# INFLUENCE OF PEPTIDE-QUERCETIN INTERACTION ON THE PHYSICOCHEMICAL PROPERTY AND ANTIOXIDANT ACTIVITY OF PHE-CYS AND QUERCETIN

**By**

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**Thesis submitted to the University of Ottawa in partial fulfilment of the requirements**

**for the**

**Master of Science degree in Nutrition and Food Biosciences**

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**April 2025**

## DEDICATION

This thesis is dedicated to the Almighty God for His abundance, blessings, grace and for seeing me through this research period.

## ACKNOWLEDGEMENTS

I would like to express my sincere thanks to my supervisor Prof. Chibuike C. Udenigwe, who bet on me and gave me a chance to explore my potential. Thank you for your patience, guidance, advice, correction, and encouragement during this period. I do not take your mentorship for granted.

My greatest appreciation goes to my mother Dr. (Mrs.) Ogunrinola for her support, prayers, encouragement, for laying the foundation of my education which kept me motivated throughout the program. To my siblings, Adeola, Mayowa, Busolami Ogunrinola for their love, you are the reason I work so hard.

To my love, Ibukun, you have been my pillar of support through this journey, thank you for everything. I can't fail to acknowledge my dear friends and lab members. I am grateful for your kindness, guidance, feedback, friendship, and mentorship throughout this project. I love you all and your friendship means the world to me. Thank you for always being there for me.

Finally, this research was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant, and the University Research Chairs Program, University of Ottawa.

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**ABTS** – 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid)

***C. elegans*** - *Caenorhabditis elegans*

**Cys** – Cysteine

**DPPH** – 2,2-Diphenyl-1-picrylhydrazyl

**ET** – Electron Transfer

**FRAP** – Ferric Reducing Antioxidant Power

**GSH** – Reduced Glutathione

**HAT** – Hydrogen Atom Transfer

**HyPer** – $H_2O_2$ Redox Sensor

**Phe** – Phenylalanine

**Phe-Cys** – Phenylalanine-Cysteine Dipeptide

**ROS** – Reactive Oxygen Species

## ABSTRACT

Interaction between peptides and polyphenols, yielding peptide-polyphenol complexes, spontaneously occurs in most food systems, and is known to have an impact on sensory, functional, and nutraceutical properties of the food products. A better understanding of peptide-phenolic compound interactions would help to control the functional properties of peptides in food products during processing, transportation, and storage. Furthermore, it is necessary to understand the binding mechanism between polyphenols and peptides, which is useful for the development of peptides as delivery systems for insoluble polyphenols in the functional foods formulation. Thus, the understanding of the binding mechanism between polyphenols and peptide would be essential to evaluate the bioavailability of polyphenols. Moreover, a rational design of polyphenol-peptides particles would ensure a positive contribution to food quality, protein nutrition, and delivery of a health-relevant dose of polyphenols to the gastrointestinal tract. Hence, the polyphenols-proteins interactions and the impact of the inhibition of peptide activities by polyphenols are two key themes which are fundamental to draw attention to the importance of polyphenols. For all the reasons cited above, an insight into the *in vitro* and *in vivo* aspect of the peptide-polyphenols interactions and about an interesting impact of the antioxidant activities is the purpose of this work.

The results indicated a clear antagonistic interaction between Phe-Cys and quercetin *in vitro*, with the peptide reducing the free radical scavenging ability of quercetin. The data suggest that molecular interactions, such as π-π stacking between the phenylalanine residue of the peptide and the aromatic rings of quercetin, limit the accessibility of quercetin's hydroxyl groups, which are essential for its antioxidant activity. Furthermore, dynamic light scattering analysis showed that the formation of peptide-quercetin complexes did not significantly alter particle size but likely contributed to the reduced antioxidant capacity observed in vitro.

To complement the *in vitro* findings, an *in vivo C. elegans* model was employed to investigate the effects of these peptide-quercetin mixtures under oxidative stress induced by juglone, a known prooxidant. Despite the antagonistic *in vitro* results, the *in vivo* experiments demonstrated that the mixtures, particularly quercetin and the quercetin-peptide combination, significantly extended the lifespan of *C. elegans* by reducing reactive oxygen species (ROS) levels. These findings suggest that while the peptide-quercetin interactions reduce antioxidant efficacy in vitro, they still confer protective benefits against oxidative stress in a biological system.

Molecular docking simulations further elucidated the interactions between Phe-Cys and quercetin, confirming the presence of π-π stacking and hydrogen bonding, which likely contributed to the observed reduction in antioxidant activity. The study highlights the complex nature of peptide-quercetin interactions, where antagonistic effects *in vitro* do not necessarily translate to reduced biological efficacy *in vivo*.

Overall, this research provides new insights into the molecular mechanisms of peptide-quercetin interactions, with implications for the development of antioxidant formulations in food and nutraceutical applications. While the antagonistic effects observed *in vitro* present challenges, the positive *in vivo* results underscore the potential of peptide-quercetin mixtures in mitigating oxidative stress and promoting health.

