

Milk whey proteins and onion extract powder interactions - antimicrobial and anticancer activities

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Abstract

The effect of modification of milk whey protein (WP) with onion extract and quercetin on their antimicrobial and anticancer activities was studied. WP was modified with onion extract powder rich with quercetin (OE) and quercetin dihydrate (Q) with concentration of (60 mg/g protein) at alkaline conditions (pH 9) and room temperature in the presence of air for 24 h. The findings showed that the quercetin of OE and Q was covalently attached to WP as illustrated by RP-HPLC and the decrease in tryptophan content. In addition, the derivatives produced between WP, OE and Q were characterized in terms of their antimicrobial and anticancer properties. The results displayed that, WP modified with OE or Q showed a significant antimicrobial activity especially against gram-positive bacteria (*Bacillus cereus*) compared to unmodified WP (control). WP modified with Q was more effective than other investigated samples. Moreover, WP modified with OE and Q showed a significant anticancer activity especially against H1299 (carcinogenic cells of human lung). The activity of WP modified with Q against MCF7-1 (carcinogenic cells of human breast) and H1299 (carcinogenic cells of human lung) was higher than those of WP modified with OE where their IC₅₀ values were 13.6 µg/ml and 18.7 µg/ml, respectively. Therefore, this study suggests that these proteins may have potential alternative antimicrobial and anticancer properties as functional ingredients, so they can be used in preparing functional and nutraceutical foods.

Keywords: Antimicrobial and anticancer activities, Onion extract, covalent modification, whey protein and functional properties

1. Introduction

Polyphenols are considered one of the main secondary metabolites groups in fruits and vegetables. Flavonoids, chemically comprises of two phenyl rings and heterocyclic ring, are account roughly 66% of plant polyphenols in the human eating regime [1]. They appear to show important anti-allergic, anti-inflammatory, and anticancer activities. Flavonoids are likewise observed to be effective as antioxidant, antimicrobial and anticancer substance [2]. Quercetin, flavonoid group, is generally spread in many vegetables, fruits, grains, and leaves. Onions, unpeeled apples, kale, citrus fruits, and berries are the rich sources of

dietary quercetin. It can be utilized as an ingredient in supplements, beverages, or foods.

According to literature, the daily intake of quercetin was usually varied depending on the country. Where, the average and median quercetin intakes were 10.3 mg/day in Germany [3], whereas the range of 6-18 mg/day was stated in the U.S. [4, 5]. Polyphenols interact with proteins via covalent and non-covalent bounds and form protein-phenolics conjugates [6-16]. Figure (1) illustrates the covalent interaction between quercetin and protein at alkaline condition in the presence of oxygen. Quercetin oxidizes to reactive quinone radical, which react with nucleophilic amino acids of proteins such as lysine or tryptophan to form protein-quercetin conjugates.

As apparent also in the same figure that a second oxidation could be happened to form a protein-quercetin quinone, that could be react with another nucleophilic amino acids of proteins, resulting in protein-quercetin polymers [17].

Proteins covalently modified with polyphenols, are thought to act as antioxidant, anti-carcinogenic, anti-microbial, anti-allergic, anti-mutagenic and anti-inflammatory, as well as reduce cardiovascular diseases [16, 18]. The mechanism by which an antimicrobial peptides achieves its function depends on many factors such as, the amino acid sequence, oligomerization, peptide concentration, net charge, a number of physicochemical properties, amphipathicity, hydrophobicity, structural folding (secondary structure, orientation and dynamics) in membranes and membrane composition [19]. There are many mechanisms of membrane-disruption suggested to clarify the action of antimicrobial peptides. Of these models are barrel-stave model [19], and detergent-type membrane lytic mechanism [20, 21].

Cancer is identified as uncontrolled growth and spread of abnormal cells, which may be encouraged by external factors (radiation, chemicals, and infectious organisms) or internal factors (mutations, hormones, and altered immunity). The carcinogenesis process consists of multiple stages of molecular biochemical alterations in the cells of the tissue where cancer develops. The three main stages in carcinogenesis are: (i) initiation, in which free radicals induce genetic changes; (ii) promotion, which is a relatively slower stage of carcinogenesis and involves selective and sustained preneoplasia, causing specific expansion of initiated cells to a benign tumor; and (iii) tumor progression, in which a high degree of genetic instability prompts chromosomal alterations [22].

