was purchased from a nearby supermarket, packaged in low-density polyethylene (LDPE) containers, and delivered to the lab in less than an hour. The animal was slaughtered two hours before being purchased, in accordance with customary halal practices. However, to achieve the ideal softness and palatability in our products, the beef was collected in an 80:20 ratio of flesh to fat. The meat was thoroughly minced in the lab (MK-MG1360W, Panasonic, Malaysia) after being thoroughly cleaned in tap water. The minced beef was then split into three parts, each weighing around 1.5 kg, and placed in three clean dishes. Three types of products were made from these separated portions: Control (contained only spices but no ethanol extract or butylated hydroxyl toluene (BHT), Product with Ethanolic Extract (PEE; contained spices and 0.2 % ethanol extract but no BHT), and Product with Commercial Preservative (PCP; contained spices and 0.1 % BHT but no ethanol extract). The composition of these products is presented in Table 1. The beef samples were thoroughly mixed after adding all of the ingredients, and then they were physically shaped into 54 patties (18 patties for each treatment), each weighing approximately 83 gm. Finally, these patties were refrigerated at 4 °C ± 1 °C after being properly wrapped in low-density polythene wrap. To examine the

Table 1
Ingredients used for beef patties preparation.

SL. No.	Ingredients	Control	(PEE)	(PCP)
1	Minced beef	1500 g	1500 g	1500 g
2	Onion paste	300 g	300 g	300 g
3	Garlic paste	5 g	5 g	5 g
4	Ginger paste	5 g	5 g	5 g
5	Hot spices	10 g	10 g	10 g
6.	Black pepper	12.5 g	12.5 g	12.5 g
7	Egg	1 piece	1 piece	1 piece
8	Bread	1 slice	1 slice	1 slice
9.	Salt	9 g	9 g	9 g
10	Oil	5 mL	5 mL	5 mL
11	Red chili powder	4.5 g	4.5 g	4.5 g
12	*P. chaba* ethanol extract	–	3 g	–
13	BHT	–	–	1.5 g

BHT = Butylated hydroxytoluene. PEE: Product with Ethanolic Extract, PCP: Product with Commercial Preservative.

effect of extract in meat products, free fatty acid, antioxidant content, and oxidative stability were measured at 0, 6, 16, and 33 days.

2.8.2. Analytical methods

The moisture, total ash, total fat, and total protein contents of our prepared beef patties were determined as per the standard procedures of Association of Official Analytical Chemists (AOAC) (Aoac, 2005). The following equation was used to calculate the total carbohydrate content:

Total carbohydrate = 100 – Sum of percentages of ash, protein, fat, and moisture contents.

Therefore, the energy values of our beef patties calculated by Atwater's conversion factor are estimates instead of actual values. However, for the measurement of free fatty acid (FFA), chloroform–methanol solvent extraction system described by Folch et al. was followed (Folch et al., 1957). Finally, the oxidative stability of fat content was evaluated following the American Oil and Chemists Society (AOCS) cd 12b-92 method (Sultana et al., 2017; Determine oxidative stability of oils and fats based on AOCS Cd 12b-92 | Scientist Live, 2022).

2.9. Statistical analysis

SPSS software was used to perform the Statistical analysis. Each experimental value is represented as a mean ± standard error of the mean (SEM). Using two-way ANOVA, the effect of *P. chaba* ethanolic extract on the physicochemical qualities of beef patties was evaluated. The experiments were carried out a total of four times (n = 4). In all cases of analysis, statistical significance was considered to be as $p < 0.05$.

3. Results

3.1. Phytochemical screening and total antioxidant contents of P. Chaba extract

The results revealed that the ethanolic extract of *P. chaba* had a relatively higher flavonoid and phenol content than the methanolic extract, and that it also had a significantly higher level of tannin and antioxidant content. The levels of flavonoid, phenol, and tannin content in the ethanolic extract were 4.64 ± 0.17 mg QE/g, 5.02 ± 0.42 mg GAE/g and 5.88 ± 0.39 mg TAE/g respectively, while they were 3.07 ± 0.36 mg QE/g, 3.97 ± 0.21 mg GAE/g and 4.76 ± 0.12 mg TAE/g in the methanolic extract (Fig. 1A - C). Both the ethanolic and methanolic extracts had antioxidant levels of 20.742 ± 2.80 mg AAE/g and 18.72 ± 1.55 mg AAE/g, respectively (Fig. 1D).

3.2. Analysis of phenolic contents by HPLC

Figs. 2, 3A and B show the HPLC results for standards, methanolic, and ethanolic extracts, respectively. In both ethanolic and methanolic extracts, eight polyphenols were detected, including CH, Van, Syr, CafA, CA, RH, TA, QH out of 16 standard polyphenolic compounds screened (Fig. 3). Syr, CA, RH, TA, QH were all found to be high in ethanolic extract. CH, CafA, and Van were found in higher amounts in methanolic extract than in ethanolic extract (Table 2). However, QH was discovered to be the most abundant enriched polyphenol in both extracts.

