

RESEARCH ARTICLE

Antioxidant activity of garden cress seed (*Lepidium sativum*) protein hydrolysate incorporated Kesar flavoured milk

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Abstract: The present study investigates the application of garden cress protein hydrolysates (GCPH) derived from garden cress protein concentrate (GCPC) using papain (1:25, w/w). The effects of pH (6.0, 6.5, 7.0), temperature (50, 55, 60°C), and hydrolysis time (0, 30, 60, 90, 120, 150, and 180 min) on the degree of hydrolysis (DH) and antioxidant activity were assessed. Optimal conditions (pH 6.5, 55°C, 120 min) yielded a DH of $13.6 \pm 1.1\%$ and DPPH radical scavenging activity of $60.03 \pm 0.96\%$. GCPH was incorporated into Kesar flavored milk at 1% and 2% concentrations. The 2% GCPH addition significantly enhanced antioxidant activity to 0.45 ± 0.01 mM Trolox/L, compared to control and 1% GCPH. However, the 2% GCPH imparted a slight bitterness, which was mitigated by increasing the flavor concentration to 3%. These results indicate that GCPH can be effectively used as a functional additive to enhance the antioxidant properties of milk and milk products, providing potential health benefits and improved product quality.

Keywords: Flavoured milk, antioxidant activity, garden cress seed, protein, hydrolysate.

Introduction

Garden cress (*Lepidium sativum*) belonging to *Brassicaceae* family is used as an important medicinal plant in India. The seeds contain 33-54 % carbohydrate, 22-25 % protein and 17-27 % lipids and various biologically active compounds exhibiting various

health benefits (Gokavi et al. 2004; Azene et al. 2022). In recent years, there has been an increasing interest in the isolation and modification of the proteins from seeds and legumes to improve the bio-functional properties for its exploitation in food systems (Yadav et al. 2022).

Reactive oxygen species (ROS) produced during oxidative metabolism are considered as a causative factor in several lifestyle-mediated diseases (Hernández-Ledesma et al. 2005). The body has its own defense system to neutralize the free radical oxygen species, oxidative stress occurs when reactive oxygen species exceed the body's antioxidant resistance mechanism (Lobo et al. 2010). The hydrolysates obtained by enzymatic hydrolysis of plant protein has been reported to have antioxidant properties and considered as promising dietary supplement for improvement of antioxidant defense mechanism and a practical approach to reduce the oxidative stress in the body (Daliri et al. 2017; Rizzello et al. 2017; Sarmadi and Ismail, 2010). Hydrolysis of protein from *Bunium persicum* BioSS using alcalase shown to improve the antioxidant properties and metal chelating activity (Shahi et al. 2020). Bagul et al. (2018) reported maximum degree of hydrolysis of 39.49 % and radical scavenging activity of 42.92 % in tamarind protein hydrolysate obtained under optimized hydrolysis conditions with papain-to-protein ratio, hydrolysis time, temperature and pH of 1:5, 3h, 65 °C and 6.0, respectively. In the recent study on hydrolysis of garden cress protein concentrate with alcalase exhibited a broad range of antioxidant activities ranging between 11.18 % to 69.25 % DPPH inhibition and Fe²⁺ chelating activity of 4.32 % to 21.76 %, respectively Mulla and Ahmed (2019). However, no reported literature available on utilization of garden cress protein hydrolysate (GCPH) in beverages. Therefore, the potential of GCPH as an antioxidant ingredient in beverage system needs to be studied for better utilization in functional beverages. Milk-based beverages are a rich source of proteins as well as a high calcium and phosphorus content. They are convenient with good nutritional and easily digestible health foods connected with high protein content. Further, the stability of plant proteins hydrolysate over a wide range of pH makes them suitable for incorporation in healthy beverages. The addition of antioxidant rich GCPH could increase the antioxidant properties and health benefits of flavoured milk.

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Strawberry and chocolate flavoured milk supplemented with 2 % whey protein hydrolysate (flavourzyme, alcalase and corolase) showed an increase in the antioxidant activity (Mann et al. 2014). As a result of the harmful effects of synthetic antioxidants on human health, several workers have studied the production of natural antioxidants from plant proteins for its utilization in food systems (Park et al. 2001; Lourenço et al. 2019). Hence, the present work was carried out to optimize the hydrolysis condition to prepare GCPH with high antioxidant activity and to evaluate the antioxidant potential of Kesar flavoured milk incorporated with GCPH.