# CHAPTER 1 – INTRODUCTION

## 1.1 Background:

Peptides, consisting of active amino acid residues, are now popular as antioxidants owing to their advantages related to absorption and safety. The antioxidant activity is usually attributed to such properties as the amino acid composition, active amino acid position, molecular mass, and spatial structure of the peptides (Shi et al., 2022). An antioxidant is a molecule/substance that delays, prevents or removes oxidative damage to a target molecule or that directly and indirectly scavenges reactive oxygen species (ROS) and acts to up-regulate antioxidant defenses (Gulcin, 2020). Antioxidants have been used widely to protect the human body against ROS oxidative damage and chronic disease. Antioxidants have become a crucial group of food additives, due to their unique ability to extend the shelf life of food products while having no negative impact on their sensory or nutritional aspects (Shahidi & Ambigaipalan, 2015). The growing interest in food-derived antioxidants motivates the ongoing investigations on bioactive peptides with antioxidant properties, especially those containing 2–20 amino acid residues from various dietary peptides (C. Chen et al., 2020; Pérez-Gregorio et al., 2020). Peptides (especially short peptides) are of great interest due to their excellent biocompatibility, ease of synthesis and functionality, as well as the ability to fine-tune their structures and functions according to environmental conditions, thanks to their controllable bioactivity (Acet et al., 2023). Because dipeptides possess good properties such as permeability, solubility, and bioavailability, some of them could bind to membrane receptors (Attah et al., 2022). Antioxidant dipeptides are effective for defense free radicals and reactive oxygen species (ROS); therefore, they can resist and avoid oxidative damages of cellular components (Shan et al., 2019). However, using peptides alone may show some drawbacks, such

as the undesirable stability of peptide-stabilized emulsions during storage, and the diminished bioactivities and the occurrence of peptide-induced celiac disease during digestion. The addition of polyphenols may help to solve the above issues by the formation of peptide-quercetin complexes (Lin et al., 2023). In recent years, peptides as a source of antioxidants have received much attention since they scavenge free radicals, inhibit lipid peroxidation, chelates transition metal ions, as well as their additional nutritional value (Sarmadi & Ismail, 2010).

### 1.2 Food derived antioxidant peptides:

Food protein-derived peptides and protein hydrolysates have gained considerable interest as prospective ingredients for the formulation of functional food and nutraceutical products (Udenigwe & Aluko, 2011). Among the bioactivities, antioxidative activity of food peptides has potential application in reducing oxidative deterioration of food products and in preventing oxidative stress-related health condition (Nwachukwu & Aluko, 2019). Antioxidant activities of free amino acids have been explored, and several amino acids have been proposed to contribute to the antioxidant activity of food protein hydrolysates and peptides. These amino acids include Tryptophan (Trp), Tyrosine (Tyr), Methionine (Met), Cysteine (Cys), Histidine (His), Phenylalanine (Phe), Leucine (Leu), Valine (Val), Alanine (Ala), and Proline (Pro) (Thamnarathip et al., 2016; Udenigwe & Aluko, 2011). Moreover, peptides typically have significantly higher antioxidant activities than their parent peptides and constituent amino acids (Z. Xie et al., 2008). This is due to increased accessibility of the redox-active and functional side chain (R-group) of peptides to reactive species and the electron-dense peptide bonds (Udenigwe & Aluko, 2011). Antioxidant peptides of various chain lengths have been identified in various protein hydrolysates obtained via enzymatic hydrolysis and fermentation. Antioxidant activities of the peptides reported

include activation of cytoprotective and antioxidant enzymes, inhibition of intracellular ROS production and activation of antioxidant system via oxidative signaling pathways (Okagu & Udenigwe, 2022). The antioxidant activity of peptides can be influenced by a number of factors and the interactions among these factors: amino acid composition (Gallego et al., n.d.), peptide chain length (N. Xie et al., 2014), molecular weight (Yang et al., 2017), type and characteristics of the amino acids at the C- and N-terminals (Zheng et al., 2016), spatial conformation (Ma et al., 2018), and quantity of certain amino acids such as hydrophobic amino acids (Torres-Fuentes et al., 2019), negatively charged acidic amino acids (He et al., 2012) and aromatic amino acids (Ketnawa et al., 2018).

Many bioactive peptides derived from food peptides have been reported to display significant antioxidant activity by scavenging free radicals *in vitro* and in vivo (Du et al., 2019). These *in vitro* assays include free radical diphenylpicrylhydrazyl (DPPH), ferric reducing activity power assay (FRAP), and 2,20 -azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS). DPPH assay can be applied for the estimation of the antiradical activity of functional foods such as herbal extracts and natural or synthetic pure compounds due to high stability, experimental feasibility, and low cost of DPPH radical (Han et al., 2017). FRAP, which evaluates the total antioxidant activity in the reaction of reduction of ferric tripyridyl triazine (Fe III TPTZ) complex to blue ferrous tripyridyl triazine (Fe(II)-TPTZ) form. The reaction has a place at low pH and in the presence of an antioxidant, which can be monitored by measuring the change in absorption at 593 nm (Elsa Madhu et al., 2020). $ABTS^{\bullet+}$ for the evaluation of antioxidant properties of natural compounds can be applied. Apart from good solubility in organic solvents, ABTS assay can be implemented over a wide range of pH values (Re et al., 1999).

The antioxidant activity of peptides is highly dependent on its primary structure, which is subjective to its amino acid composition, implying the importance of knowledge regarding the relationship between antioxidant activity and the structure of bioactive peptides (Du et al., 2019). Depending on the reactions involved, these assays can roughly be classified into two types: assays based on hydrogen atom transfer (HAT) reactions, like ORAC assays and assays based on electron transfer (ET), like DPPH, ABTS, and FRAP assays (Du et al., 2019). The antioxidant capacities of dipeptides depend on their composition as well as on the conditions of the assay (Du et al., 2019). Dipeptides containing the Cys residue were shown to have antioxidant effects, as indicated by the results of the DPPH and FRAP assays. Lys may play an important role in the synergistic FRAP activity of dipeptides compared to other amino acids, such as His and Glu; the synergistic effects of dipeptides in the FRAP assay are like those of their constituent amino acids (Du et al., 2019). Important interactions occur between peptides such as electrostatic and hydrophobic interactions, hydrogen bonding and π-π stacking can be summarized as self-assembly mechanism. The antioxidant properties are strongly related to the chemical structure of the compounds, i.e., the number of hydroxyl groups, their mutual position in the aromatic ring, and the degree of their esterification. Small amino acids like dipeptides can be absorbed by the human cells where they act as antioxidants (Sun et al., 2019).

