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Antioxidants, antimicrobial and anticancer activities of whey protein isolate covalently modified with chlorogenic and rosmarinic acids

Essam Elsebaie[1] and Mostafa Ali[1,2]

[1]*Department of Food Technology, Faculty of agriculture, University of Kafrelsheikh, Kafrelsheikh, Egypt*
[2]*Department of Special Food Chemistry, Faculty of Chemistry and Food Chemistry, Techn. Univ. Dresden, Germany*

Abstract. Antimicrobial and anticancer activities of the milk whey protein isolate covalently modified with phenolic compounds, chlorogenic acid and rosmarinic acid were studied. The findings indicated that, the oxidized phenolic compounds, at alkaline conditions (pH9) in open air and at room temperature, were covalently bound to whey protein as confirmed by the decrease in tryptophan content. Where, the ratio of tryptophan decrease was higher in the whey protein modified with rosmarinic acid compared to which modified with chlorogenic acid. Moreover, these interactions were characterized by the RP-HPLC and the results showed that the amount of covalently attached rosmarinic acid to whey protein was higher than the amount of chlorogenic acid. WP modified by RA and CA showed a change in the WP modified with RA showed better antioxidant activity when compared with the unmodified WP and WP modified with CA.

Keywords: covalent modification, whey protein, rosmarinic acid, chlorogenic acid, anticancer, antimicrobial, and functional properties

Introduction

Due to its superior nutritional and functional qualities when compared to other commercial protein sources, whey protein isolate (WP) is one of the principal whey protein products utilised on a large scale in the food industry. It includes β-lactoglobulin with a molecular weight of 18.367 kDa (A form), 18.281 kDa (B form), α-lactalbumin with a molecular weight of 14.175 kDa (A form), 14.175 kDa (B form), and bovine serum albumin with a molecular weight of 66.267 kDa [1-4].

Due to its capacity to bind a variety of molecules, WP can be employed as a carrier for bioactive substances like phenolic compounds [5-8]. Phenolic chemicals are plant ingredients, including several that are widely ingested by humans. Data from human and animal research indicate that dietary phenolic compounds have essential roles in the prevention of a variety of human disorders, including cardiovascular disease, some forms of cancer, and osteoporosis prevention.

Polyphenols are the most abundant secondary metabolite in fruits and vegetables. They appear to have anticancer, anti-inflammatory, and antiallergic properties. Phenolic chemicals interact with proteins via covalent or non-covalent interactions, resulting in protein-phenolics conjugates [1, 9-12].

It is believed that proteins that have been covalently modified with polyphenols lessen the risk of cardiovascular disease and have anti-microbial, anti-inflammatory, anti-carcinogenic, antioxidant, and anti-allergic properties [13, 14]. In a recent study conducted by the author and Elsharkawy in 2018, modified WP containing chlorogenic acid and rosmarinic acid shown a strong antiviral impact on tomato plants infected with the Tobacco Mosaic Virus (TMV). The integration of phenolics in protein, such as dairy proteins, is being investigated as a technique to generate oral nutraceutical formulation due to their antioxidants, even if interactions between polyphenols and proteins may cause a loss of their nutritional qualities [4, 5, 10, 11, 15].

In the developing nations, the rise in non-communicable illnesses has become a serious public health issue. One of the main contributing causes of chronic diseases such cancer, mellitus, neurodegenerative disorders, diabetes and cardiovascular diseases has been recognised as oxidative stress-induced diseases [16]. Cancer has been named as one of the diseases that poses the

Corresponding authors: Essam.ahmed@agr.kfs.edu.eg

greatest threat to humanity. In 2025, there are expected to be 1.8 million new instances of cancer. With 0.3 million annual fatalities, cancer is the leading cause of death in India. The Indian population has been affected by all types of cancer, including those of the mouth, oesophagus, lungs, breast, stomach, rectum, liver, prostrate, cervix, and blood. About 12.3% of newly diagnosed cases of cancer in 2018 were lung and breast cancers. According to information gathered from the WHO mortality database, lung and breast cancer mortality is anticipated to be high in high-income nations by 2030, and lung cancer may surpass breast cancer, making prevention the main factor in lowering incidence [17].

