Ultrasonics as a tool for development of pine-needle extract loaded bee wax edible packaging for value addition of Himalayan cheese

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ABSTRACT

In the present study, Himalayan cheese, kradi was coated with beeswax loaded with pine needle extract (PNE) to increase its shelf life and nutraceutical potential. PNE was extracted via ultrasonication and incorporated into beeswax at concentrations, 2:1, 1:1, and 2:3 (grams of beeswax to mL of PNE). The dispersion of PNE in the coatings was carried out using an ultrasonic probe at a frequency of 20 kHz for 15 min and at power rating of 500 W. The coatings were characterised using scanning electron microscopy, light microscopy, dynamic light scattering (DLS), fourier transmission infrared spectroscopy. DLS revealed a hydrodynamic diameter and zeta potential of 12.11 ± 0.41 µm and −19.32 ± 0.61 mV for coating loaded with highest concentration of PNE. The bioactivities of the coating including antioxidant, anti-diabetic and antibacterial assays revealed significantly higher values with the increase in PNE concentration. Shelf life and sensory evaluation study including microbiological and sensory analysis revealed inhibition of mould growth and good score of texture and appearance with the increase in concentration of PNE. The study provides a future perspective for application of beeswax loaded PNE coatings in cheese industry.

1. Introduction

Cheesemaking is one of the oldest crafts mastered by homo sapiens. The evidence of cheese and caseiculture dates back to 6th millennium BC and are evident from the tomb art of Ancient Egypt [1]. But till date, high perishability of the cheese makes it a critical product for preservation. Various synthetic packaging materials have been used to enhance the shelf life and microbiological safety of the cheese, but owing to their environmental and health concerns the use of synthetic packaging materials is restricted. In recent years, biobased packagings are being developed to reduce the usage of synthetic packaging materials [2]. Also, the customer preference for natural antioxidants, which can also act as a shelf-life booster in various food products has directed the research towards plant-based extracts which can be a source of phenolic antioxidants and antimicrobials [3]. Extracts from pine needles could be a potential source of anti-browning, antioxidant and antimicrobial agents [4]. Pine needle extract (PNE) have been used as a preservative in cheese to enhance the microbiological and sensory profile of the cheese [5].

Beeswax obtained from the honey combs is a complete mixture of several chemical compounds which has food applications such as, glazing and coating, carrier for food additives and texturizer for chewing gums [6]. Beeswax has also been used as a coating in kashar cheese to provide prevention against moisture loss, and maintenance of better texture [7]. The coating so developed was used to enhance the shelf life of Himalayan cheese kradi/kalari. Kradi is an important traditional artisan cheese made from the butter milk found in the Himalayan region of Jammu and Kashmir, India. It is a fresh cheese with smooth, bright-white surface and undergoes rapid spoilage within 5 to 7 days under ambient conditions. So, in order to control the spoilage and preserve its texture and flavour, a packaging system loaded with active ingredients needs to be developed to enhance its shelf life and nutraceutical potential [8]. In a study by Mushtaq, Gani, Gani, Punoo, & Masoodi [8], zein films loaded with different concentrations of pomegranate peel extract were developed as a packaging material for Himalayan cheese, kradi. This study presents incorporation of PNE in beeswax to form an edible coating for Himalayan cheese, kradi via ultrasonication. The technique of ultrasonication is based on the principle of dispersing particles in the liquid medium. The process of cavitation involves rapid formation, growth and collapse of bubbles in the liquid which helps in dispersion of particles in the liquid. Eslamian [9], reported that high pressure generated from the propagation of ultrasound in liquid medium
resulted in efficient dispersion of nanoparticles within the matrix, thus producing better-performing composites for packaging. Similarly, ultrasonication resulted in better dispersion of ZnO nanoparticles in a fluid having different concentrations of graphene oxide to form a composite fiber with improved anti-bacterial and mechanical properties [10]. Thus same principle has been applied to incorporate PNE in the bee wax to form edible coating for kradì.

The research starts with the physical characterisation of the bee wax, and nutraceutical evaluation of the PNE. Following incorporation of PNE with bee wax using ultrasonication, the coatings so developed were studied for their physical characteristics using, scanning electron microscopy (SEM), light microscopy, dynamic light scattering (DLS), Attenuated total reflection-Fourier transform infrared-spectroscopy (ATR-FTIR), and texture analyser. Nutraceutical characterisation of the coating material included antioxidant, anti-bacterial, and anti-diabetic studies. Finally, kradì is coated with the so-prepared PNE enriched bee wax coating material, to prolong the shelf life and enhance the nutraceutical potential.

2. Materials and methods

Beeswax and kradì were purchased from the local markets of Srinagar, India, and pine needles were collected from local species of pine tree (Pinus wallichiana). The beeswax was melted and strained to remove the coarse impurities, and the pine needles were washed to remove the dirt before blending, for the extraction of bio-actives. All the chemicals used in characterisation were of analytical grade.