An estimated 12.7 million people were diagnosed with cancer across the world in 2008, and 7.6 million people died from the cancer during the same year [23]. Lung (12.7%), breast (10.9%), and colorectal (9.7%) cancers were the most commonly diagnosed cancers world-wide in 2011 [24]. Breast cancer is one of the most common malignancies and it has a serious impact on female health [25]. However, Deng et al. [26] found that quercetin induced the apoptosis of MCF-7 cells and inhibited the proliferation of the MCF-7 breast cancer cells depending on the time of concentration of quercetin used.

Lung cancer is the leading cause of cancer death in many countries [27]. Quercetin is considered one of the most dietary flavonoids, that has diverse biological activities, moreover it has been reported to inhibit the growth of several human cancers including leukemia, breast and lung cancers [28].

As mentioned above, the antimicrobial and anticancer effects of polyphenols, including flavonoids, and native whey proteins have been reported by many researchers, but till now no studies were done to study the antimicrobial and anticancer effects of proteins-phenolics conjugates. Therefore, the main objectives of this study were to characterize the covalent interactions between whey protein and onion extract powder rich with quercetin (OE) and quercetin dihydrate (Q), at alkaline conditions (pH 9) and room temperature. The second target is to investigate the effect of covalent modification of whey protein with OE and Q on their antimicrobial and anticancer properties.

2. Materials and Methods

Materials: Whey protein isolate (WP, 97.7% protein) was purchased from (BiPRO, Davisco Foods International, Inc., Eden Prairie, USA). Onion extract powder rich with quercetin (OE, *Allium cepa* L) was purchased from (Rudolf Wild GmbH & Company KG). The HPLC with diode-array detection was used to determine the flavonoid contents of the OE and the data of quantification showed that it contained 95.49 ± 1.5 % quercetin on dry weight basis. Quercetin dihydrate (Q, 97%; 2-(3,4 - dihydroxyphenyl) - 3,5,7 - trihydroxy - 4H chromen - 4 - one dehydrate) was obtained from Alfa Aesar (Germany). 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid, ABTS), and 6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carbon acid (Trolox) were purchased from Sigma-Aldrich (Seelze, Germany).

Microorganisms and cancer cells: Bacterial strains representing Gram positive, *Bacillus cereus* (DSM351) besides Gram negative, *Escherichia coli* (ATCC25922), mold strain (*Aspergillus niger*) and yeast strain (*Saccharomyces cerevisiae*) were supplied by Microbiology Department, Faculty of Agriculture, Kafrelsheikh University, Egypt. Drug cytotoxicity MCF7-1 (Breast) and drug cytotoxicity H1299-1 (Lung) were obtained from National Institute of Oncology, Cairo, Egypt.

Methods: Covalent modification of WP with onion extract and quercetin: Covalent modification of WP with OE and Q was done as designated by Ali et al. [16] with minor changes. 2g of WP was suspended in 90 ml of distilled water for 1 h. The solution pH was adjusted to 9 by 2N NaOH. Afterward, 120 mg of OE and Q dissolved in 10 ml of ethanol was added followed by re-adjustment of pH to 9. The solutions were continuously stirred at room temperature for 24 h in open flasks. The solutions were then dialyzed against distilled water for one day at room temperature followed by freeze – drying. In parallel, the unmodified WP protein was prepared with the same method but without adding OE and Q.

Characterization of modified proteins. The change in tryptophan content: The tryptophan content of unmodified WP and WP conjugates was measured as described in [16]. Proteins solutions (1 mg/ 1 ml 8M urea) were prepared and the fluorescence intensities were measured using a Varian Cary Eclipse fluorescence spectrophotometer (Varian Australia PTY Ltd.). All measurements were done at room temperature using an excitation at 290 nm and slit 2.5 nm and emission at wavelength range between 300 to 700 nm with slit 5 nm. The tryptophan content was calculated from the highest intensities of peak using Tryptophan standard curve (4 – 20 nM).