Therefore, the main objectives of this pilot work were to characterize the covalent interactions between whey protein isolate and rosmarinic or chlorogenic acids, at alkaline conditions (pH 9) in the open air and room temperature. Moreover, to study the antimicrobial and anticancer activities of these modified proteins.

Materials. Chlorogenic acid (hereinafter designated as CA) and rosmarinic acid (hence coded as RA) were provided by Sigma Chemicals Co. (Seelze, Germany). The whey protein isolate (hereinafter coded as WP) utilized for the model of modification was acquired from (BiPRO, Davisco Foods International, Inc., Eden Prairie, US) and contained 97.7% protein (75% β-Lactoglobulin on dry matter basis). For HPLC, grade methanol and acetic acid were used. All another chemicals and solvents were also analytical grade.

Methods.

Model of modification. The WP solution (2% dest. water) was maintained at room temperature in the presence of air with CA and RA to evaluate the interaction between WP and phenolic compounds, CA and RA (1.6 mM). NaOH was used to adjust the pH of the combinations to 9. The mixtures were agitated for 24 hours before being dialyzed at room temperature against dest. water for 24 hours. Lastly, all mixtures were freeze-dried and kept at - 20ºC until analysis. Under the same circumstances, the unmodified WP protein (control) was produced without the addition of CA and RA. The proteins were labelled as UWP, which stands for unmodified whey protein, WP-CA, which stands for modified whey protein with chlorogenic acid, and WP-RA, which stands for modified whey protein with rosmarinic acid.

Determination of tryptophan content. The tryptophan content of unmodified WP and WP conjugates was measured using the method outlined by Ali and Elsebaie [14]. In a brief, one milligramme of proteins were dissolved in urea (8M). A Varian Cary Eclipse fluorescence spectrophotometer was used to record the fluorescence intensity (Varian Australia PTY Ltd.). All measurements were performed at room temperature with an excitation wavelength of 290 nm and a slit of 2.5 nm and an emission wavelength range of 300 to 700 nm with a slit of 5 nm. The tryptophan content was calculated using peak intensities. 4 - 20 nM Tryptophan was used to create the calibration curve.

Determination of amount of CA and RA covalently bound to proteins using RP-HPLC. The amount of covalent bound CA and RA by WP proteins was determined as described in [4] with a slight modification. In one ml of 8 M urea solution, 4 mg of UWP, WP-CA, and WP-RA proteins were dissolved. The protein solutions were then precipitated with 20% trichloroacetic acid (TCA). The precipitated protein was then redissolved in 1 mL of 8 M urea. At 37°C, the HPLC (Agilent 1100 Series with a diode-array detector) and PLRP-S column (300, 8 m, 150 x 4.6 mm, Agilent Technologies, Santa Clara, USA) were used to accomplish RP-HPLC. The eluent (A) was 0.1 percent trifluoroacetic acid (v/v), and the eluent (B) was acetonitrile. The gradient was completed as follows: 10-18% B, 1-22 min; 18-80% B, 22-30 min; 80 percent B, 30-33 min; 80-10% B, 33-35 min; 10 percent B, 35-42 min. The whole run duration was 42 minutes. A volume of 50 l was injected at a rate of 0.6 ml/min. The detection wavelengths of 280 and 350 nm, as well as the calibration curve (10-100g quercetin/ml), were employed.

Determination of antioxidative capacity by DPPH assay. The antioxidative capacity of proteins was determined by DPPH assay as described by Binsan et al. (2008), with a slight modification. 0.15 mM of 2,2-diphenyl-1-picryl hydrazyl (DPPH) in 95% ethanol was added to 0.1% protein solution (in 5 mM PBS buffer pH 7.2) in ratio of (1:1, v/v). The mixture was mixed and stored in the dark for 30 min at room temperature. The absorbance of the resulting solution was measured at 517 nm using a spectrophotometer (Helios Gamma; Thermo Fisher Scientific). The blank was prepared in the same manner, except that 5 mM PBS buffer pH 7.2 was

used instead of the sample. The calibration curve was prepared using trolox in the range of 12.5 to 100 µM. The activity was expressed as µM TE/g protein.