2.1. Extraction of bio-actives from pine needles

The clean pine needles were crushed and blended using a food processor in the proportion of 1:20, grams of pine needles to mL of distilled water. The pine needle pulp was then ultrasonicated for 20 min at a pulse mode of 1 s ON and 1 s OFF using ultrasonic probe sonicator (Cole-Parmer, 04711–35), with a frequency of 20 kHz, and power rating of 500 W. The bio-actives from the pulp were separated into supernatants and precipitate using a centrifuge (Eppendorf 5810R) for 15 min at 4000 rpm. The supernatant was then filtered using cellulose filter paper with 11 µm particle retention. The extracted supernatant i.e., PNE loaded with bio-actives, was stored at 4 °C for further analysis.

2.2. Preparation of enriched-edible coating

Pure beeswax and three other samples, with grams of beeswax to mL of PNE of proportions 2:1, 1:1, and 2:3, were prepared for the study (say, control, C1, C2 and C3, respectively). The samples were prepared by adding a measured quantity of beeswax and PNE in beakers, at said ratios and placing in a water bath at a temperature of 65 °C. At this temperature, layers of immiscible fluids, molten wax and PNE were observed. To incorporate the PNE in beeswax, ultrasonication was carried out for 15 min. This was done by inserting an ultrasonic probe sonicator (Cole-Parmer, 04711–35), with frequency set to 20 kHz, power rating of 500 W and a pulse mode of 1 s ON and 1 s OFF, into all four beakers i.e., pure beeswax and samples with different concentrations of PNE, which were already heated in the water bath, for 15 min each. Thus, PNE encapsulated beeswax coatings were prepared.

2.3. Coating of cheese

Initially, kradì was cut into small cuboids of dimensions 2 × 2 × 0.7 cm and dipped in the prepared coating, placed in the water bath maintaining a temperature range of 65–75 °C. The temperature was maintained in this range so that the wax remains a liquid and the Himalayan cheese, kradì, will not disintegrate. The coating was done in triplicates. The kradì samples coated with coating materials, control, C1, C2 and C3 were designated as, C<Cₚ, CₐKCₜ, C₂ₖ, and C₃ₖ, respectively. The 12 coated samples along with 3 uncoated samples were left at room temperature for 14 days, for shelf study.

2.4. Ethanolic extraction

Characterisation of wax by itself is a complicated task, because of its tendency to solidify at room temperature. Even though beeswax can be dissolved in organic solvents, turbidity and precipitance during reactions is common, which causes problems during spectral analysis. Thus, extraction of bio-actives is pertinent, the samples, control, C1, C2 and C3 were subjected to ethanolic extraction [11]. The wax samples (in grams), ethanol (in mL) and water (in mL), were mixed in a ratio 5:96:4 and placed in a water bath at a temperature of 70 °C for 6 h. The ethanolic extract was separated by centrifugation (Eppendorf 5810R) for 15 min at 4000 rpm. The precipitate so obtained was freeze-dried and stored in air tight containers. The ethanolic extracts of the samples, control, C1, C2 and C3, were named Eₐ, EₐC₁, EₐC₂, EₐC₃, respectively. The ethanolic extracts were used for nutraceutical characterization.

2.5. Physical characterisation of the coating

Different physical characterisation techniques were performed to check the impact of ultrasonication on particle size, encapsulation, and to analyse the bio-activity of the coating.

2.5.1. Scanning electron microscopy

The surface morphology of different samples was observed with the help of a scanning electron microscope (Hitachi Se 3600 N-Tokyo, Japan). The surface of the coating was sputtered with gold to obtain quality SEM images.

2.5.2. Light microscopy

The samples of coating materials were dispersed in a glycerol water mixture of the ratio of 1:1 and a drop of suspension was observed using the light microscope (Leedz MicroImaging EAPRIME Inverted – 900) at 40x magnification.

2.5.3. Particle size and zeta potential

The particle size and ζ-potential of coatings (control, C1, C2 and C3) were measured with a Zetasizer (Litesizer 500 Anton Paar, Austria). 0.01 g of the sample was dispersed in 100 mL of distilled water using a sonicator bath at 40 kHz for 5 min. Then the prepared dispersions were put in the cuvette to observe the particle size using the Zetasizer. ζ-potential was measured by mixing 0.01 g of sample in 100 mL of 0.1 mM potassium chloride, at a neutral pH.

2.5.4. Attenuated total Reflectance-Fourier transform infrared-spectroscopy

ATR-FTIR spectra of PNE, Eₐ, EₐC₁, EₐC₂, and EₐC₃, were observed using an ATR-FTIR spectrometer (Gary 630 FTIR Spectrometer, Agilent Technologies, Inc., USA). The spectra were observed between 650 and 4000 cm⁻¹, at room temperature, and the data acquisition was done using Agilent Resolution-Pro Micro Lab (software version B.05.2).