The amount of quercetin covalently bound to proteins: The amount of covalent bound quercetin by WP proteins was determined using RP-HPLC method as outlined by Ali et al. [16]. 4 mg of modified and unmodified proteins were dissolved in 1ml of 8 M urea solution. 1mL of 20 % trichloroacetic acid (TCA) was mixed with 1 mL of protein solution to precipitate proteins and remove the free Q. Then, the precipitated protein was re-dissolved in 1 ml of 8M urea. RP-HPLC was achieved with the HPLC (Agilent 1100 Series with a diode-array detector) and PLRP-S column (300 Å, 8 m, 150 x 4.6 mm, Agilent Technologies, Santa Clara, USA) at 37°C. 0.1 % trifluoroacetic acid (v/v) was used as eluent (A), and acetonitrile as eluent (B). The gradient was done as follows: 10-18 % B, 1-22 min; 18–80% B, 22-30 min; 80 % B, 30-33 min; 80-10% B, 33-35 min; 10 % B, 35-42 min. Total run time was 42 min. 50 µl was injected with a flow rate of 0.6 ml/min. The wavelengths of detection were applied at 280 and 350 nm, and standard curve using (10–100µg quercetin /ml) was prepared.

Determination of antimicrobial activity of proteins: Antimicrobial activities of investigated protein samples were measured using the agar diffusion method as described by Hammer et al. [29]. Tests were conducted at the following protein concentrations: 12.5, 25, and 50mg /1ml. The bacterial strains were cultured on nutrient agar, while yeast and mold were inoculated on potato-dextrose agar (PDA). The inhibition zones diameter was taken as measured of the antimicrobial activity.

Determination of anticancer activity of proteins: Potential cytotoxicity of investigated protein samples at (12.5, 25 and 50 mg/ml) was tested using the Natural red Uptake (NRU) assay as outlined by Benassi et al. [30].

Statistical Analysis: All experiments were performed at triplicates 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.

3. Results and discussion

The change in tryptophan content of modified proteins: The covalent interactions between WP and OE and Q were characterized by the change in the quenching of fluorescence intensity, which is an indicator of tryptophan content. The intrinsic emission fluorescence spectra of UWP, WP-OE and WP-Q between 300 to 700 nm was studied and the data showed significant decrease in the fluorescence intensity of modified proteins compared to control. β -lactoglobulin is the main fraction of WP and it has two tryptophan residues [31]. As shown in (Figure 2A), the derivatization of WP with OE and Q caused a significant decrease in the tryptophan content, where the values were 57.57, 11.62 and 8.74 nmol /mg protein for UWP, WP-OE and WP-Q, respectively. This means that, up to 79.82 and 84.82 % of tryptophan content were missed when WP incubated with OE and Q (60 mg/g protein), respectively. A comparable phenomenon was recorded by Ali et al. [11], who found that the content of tryptophan in beta-lactoglobulin was decreased when incubated with green coffee extract for 24 h at pH 9. It can be assumed that quercetin induced preferable to directly interact with quinone formed from the oxidation of quercetin. This hypothesis is supported by the findings of [11]. 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 quercetin: The amount of covalent attached quercetin to WP after incubation with OE and Q, at alkaline conditions for 24h and at room temperature, was calculated using a RP-HPLC method (roughly method). This method depends on the, native WP that can be measured at wavelength of 280 nm, but it cannot measure at 350 nm (the maximum quercetin wavelength) while modified WP with quercetin can be measured at 350 nm (the chromatograms were shown in Figure3).

Proteins were dissolved in 8M urea because in the presence of urea, the denaturation of protein helped

to destroy the non-covalent bound [32]. This confirms that, the bond that formed between WP protein and quercetin, via tryptophan as mentioned above was covalent bond. This may be attributed to the oxidation of quercetin 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 (Figure2B) demonstrated that the amount of quercetin covalently bound to WP after incubation with OE was lower than that incubated with Q, where the values were 6.61 and 8.11 ($\mu\text{g}/\text{mg}$) for WP-OE and WP-Q, respectively.