Proteins antimicrobial activity determination. The agar diffusion technique, as reported by Ali and Elsebaie [14], was used to assess the antimicrobial activity of the protein samples under investigation. 12.5, 25, and 50 mg of protein per millilitre were used in the tests. While yeast and mould were injected on potato-dextrose agar, the bacterial strains were grown on nutritional agar (PDA). The antibacterial activity was assessed by the diameter of the inhibitory zones.

Proteins anticancer activity determination. Using the Natural Red Uptake (NRU) assay described by Ali and Elsebaie [14], the potential cytotoxicity of the examined protein samples at (12.5, 25 and 50 mg/ml) was evaluated.

Statistical Analysis. All experiments were performed at least 3 times and the results are given as means of their standard deviations. The differences between the samples were analyzed using an analysis of variance (ANOVA) and a post-hoc Tukey test using SPSS software (SPSS, version 18). Results of $p < 0.05$ were considered statistically significant.

Results and discussion

The change in the tryptophan content of modified proteins. The covalent and noncovalent interactions between WP and CA and RA were characterized by the change in the quenching of fluorescence intensity, which is an indicator of tryptophan content. Two procedures were used to determine the amount of total and exposed (surface) tryptophan. Samples were dissolved in 5 mM PBS buffer pH 7.2 or 8M urea, for surface and total tryptophan determination, respectively. The intrinsic emission fluorescence spectra of UWP, WP-CA and WP-RA between 300 to 700 nm was studied and the data showed significant decrease in the fluorescence intensity of modified proteins compared to control (Figure is not shown). β-lactoglobulin is the main fraction of WP and it has two tryptophan residues [18]. As shown in Table 1, the derivatization of WP with CA and RA caused a significant decreased in the tryptophan content, where the values were 61.84, 25.5 and 9.3 nmol/mg protein for UWP, WP-CA and WP-RA, respectively. This means that, up to 58.72 and 85.03 % of tryptophan content were missed when WP incubated with CA and RA (60 mg/g protein), respectively. A comparable phenomenon was recorded by [4] and Ali and Elsharkawy (2018) who found that the content of tryptophan in beta-lactoglobulin and whey protein were decreased when incubated with green coffee extract and chlorogenic and rosmarinic acids for 24 h at ph 9. Moreover, the enzymatic and alkaline modifications of proteins with commercial and natural phenolic compounds decreased the available amino acids: lysine, cysteine, and tryptophan contents [11, 19]. It can be assumed that wp induced preferable to direct interact with quinone formed from the oxidation of rosmarinic acid. This hypothesis is supported by [4, 20]. Since the tryptophan of WP involved in this reaction, the nutritional consequence of protein is the limited availability of the essential amino acid tryptophan.

The amount of covalent bound CA and RA. The amount of covalent attached CA and RA to WP after incubation at alkaline conditions for 24h and at room temperature, was calculated using a RP-HPLC method.

This method depends on that, native WP can be measured at wavelength 280 nm, but it can't measure at 325 and 330 nm (the maximum CA and RA wavelength, respectively) while modified WP with CA and RA can be measure at 325 and 330 nm (the chromatograms were shown in Figure 1 A, B, C and D**,** respectively). Proteins were dissolved in 8M urea because in the presence of urea, protein is denaturated and causes destroy the non-covalent bound [21]. This confirms that, the bond that was formed between WP protein and CA and RA, via tryptophan as mentioned above or lysine, cysteine, was covalent bound.