Over the years, dipeptides are attracting much interest as post-amino acids, which have residues in common with long-chain amino acids but differ in physiological properties and functions from those of long-chain amino acids (Liu et al., 2021). Dipeptides, which are comprised of two amino acids linked by a peptide bond, are much more diverse than monomers of peptides. Antioxidant dipeptides also mainly act as radical scavengers and dipeptide containing Tyr, Trp, Cys or Met with electron/ hydrogen donating ability are regarded as the driving force for its enhanced radical

scavenging activity while Phe and Lys have the lower antioxidant activities than those of Tyr, Trp, Cys and Met. The one of interesting functions of dipeptides is that the dipeptides which has radical scavenging activity are not only the dipeptides containing antioxidant amino acids (such as Trp, Tyr, His, Met, Cys) but also not containing (Ala-Leu and Val-Pro) (Ozawa et al., 2022).

### 1.3 Effect of oxidation on antioxidant properties:

Antioxidant amino acids contained in a protein sequence may act as protective agents against radicals. Particularly, Met amino acid is readily oxidized to Met−sulfoxide. Then, the reduction can be catalyzed by methionine sulfoxide reductases, providing to the protein of an intrinsic antioxidant system that may participate in cellular processes and preventing the surrounding residues from reacting with pro-oxidative species (Stadtman & Levine, 2003). This constitutes promising prospects about the potential of the generation of peptides with antioxidant properties in dry-cured ham, which may prevent residues from the oxidative stress during the processing, ameliorating alteration of taste, and confer antioxidant bioactivities beneficial for the consumer's health (Heres et al., 2022). Since oxidation leads to residue modifications and properties alterations, this may suppose the modification, loss of function or gain of function of bioactive peptides. According to Papuc et al. 2017 results, it seemed the antioxidant power may be upgraded or downgraded by oxidation depending on the assay. It is known oxidation alters hydrophilicity of the molecules (Papuc et al., 2017), and this may have an impact on their capacity to access the radicals depending on their hydrophobic nature. Apparently, oxidation of the dipeptide benefits the reaction with hydrophobic compound DPPH and with ($Fe^{3+}$)-ligand complex in acidic conditions (Heres et al., 2022; Papuc et al., 2017). However, the dipeptide modification affects proton transfer to the hydrophilic $AAPH^{\cdot+}$ radical and the electron transference to $ABTS^{\cdot+}$. Thus, the dissimilar effects of oxidation could possibly be due to the difference in the molecular structures of the

radicals and to the nature of the chemical reactions (Heres et al., 2022). Dry-cured ham-derived peptides have been reported to exert alleviative effects on the generation of reactive free radicals (Toldrá et al., 2020). But as an interesting example, it has been proved that Trp-containing dipeptides have remarkable quenching bioactivity and a higher stability than the better-known antioxidant and naturally-dry-cured ham-occurring peptide carnosine (Vistoli et al., 2013).

### 1.4 Polyphenols:

Plant-based foods such as vegetables, fruits, coffee, tea, and cereals often contain relatively high levels of bioactive polyphenols. These polyphenols may have health benefits due to the broad spectrum of bioactivities they exhibit such as antioxidant, anticancer, antimicrobial, and anti-inflammatory activities (X. Chen et al., 2016; Quan et al., 2019). Numerous studies have reported that polyphenols can interact with other compounds in foods, like carbohydrates, peptides, lipids, and metal ions, which alters their bioavailability and bioactivity. Flavonoids can be divided into flavanols, flavonols, anthocyanidins, flavones, flavanones, and chalcones. Non-flavonoids include stilbene, phenolic acids, saponin, and tannins. Among the important biological properties exhibited by plant polyphenols, their antioxidant activity has raised a great interest (Pérez-Gregorio et al., 2020; Stagos, 2020). Thus, antioxidant compounds such as plant polyphenol have been reported to be used for the prevention and/or treatment of this disease afflicting millions of people worldwide. However, one of the main problems regarding the use of polyphenols as antioxidant supplements is their low availability (Stagos, 2020). The dosage of antioxidant compounds used as food preservatives should be regulated, and the functionality should be evaluated to ensure stability. For example, polyphenolic extracts from *Rosmarinus officinalis* L. (rosemary) have been widely used in the food industry as an antioxidant additive. The antioxidant activity of polyphenols

is characterized by the phenolic structure and those compounds with catechol like moieties and the ability to delocalize unpaired electrons have the strongest activity (Croft, 2020). Quercetin is a natural flavonoid that has been promoted to an excellent antioxidant in many studies (X. Chen et al., 2016) and possesses anti-inflammatory properties (Hisanaga et al., 2016), can prevent cardiovascular disease, has antiulcer, antiallergy, and anti-proliferative effects (Al-Jabban et al. 2015), and regulates gene expression. Quercetin also possesses key structural features for effective free radical scavenging, namely ortho-dihydroxy substitution in the B-ring (or the catechol structure) and enol moiety in the A-ring, which leads to the formation of an additional H-bond with the 4-keto group (Rasouli et al., 2017; Su et al., 2022a). However, the poor water solubility of quercetin limits its function and efficacy (Su et al., 2022a).