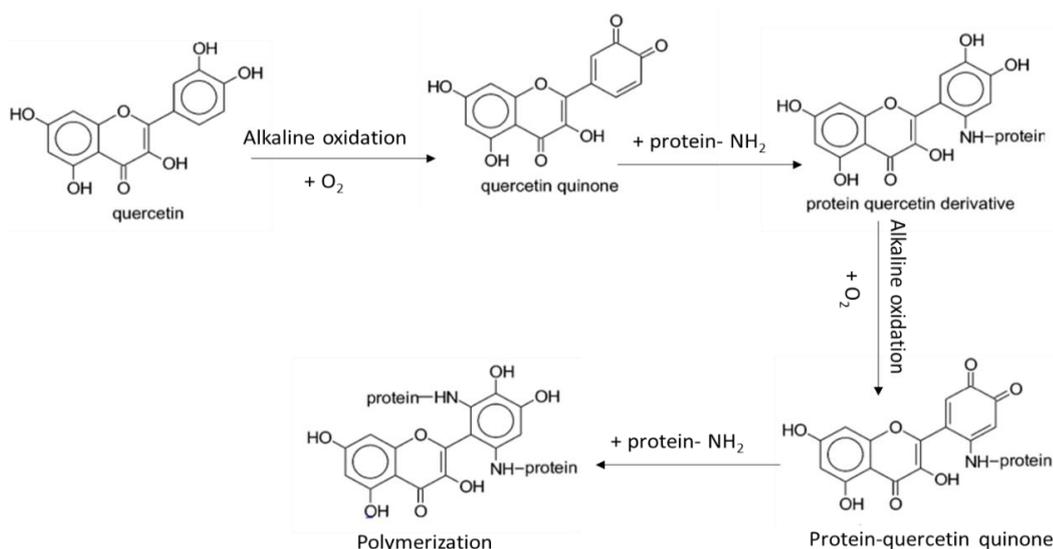
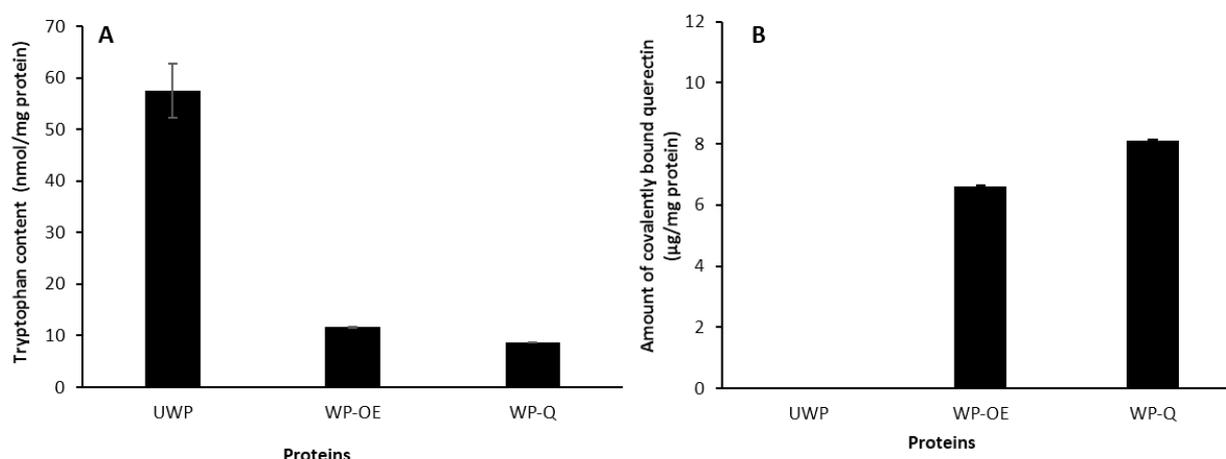
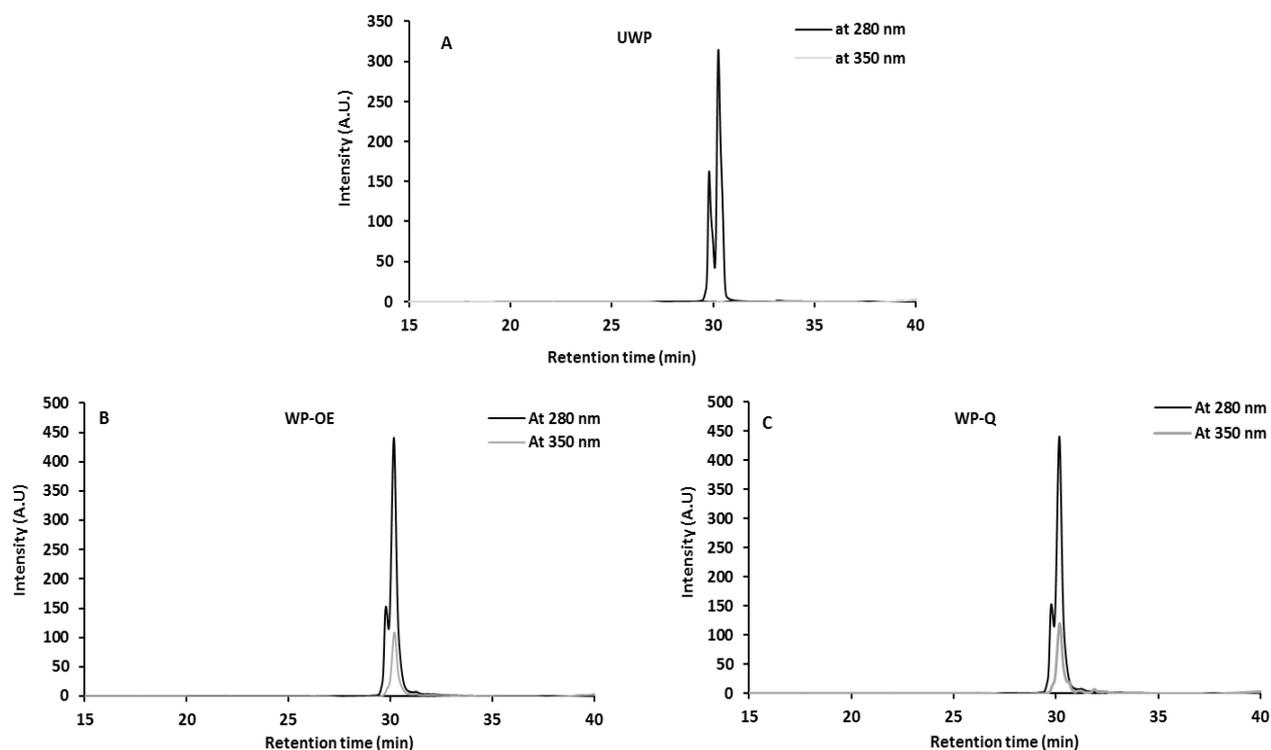