Table 1. The change in tryptophan content and amount of phenolics covalently bound to whey protein isolates

Parameters / Proteins	UWP	WP-CA	WP-RA
Urea (nmol/mg protein)	61.84±4.63^{a}	25.5±0.1^{b}	9.3±0.2^{c}
% of remaining	100.0±7.48	41.28±0.27	14.97±0.46
PBS (nmol/mg protein)	25.06±2.11^{a}	6.5±0.0^{b}	0.6±0.0^{c}
% of remaining	96.19± 7.36	29.90±0.1	2.54±0.02
Amount of covalent bound phenolics (nmol/mg protein)	0.0±0.0	18.54±0.48^{b}	57.25±0.69^{a}

UWP; unmodified protein, WP-CA; modified WP with chlorogenic acid and WP-RA; modified WP with rosmarinic acid.

Table 2. Antimicrobial activity of whey proteins modified with chlorogenic acid and rosmarinic acid

Samples	Concentration (mg/ml)	Diameter of inhibition zones (mm)			
		Escherichia coli	*Bacillus cereus*	*Aspergillus niger*	*Saccharomyces cerevisiae*
UWP	12.5	0	0	0	0
	25	0	0	3gB	4gA
	50	6fD	7fC	8fB	9dA
WP-RA	12.5	7eC	8eB	9eA	8eB
	25	10bC	12cA	12cA	11bB
	50	11aD	15aA	14aB	13aC
WP-CA	12.5	6fC	7fB	8fA	6fC
	25	8dC	11dA	10dB	8eC
	50	9cC	13bA	13bA	10cB

UWP; unmodified whey protein, WP-RA; modified whey protein with rosmarinic acid and WP-CA; modified wheyprotein with chlorogenic acid.
Means with different superscripts uppercase letters in a row are significantly different at P≤ 0.05.
Means with different superscripts lowercase letters in a column are significantly different at P ≤ 0.05.

The explanation for that is oxidation of CA or RA under alkaline conditions leads to form reactive *o*-quinone, which can be react with nucleophilic compounds such as amino acid side chains, thereby covalently modifying the proteins. The results presented in Table 1 demonstrated that the amount of RA covalently bound to WP after incubation was significantly higher than which incubated with CA, where the values were 57.25 and 18.54 (nmol /mg protein) for WP-RA and WP-CA, respectively.

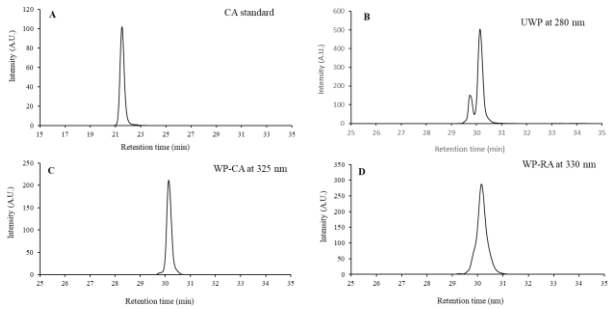

Figure 1. RP-HPLC chromatogram for CA standard (A), UWP (B), WP-CA (C) and WP-RA (D) at wavelengths, 325, 280, 325 and 330 nm, respectively

The change in antioxidative capacity of proteins. The total antioxidant capacity of unmodified and modified WP with CA and RA after alkaline treatment was determined using DPPH assay (Figure 2). The two different phenolics lead to a significant increase in the antioxidative capacity of the WP compared to the UWP. WP-RA exhibited a higher antioxidative capacity than WP-CA and UWP (372.10, 154.90, and 43.40 µM TE/ g protein, respectively).

The higher antioxidative capacity of the WP-RA than the WP-CA is probably caused by the higher amount of RA covalently attached to WP as mentioned above. An increasing antioxidative capacity of modified WP with increasing covalent attachment of a phenolic compound was also observed previously [1, 2, 5, 6, 11]. The antioxidative capacity of WP-RA and WP-CA conjugates formed after incubation at alkaline conditions with WP compared to control may be related to its ability to become a free radical, moreover to the hydroxyl groups of the catechol moieties of phenolic compounds.