### 1.5 Mechanism of peptide-polyphenol interaction:

Peptides and polyphenols can interact together via either non-covalent or covalent bonds (You et al. 2014). However, the complexes formed by covalent bonds are preferably used in food applications owing to their stronger and more permanent interactions with high stability (Quan et al., 2019). Non-covalent interactions between peptides and polyphenols are normally reversible interactions and weaker than covalent counterparts (Feng et al., 2023). This may typically include hydrophobic interactions, hydrogen bonds, electrostatic interactions, and van der Waals interactions, which are reversible and weaker than covalent interactions (Feng et al., 2023). When peptides and polyphenols interact with each other, alterations in the total strength of molecular interactions cause changes in the heat of the system (Feng et al., 2023). Hydrogen bonding interactions are one of the main drivers of polyphenols binding to peptides. As for polyphenols, they act as hydrogen donors, and their hydroxyl groups can form hydrogen bonds through interactions between the C=O groups of the amide group on the peptide chain, the oxygen or

nitrogen on the side chains of amino acid residues, especially hydroxyl (–OH) and amino (–NH2) groups (Feng et al., 2023). Hydrophobic interactions are one of the main driving forces of polyphenol–protein binding and it relies on the fact that the non-polar aromatic ring in the phenolic compounds interacts hydrophobically with the hydrophobic amino acid residues of peptides (alanine, cysteine, glycine, isoleucine, leucine, methionine, phenylalanine, tyrosine, tryptophan, and valine) (Feng et al., 2023). Electrostatic interactions occur as an attraction force that is created between two completely or partially ionized species with opposite charges. Electrostatic interactions between peptides and phenolics usually involve the deprotonation of some phenolic acids with low pKa values (e.g., cinnamic acid derivatives such as ferulic acid) under neutral conditions (Feng et al., 2023).

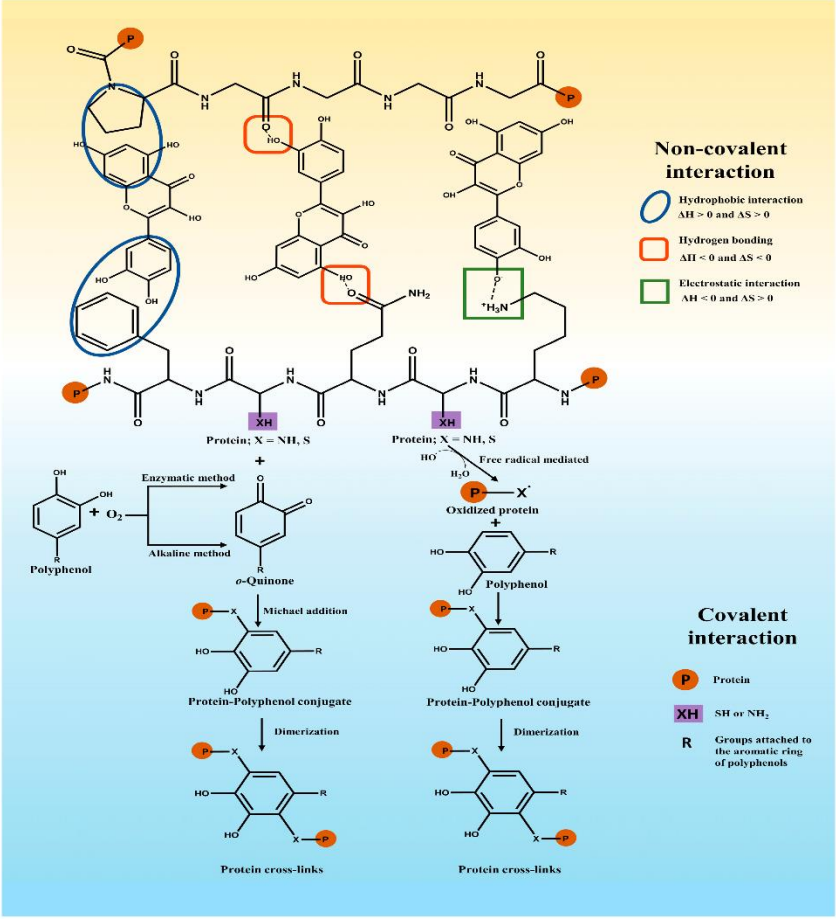

**Figure 1.1.** Major non-covalent interaction forces (the top half) between polyphenols and proteins and covalent bonding of polyphenols to proteins (the bottom half) (Feng et al. 2023).

The weak interactions between molecules resulting from electron oscillations and the interaction of dipole moments are comparatively attracted by van der Waals forces. Typically, van der Waals forces are produced in conjunction with other interactions. For example, rosmarinic acid interacted with β-lactoglobulin or α-lactalbumin with the driving forces of hydrogen bonds, hydrophobic forces, and van der Waals force (Lu et al. 2022). The binding of ferulic acid/quercetin/vanillic acid to β-lactoglobulin involved various non-covalent interactions such as hydrogen bonds, van der Waals interactions, and hydrophobic interactions (Ojha et al., 2012). Since the non-covalent interaction of the complexes is reversible, it is possible to achieve the binding of polyphenols to proteins during preparation and release of polyphenols during digestion.

When covalent binding between polyphenols and peptides occurs, it is an irreversible interaction because of the chemical reactions involved. The process mainly concerns the oxidation of polyphenols to strongly electrophilic quinones. Then, the interaction of the quinones with nucleophilic amino acid (cysteine, lysine, methionine, and tryptophan) residues on peptides or peptides via Michael addition forms covalent cross-linking (Le Bourvellec & Renard, 2012). Since the covalent interaction between polyphenol–protein complexes are irreversible, which makes the complexes more stable. At the same time, covalent binding can mediate the high grafting rate of polyphenol binding to proteins so that polyphenol–protein complexes with higher antioxidant properties can be obtained.