3.3. Antioxidant activity assay

Fig. 4 depicts the percentage (%) of DPPH inhibition against increasing concentration of ascorbic acid, ethanolic and methanolic extracts. The antioxidant activity of both the ethanolic and methanolic extracts was high. Logarithmic regression was used to calculate the IC50 values of the extracts. The ethanolic extract (234.23 μg/ml) had a lower IC50 value than the methanolic extract (312.56 μg/ml), indicating that it had greater DPPH scavenging properties.

3.4. Antibacterial activity

Both extracts had antimicrobial activity against all microorganisms at the maximum dose (0.1 g/ml) (Table 3). Among the extracts, the 0.1 g/ml methanol extract had the highest antibacterial activity against Gram-negative *S. typhimurium*, forming a clear zone of inhibition of 22.5 ± 0.5 mm (Fig. 5). The same extract had a zone of inhibition of 19.5 ± 0.5 mm against Gram-positive *B. cereus*. Overall, the methanolic extract outperformed its ethanolic counterpart in terms of antibacterial activity. At a concentration of 0.02 g/ml of the ethanolic extract, no zone of inhibition was detected against *S. typhimurium* and *E. coli*. When the methanolic extract's concentration was reduced from 0.1 g/ml to 0.05 and 0.02 g/ml, no antibacterial activity against *E. coli* was found.

3.5. Proximate composition of P. chaba extract

After the stipulated storage period, there was a slight reduction in moisture content, which was comparable and can be interpreted as Control > PCP > PEE (Table 4). In terms of ash, protein, and fat content, no significant differences were detected across the products. However, among all nutrients, protein had the highest percentage (about 30%) in all products. In addition, PEE and PCP had a little larger energy content than control.