Figure 1. Reaction of quercetin with free amino groups of protein after quinone formation adapted from [17]



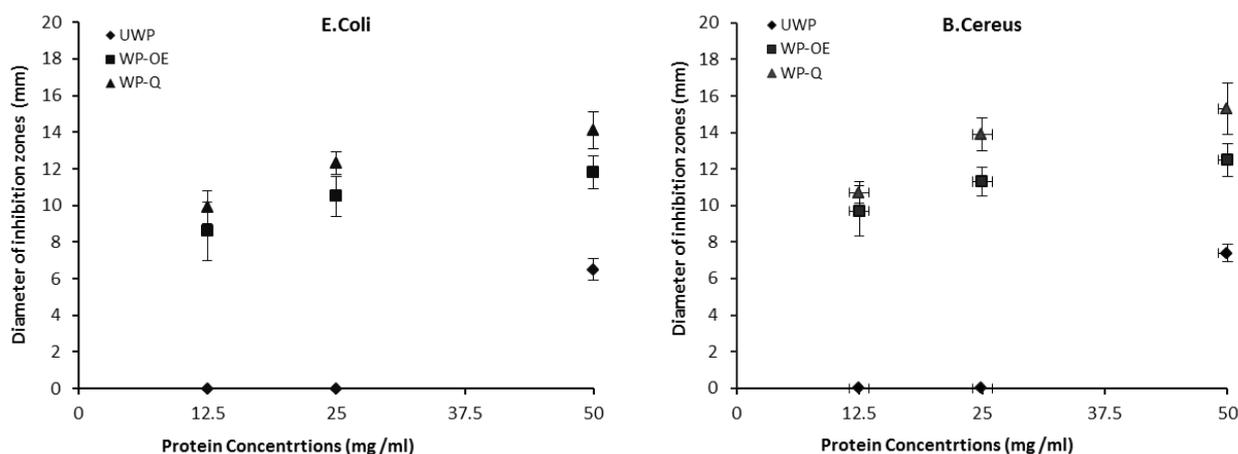
Where: UWP; unmodified whey protein, WP-OE; modified whey protein with onion extract and WP-Q; modified whey protein with quercetin