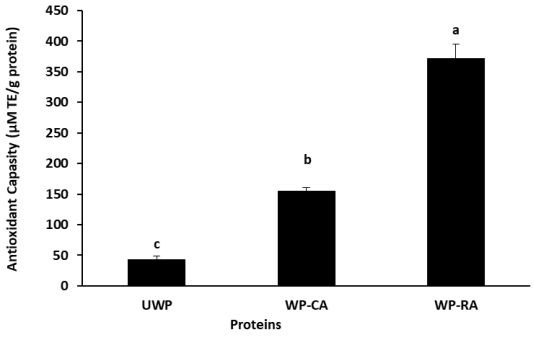

Figure 2. Total DPPH antioxidant capacity of unmodified and modified WP with CA and RA after alkaline treatment

Another explanation is the hydroxyl groups of of phenolic compounds which are produced from the reduction of free quinones of oxidized phenolic compounds, could provide hydrogen to DPPH radicals [11, 22].

Antimicrobial activity of modified proteins. The antimicrobial activity of UWP,WP-CA, and WP-RA solutions were studied at different concentrations (12.5, 25, 37.5 and 50 mg/ml) against two pathogenic bacterial strains, Gram positive, *Bacillus cereus* (DSM351) besides Gram negative, *Escherichia coli* (ATCC25922) and one fungal strain (*Aspergillus niger* MTCC 282). The selection of these microbial strains depended on their

applicable objectives from previous studies. The antimicrobial possibilities for protein solutions were evaluated by measuring the microbial growth inhibition zones. The antimicrobial activity data obtained are shown in Table 2.

The antimicrobial activity values for the protein solutions were linearly increased as a function of increasing the concentration (mg/ml). The results presented in Table 2 indicate that for UWP solution, a concentration of 50 mg/ml led to an inhibition of the growth of all types of microbes under study, while a concentration of 25 mg/ml led to an inhibition of the growth of fungi and yeasts without showing any inhibitory effect on both gram-positive and gram-negative bacteria. Also, the results presented in the same table indicate that all the concentrations used (12.5, 25.0, and 50.0 mg/ml) of WP-RA and WP-CA solution led to an inhibition of both gram-positive or gram-negative bacteria, fungi, and yeasts. The inhibition values obtained by WP-RA solution were higher than those obtained by WP-CA solution at the same concentration. It is also apparent from the same table that the inhibitory impact of all protein solutions on gram-positive was relatively higher than those of gram-negative bacteria. In this connection, the differential sensitivity of gram-positive (more sensitive) and gram-negative bacteria to phenolic compounds was due to the different structures of their respective cell walls. Our obtained data were in the same trend obtained by Ali and Elsebaie (2018) [14]. Generally, the antimicrobial activity of WP-RA was higher than WP-CA this may be due to its higher antioxidant activity and content of polyphenols.

The change in anticancer activity of modified proteins. Whey proteins modified with RA and CA were evaluated for their anticancer activity *in vitro* disease orient anti-tumer screening using neutral red up take (NRU)assay including two human tumor cell lines representing their different cancer types, where MCF7-1(Human breast)and H1299(Human lung carcinoma)cancer cell lines are represented. As shown in Figure 3 the WP-RA and WP-CA appeared that, various cytotoxic potentials against the investigated two lines of human cancer cells. Generally, the decrement in the cell lines tumors was observed as a dependent to the dose. However, the dead cells were increased by increasing the concentration of modified proteins.

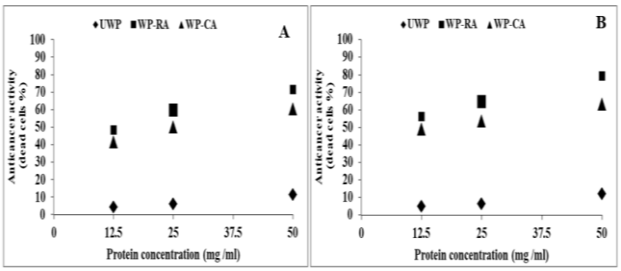

Figure 3. Anticancer activity of whey proteins modified with chlorogenic acid and rosmarinic acid against MCF7-1 (A) and H1299 (B) (UWP; unmodified whey protein, WP-RA; modified whey protein with rosmarinic acid and WP-CA; modified wheyprotein with chlorogenic acid)

It is clear from Figure 3 that the higher the concentration of the protein used, the higher the rate of cancer cell death, whether it was MCF7-1 or H1299. For breast cancer cells (MCF7-1), we note that when using a solution of each of UWP, WP-RA, and WP-CA with a concentration of 12.5 mg/ml, the percentage of cancer cell death was 4.2, 3, 48, and 41.6%, respectively. While the death rate of cancer cells when using a concentration of 50 mg/ml was (11.3%) in the case of UWP, (71.4%) in the case of WP-RA and (60.5%) in the case of WP-CA.