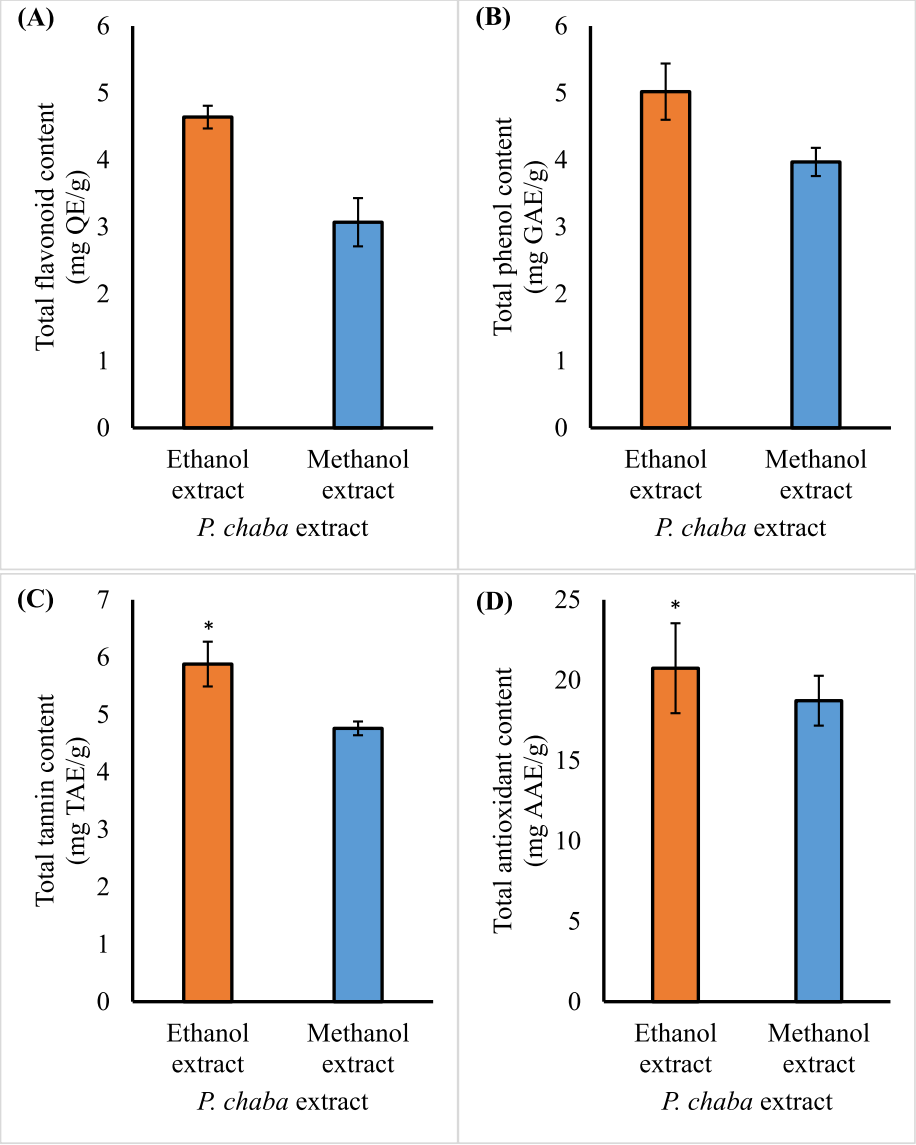

Fig. 1. Quantitative determination of phytochemicals and antioxidant content in *P. chaba* stem extract. The relative amounts of (A) TFC, (B) TPC, (C) TTC, and (D) TAC in various extracts are presented. The data represent the mean value ± SD (n = 4). Statistical significance, which was determined as $p < 0.05$, is indicated by an asterisk (*).

3.6. Profiling free fatty acid (FFA) content in beef patties

FFA is formed when lipids are degraded by bacteria or enzymes (Das et al., 2008). Determination of FFA reveals the stability of fat contents of products during storage. With the progression of the refrigerated storage period in the current study, the mean value of FFA % increased among all products (Fig. 6A). At day 33, the FFA % in the control product was almost five times higher than it was at day 0. During the storage period, the rate of FFA % rise in both PEE and PCP remained nearly constant. This indicates that the fat content of the PEE and PCP decreased at a slower rate than the control over the 33-day storage period.

3.7. Total antioxidant content in beef patties

The PCP had the highest antioxidant content (29.36 ± 0.26 mg AAE/g), followed by PEE (25.24 ± 0.16 mg AAE/g) and Control (20.64 ± 0.52 mg AAE/g) (Fig. 6B). All the products had a gradual decrease in antioxidant content, with control having the highest reduction (85.65%). PEE and PCP, on the other hand, showed a 60.18 % and 40.40 % decline in antioxidant content. As a result, it implies that PEE had greater antioxidant content than the control, preventing ongoing oxidation.

3.8. Evaluation of oxidative stability of beef patties

For all beef products, the oxidative stability test revealed a gradual decrease in the temporal duration of oxidative stability, which was reported as Induction Time (Fig. 6C). In comparison to the PEE, which had a more favorable outcome, the control had a quick decline in oxidative stability. PCP had the highest oxidative stability (7.10 ± 0.09 h) followed by PEE (6.27 ± 0.14 h) and Control (4. 36 ± 0.23 h). The order of induction time reduction in terms of higher to lower can be interpreted as control (90.01 %) > PEE (67.27 %) > PCP (43.99 %).

4. Discussion

Plant extracts have been found to exhibit several pharmacological and biological properties, including antioxidant, antimicrobial,

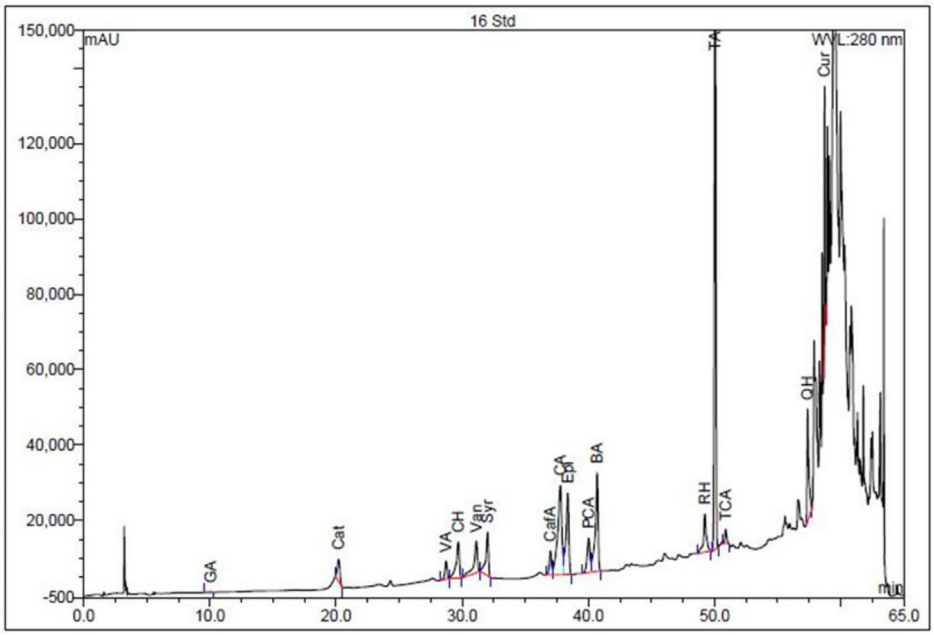

Fig. 2. HPLC chromatogram of 16 mixed polyphenol standard solutions. GA, Cat, VA, CH, Van, Syr, CafA, CA, EC, PCA, BA, RH, TA, TCA, QH and Cur.