## 1.6 RuBisCO and Quercetin:

RuBisCO (Ribulose-1,5-bisphosphate carboxylase/oxygenase) is the most abundant enzyme-protein in plants and plays a crucial role in photosynthesis by fixing carbon dioxide. It is primarily known as an enzyme, but it is also a significant source of dietary protein, especially in plant-based diets. As the most abundant protein in chloroplasts, RuBisCO can serve as a sacrificial antioxidant due to its high susceptibility to oxidative damage by reactive oxygen species (ROS) like hydrogen peroxide ($H_2O_2$), superoxide ($O_2^-$), and singlet oxygen ($^1O_2$), thereby protecting more critical cellular components (Morita et al., 2016). Its large subunit contains amino acid residues, such as cysteines and methionine's, that are prone to oxidation, which can cause structural changes or inactivation (Muthuramalingam et al., 2013). This oxidized or damaged RuBisCO is often targeted by the plant's proteolytic systems, including chloroplast proteases, for degradation and replacement—a process that indirectly contributes to stress tolerance (Feller et al., 2008). Moreover, the degradation products of RuBisCO, including peptides and free amino acids, have been shown to possess ROS-scavenging capacity (Kim et al., 2013). Beyond its enzymatic role, RuBisCO is recognized as a promising source of high-quality plant-based protein due to its balanced essential amino acid profile (Yee et al., 2021). While RuBisCO itself is not classified as an antioxidant, hydrolysis of this protein can produce bioactive peptides with antioxidant activity, which help neutralize reactive oxygen species and reduce oxidative stress (~~Sheih et al., 2009~~). Furthermore, RuBisCO supports efficient photosynthesis, thereby indirectly aiding in the plant's overall oxidative stress management. Owing to these properties, RuBisCO is being investigated for potential applications in functional foods, nutraceuticals, and sustainable protein sources for human nutrition (Henrikson, 2013). Recent research has demonstrated that RuBisCO protein (RBS) exhibits effective antioxidant activity, particularly in food systems such as flaxseed oil-in-

water emulsions, where both native RuBisCO and its Flavourzyme-hydrolyzed form (H-RBS) significantly reduced lipid oxidation rates during storage, with the native form showing superior long-term oxidation inhibition. This dual functionality as both an antioxidant and emulsifier make RuBisCO particularly valuable for food product development applications (Lam Hon Wah et al., 2025). RuBisCO-derived peptides exhibit diverse bio-functional properties with significant applications in food and health. Enzymatic hydrolysis of RuBisCO produces bioactive peptides like the antimicrobial pentapeptide Arg-Asp-Arg-Phe-Leu, which inhibits lipid oxidation in meat products (). These peptides also demonstrate multifunctional health benefits, including antihypertensive, anti-cancer, and anti-atherosclerotic effects through mechanisms like δ-opioid receptor activation. The combination of these functional properties with RuBisCO's high nutritional quality and non-allergenic nature makes its derived peptides promising ingredients for sustainable food innovation and nutraceutical development.

Quercetin is a naturally occurring flavonoid found in a variety of fruits, vegetables, and beverages such as apples, onions, berries, red wine, and green tea. It is one of the most abundant dietary flavonoids and is known for its potent antioxidant properties (Li et al., 2016). As an antioxidant, quercetin performs multiple biological activities that help protect cells from oxidative stress. Its primary function lies in its ability to scavenge free radicals—unstable molecules that can cause cellular damage through oxidation. By donating electrons, quercetin neutralizes these radicals, thereby reducing oxidative stress and the potential damage to lipids, proteins, and DNA (Boots et al., 2008). In addition to direct radical scavenging, quercetin also exhibits metal-chelating properties. It can bind to transition metal ions such as iron and copper, which are catalysts in free radical-generating reactions, thereby inhibiting the Fenton reaction and limiting oxidative damage (Bors et al., 1990). Moreover, quercetin enhances the activity of endogenous antioxidant enzymes.

Studies have shown that quercetin increases the expression of antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase, contributing to cellular protection (Knekt et al., 2002). Another key antioxidant mechanism of quercetin is the inhibition of lipid peroxidation. Lipid peroxidation damages cellular membranes and plays a role in the pathogenesis of many chronic diseases. By stabilizing lipid structures, quercetin helps maintain membrane integrity (Formica & Regelson, 1995). Due to these antioxidant mechanisms, quercetin is believed to offer protective effects against a wide range of chronic conditions including cardiovascular diseases, neurodegenerative disorders, and certain cancers. For example, it helps prevent the oxidation of low-density lipoprotein (LDL) cholesterol, a key event in the development of atherosclerosis (Egert et al., 2008). It also demonstrates anti-inflammatory and neuroprotective effects, which are closely tied to its antioxidant properties (Li et al., 2016). Despite its promising biological effects, quercetin's bioavailability is relatively low. It undergoes extensive metabolism in the gut and liver, which can limit its efficacy (Manach et al., 2005).

Oxidative stress is a key contributor to many chronic diseases, driving the search for effective natural antioxidants. RuBisCO-derived peptides, obtained through enzymatic hydrolysis of the photosynthetic enzyme RuBisCO, have shown strong antioxidant activity due to their amino acid composition. Quercetin, a plant flavonoid, is also a well-known antioxidant but has limitations such as poor bioavailability. Combining these two bio-actives may offer synergistic effects, enhancing radical scavenging, metal chelation, and overall antioxidant efficacy. This strategy has potential applications in functional foods, nutraceuticals, and natural preservatives, making it a promising area for further research and development.

## 1.7 Conclusion:

The interaction of peptides and polyphenols mainly results from non-covalent (H-bonding, electrostatic interactions) or covalent bonds taking place mostly based on the oxidation of peptides or polyphenol by enzymatic or non-enzymatic pathways. Moreover, peptide-quercetin interaction greatly depends on environmental conditions such as temperature and pH as well as on the conformation or type of peptides (size, conformation, and charge of the peptide) and polyphenols (size, length, and flexibility). The antioxidant capacity of peptide-quercetin complexes is also affected by the structure peptides like that of polyphenols. The peptide-quercetin conjugates with higher thermal stability, and better antioxidant activities can be used as novel food additives for improvement of functionalities and quality of food products. Peptides and polyphenol possess their distinct functional properties. Interaction of these components generally affects the functional attributes of food products and eating quality. Nevertheless, the complexation of peptides and polyphenols from different food sources should be intensively investigated to develop new food ingredients or products with better nutritional, functional, sensory qualities as well as bioactivities.