It is also clear from the same figure that by increasing the concentration of the used protein solution from 12.5 to 50 mg/ml, an increase in the percentage of H1299 cancer cell deaths occurred. The results also indicate that the highest death rate of H1299 cancer cells was at a concentration of 50 mg/ml, which was 79.2% (WP-RA), then 63.4% (WP-CA), and then 11.9% (UWP). Also, from the results, it is clear that the IC50 values of WP-R A solution are 12.62 and 10.65 mg/ml for both MCF7-1 and H1299, respectively. Also, the IC50 values for WP-C A solution were 24.8 and 15.04 mg/ml for MCF7-1 and H1299, respectively.

However, chlorogenic acid and rosmarinic acid act an important role in reducing breast cancer by regulating the mRNA expression in MCF-7 cell, which may be the mechanism of its antitumor effect as reported by [23], Hossan, Rahman [24].

Conclusion

According to our knowledge, there are no more studies were done to study the covalent modification of whey protein with rosmarinic acid and the antimicrobial and anticancer properties of formed conjugates. Consequently, this study was conducted to give an evidence for covalent

interactions between both, CA and RA, and whey protein. These interactions were characterized using quenching fluorescence intensity and RP-HPLC. Moreover, the effects of these interactions on antimicrobial and anticancer properties of proteins were studied. The results of this study demonstrated that, the covalent interactions between WP and CA or RA caused a significant increase in antioxidant, antimicrobial and anticancer activities. Finally, the consumers can be benefit from a health promoting effect of conjugated phenolic compounds. Therefore, we can recommend that, these modified proteins can be used to develop nutraceutical foods which can meet the modern consumer needs.

Compliance with Ethics Requirements: Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human or animal subjects (if exist) respect the specific regulation and standards.