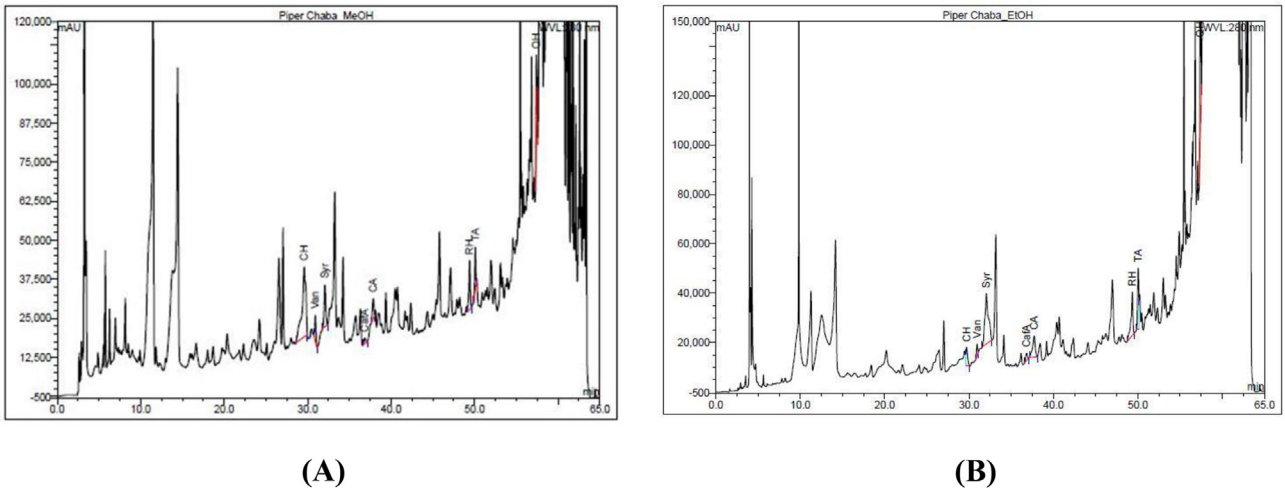

Fig. 3. HPLC separation of polyphenolic compounds identified in (A) methanol and (B) ethanol extracts prepared from stems of *P. chaba*. Peaks. CH, Van, Syr, CafA, CA, RH, TA, QH.

Table 2
Quantity of polyphenols identified in *P. chaba* stem extracts (mean ± SD).

Polyphenol	Ethanolic extract	Methanolic extract	P-value
Catechin hydrate	122 ± 0.8	841 ± 1.23	0.000004
Vanillin	31.2 ± 0	60.4 ± 0.89	0.00071
Syringic acid	406.8 ± 2.1	123.4 ± 1.21	0.000067
Caffeic acid	277.2 ± 0.76	530.8 ± 0	0.003463
Chlorogenic acid	104 ± 3.4	47.4 ± 0	0.00787
Rutin hydrate	403.8 ± 0.65	275.2 ± 2.89	0.00114
Tannic acid	180.2 ± 0.2	73.6 ± 1.2	0.0005
Quercetin hydrate	2704 ± 2.9	1672 ± 1.39	0.0145

antidiabetic, and anticancer properties. Antimicrobials and antioxidants isolated from natural sources are not only considered safe, but also compostable (Islam et al., December 2019; Salehi et al., 2019; Ben et al., 2011; Gadekar et al., 2014). In this study, extracts prepared from the stem of *P. chaba* were examined for their ability to preserve raw beef patties during storage. In recent decades, researchers have focused on agriculture products and by-products as a potential source of natural preservatives due to high antioxidant and antimicrobial content (Gutiérrez-Del-río et al., 2021; Salehi et al., 2019; Kalem et al., 2017; Hardur Ven et al., 2020). The antioxidant properties of *P. chaba* are due to the presence of phytochemicals, including tannin, phenol, and flavonoid content (Taufiq-Ur-Rahman et al., 2005; Salehi et al., 2019; Jadid et al., 2018; Islam et al., 2015; Naz et al., 2012). Phytochemicals were found in both extracts, however they were more soluble in ethanol than in methanol. Overall, the ethanol extract performed better in terms of antioxidant activity, which may be attributed to the higher concentration of phytochemicals present in it. Our results also supported a previously conducted study that claimed ethanol as a better solvent than methanol for phenolic, flavonoid, and other phytochemicals extraction (Do et al., 2014). However, before adding the plant extracts to the meat products for in situ