Figure 2. The change in tryptophan content (A) and amount of quercetin covalently bound to whey proteins (B).



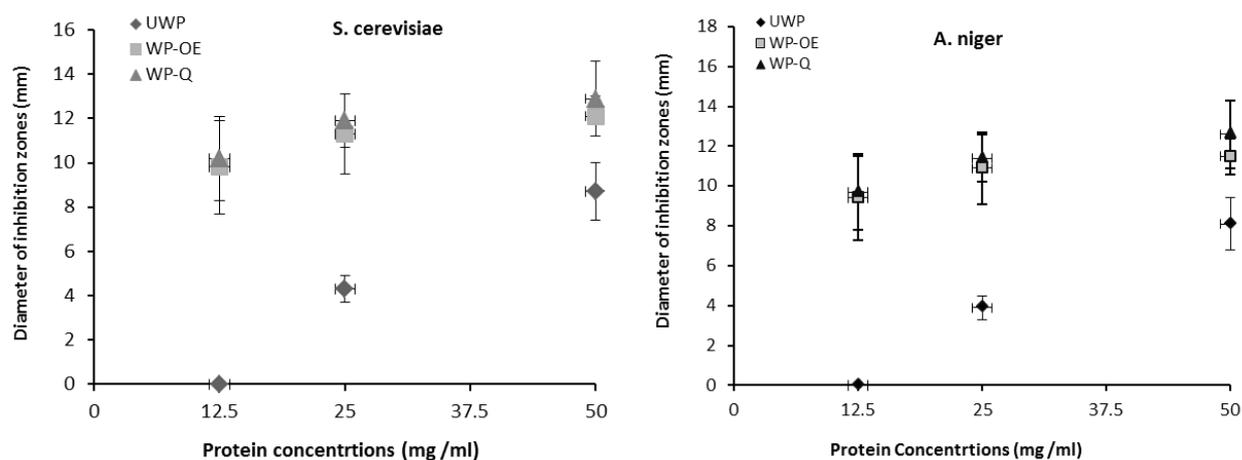
Where: UWP; unmodified whey protein, WP-OE; modified whey protein with onion extract and WP-Q; modified whey protein with quercetin.

Figure 3. RP-HPLC chromatogram for UWP (A), WP-OE (B) and WP-Q (C) at wavelengths 280 and 350 nm.



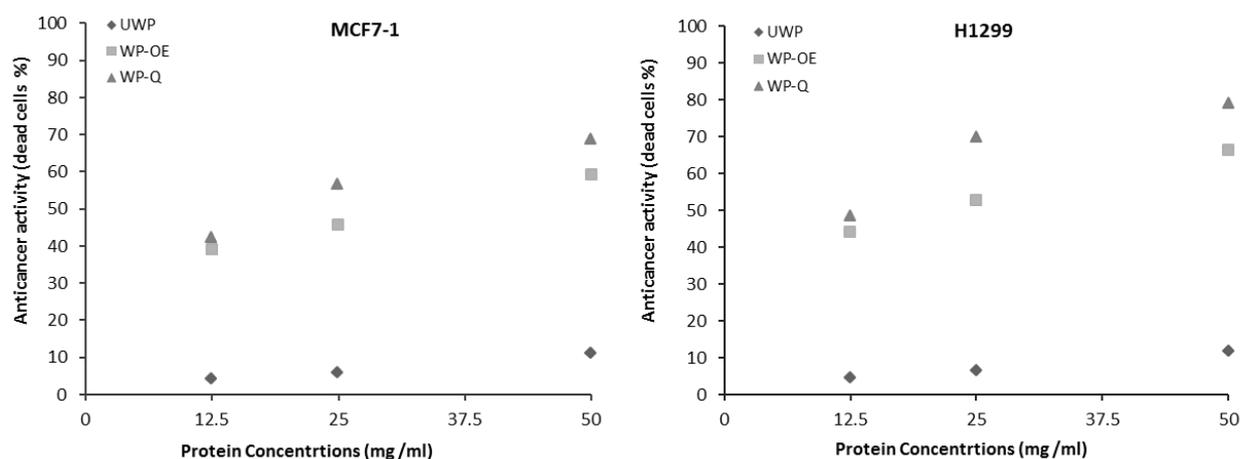
Where: UWP; unmodified whey protein, WP-OE; modified whey protein with onion extract and WP-Q; modified whey protein with quercetin.