2.5.5. Thermal properties

The thermal properties of control, C1, C2, and C3, were studied using differential scanning calorimetry (DSC). 3.5 mg of sample and 8 µL of distilled water were taken in the platinum pans and loaded into the calorimeter (DSC-1, Mettler Toledo, USA). The thermal properties were studied between the range of 20 °C to 240 °C at a rate of 10 °C/minute.

2.5.6. Texture investigation

Texture profile analysis (TPA) of the coated (control, C1, C2 and C3) and uncoated cheese samples were performed using a texture analyser (TA-XT plus texture analyser, Stable micro systems, UK). The analysis was performed based on the method given by Bourne [12].
2.6. Physicochemical characterisation

The physicochemical characterisation of control, C1, C2, and C3, was studied in terms of water activity and colour.

2.6.1. Water activity

The water activity ($a_w$) of the samples was checked using a water activity meter (Aqua lab CX-2, Decagon Devices, Inc, USA). Shreds of fresh and coated cheese samples (control, C1, C2, and C3) were placed on the sample drawer and $a_w$ values were observed.

2.6.2. Colour characterisation

Colour is one of the important parameters which determine the likeliness of cheese. The colour of the coatings and the cheese (kraidi) were determined using a colour-flex Spectro-colourimeter (D25 NC, Hunter Associates Laboratory, USA). The colours of the sample were determined in terms of 'L', 'a', and 'b', where, 'L' value indicates the lightness (0–100 represents dark to light), 'a' value gives the degree of red to green (positive 'a' value indicates redness), 'b' value indicates the degree of yellow to blue colour (positive 'b' value indicates yellowness) [13].

2.7. Nutraceutical characterisation of the coating

The beeswax itself has nutritional value, and in this study PNE is also incorporated in the beeswax, which could enhance the nutraceutical value of the coating and the cheese so coated.

2.7.1. Antioxidant properties

The antioxidant property of the coating was determined based on the radical scavenging activity of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and the metal chelation activity of the ferrous ion.

2.7.1.1. DPPH scavenging activity. The DPPH scavenging ability of samples, $E_0$, $E_{C1}$, $E_{C2}$, and $E_{C3}$, was evaluated to know the change in the antioxidant potential of the coating with different concentrations of PNE. The assay was conducted based on the method of Noor, et al [14]. The radical scavenging activity was calculated using the formula,

$$\text{Percentage DPPH radicals scavenging} = \left(1 - \frac{\text{Absorbance of sample at } 517 \text{ nm}}{\text{Absorbance of control at } 517 \text{ nm}} \right) \times 100$$

The absorbance was observed using a spectrophotometer (Bio-Spectrometer-basic, Eppendorf, Germany).

2.7.1.2. Metal chelating activity. The metal chelating ability of the samples, $E_0$, $E_{C1}$, $E_{C2}$, and $E_{C3}$ was carried out based on Tang, Kerry, Sheehan, & Buckley [15]. The metal chelation ability was calculated using the formula,

$$\text{Percentage metal chelation} = \left(1 - \frac{\text{Absorbance of sample at } 562 \text{ nm}}{\text{Absorbance of control at } 562 \text{ nm}} \right) \times 100$$

The absorbance was observed using a spectrophotometer (Bio-Spectrometer-basic, Eppendorf, Germany).

2.7.2. Anti-diabetic activity

The anti-diabetic activity of the samples was estimated by observing the inhibition activity of $\alpha$-amylase, based on the method proposed by Anga, et al. [16]. The sample solutions were prepared by adding 10 mg of samples to 1 mL of distilled water. The assay included 50 $\mu$L of sample solution, 50 $\mu$L of 0.02 M sodium phosphate buffer and 50 $\mu$L of $\alpha$-amylase (40 U/mL in distilled water). The sample solutions were incubated for 10 min at a temperature of 37 °C. After incubation 40 $\mu$L of starch solution is used as a substrate and again incubated for 15 min. Finally, 40 $\mu$L of HCl is added to cease the reaction. The change in colour was analysed after adding 5 mM of iodine reagent. The absorbance of the samples was observed at 620 nm using a spectrophotometer. Acarbose was used as a standard. The percentage inhibition of $\alpha$-amylase was calculated using the formula,

$$\text{Percentage of } \alpha - \text{amylase inhibition} = \left( \frac{A_0 - A_1}{A_1 - A_2} \right) \times 100$$

where, $A_0$ is the absorbance of the sample, $A_1$ is the absorbance of negative control, and $A_2$ is the absorbance of positive control.

2.7.3. Anti-bacterial properties

The anti-bacterial activity of the PNE encapsulated beeswax was studied against gram positive bacteria Bacillus pumilus (MCC2703) and Bacillus cereus (MCC2236), using agar disc diffusion method. Cell suspension of 0.1 mL, with density $10^8$–$10^9$ colony-forming unit per mL, of the culture was inoculated on nutrient agar. Then, sterile disks of 10 mm diameter were placed on the prepared agar plate and soaked with 50 $\mu$L of sample extracts. The plates were incubated for 24 h at 37 °C, along with erythromycin discs as positive control. Finally, the incubation zone diameters (in mm) were noted to know the bacterial growth and the degree of inhibition of the extract [17].