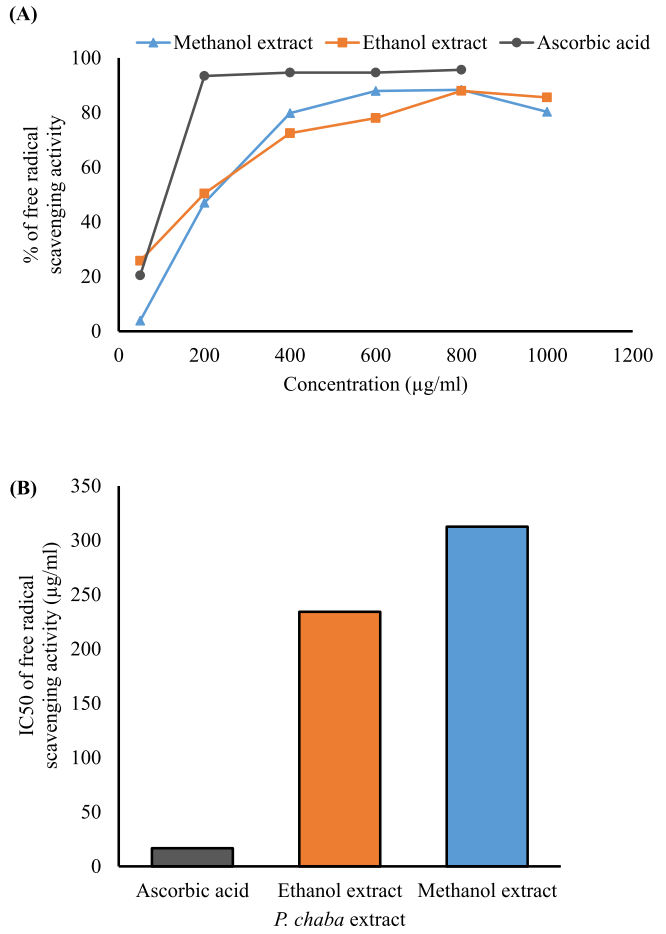

Fig. 4. DPPH free radical scavenging activity of *P. chaba* extracts. (A) A dose–response curve is plotted for the percentage of DPPH scavenging by the ascorbic acid, methanol, and ethanol extract. (B) Comparison of ascorbic acid and different extracts in terms of IC50 values.

measurement of antioxidant and free fatty acid concentrations as well as oxidative stability, each plant extract's antimicrobial and antioxidant properties were assessed in vitro after quantifying specific polyphenols using HPLC.

Polyphenols are one of the most investigated natural compounds due to their health benefits (Del Rio et al., 2010). Anticarcinogenic, antimicrobial, anti-arthritic, antihypertensive, cardioprotective, anti-inflammatory, anti-allergic, and antioxidant effects have been associated to polyphenols produced from diverse natural sources (Dai and Mumper, 2010). Here we have identified a total of eight polyphenols in both ethanol and methanol extracts of *P. chaba* (Table 2), including catechin hydrate (CH), vanillin (Van), syringic acid (Syr), quercetin hydrate (QH), caffeic acid (CafA), chlorogenic acid (CA), rutin hydrate (RH), and tannic acid (TA) using HPLC. The presence of these polyphenols can thus be linked to the antioxidant and antibacterial effects of *P. chaba* stem extracts observed in our study. However, these bioactive compounds were divided into two categories. Syr, CA, RH, TA, and QH were found to have high in ethanolic extract, whereas CH, CafA, and Van were found to have high in methanolic extract. This could be owing to the polyphenols' varying solubility in different solvents. Phytochemicals belonging to classes of alkaloids, phytosterol, epoxylignane were previously been screened in *P. chaba* (Islam et al., 2015) but there was insufficient information on the polyphenolic profile of methanol and ethanol extract from the same research. Hence, our research could be a first in this field.