## 1.7 Study Hypothesis and Objective:

### 1.7.1 Hypothesis:

The hypothesis of this study is that the RuBisCo dipeptide (Phe-Cys) and quercetin complex will exhibit increased antioxidative activity due to their synergistic effect, and the hydroxyl group of the polyphenol and sulfhydryl group of Cys, which are known to be strongly antioxidative, which would potentially reduce the induced oxidative stress in a nematode model.

### 1.7.2 Objectives:

The aim of this study was to assess the antioxidant capacity of the complexes formed between quercetin and the dipeptide (Phe-Cys) and to investigate if this complex creates a synergistic, additive or antagonist effect. The specific objectives of this project include **a)** *in vitro* evaluation of the antioxidative activities of the peptide-quercetin mixture, **b)** determination of the antioxidant effect of the peptide-quercetin mixtures using the *Caenorhabditis elegans* models, and **c)** identification of the physicochemical properties of the peptide-quercetin complexes.

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# CHAPTER TWO

## Peptide-quercetin Interactions: Antagonistic Effect of RuBisCo dipeptide (Phe-Cys) on Antioxidant Properties of Quercetin in *Caenorhabditis elegans*

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**ABSTRACT:**

Both peptides and polyphenols play an important role in functional properties and quality of food products. However, little is known about their combined antioxidant capacity when interacting within food matrices. This study investigated the antioxidant properties of complexes formed by quercetin and dipeptide Phe-Cys *in vitro* and *in vivo* using the *Caenorhabditis elegans* biological models. Dynamic light scattering, spectroscopic, and molecular docking analyses revealed the formation of peptide-quercetin complexes, with the peptide phenylalanine residue interacting non-covalently with quercetin. The well-established model organism *Caenorhabditis elegans* was used to assess the protective effects of quercetin, peptide and the peptide-quercetin complex, *in vivo*. The worms exposed to quercetin enhanced the resistance to oxidative stress and prolonged the mean lifespan of *C. elegans*. The peptide-quercetin interaction resulted in antagonistic radical scavenging activity *in vitro* and antioxidative effects *in vivo* based on nematodes mean lifespan and survival rate, and intracellular reaction oxygen species production. The findings provide

valuable insights into food matrix interactions that affect antioxidant capacity of nutraceutical mixtures in formulated products.

**KEYWORDS:** dietary polyphenol, bioactive peptides, antioxidants, *Caenorhabditis elegans*, quercetin

## 2.1 INTRODUCTION:

Antioxidant peptides are molecules that can interact and neutralize free radicals by donating an electron to blow up free radicals. Free radicals are unstable molecules seeking electrons from surrounding atoms for stability. It can be derived from normal essential metabolic processes in the body. Some structural requirements for high antioxidant activity of peptides include the presence of Cys, His, and Phe which respectively possess sulfhydryl, imidazole, and aromatic groups, and can participate in electron transfer, metal chelation and ROS trapping (Je et al., 2015).

Natural antioxidants, such as polyphenols, are finding increasing applications in functional foods designed to improve human health, wellbeing, and performance (Kandemir et al., 2022). Polyphenols can bind to peptides either covalently or non-covalently (Dueñas et al., 2011; Feng et al., 2023). Quercetin is a flavonol-based polyphenol that exhibits a broad range of potentially beneficial health effects in several cellular and animal models (Wang et al., 2016). It is a strong antioxidant due to its ability to scavenge free radicals and bind transition metal ions which allows it to inhibit lipid peroxidation (Dueñas et al., 2011). However, the bioavailability of quercetin is often relatively low (less than 10%) because of its poor water-solubility and absorption profile. The bioavailability of quercetin depends on its chemical structure, physiochemical properties, and food matrix effects (Dueñas et al., 2011; Wang et al., 2016).

Attention has not been directed to interactions of the phenolic compounds with the other components also present in food or in the organism (e.g., proteins), which may be in position to influence the antioxidant activity (Rohn et al., 2004). Polyphenols have a significant affinity for proteins and peptides. For example, polyphenol binding to salivary protein leads to an insoluble complex, interacting irreversibly with gut enzymes and dietary proteins (Richard et al., 2006).

In recent investigations, it was shown that protein–polyphenol complexes may interact based on non-covalent interactions such as π-bonding, hydrogen bonding, and hydrophobic or ionic interactions and are formed by multiple weak interactions between peptide side chains and polyphenol aromatic rings (Parolia et al., 2022; Richard et al., 2006). There is limited understanding of the mechanisms of peptide-quercetin interaction or the resulting effect on their antioxidant capacities. Recent studies have demonstrated synergistic, additive, or antagonistic antioxidant effects of the protein-polyphenol complexes, depending on the protein/phenolic ratio and the type of polyphenol and peptides used. It was hypothesized in this study that the peptide-quercetin interactions would enhance antioxidant capacity of the mixture due to additive effects. In our current research, we focused on protein–polyphenol interactions, which are likely to be a factor in relation to their bioavailability.