2.8. Shelf life and sensory evaluation

The nutraceutical enhancement and value-addition of the coating on one hand, another important aspect is to improve the shelf life and likeability of the coated material, i.e., kraidi in this case. The study concentrates on the self-life by studying the changes in the microflora and the overall acceptability of the coated cheese.

2.8.1. Microbiological analysis

Microbial analysis of the fresh cheese and the coated cheese samples after 14 days on the shelf, at room temperature, was carried out using the serial dilution method. The cheese samples were diluted with NaCl saline solution (0.9%), by homogenisation. The analysis was conducted in two different temperature ranges 40–45 °C and 30 °C to ensure the optimum growth of mesophilic aerobes and LAB [18].

2.8.2. Sensory evaluation

Competitive evaluation of the sensory attributes like, flavour, texture, colour, appearance, and overall acceptability of the uncoated and the coated cheeses (control, C1, C2 and C3) were evaluated using the standards framed by the International Dairy Federation [19]. The evaluation panel comprised of 13 members and were asked to score the said parameters on a scale of 1 to 9 (1 being very poor and 9 being excellent). The results were calculated by pooling the evaluators' responses for each criterion, and finally the sensory characteristics of the samples were expressed in percentage [20].

3. Results and discussions

3.1. Physical characterisation

3.1.1. Scanning electron microscopy

Fig. 1 sections I, II, and III shows the morphological structures of beeswax at three different magnifications 3000, 5000 and 10000x. The figures show a flaky and layered appearance, which could be due to the melting and uneven freezing of the wax. Similar layered morphological patterns of the beeswax were observed by Espolov, Ukibayev, Myrzakozha, Perez-Lopez, & Ermolayev [21]. Sections A, B and C, which are the SEM images of sample C3, the sample with the highest concentration of PNE, at magnifications 3000, 5000 and 10000x respectively. The reduction of flakiness and layers was observed in A, B and C, this could be the impact of ultrasonication and also the appearance of small globules could be due to the successful incorporation of PNE in beeswax.
Similar structures were observed in the study where beeswax is used as the wall material to encapsulate thiamethoxam and kaolin [22].

3.1.2. Light microscopy

The effect of PNE incorporation in beeswax was observed using light microscopy, and is represented in Fig. 2. Representative image of sample C3 is selected for its maximum concentration of PNE. The optical image of control (Fig. 2 A) appears to be flaky and this correlates with the observations of SEM micrographs of the same sample. As for sample C3, the image displayed in Fig. 2 B shows reduction in flakiness and appearance of small PNE droplets, confirming the successful encapsulation. This phenomenon might be due to ultrasonication.

3.1.3. Particle size and zeta potential

The hydrodynamic diameter, zeta potential and polydispersity index of the samples are presented in Table 1. The hydrodynamic diameter of the control displayed significantly higher (p ≤ 0.05) value of 22.05 ± 1.25 μm and with ultrasonication the diameter decreased significantly (p ≤ 0.05) in samples C1, C2 and C3. There is a small increase in the hydrodynamic diameters with the increase in amount of PNE extracts.

| Sample | Hydrodynamic diameter (μm) | Zeta potential (mV) | Polydispersity index (%) |
|--------|---------------------------|--------------------|-------------------------|
| Control | 22.05 ± 1.25<sup>c</sup> | -12.08 ± 0.22<sup>c</sup> | 50.50 ± 1.69<sup>c</sup> |
| C1     | 09.75 ± 0.23<sup>a</sup> | -15.33 ± 0.34<sup>b</sup> | 60.89 ± 0.03<sup>a</sup> |
| C2     | 11.26 ± 0.11<sup>a</sup> | -18.11 ± 0.09<sup>b</sup> | 62.10 ± 0.11<sup>c</sup> |
| C3     | 12.11 ± 0.41<sup>b</sup> | -19.32 ± 0.61<sup>b</sup> | 62.89 ± 1.23<sup>c</sup> |

Table 1
Hydrodynamic diameter, zeta potential and polydispersity index of pine needle extract enriched beeswax coatings.

Values expressed are mean ± standard deviation. Mean in the same columns with different superscripts are significantly different as p ≤ 0.05. C1, C2 and C3 represents pine needle extract enriched beeswax coating at concentrations of 1:2, 1:1 and 2:3, respectively.
which could be due to the aggregation of polyphenols, encapsulated. The zeta potential of all the samples was found to be negative. Stronger Vander Waals forces are responsible for agglomeration of particles. Ultrasonication is responsible for the increase in electrostatic repulsion between particles [23]. The relation between electrostatic repulsion and Vander Waals’ forces is that they are inversely proportional. This phenomenon can be observed with the increase in zeta potential of C1, C2 and C3 from that of the non-ultrasonicated control. The weaker the impact of Vander Waals’ forces higher the stability of the coating. Each sample displayed a polydispersity index greater than 40%, which indicates wide particle size distribution and heterogeneity [24].