The in vitro antioxidant study was conducted using the DPPH assay, which has been used in the most number of studies to date and is recognized as a simple, sensitive, reliable, and rapid approach (Moon and Shibamoto, 2009). In the present study, we found comparatively better DPPH scavenging activity in ethanolic extract than its methanolic equivalent. Previously, it was reported that the antioxidant property of plant extract relies on the presence of phenolic, flavonoid, and other phytochemical compounds (Conde-Hernández and Guerrero-Beltrán, 2014). Our study justifies the fact as ethanolic extract contained higher phytochemical content and exhibited greater free radical scavenging activity. Most of the phenolic compounds detected were also found enriched in ethanolic extract. It is known that phenolic compounds have inhibitory effect on hydrogen peroxide-induced lipid peroxidation in biological homogenates (Yang et al., 2009). By contributing electrons, the extract's phenolic components can also neutralize hydrogen peroxide by scavenging it (Ebrahimzadeh). According to the previous study, phenolic compounds are potent hydrogen donors, which give them the feature of being good antioxidants (Yen et al., 1993). The redox characteristics of phenolic compounds, which can be useful in adsorbing and neutralizing superoxide anion ($O_2^{-\bullet}$), hydroxyl radical, or peroxy radicals, quenching singlet and triplet oxygen, or dissolving peroxides, are primarily responsible for their antioxidant action (Osawa, 1994). By converting free radicals and reactive oxygen species into more stable products, phenolic contents seem to serve as potent electron and hydrogen atom donors, interrupting the radical chain reaction that leads to lipid peroxidation (Das et al., 2011). Therefore, the findings of our antioxidant activity might be attributed to these modes of

Table 3
Antibacterial activity assay of *P. chaba* stem extracts (mean ± SD).

Bacteria	Concentrations	Ethanol extract	Methanol extract	Positive control Amp (0.1 g/mL)	P-value
Bacillus cereus	0.02 g/mL	6.0 ± 0	8.5 ± 0.5		
	0.05 g/mL	7.5 ± 0.5	9.5 ± 0.5	22.5 ± 0.5	0.0029
	0.1 g/mL	17.5 ± 0.5	19.5 ± 0.5		
Staphylococcus aureus	0.02 g/mL	5.5 ± 0.5	7.5 ± 0.5		
	0.05 g/mL	8.5 ± 0.5	11.0 ± 1.0	19.5 ± 0.5	0.0432
	0.1 g/mL	10.0 ± 0	16.5 ± 0.5		
Salmonella typhi	0.02 g/mL	6.0 ± 1.0	10.5 ± 0.5		
	0.05 g/mL	13.5 ± 0.5	19.0 ± 0	29.5 ± 0.5	0.0446
	0.1 g/mL	10.5 ± 0.5	22.5 ± 0.5		
Escherichia coli	0.02 g/mL	NZ	NZ		
	0.05 g/mL	6.0 ± 0.81	NZ	23.0 ± 0.82	0.236
	0.1 g/mL	11.0 ± 0.8	11.5 ± 0.5		

NZ = No zone of inhibition; Amp = Ampicillin;
Negative control is made up of ethanol and methanol solvents.

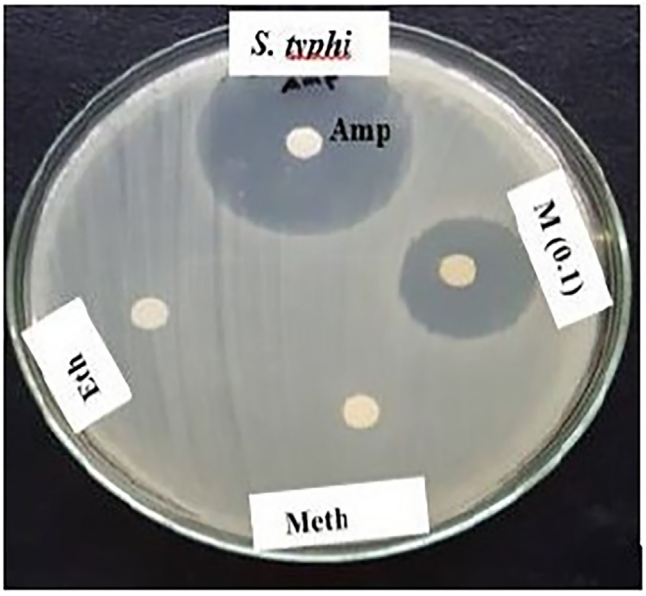

Fig. 5. Inhibition zone created by *P. chaba* methanolic extract against *S. typhi*. M = Methanolic extract, Amp = Ampicillin, Meth = Methanol, and Eth = Ethanol.

Table 4
Proximity analysis of beef patties (mean ± SD).

Parameters	Control	PEE	PCP
Moisture (%)	44.15 ± 0.89	42.88 ± 0.49	43.84 ± 0.193
Ash (%)	4.49 ± 0.5	4.16 ± 0.4	3.03 ± 0.6
Protein (%)	30.63 ± 0.55	31.67 ± 0.68	30.85 ± 0.98
Carbohydrate (%)	17.13 ± 1.27	17.67 ± 1.01	18.68 ± 0.54
Fat (%)	3.5 ± 0.3	3.6 ± 0.21	3.5 ± 0.24
Energy (Kcal)	223.30 ± 3.95	230.12 ± 4.7	230.38 ± 4.49

PEE = Product with Ethanolic Extract; PCP = Product with Commercial Preservative.

action and also suggests that the ethanolic extract of *P. chaba* might be a potential source of natural antioxidants.