Materials and methods

Materials

Garden cress seeds, milk and Kesar flavour were obtained from the local market in Hisar, India. The commercial enzyme papain (e⁺ 30000.0 U/mg) received from NDRI, Karnal, India. 2,2-diphenyl-1-picrylhydrazyl (DPPH), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), sulphuric acid, sodium hydroxide, hydrochloric acid, copper (II) sulfate, potassium sulfate, ethanol, methyl blue and red, sucrose, Folin phenol reagent were purchased from Sigma-Aldrich, India.

Defatted garden cress seeds powder

Garden cress seeds were defatted by cold press, dried at $55 \pm 2^\circ\text{C}$ for 48 h and ground into a fine powder, then passed through 45-mesh standard sieve and stored in air-sealed plastic bags at 4°C until further use.

Preparation of protein concentrate

Garden cress protein concentrates (GCPC) were prepared according to the method described by Gaafar et al. (2013), with slight modifications. Briefly, 100 g of defatted garden cress seed flour was dispersed in distilled water (1:20). Suspension was adjusted to pH 9.0 with 1 N NaOH. The mixture was stirred for 60 min at room temperature using a magnetic stirrer and centrifuged (Model: MAX 5200 RPM, Biogen Scientific, Meerut, UP, India) at 5000 rpm for 20 min. The supernatant was collected and subsequently filtered through Whatman No.1 filter paper to eliminate any insoluble components. The filtrate was acidified to 4.5 pH with 1N HCl. The protein precipitate recovered by centrifugation at 5000 rpm for 20 min and washed with water (pH 7.0). The obtained GCPC were dried using freeze dryer (Model: -80 DEG C, Lark, Padi, TN, India) and packed in an air-sealed container and stored at 4°C until further use.

Estimation of crude protein

Total nitrogen content of GCPC was determined by Kjeldahl AOAC (2006) with some modifications. Briefly, 0.5 g of GCPC

was digested with 15 ml nitrogen free concentrated sulphuric acid by using 2.4 g of mixture ($\text{CuSO}_4:\text{K}_2\text{SO}_4$ as 1:4) until the color was transparent greenish and diluted up to 100 ml. Digested sample of 10 ml was taken in distillation flask, 10 ml of 40 % NaOH was added and connected to the distillation unit. The ammonia released was absorbed in 25 ml of 4 % boric acid solution added with mixed indicator (equal volume of 0.1 % methylene blue solution and 0.2 % of methyl red in ethanol). The distillate was titrated against 0.02 N sulphuric acid till the end point of purple colour change was attained. A blank was instantaneously run similar to the sample using 0.5 g nitrogen free sucrose. The total nitrogen was calculated using the formula given below.

$$\text{Total nitrogen (\%)} = \frac{1.4 \times (\text{Sample reading} - \text{Blank reading}) \times N \times 100}{W}$$

Where, W = Sample weight (g), N = Normality of sulphuric acid. The protein content (%) was calculated by multiplying % Nitrogen content by 6.25.

Preparation of protein hydrolysate

The garden cress protein hydrolysates (GCPH) were prepared according to the method with some modifications (Gao et al. 2014; Nwachukwu and Aluko, 2019). The hydrolysis conditions were optimized to obtain hydrolysate with enhanced antioxidant activity from GCPC with 72.61 % protein. The papain to protein ratio was maintained at 1:25 (w/w). In brief, GCPC (5 % w/v) were suspended in distilled water and pH was maintained at 6.0, 6.5, and 7.0 by the addition of 0.1 M HCl or 0.1 NaOH. Thereafter, hydrolysis was performed at 50, 55, and 60°C . Seven samples were drawn at 0, 30, 60, 90, 120, 150, and 180 min, respectively. At the end of the hydrolysis, the enzyme was inactivated by heating to 90°C in a hot water bath for 10 min followed by cooling to room temperature, freeze dried and stored at -18°C . The hydrolyses of protein concentrate was performed in triplicates.