References

1. Ali, M., *Chemical, structural and functional properties of whey proteins covalently modified with phytochemical compounds.* Journal of Food Measurement and Characterization, 2019. **13**(4): p. 2970-2979.
2. Ali, M. and M.M. Elsharkawy, *Characterization of Whey Protein Isolate Covalently Modified with Phenolic Compounds – 1: Antioxidant and Antiviral Activities.* Journal of Food and Dairy Science, Mansoura University, 2018. **9**(12): p. 385-393.
3. Keppler, J.K., et al., *Characterization of the covalent binding of allyl isothiocyanate to β-lactoglobulin by fluorescence quenching, equilibrium measurement, and mass spectrometry.* Journal of Biomolecular Structure and Dynamics, 2014. **32**(7): p. 1103-1117.
4. Ali, M., et al., *Milk Whey Protein Modification by Coffee-Specific Phenolics: Effect on Structural and Functional Properties.* Journal of Agricultural and Food Chemistry, 2013. **61**(28): p. 6911-6920.
5. Ali, M. and S.G. Arafa, *Characterization of Whey Protein Isolate Covalently Modified with Phenolic Compounds 2: Physiochemical and Emulsifying Properties.* Journal of Food and Dairy Science, Mansoura University, 2019. **10**(1): p. 1-8.
6. Ali, M., et al., *Covalent Whey Protein - Rosmarinic Acid Interactions: A Comparison of Alkaline and Enzymatic Modifications on Physicochemical, Antioxidative, and Antibacterial Properties.* Journal of Food Science, 2018. **83**(8): p. 2092-2100.
7. Keppler, J.K., et al., *Functionality of whey proteins covalently modified by allyl isothiocyanate. Part 1 physicochemical and antibacterial properties of native and modified whey proteins at pH 2 to 7.* Food Hydrocolloids, 2017. **65**: p. 130-143.
8. Cao, Y. and Y.L. Xiong, *Interaction of Whey Proteins with Phenolic Derivatives Under Neutral and Acidic pH Conditions.* Journal of Food Science, 2017. **82**(2): p. 409-419.
9. Gan, J., et al., *Interactions of β--Conglycinin (7S) with Different Phenolic Acids - Impact on Structural Characteristics and Proteolytic Degradation of Proteins.* International Journal of Molecular Sciences, 2016. **17**(10): p. 1671.
10. You, J., Y. Luo, and J. Wu, *Conjugation of Ovotransferrin with Catechin Shows Improved Antioxidant Activity.* Journal of Agricultural and Food Chemistry, 2012. **62**(12): p. 2581-2587.
11. Aewsiri, T., et al., *Antioxidative activity and emulsifying properties of cuttlefish skin gelatin modified by oxidised phenolic compounds.* Food Chemistry, 2009. **117**(1): p. 160-168.
12. Prigent, S.V.E., et al., *Covalent interactions between amino acid side chains and oxidation products of caffeoylquinic acid (chlorogenic acid).* Journal of the Science of Food and Agriculture, 2008. **88**(10): p. 1748-1754.
13. Von Staszewski, M., A.M. Pilosof, and R.J. Jagus, *Antioxidant and antimicrobial performance of different Argentinean green tea varieties as affected by whey proteins.* Food Chemistry, 2011. **125**(1): p. 186-192.
14. Ali, M. and E. Elsebaie, *Milk whey proteins and onion extract powder interactions-antimicrobial and anticancer activities.* Journal of Agroalimentary Processes and Technologies 2018. **20**(3): p. 152-160
15. Ali, M., et al., *Characterization and Modeling of the Interactions between Coffee Storage Proteins and Phenolic Compounds.* Journal of Agricultural and Food Chemistry, 2012. **60**(46): p. 11601-11608.
16. Wanyo, P., N. Meeso, and S. Siriamornpun, *Effects of different treatments on the antioxidant properties and phenolic compounds of rice bran and rice husk.* Food chemistry, 2014. **157**: p. 457-463.
17. Avanthi, P. and N. Vigasini, *In vitro Antioxidant and Anticancer Activity of Ethanolic Extract of Defatted Rice Bran on MCF-7 (Breast) and A549 (Lung) Cancer Cell Lines.* Asian Journal of Biological and Life Sciences, 2021. **10**(1): p. 211.
18. Jia, Z., et al., *Effect of covalent modification by (-)-epigallocatechin-3-gallate on physicochemical and functional properties of whey protein isolate.* LWT - Food Science and Technology, 2016. **66**: p. 305-310.
19. Rawel, H.M., et al., *Determining the binding affinities of phenolic compounds to proteins by quenching of the intrinsic tryptophan fluorescence.* Molecular Nutrition & Food Research, 2006. **50**(8): p. 705-713.
20. Rawel, H.M., et al., *Interactions of different phenolic acids and flavonoids with soy proteins.* Int J Biol Macromol, 2002. **30**(3-4): p. 137-50.
21. Guharay, J., B. Sengupta, and P.K. Sengupta, *Protein–flavonol interaction: Fluorescence spectroscopic study.* Proteins: Structure, Function, and Bioinformatics, 2001. **43**(2): p. 75-81.
22. Rohn, S., H.M. Rawel, and J. Kroll, *Antioxidant activity of protein-bound quercetin.* J Agric Food Chem, 2004. **52**(15): p. 4725-9.
23. Nwafor, E.-O., et al., *Chlorogenic acid: Potential source of natural drugs for the therapeutics of fibrosis and cancer.* Translational Oncology, 2022. **15**(1): p. 101294.
24. Hossan, M.S., et al., *Rosmarinic acid: A review of its anticancer action.* World J. Pharm. Pharm. Sci, 2014. **3**(9): p. 57-70.