3.1.4. ATR-FTIR spectroscopy

Fig. 3 shows the FTIR spectra of the PNE, E_C, E_C1, E_C2, and E_C3. The spectra observed by the control, i.e., pure beeswax correlates with the spectra observed by Baglioni, et al. [25]. Similarity in the fingerprint regions of all the samples shows the preservation of the properties of beeswax even after the addition of PNE. The peak at 1735 cm\(^{-1}\) observed in control, shrinks as the concentration of PNE increases, in the contrary the peak at 1707 cm\(^{-1}\) grows as the concentration of PNE increases. These phenomena could be due to the stretching of C-O bonds and are observed in the highlighted section A of the Fig. 3. Section B shows how the trough observed from 3000 – 3750 cm\(^{-1}\) in the spectra of the PNE, impacted the other samples. In the range from 3000 – 3750 cm\(^{-1}\) there is no noticeable change in the spectra of the control sample, but small depressions can be observed in the spectra of E_C2 and E_C3, which may be due to the stretching of O-H bonds. Hence, shows successful encapsulation of PNE by the beeswax. Such interactions between polyphenols and proteins were also observed by Zou, Li, Percival, Bonard, & Gu [26].

3.1.5. Thermal properties

The thermal parameters of the samples are presented in Table 2. C1 displayed a significant decrease (p ≤ 0.05) in onset (T_o), peak (T_p), end set (T_c) and heat of enthalpy (ΔH) than control, even after the addition of PNE. This decrease could be the impact of ultrasonication, which breaks down the molecular structures [27]. Further, the decrease could be due to the entrapment of PNE in the beeswax matrix which decreases the associative forces between the molecules of beeswax, there by resulting in weakened structure [28]. The subsequent increase of the said values from C1 to C3 indicates that the coatings were stable over a wide range of temperature.

| Thermal properties | Control | C1      | C2      | C3      |
|--------------------|---------|---------|---------|---------|
| T_o (°C)           | 58.12 ± 0.34 | 56.17 ± 0.33 | 59.39 ± 0.06 | 63.34 ± 0.99 |
| T_p (°C)           | 68.95 ± 1.09 | 65.32 ± 0.18 | 68.99 ± 1.05 | 72.32 ± 0.45 |
| T_c (°C)           | 72.09 ± 1.23 | 68.33 ± 0.19 | 74.86 ± 0.02 | 79.25 ± 0.06 |
| ΔH (J/g)           | 12.46 ± 0.89 | 11.33 ± 0.38 | 14.36 ± 0.89 | 19.34 ± 1.66 |

Values expressed are mean ± standard deviation. Mean in the same columns with different superscripts are significantly different as p ≤ 0.05. C1, C2 and C3 represents pine needle extract enriched beeswax coating at concentrations of 1:2, 1:1 and 2:3, respectively.
3.1.6. Texture investigation

The textural characteristics of fresh and coated cheese samples at different time intervals are shown in Table 3. The hardness value of the coated cheese increased significantly ($p < 0.05$), but at the same time the hardness value was significantly lower than fresh cheese after 14 days. This shows the preservative effect of coating on retention of moisture. The cohesiveness values displayed a significant increase ($p < 0.05$) both after 0 and 14 days. Also, the cohesiveness of the cheese after 14 days (0.53 ± 0.05) displayed a significant decrease ($p < 0.05$) than fresh cheese (0.93 ± 0.05, 0.93 ± 0.01, 0.92 ± 0.08 and 0.91 ± 0.04 for Cx, C1x, C2x, and C3x respectively), this shows the preservation of cohesiveness due to the coating. Similarly, the values of springiness, adhesiveness, gumminess, chewiness and resilience, displayed values which might signify undesirability of the coating, but after weighing those values with the observations after 14 days, it can be inferred that the benefits of the coating outweigh the shortcomings.

3.2. Physico chemical characterisation

3.2.1. Water activity

The values of aw for fresh and coated cheese are presented in Table 3. The aw of fresh kradi on day 0 was observed to be 0.980 ± 0.036, whereas the values for coated cheese sample showed a non-significant increase on the same day, this could be due to the addition of PNE into the beeswax, which may add to the increased moisture content of the samples. On 14th day of storage, the aw values decreased in fresh as well-coated samples, the decrease could be due to the loss of moisture from the cheese samples. The decrease was significantly higher ($p < 0.05$) in fresh kradi, as compared to the coated ones, signifying the positive effect of coating in retaining the moisture of cheese samples. This can in turn retain tenderness and juiciness of the cheese. Similar results of higher aw of cheese samples coated with beeswax were also reported by Bucio, Moreno-Tover, Bucio, Espinosa-Davila, & Anguebes-Franceschi [29].