Degree of hydrolysis (DH)

$$\text{DH (\%)} = \frac{100 \times \text{Concentration of soluble protein in TCA (10 \%) mg}}{\text{The total content of protein in mg}}$$

The DH was determined following the method (Hoyle and Merrit, 1994; Sonawane et al. 2017) with modifications. After completion of hydrolysis, added equal volume of 2.0 ml of 20 % Trichloro-acetic acid (TCA) with GCPC solution and hydrolysate, respectively. After incubation at room temperature for 30 min, the mixture was centrifuged at 7000 rpm for 10 min. For measurement of DH, 1 ml of supernatant was added to 5 ml of alkaline reagent followed by addition of 0.25 ml of 1.0 N Folin phenol reagent. The mixture was kept in dark for 10 to 15 min for color development and intensity of the blue

colour was measured at 660 nm using dual-beam spectrophotometer (Model: 2203, Systronics, Ahmedabad, GJ, India). The total soluble protein was obtained by tyrosine standard curve (0.05 - 0.5 mmol/L). The DH was calculated using the formula given below.

Antioxidant activity of GCPH

$$\text{DPPH (\% Inhibition)} = \frac{(\text{Blank reading} - \text{Sample reading}) \times 100}{\text{Blank reading}}$$

Antioxidant activity was measured by scavenging free radicals using 2,2-diphenyl-1-picrylhydrazyl (DPPH) according to the method described by Brand-Williams et al. (1995). A stock solution of DPPH (100 mM) was prepared in methanol. The supernatant from TCA precipitated GCPC and GCPH were appropriately diluted with distilled water. Diluted samples (100 µL) were added to 3.9 ml of diluted DPPH solution (1:25 in methanol) and kept in dark condition for 30 min, absorbance was recorded at 517 nm. For blank determination, 100 µL methanol was taken as a replacement for the TCA supernatant and absorbance was measured instantaneously against methanol. The antioxidant activity of the hydrolysate was calculate using the formula given below.

Preparation of Kesar flavoured milk

Freeze dried GCPH prepared under optimal hydrolysis conditions (papain/protein 1:25 w/w) ratio, 6.5 pH, 120 min, 55 °C) was used for the preparation of Kesar flavoured milk. The method of De, 2008 with minor modifications was followed. Homogenized toned milk (3.0 % fat, 8.5 % SNF) was pre-heated to 45 °C. Added 8 % sugar followed by GCPH (1 % and 2 %), 2 % Kesar flavour and mixed. The mixture was filled in a sterilized glass bottle, cap sealed and heated to 88 °C / 7 min, cooled and stored at 5 ± 2 °C. Control Kesar flavoured milk was prepared without addition of the GCPH.

Antioxidant activity of Kesar flavoured milk

Antioxidant activity of Kesar flavoured milk was measured using DPPH scavenging of free radicals by the method of Brand Williams et al. (1995) with modifications. The DPPH solution (100 mM) was prepared and diluted to 1:25 with methanol. The appropriately diluted sample of 100 µL was added to 3.9 ml diluted DPPH solution. The content was mixed and incubated for 30 min under dark condition. The decrease in the absorbance was recorded at 517 nm. For blank determinations, methanol (100 µL) was taken as a replacement of sample and absorbance was measured instantaneously against methanol. The percent inhibition was calculated and Trolox equivalent antioxidant capacity (TEAC) was determined using a standard curve generated by plotting percent inhibition against Trolox concentration (100-1000 µM). The antioxidant activity was expressed in the terms of mM Trolox/L of the flavoured milk.

Sensory evaluation

The sensory analyses of GCPH incorporated Kesar flavoured milk was carried out using 9-point hedonic scale by panel of 21 semi trained judges. The panel members were asked to record scores for flavour, colour, mouth feel, sweetness, and overall acceptability.

Statistical analysis

The data shown in the tables and figures are the mean of at least triplicates. The standard deviation is shown by error bars. Analysis of variance (ANOVA) was used to test for significance, and the comparison of mean was done using the critical difference value at 5 % level of significance. The data was analyzed using Microsoft Excel (Microsoft Office 365).