The antimicrobial property of *P. chaba* stem extract was determined by evaluating the extract's ability to suppress the growth of four potent foodborne pathogens, namely *S. typhi*, *S. aureus*, *E. coli*, and *B. cereus* using the disc diffusion method. At the highest doses of both extracts, strong antibacterial activity was observed. Inhibitory zones were detected against Gram-positive bacteria at all doses of both extracts. In Gram negative bacteria, however, no obvious inhibitory zone was found at low concentrations of the extracts. Based on the inhibition zones observed, *S. typhi*, however, was shown to be the most sensitive in methanolic extract, followed by *B. cereus*, *S. aureus*, and *E. coli*. Furthermore, ethanolic extract exhibited a different order in terms of higher to lower sensitivity (i.e., *B. cereus* > *S. typhi* > *E. coli* > S. *aureus*). The antibacterial activity found for methanol extract against *B. cereus*, *S. typhi*, and *S. aureus* was close to that of standard ampicillin, suggesting that the extract had strong antibacterial potential. Overall, the methanolic extract had better antibacterial action against both types of bacteria, especially *S. typhi*. This might be owing to the bacteria's great susceptibility to the polyphenols present in abundance in this extract, such as CA, vanillin, and CH. Many reports suggest the efficient use of vanillin as antimicrobial agent in food preservation (Das et al., 2011). The aldehyde group and the side group of the benzene ring are responsible for the antibacterial activity of vanillin. Due to vanillin's hydrophobic nature, the majority of its antimicrobial mechanisms are based solely on its capacity to damage the cytoplasmic membrane of microorganisms through interactions

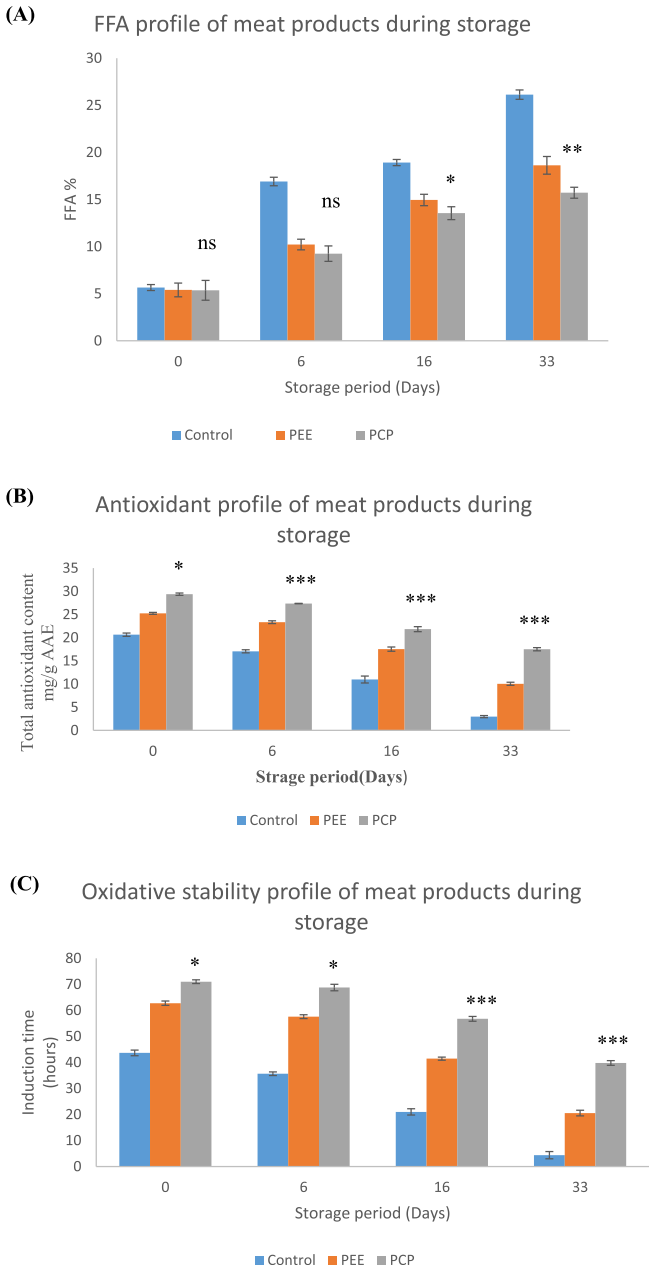

Fig. 6. Effect of *P. chaba* ethanolic extract on physicochemical quality of beef patties during refrigerated storage. (A) FFA % (B) antioxidant content, and (C) oxidative stability profile of beef patties treated with *P. chaba* ethanolic extract vs control and commercial preservative BHT. Data are expressed as mean ± SEM, n = 4. Two-way ANOVA was used to perform statistical analysis. Statistical significance was considered as ns. not significant, * $P \leq 0.05$, ** $P \leq 0.01$, and ***$P \leq 0.001$.

with lipids, proteins, or both structures, resulting in the depletion of the gradient ionic and the suppression of oxidative metabolism (Fu et al., 2007). Thus, in accordance with previous findings (Rawat et al., 2016; Ouerghemmi et al., 2017), our antibacterial assay reflected the intricacy of the events involved in antibacterial activity, which is dosage and species dependent.