3.2.2. Colour characterisation

Fig. 4 shows the values of ‘L’, ‘a’, and ‘b’ of samples (fresh cheese, uncoated cheese after 14 days, control, C1, C2 and C3) and respective colour graphs in section (1) to (6). Section (A) of Fig. 4 shows the change in lightness of various samples. Fresh cheese had the lightness value or ‘L’ value of 59.74, and after 7 days the value decreases to 47.38. The yellowish colour of the beeswax gives a lower ‘L’ value of 45.2 in control sample, the lightness increases with the increase in concentration of PNE. The lightness value of the fresh and C3 were close, 59.74 and 60.87 respectively, this shows adding more PNE gives lightness to the cheese by neutralising the typical yellowishness of the beeswax. This neutralisation phenomenon could be credited to ultrasoundation and encapsulation of PNE into beeswax.

3.3. Nutraceutical characterisation

3.3.1. Antioxidant properties

Table 4 shows the antioxidant activity of cheese (kradi), Ec, Ec1, Ec2, and Ec3 based on the DPPH radical scavenging, and metal chelating potential. The antioxidant activity of beeswax (10.11 ± 0.66 % and 27.36 ± 0.93 %, for DPPH radical scavenging and metal chelating activity) was significantly lower ($p < 0.05$) than fresh cheese (12.32 ± 0.03 % and 32.99 ± 0.01 %, for DPPH radical scavenging and metal chelating activity), however with the increase in the amount of PNE in the coating material antioxidant activity increased significantly ($p < 0.05$). The DPPH radical scavenging activity increased from 14.12 ± 0.41 % to 19.12 ± 0.65 %, and the metal chelating activity increased from 34.19 ± 0.42 % to 37.22 ± 0.23 %, as the concentration of PNE increases from Ec1 to Ec3. The antioxidant activity of PNE could be attributed to hydrogen donating ability, arising from the phenolic compounds such as flavonoids, and phenolic non flavonoids [30]. As the concentration of PNE increased in the coating it might increase the O–H groups that could scavenge the free radicals to stabilise and block radical chain reactions. The stretching of hydroxyl bonds observed on ATR-FTIR spectra of Ec2, Ec2, and Ec3 (Fig. 3), due to the impact of the PNE, also correlates with the numbers observed on Table 4.

3.3.2. Anti-diabetic activity

The anti-diabetic activity of kradi, Ec, Ec1, Ec2, and Ec3 were evaluated in terms of α-amylase inhibition activity (observations are shown in Table 4), which was significantly higher ($p < 0.05$) for cheese (18.12 ± 0.41 %) as compared to the pure beeswax (10.23 ± 0.63 %). However, with the encapsulation of PNE in coating the α-amylase inhibition activity increased significantly from 10.23 ± 0.63 % to 16.80 ± 0.01 %. The anti-diabetic potential of pine needles could be attributed to the presence of phenolics and diterpenoids [31, Singh, Kumar, & Dass [32] reported considerable fall in fasting glucose levels in diabetic rats upon incorporation of pine enriched oral treatment. Yang, et al. [33] reported isolation of α-amylase inhibitor, longifolene which had an IC50 value of 79.85 ± 0.23 μg/mL against α-amylase, which could be the reason for the increase in anti-diabetic activity, as the concentration of PNE was increased.

Table 3

| Storage time (days) | Kradi | Cx | C1x | C2x | C3x |
|---------------------|-------|----|-----|-----|-----|
| **Water Activity**  |       |    |     |     |     |
| 0       | 0.980±0.68 | 0.971±0.975 | 0.980±0.975 | 0.978±0.975 | 0.975±0.975 |
| 14      | 0.053±0.037 | 0.045±0.037 | 0.051±0.037 | 0.051±0.037 | 0.051±0.037 |
| **Hardness (kg)**  |       |    |     |     |     |
| 0       | 15.98±16.34 | 16.98±16.54 | 16.00±16.54 | 16.54±16.54 | 16.60±16.54 |
| 14      | 21.34±17.38 | 17.44±17.25 | 17.00±17.25 | 17.25±17.25 | 17.00±17.25 |
| **Cohesiveness**   |       |    |     |     |     |
| 0       | 0.83±0.95   | 0.93±0.92   | 0.91±0.91   | 0.91±0.91   | 0.91±0.91   |
| 14      | 0.53±0.93   | 0.93±0.92   | 0.91±0.91   | 0.91±0.91   | 0.91±0.91   |
| **Springiness (%)**|     |    |     |     |     |
| 0       | 0.97±0.84   | 0.82±0.80   | 0.80±0.80   | 0.80±0.80   | 0.80±0.80   |
| 14      | 0.65±0.80   | 0.79±0.77   | 0.75±0.76   | 0.75±0.76   | 0.77±0.77   |
| **Adhesiveness (g/sec)** |     |    |     |     |     |
| 0       | 43.56±44.31 | 44.36±59.74 | 43.11±44.00 | 43.00±44.00 | 40.11±44.00 |
| 14      | 28.22±20.92 | 33.14±33.56 | 28.34±28.95 | 37.16±37.16 | 37.16±37.16 |
| **Gumminess**      |       |    |     |     |     |
| 0       | 14.58±15.36 | 14.95±14.34 | 14.00±14.34 | 14.00±14.34 | 14.00±14.34 |
| 14      | 0.94±0.14   | 0.41±0.91   | 0.16±0.91   | 0.16±0.91   | 0.16±0.91   |
| **Chewiness**      |       |    |     |     |     |
| 0       | 12.98±14.55 | 14.35±14.28 | 13.99±14.28 | 13.99±14.28 | 13.99±14.28 |
| 14      | 0.735±0.15  | 0.14±0.07   | 0.05±0.07   | 0.05±0.07   | 0.05±0.07   |
| **Resilience (%)** |     |    |     |     |     |
| 0       | 0.77±0.81   | 0.81±0.77   | 0.79±0.76   | 0.79±0.76   | 0.77±0.77   |
| 14      | 0.49±0.86   | 0.63±0.62   | 0.59±0.59   | 0.59±0.59   | 0.59±0.59   |