Figure 4. Antibacterial activity of whey proteins modified with onion extract powder and quercetin dihydrate against *E. coli* and *B. Cereus*.



Where: UWP; unmodified whey protein, WP-OE; modified whey protein with onion extract and WP-Q; modified whey protein with quercetin.

Figure 5. Antifungal activity of whey proteins modified with onion extract powder and quercetin dihydrate against *S. cerevisiae* and *A. niger*.



Where: UWP; unmodified whey protein, WP-OE; modified whey protein with onion extract and WP-Q; modified whey protein with quercetin.

Figure 6. Anticancer activity of whey proteins modified with onion extract powder and quercetin dihydrate against cancer cells; MCF7-1 (Human breast) and H1299 (Human lung carcinoma).

The change in antimicrobial activity of modified proteins: Antibacterial activity of UWP, WP-Q, and WP-OE solutions exhibited different inhibition levels against *E. coli* and *B. Cereus* as shown in (Figure 4). In the dose response study, the inhibition zone increased with increasing concentration of solutions. Low concentration (12.5 mg/ml) inhibited weakly the development of bacteria; however, *B. Cereus* was more sensitive than *E. coli*. At high concentrations (50 mg/ml), UWP solution exhibited marked inhibition activity against bacteria, and inhibition of WP-Q solution was the strongest among the different tested samples (UWP and WP-OE solutions). These results are in agreement with

data published recently by Ali et al. [16] who found that the whey protein isolate modified enzymatically by rosmarinic acid showed antibacterial activity. The activity appears to be related to damage occurred in the cytoplasmic membrane and other structures, reduction of the pH gradient across the cytoplasmic membrane; permeabilization (generally by hydrophobic compounds able to disintegrate the lipopolysaccharide layer of the outer membrane of Gram-negative organisms); inhibition of extracellular microbial enzymes; inhibition of oxygen consumption and disruption of the membrane-associated respiratory chain, etc. In this connection, the differential sensitivity of Gram-

positive (more sensitive) and Gram negative bacteria to phenolic compounds was due to the different structure of their respective cell walls [18, 33].

The effect of UWP, WP-Q, and WP-OE solutions as antifungal activity on *S. cerevisiae* and *A. niger* are illustrated in (Figure 5). According to the dose response study, no inhibition was detected against *S. cerevisiae* and *A. niger* at low concentrations (12.5 mg/ml) UWP. However, a marked inhibition was observed with higher concentrations. On the other hand, all concentrations of WP-Q and WP-OE solutions exhibited potent inhibitory activity against *S. cerevisiae* and *A. niger*. Statistically, there was a significant difference between concentrations 12.5, 25.0 and 50.0 mg/ml of each investigated solution. The sensitivity of *S. cerevisiae* to UWP, WP-Q and WP-OE solutions was in the same trend with those of *A. niger*. However, WP-Q solutions gave higher inhibitory effect against *S. cerevisiae* and *A. niger* than WP-OE (Fig. 5). These results were in harmony with those obtained by Santos et al. [34].