Around 76 million people were found to be affected by foodborne infections in the United States alone, resulting in roughly 5000 fatalities per year. Hence, to extend the keeping quality of prepared foods, artificial preservatives such as sulfur dioxide, calcium propionate, BHT, and BHA, among others, are used (Seetaramaiah et al., 2011). Antioxidants, antimicrobials, and antibrowning agents are the three most often used preservatives.

However, because prolonged use of these preservatives can result in the accumulation of chemical residues, which can cause major adverse effects, as well as the development of microbial resistance, medicinal plants with higher polyphenol levels are frequently sought (Rawat et al., 2016; Seetaramaiah et al., 2011; Anand and Sati, 2013), because these plants have the potential to replace synthetic preservatives. The presence of a higher quantity of polyphenols and phytochemicals in the ethanolic extract of *P. chaba*, as well as strong antibacterial and antioxidant activities, prompted us to test it in situ on raw beef patties to determine its preservation activity in our study. Plant extracts with high antibacterial activity were previously used to protect food from food-borne diseases (Gutiérrez-Del-río et al., 2021; Gadekar et al., 2014; Kalem et al., 2017; Seetaramaiah et al., 2011).

In the proximity analysis, all the preparations had a composition that was more or less similar. This could be due to the usage of the same percentage of lean beef. A prior study found that natural preservatives have no influence on the proximate composition of meat products (Wills et al., 2006). The moisture content was found to be somewhat lower in the current study, which could be due to evaporation loss. El-Nashi et al. reported a similar reduction in moisture content when pomegranate peel powder was used to store beef sausage at refrigerated temperature (El-Nashi et al., 2015).

Rancidity (the formation of typical off-flavors in food) develops, when lipolytic enzymes break down glycerides and produce FFA (Huis In't Veld JHJHI, 1996). The quality of meat can therefore be determined by measuring the amount of FFA formed. In our study, the FFA values in all formulations increased over time throughout the storage period. According to previous studies, the FFA concentration in meat products may increase over time (Das et al., 2008; Culea et al., 2010). However, both the PEE and the PCP had much reduced FFA % as compare to the control product. That is, the fat content in the PEE degraded less than the control during the storage period. This is in line with prior research that adding antioxidants from natural sources reduced the level of FFA in mutton patties (Malav et al., 2015) and chicken nuggets (Reddy, 2015) when compared to the control product.

A gradual decrease in antioxidant content of the prepared beef patties was observed in our study, which supported the findings of Mancini et al (Mancini et al., 2015). Among the three categories of the prepared products, maximum level of antioxidant reduction was found for the control group (85.65 %), followed by PEE (60.18 %) and PCP (40.40 %) on the last day of storage. The results indicate that the PEE has more antioxidant content than the control product to prevent oxidation from continuing. Sultana et al. found similar results in a study, reporting clove oil and oleoresin as sources of potent antioxidants for beef product preservation at 4 °C (Sultana et al., 2017).

In our oxidative stability study, the control product exhibited a rapid decrease in oxidative stability, but the PEE had a more favorable effect. These findings clearly stated that, when employing *P. chaba* ethanol extract as a natural source of antioxidant, the PEE is better in terms of oxidative stability than the control, validating the findings of Loizzo et al (Polash et al., 2017) and Soyer A. et al (Soyer et al., 2010). According to Ali et al. antioxidant-rich phenolic extracts can interact with lipidperoxy- or lipidoxy-free radicals (produced during lipid oxidation) and prevent them from self-degrading further (El Hadj et al., 2014). They also found that reducing the oxidation process with plant-based phenolic extracts could help fish flesh products last longer.

Rojas and Brewer, carried out a similar investigation on raw meat preservation using natural antioxidants such as oleoresin rosemary extracts, grape seed extracts, and water-soluble oregano extracts (Rojas and Brewer, 2007). Meat products treated with rosemary extract, in particular, showed a considerable amount of antioxidant activity in terms of TBARS value. With the support of these previous findings, our study concluded that *P. chaba* ethanolic extract had a significant impact on avoiding lipid oxidation, thereby increasing the oxidative stability of raw beef products.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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