Cx, C1x, C2x, and C3x represents kradi coated with coating materials, control, C1, C2 and C3, respectively. Values expressed as mean ± standard deviation. Mean in the same rows with same superscripts are non-significantly different.
3.2.3. Colour characterisation

Fig. 4. Sections (1), (2), (3), (4), (5) and (6) show the colour observed on fresh, uncoated, control, C1, C2 and C3 samples respectively. Sections (A), (B) and (C) show the change on fresh, uncoated, control, C1, C2 and C3 samples in L, a, and b values, respectively.

Table 4 Nutraceutical characterisation of kradi and coating materials.

| Name of the assay                | Cheese | EC | EC1 | EC2 | EC3 |
|----------------------------------|--------|----|-----|-----|-----|
| DPPH radical scavenging activity (‰) | 12.32 ± 0.03b | 10.11 ± 0.66a | 14.12 ± 0.41c | 17.11 ± 0.03d | 19.12 ± 0.65e |
| Metal chelating activity (‰)     | 32.99 ± 0.01a | 27.36 ± 0.92b | 34.19 ± 0.42c | 36.26 ± 0.39d | 37.22 ± 0.23e |
| Alpha-amylase inhibition activity (‰) | 18.12 ± 0.41d | 10.23 ± 0.63a | 12.05 ± 0.42b | 13.11 ± 0.05c | 16.80 ± 0.01c |
| Antibacterial activity in IZD Bacillus pumilus (mm) | 0.07 ± 0.05b | 0.35 ± 1.00b | 0.15 ± 0.50c | 0.44 ± 0.44d | 0.26 ± 0.46e |
| Antibacterial activity in IZD Bacillus cereus (mm) | 0.49 ± 0.76b | 11.65 ± 0.05c | 14.23 ± 0.05c | 18.64 ± 0.22d | 23.64 ± 0.03e |

Values expressed are mean ± standard deviation. Mean in the same rows with different superscripts are significantly different as p ≤ 0.05. EC, EC1, EC2, and EC3 represents the ethanolic extracts of control and PNE enriched beeswax coating at concentrations of 1:2, 1:1 and 2:3, respectively.

3.3.3. Anti-bacterial activity

The antibacterial activity of kradi, EC, EC1, EC2, and EC3 was evaluated against gram positive bacteria Bacillus pumilus and Bacillus cereus. The observations are given in Table 4, which shows an increase in the zone of inhibition as the amount of PNE increases, (15.35 ± 0.50% and 14.23 ± 0.05 % to 25.06 ± 0.48% and 23.64 ± 0.03% for Bacillus pumilus and Bacillus cereus [34]. The antimicrobial activity of the cheese could be due to the peptides generated by proteolytic enzymes during fermentation [35].

3.4. Shelf life and sensory evaluation

3.4.1. Microbiological analysis

The average count of microbes during storage at 0 and 14 days for fresh and coated cheese is presented in Table 5, L. plantarum contribute to the flavour, texture and keeping quality of kradi, and to determine the negative impact of coating on this species, LAB counts were made to
C₀ represents kradi coated with coating materials, control, C₁, C₂ and C₃ respectively. Values expressed are mean ± standard deviation. Mean in the same rows with different superscripts are significantly different as p ≤ 0.05. ND represents not detected.

assess the same. The initial LAB count was 6.85 ± 0.23 which displayed no significant difference after coating. However, for fresh kradi LAB count decreased drastically, while the coating significantly preserved the LAB count. C₀ exhibited highest logarithmic value after 14 days at room temperature. Similar effect of beeswax coating on the survival of LAB was also reported by Yilmaz & Dagdemir [7]. As the PNE concentration of the coatings increased from C₁₀ to C₃₀ the LAB counts decreased non significantly, which could be attributed to the anti microbial properties of pine needles. For mould counts, coated cheese displayed a significantly lower value and reduced the logarithmic value to 2.95 ± 0.08, signifying that the coatings prevented growth of moulds, which can be detrimental to the self-life of kradi. Similar observations were also reported by Mahajan, Bhat, & Kumar [5], for cheese samples coated with PNE. The authors inferred the reduction in mould count to the antifungal properties of pine needles. Similarly, the mesophilic aerobic count increased significantly (p ≤ 0.05) after 14 days in fresh kradi, whereas the coated cheese sample showed a decreased count. Coliforms were not detected in any of the samples, indicating that cheese and coatings were prepared under hygienic conditions.