The dipeptide Phe-Cys was derived from RuBisCO, which is the most abundant enzyme-protein in plants and plays a crucial role in photosynthesis by fixing carbon dioxide, was predicted to be bioactive; the two amino acid residues, Phe and Cys make peptide a strong antioxidant (Je et al., 2015). Based on previous study done by Je e al. (2015), the dipeptide (Phe-Cys) displayed moderate antioxidative effect due to its ability to directly scavenge free radicals, due to the presence of the sulfhydryl group of Cys in the peptide, based on effects *in vitro* and in cultured hepatic cells. However, there is a dearth of information on the antioxidative effect of Phe-Cys or

similar dipeptides in the presence of other food antioxidants such as polyphenols. Therefore, the objective of this study was to investigate the antioxidant capacity of the complexes formed between quercetin and the RuBisCo dipeptide (Phe-Cys) using *in vitro* radical scavenging assays and the *Caenorhabditis elegans* biological model, as well as their nature on interactions by dynamic light scattering, UV/Vis spectra, and *in silico* analysis.

## 2.2 METHODOLOGY:

### 2.2.1 Materials:

The peptide (Phe-Cys) was synthesized and supplied as a white powder by GenScript Biotech (Piscataway, NJ, USA). The experimental mass of the peptide was 269.1 Da (calculated 268.3 Da) and purity was determined to be > 95% after observing a peak by analytical reverse-phase high performance liquid chromatography (Je et al., 2015). 2,2-diphenyl-1-picrylhydrazyl (DPPH), dimethyl sulfoxide (DMSO), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ethanol, methanol, potassium persulphate, quercetin, sodium phosphate buffer, ammonium thiocyanate, ferrozine (1,10-phenanthroline and 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulphide acid sodium salt), hydrochloric acid (HCl), trichloroacetic acid (TCA), ferric chloride, tris-hydrochloride buffer, sodium chloride, calcium chloride, magnesium sulfate phosphate buffer, disodium phosphate, monopotassium phosphate, sodium hydroxide, juglone (5 hydroxy-1,4-naphthoquinone) were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). The wild-type *Caenorhabditis elegans* N2 strain and *Escherichia coli* OP50 were obtained from the *Caenorhabditis* Genetics Center (CGC), University of Minnesota (Minnesota, MN, USA). Milli-Q water was obtained using the Milli-Q water supply system (Millipore Corporation, Billerica, MA, USA) with a total organic carbon level ≤5 ppb and resistivity of 18.2 MΩ·cm at 25 °C. All

chemicals and reagents were analytical grade and double distilled water (Millipore) was used in the preparation of all reagents.

### 2.2.2 Sample Preparation:

A serial dilution solution of the dipeptide was prepared in DMSO. Different concentrations of the peptide (with 10-fold serial dilution factors of 1, 25, 50, 100, 250, 500 and 1000) were mixed with equal volume of polyphenol (quercetin) in DMSO. The final concentration of peptide in the final mixtures were 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, and 1 mM while that of the polyphenol was 0.1 mM. The peptide, polyphenol, and peptide-quercetin mixtures were evaluated for antioxidant capacity using four *in vitro* assays and a biological model.

### 2.2.3 Characterization of Molecular Interaction Assay:

#### 2.2.3.1 Dynamic Light Scattering (DLS) Analysis:

The average particle size, size distribution, and polydispersity index of the peptide, polyphenol, and peptide-quercetin samples at 1:1 molar ratio was determined by DLS with Nano-ZS Zetasizer (Malvern Instruments Ltd., Malvern, UK at a Refractive index of 1.477, and absorption of 0.001). Dilute samples of 1 mL each were prepared to avoid multiple scattering effects, i.e. 0.05 mM peptide, 0.05 mM quercetin, and a mixture containing 0.05 mM peptide and 0.05 mM quercetin. DMSO at a pH 7, refractive index of 1.477, viscosity of 2.0 cP and dielectric constant of 48.90 was used. Triplicate analysis was performed at 25°C.

#### 2.2.3.2 UV/Vis Spectroscopy Analysis:

The UV/Vis spectra of the samples were obtained using a microplate reader (Tecan, Stockholm, Switzerland). Briefly, absorption spectra of 3 samples of 200 µL each were recorded from wavelength 250 to 400 nm, i.e., 0.05 mM peptide, 0.05 mM polyphenol, and a mixture of 0.05 mM

peptide and 0.05 mM polyphenol samples. The sample absorbance was subtracted from the blank reading. Each spectrum presented represents the average of three replicates.

#### 2.2.3.3 Molecular Docking Analysis:

The molecular docking study was carried out according to the method of Obeme-Nmom et al. (2023) to assess the non-colavent interactions between the peptide and polyphenol. Briefly, PHE-CYS peptide 3D structure was obtained from the AlphaFold structure of tRNA-dihydrouridine synthase (UniProt ID: A0A1J5IYG2) with amino acid residues (aa:221-222) present on the active site. The structures of the ligands (quercetin) were obtained NCBI PubChem Compound database (http://www.pubchem.ncbi.nlm.nih.gov/compound) in SMILES (Simplified Molecular Input Line Entry Specification) format and their 3D structure were optimized using ACDLab/Chemsketch software and saved in .mol format. PyMol software was used for ligand file conversion from .mol to .pdb. Both ligand and protein were prepared for docking using AutoDock Tools (ADT) v1.5.6 (Morris et al. 2009) at default settings, and the output file was saved in pdbqt format. The docking parameters: center grid box (-8.057× -11.905 × 13.777 points), size (24 × 24 × 24 points), and spacing (0.375 Å). Molecular docking program AutoDock Vina v1.2.3 (Eberhardt et al., 2021; Trott & Olson, 2010) was employed for the docking experiment. After docking, close interactions of binding of the target with the ligands were analyzed and visualized using PyMol and ADT.