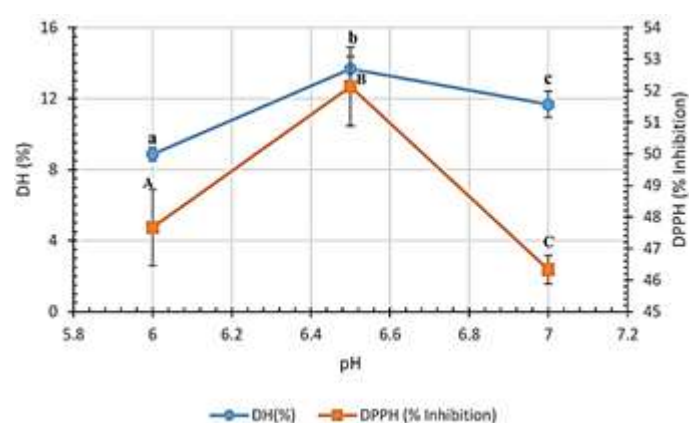
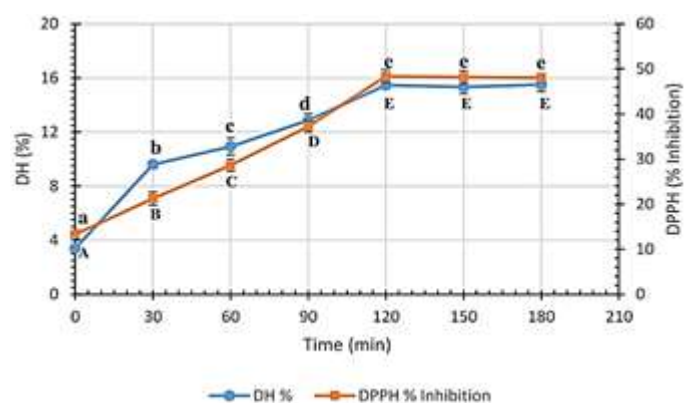
Results and Discussion

Effect of hydrolysis time on DH and antioxidant activity

The effect of hydrolysis time (0-180 min) on DH and antioxidant activity at 1:25 (w/w) papain/protein ratio, 50°C temperature and 6.5 pH was investigated (Fig. 1a). It was observed that increase in DH and antioxidant activity between hydrolysis time of 0 min to 120 min was significant ($P < 0.05$). As the hydrolysis time changed from 120 min to 180 min, no significant ($P > 0.05$) changes were noticed in DH and antioxidant activity of the hydrolysate. The hydrolysate showed maximum antioxidant activity of 48.38 % at papain/protein ratio of 1:25 (w/w), 50 °C temperature, 6.5 pH and a hydrolysis time of 120 min. From the results, the hydrolysis time was fixed at 120 min to evaluate the effect of pH and temperature on DH and antioxidant activity. Mahdavi Yekta et al. (2019) observed an increase in antioxidant activity in the quinoa protein hydrolysate as the hydrolysis time increased. According to Ibrahim et al. (2020), the degree of hydrolysis affects the antioxidant activity in defatted chia protein. The optimal DH (14.33 %) and antioxidant activity (75.89 %) of *Chlorella pyrenoidosa* protein were reported by Wang and Zhang (2012).

Effect of pH on DH and antioxidant activity

The effect of pH (6.0, 6.5, 7.0) on the DH and antioxidant activity of hydrolysate at 1:25 (w/w) papain/protein ratio, 120 min, 50 °C temperature were investigated and illustrated in Fig. 1b. A significant difference in DH and antioxidant activity were observed between pH 6.0 to 7.0 ($P < 0.05$). At 6.5 pH, found high antioxidant activity of 52.1 % with corresponding DH of 13.8 % as compared to other pH. Furthermore, the pH 6.0, DH and antioxidant activity were 8.8 % and 47.8 %, respectively. Interestingly, DH and antioxidant activity were decreased as the pH raised from 6.5 to 7.0. This could be due that the denaturation of the enzyme structure with loss of activity (Noman et al. 2018). These results were similar with the findings of Islam et al. (2021)



who reported a rise in DH from 9.28 % to 11.06 % with increased pH of 5.0 to 6.0. They also observed a lower degree of hydrolysis at high pH in Grass Turtle (*Chinemys reevesii*) protein hydrolysates prepared using papain.