The change in anticancer activity of modified proteins: Whey proteins modified with OE and Q were evaluated for their anticancer activity *in vitro* disease orient anti-tumor 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 6), the investigated solutions revealed different cytotoxic activities toward the two human cancer cell lines investigated. In general, a dose dependent decrease in the survival of the two tumor cell lines was observed. However, the dead cells were increased by increasing the concentration of all investigated samples. The highest MCF7-1 dead cell percentage was recorded by WP-Q (68.9%) at a concentration of (50 mg/ml) while WP-Q was 42.4% and 56.6% at concentrations of 12.5 and 25 mg/ml, respectively. Meanwhile, in WP-OE the MCF7-1 dead cell was 38.9%, 45.6% and 59.1% at concentrations of 12.5, 25 and 50 mg/ml, respectively. The lowest MCF7-1 dead cell was recorded in UWP (4.2%) at a concentration (12.5 mg/ml), (5.9%) at a concentration of (25 mg/ml) and (11.3%) at a concentration (50 mg/ml).

The same trend was observed in H1299 cells as in MCF7-1 cells. The highest dead cells percentage of H1299 was recorded by WP-Q (79.1%) at a concentration of (50 mg/ml) while WP-Q was

48.6% and 69.8% at concentrations of 12.5 and 25 mg/ml, respectively. Besides, WP-OE recorded dead cells (44.2%) at a concentration of (12.5 mg/ml), (52.7%) at a concentration of (25 mg/ml) and (66.3%) at a concentration of (50 mg/ml). Furthermore, the lowest H1299 dead cell count was observed by UWP at (4.8%), (6.5%) and (11.9%) at concentrations of (12.5 mg/ml), (25 mg/ml) and (50 mg/ml), respectively. Of all investigated samples, WP-Q exhibited the strongest cytotoxicity toward cancer cells. Moreover, UWP and WP-OE possessed less cytotoxic activities toward MCF7-1 and H1299 cell lines.

In addition, the data in the same figure revealed that WP-Q exhibited excellent cytotoxic activity against MCF7-1 (Human breast) ($IC_{50} = 18.7$ mg/ml) and H1299 (Human lung carcinoma) ($IC_{50} = 13.6$ mg/ml), compared with WP-OE which had ($IC_{50} = 33.2$ mg/ml) for breast cancer (MCF7-1) and ($IC_{50} = 21.7$ mg/ml) for human lung cancer (H1299). Meanwhile, UWP recorded ($IC_{50} = 251.3$ mg/ml) and ($IC_{50} = 248.2$ mg/ml) for breast cancer (MCF7-1) and human lung cancer (H1299), respectively. The IC_{50} values indicated that the anticancer activity of WP-Q against both cell lines was quite high in case of WP-OE and UWP. Generally, MCF7-1 cells were less sensitive than H1299 cell lines toward the investigated samples.

However, quercetin plays an active part in reducing breast cancer by regulating the expression of surviving mRNA in MCF-7 cell, which may be the mechanism of its antitumor effect as reported by Deng et al. [26]. Moreover, Nguyen et al. [35] found that quercetin inhibited proliferation through G2/M arrest of the cell cycle and induced apoptosis via caspase-3 cascade in the human lung cancer cell line NCL-H209.

Anticancer potential of milk whey protein is believed to derive from antioxidant, detoxifying and immune enhancing effects of glutathione, which is the major endogenous antioxidant produced by cells, providing protection for RNA, DNA and proteins. In addition, *in vitro* study encouraged and demonstrated inhibition of growth in human breast cancer cells when treated with bovine serum albumin proteins [36].

4. Conclusion

According to our knowledge, there are not enough studies done to study the covalent modification of whey protein with onion extract.

Consequently, this study was conducted to give an evidence for covalent interactions between both, onion extract rich with quercetin and quercetin dihydrate, and whey protein. These interactions were characterized using quenching fluorescence intensity and RP-HPLC. Moreover, the activities of these interactions as antimicrobial and anticancer were studied. The results of this study demonstrated that, the covalent interactions between WP and quercetin or onion extract caused a significant increase in antimicrobial and anticancer activities. Finally, the consumers can get a benefit from the health promoting effect of these conjugated phenolic compounds. Therefore, it can recommend that, these modified proteins can be used to develop nutraceutical foods, which can meet the modern consumer needs.

Acknowledgements: The authors would like to thank Egyptian government - Kafrelsheikh University for financial support and members of food technology division, Kiel University, Germany, for their practical support analysis and providing us with milk whey protein.

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.

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