3.4.2. Sensory evaluation

The scores for sensory characteristics for fresh and coated samples are given in Table 6. Texture, flavour, taste, appearance and overall acceptability of the samples were evaluated using a 9-point hedonic scale. The appearance and texture displayed significantly (p ≤ 0.05) higher scores when the amount of PNE in the coating increased. Contrary, the flavour and taste scores displayed significantly lower values. This could be attributed to the characteristic odour of beeswax and pine needles. Similar reduction in flavour and taste scores were observed by Yilmaz & Dagdemir [7], for cheese samples coated with beeswax. Mahajan, Bhat, & Kumar [5], reported a similar decrease in overall acceptability of the cheese preserved using PNE.

4. Conclusion

Recently edible coatings are gaining considerable attention as compared to non edible coatings in terms of their ability to prevent moisture loss, microbial spoilage and for its eco friendliness. In this context, the present study explores the use of beeswax and PNE for the enhancement of the self-life and nutraceutical potential of the Himalayan cheese, kradi. The coatings developed were characterised for physical, morphological, physico-chemical, and nutraceutical properties. The DPPH, metal chelating, alpha-amylase inhibition and antibacterial potential of the coatings increased with the increase in PNE concentration. The coated cheese samples displayed retention of water activity, enhancement of colour, texture, LAB counts, whereas the mould counts showed a significant inhibition. However, in terms of taste and flavour the panelists marked low scores. Overall, the coatings have a tremendous nutraceutical and self-life characteristics, which could outweigh the negative implications of off odours and characteristic tastes of PNE and beeswax. The designed coatings have great potential to be used in the cheese making industry.

CRediT authorship contribution statement

J.L.H. Jenno: Formal analysis. Nairah Noor: Investigation, Writing – original draft. Mehwesh Mushtaq: . Adil Gani: Supervision, Resources.

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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Table 5

Average count (log10 cfu g⁻¹) of microorganisms during storage of fresh and coated cheese.

| Storage time (days) | Kradi | C₀ | C₁₀ | C₂₀ | C₃₀ |
|---------------------|-------|----|-----|-----|-----|
| L. planturn          |       | 6.85 ± | 6.53 ± | 6.48 ± | 6.23 ± | 6.11 ± |
|                     |       | 0.23 ± | 0.04² | 0.17³ | 0.19³ | 0.03³ |
|                     |       | 0.14² | 0.2³ | 0.82³ | 0.35³ | 0.09³ |
| Mesophilic aerobes   |       | 4.36 ± | 4.23 ± | 4.11 ± | 4.00 ± | 4.00 ± |
|                     |       | 0.11³ | 0.48³ | 0.03³ | 0.42³ | 0.35³ |
|                     |       | 0.17³ | 0.19³ | 0.06³ | 0.13³ | 0.05³ |
| Coliforms            |       | ND | ND | ND | ND | ND |
|                     |       | ND | ND | ND | ND | ND |
| Moulds              |       | 3.51 ± | 3.41 ± | 3.40 ± | 3.38 ± | 3.37 ± |
|                     |       | 0.33³ | 0.25³ | 0.11³ | 0.05³ | 0.11³ |
|                     |       | 0.12³ | 0.13³ | 0.02³ | 0.11³ | 0.08³ |

Table 6

Sensory characteristics of fresh and coated kradi.

| Parameters | Kradi | C₀ | C₁₀ | C₂₀ | C₃₀ |
|------------|-------|----|-----|-----|-----|
| Texture    | 8.42 ± | 4.36 ± | 4.16 ± | 5.39 ± | 6.32 ± |
|            | 0.1³ | 0.66³ | 0.22³ | 0.46³ | 0.14³ |
| Flavour    | 7.8³ | 6.34 ± | 5.69 ± | 4.18 ± | 4.63 ± |
|            | 0.16³ | 1.2³ | 0.06³ | 0.33³ | 1.6² |
| Appearance | 6.3³ | 7.1³ | 7.8³ | 8.01 ± | 8.23 ± |
|            | 0.05³ | 0.49³ | 0.34³ | 0.05³ | 0.25³ |
| Taste      | 7.3³ | 4.10 ± | 4.6 ± | 4.93 ± | 5.35 ± |
|            | 0.03³ | 0.11³ | 0.45³ | 0.44³ | 0.29³ |
| Overall    | 7.35³ | 4.39³ | 4.88³ | 5.10³ | 5.93³ |
| acceptability | 0.3³ | 0.4³ | 0.96³ | 0.05³ | 1.1³ |

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