Effect of temperature on DH and antioxidant activity

The effect of different temperature (50, 55, and 60 °C) on DH and antioxidant activity were carried out at pH 6.5 and 1:25 (w/w) papain/protein ratio, results are depicted in Fig. 1c. A significant difference ($P < 0.05$) was observed between 50 to 60 °C temperature on DH and antioxidant activity. However, the maximum antioxidant activity of 60.2 % was found at 55 °C with corresponding DH 13.6 % as compared with other temperatures. The DH and antioxidant activity of 12.8 % and 52.6 % was observed at 50 °C, further decrease in DH and antioxidant activity was observed at 60 °C. Based on these, temperature 55 °C was considered. Mahdavi Yekta et al. (2019) also reported maximum DH and antioxidant activity of quinoa protein hydrolysed with alcalase and pepsin at 55, 50 °C, respectively. Interestingly, they found no correlation between the degree of hydrolysis and antioxidant activity at different temperatures.

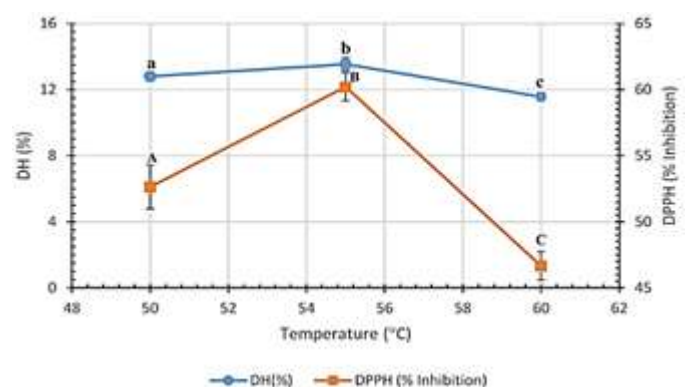


Fig. 1 (a) hydrolysis time, (b) pH, and (c) temperature on the degree of hydrolysis and antioxidant activity of supernatant of hydrolyzed garden cress protein concentrate with papain. Means $\pm$ standard deviation ($n = 3$) with different superscript letters indicate significant differences ($p < 0.05$).

Antioxidant activity of GCPH

The GCPH was prepared with optimal hydrolysis conditions (1:25 papain/protein ratio, temperature 55 °C, pH 6.5, and hydrolysis time 120 min). A significant difference ($P < 0.05$) in DH and antioxidant activity were observed between hydrolysate and unhydrolyzed GPCP (Table 1). This could be due to that, the cleavage of protein at the site of hydrophobic amino acids at optimal hydrolysis condition. The results obtained in this study are in consistent with the previous reports by Mulla and Ahmed (2019), who reported increased antioxidant activity of 10.69 ± 0.37 % and 64.91 ± 1.55 % with corresponding DH of 3.60 ± 0.30 % and 11.18 ± 0.62 % for unhydrolyzed GPCP and GCPH obtained using Alcalase enzyme. Jamdar et al. (2010) found that the antioxidant activity of peanut protein hydrolysates increased with the degree of hydrolysis. Similarly, Zhidong et al. (2013)

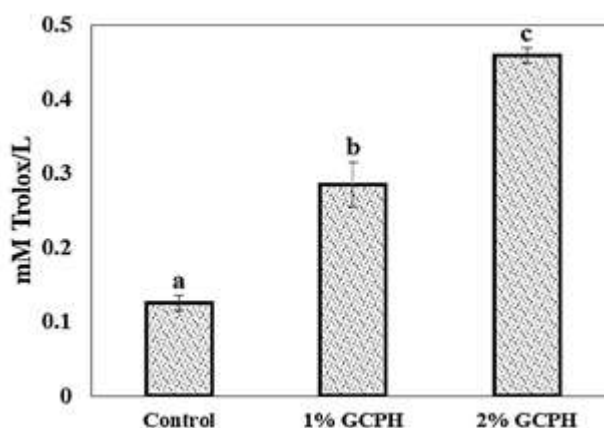


Fig. 2 Antioxidant activity of control, 1 %, and 2 % garden cress protein hydrolysate supplemented Kesar flavoured milk. Means $\pm$ standard deviation ($n = 8$) with different superscript letters indicate significant differences ($p < 0.05$).

Table 1 Degree of hydrolysis and antioxidant activity of garden cress protein and hydrolysate

Sample	Degree of hydrolysis (%)	Antioxidant activity (% Inhibition)
GCPC	2.96 ± 0.23 ^a	10.41 ± 0.52 ^c
GCPH	13.6 ± 1.10 ^b	60.03 ± 0.96 ^d

Values are means ± standard deviation (n = 5) with different superscript letters in the column indicate significant differences (p<0.05). GCPC - garden cress protein concentrate; GCPH - garden cress protein hydrolysate.

Table 2 The sensory analysis of garden cress protein hydrolysate supplemented Kesar flavoured milk evaluated using a 9-point hedonic scale (1-9)

Sensory attributes	Control	2 % GCPH (2 % flavour)	2 % GCPH (3 % flavour)
Colour	8.06 ± 0.13 ^a	7.31 ± 0.6 ^a	7.62 ± 0.15 ^a
Mouth feel	7.87 ± 0.11 ^a	6.62 ± 0.14 ^b	7.0 ± 0.09 ^{ab}
Sweetness	8.0 ± 0.6 ^a	6.66 ± 0.09 ^b	7.27 ± 0.18 ^{ab}
Flavour	7.56 ± 0.16 ^a	6.81 ± 0.22 ^a	7.35 ± 0.24 ^a
Overall acceptability	7.8 ± 0.23 ^a	6.82 ± 0.26 ^a	7.28 ± 0.12 ^a

Values are means ± standard deviation (n = 21) with different superscript letters in the row indicate significant differences (p< 0.05). GCPH - garden cress protein hydrolysate.

observed that whey protein hydrolysates exhibited higher antioxidant activity with increased hydrolysis.

Antioxidant property of Kesar flavoured milk

The antioxidant activity of the kesar flavored milk is shown in (Fig. 2). In order to optimize the addition of GCPH and based on antioxidant activity, the Kesar flavoured milk was prepared by using 1 % and 2 % GCPH with 2 % of Kesar flavour. The addition of GCPH concentration was significant (P<0.05) on the antioxidant activity in flavoured milk as compared to control. The antioxidant activities were 0.12 ± 0.01, 0.28 ± 0.03, 0.45 ± 0.01 mM Trolox/L for control, 1 % and 2 % GCPH supplemented flavoured milk, respectively. Mann et al. (2014) also found an increase in antioxidant activity up to 42.10 % and 21.70 %, after supplementation with 2 % of whey protein hydrolysate prepared using corolase and flavourzyme, respectively in strawberry flavoured milk. Similarly, Hajian et al. (2020) also observed an increase in antioxidant activity after addition of 4 % chymotrypsin camel milk hydrolysates in ice cream.

Sensory evaluation

The sensory scores obtained by hedonic scale for Kesar flavoured milk supplemented with different levels of GCPH are presented in Table 2. From the sensory evaluation, it was revealed that 2 % GCPH supplemented Kesar flavoured milk indicated slight bitter taste as compared to 1 % added GCPH. The control flavoured milk was highly acceptable due to less bitter taste. The lower flavour scores for 2 % GCPH added flavoured milk could be due to the generation of hydrophobic peptides at higher degree of hydrolysis, which might have imparted bitter taste to the flavoured milk. Based on the antioxidant activity and sensory evaluation, 2

% GCPH supplemented flavoured milk was selected and the level of flavour was increased from 2 % to 3 %.

A significant (P<0.05) difference was observed in colour, mouth feel, sweetness, flavour and over all acceptability. Nevertheless, the sensory scores of 2 % GCPH (3 % flavour) was higher as compared to 2 % GCPH (2 % flavour) added flavoured milk, indicating the contribution of addition of higher level of Kesar flavour on sensory scores. Flavored milk supplemented with 2 % GCPH and 3 % Kesar flavor was moderately liked by the sensory panel and was considered optimal on the basis of both sensory and antioxidant activity. Mann et al. (2014) reported that addition 3 % strawberry flavour was sufficient to mask the bitterness in milk beverages incorporated with 2 % flavourzyme and corolase WPHs.

Conclusions

In this study, Kesar flavoured milk was developed with the addition of garden cress protein hydrolysates (GCPH) prepared under optimal hydrolysis conditions (1:25 papain/protein ratio, pH 6.5, and 55°C). The supplementation of 2% GCPH significantly enhanced the antioxidant properties of the Kesar flavoured milk. However, at lower levels of flavor addition, GCPH supplementation negatively impacted sensory scores, imparting a bitter taste to the flavored milk. The results suggest that the sensory quality can be improved by increasing the Kesar flavor concentration to 3% in GCPH-supplemented flavored milk. Therefore, GCPH can be recommended as an antioxidant ingredient to enhance the antioxidant properties of milk and milk products.

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