### 2.2.4 *In vitro* Antioxidant Assay:

#### 2.2.4.1 DPPH radical scavenging activity (DRSA):

The DPPH scavenging assay was carried out as described by Z. Xie et al. (2008) Briefly, 100 µL of peptide, polyphenol or peptide-quercetin sample was mixed with 100 µL of 0.15 mM 2,2-diphenyl-1-picryl hydrazyl (DPPH) solution in 95% ethanol in a 96-well plate to a final assay concentration of 1 mg/mL. The solution was mixed and incubated at room temperature in the dark

for 30 min. The absorbance values of the control (Ac) and samples (As) were measured at 517 nm using a microplate reader (Tecan, Stockholm, Switzerland. Blank was prepared in the same manner except that DMSO was used instead of sample solution. The % scavenging activity of the samples was determined using the following equation:

$$DRSA\ (\%)\ = \frac{(Ac\ -\ As)}{Ac} \times 100$$

**2.2.4.2 Ferric reducing antioxidant power (FRAP):**

The reducing power of peptide samples was measured according to a previously reported method Wu & Aluko (2007) which was modified as follows. Peptide samples (100 µL) dissolved in 100 µL of 0.2 M sodium phosphate buffer at pH 6.6 or double distilled water (control) were mixed with 100 µL of 1% potassium ferricyanide solution. The final peptide concentration in the assay mixture was 1 mg/ml. The resulting mixture was heated at 50 °C and incubated for 20 min. After incubation, 100 µL of 10% of aqueous TCA was added. Thereafter, 100 µL of peptide/TCA mixture was combined with 80 µL of 0.1% ferric chloride and 400 µL of water and allowed to stand at room temperature for 10 min. The solution was centrifuged at 1,000g and 200 µL of the supernatant transferred to a clear bottom 96-well plate. The absorbance of the supernatant was measured at 700 nm using a microplate reader (Tecan, Stockholm, Switzerland). Increased absorbance of the reaction mixture indicated increased reducing power.

**2.2.4.3 ABTS radical scavenging activity:**

The ABTS scavenging assay was carried out as described by You et al. (2010) with some slight modifications. Equal volumes of 7.4 mM ABTS and 2.6 mM potassium persulphate solutions were mixed and allowed to stand for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 mL of ABTS solution with 50 mL of methanol to obtain an absorbance value of 1.1 ±

0.02 at 734 nm using a microplate reader (Tecan, Stockholm, Switzerland). Fresh ABTS solution was prepared for each assay. Sample (40 µL) was mixed with 200 µL of ABTS solution and the mixture left at room temperature for 2 h in the dark. The absorbance was then measured at 734 nm using a 96-well microplate reader. A blank reaction mixture was prepared in the same manner except that DMSO was used instead of sample solution. The radical scavenging activity was calculated as described for the DPPH scavenging assay.

#### 2.2.4.4 Metal chelating activity (MCA):

800 µL of sample was mixed with 10 µL of 2 mM $FeCl_2$ and 20 µL of 5 mM ferrozine. Mixture was vortexed and kept at 25 °C for 10 min prior to measuring the absorbance at 562 nm using a a microplate reader (Tecan, Stockholm, Switzerland). The control was a mixture composed of 800 µL of Milli-Q water, 10 µL of 2 mM $FeCl_2$ and 20 µL of 5 mM ferrozine. Chelating ability, expressed as a percentage, was calculated as follows:

$$Chelating\ ability\ (\%) = 1 - \frac{Abs\ sample}{Abs\ control} \times 100$$

### 2.2.5 *In vivo* Antioxidant Assay:

#### 2.2.5.1 *C. elegans* nematode model:

The *in vivo* antioxidant assay was carried out following the method of González-Peña et al. (2021), with some specific modifications. Briefly, the Bristol N2 wild strain of *C. elegans* were kept at 23°C ± 2°C on NGM plates containing 250 µL of *E. coli* OP50. To synchronize the stage of nematodes for future experimental assays, the *C. elegans* eggs were collected. On the third day of growth, adult stage (L2) nematodes were washed with M9 solution buffer, to avoid any contamination. Then, samples prepared in M9 buffer were centrifugated at 2602 × g at 4°C ± 1°C

for 3 min using a Lynx 4000 Centrifuge (ThermoFisher, Canada). After two rounds of washing with M9 solution buffer with 1 M NaOH and two rounds of washing with M9 solution buffer with 1 M NaOH: 5% NaClO, the eggs were placed on fresh NGM plates with *E. coli* OP50 and the samples and the plates were incubated at 22°C ± 2°C. The synchronized nematodes were separated into distinct groups for the oxidative stress resistance assay: a control group (juglone), and antioxidant groups containing peptide alone, polyphenol alone, and mixtures of peptide-quercetin. The survival to oxidative stress resistance assay was carried out as described by (Sangha et al., 2013) with slight modifications. Approximately 60 ± 5 L2 nematodes from the *C. elegans* species were exposed to the antioxidant treatments and then transferred to fresh NGM media plates containing 400 mM juglone, a dose known to induce oxidative stress death. The nematodes were then divided into their respective sample groups and examined every hour for 8 h to assess their state of death by pricking using a platinum wire for verification of movement. Similarly, nematodes in the adult stage L4 were synchronized and the obtained eggs were transferred to fresh NGM media plates containing *E. coli* and the antioxidant samples. The plates were incubated at 22°C ± 2°C until the nematodes reached the L4 stage once again. This sequence was repeated to evaluate oxidative stress resistance in three consecutive generations (G1 to G3). All experiments were conducted in triplicate.

**2.2.5.2 ROS production assay:**

To conduct a ROS production fluorescence assay with *C. elegans*, synchronized populations of worms were harvested and washed before being resuspended in M9 buffer (González-Peña et al., 2021). Eggs were placed on new NGM plates with *E. coli* OP50 bacteria and samples (100 µg/mL) and samples (200 µL) and incubated at 22 ± 2 °C until reaching the L2 stage. ROS production was stimulated through exposure to a mixed solution of 150 mM juglone stressor, Tween-20 (1%), and

PBS, pH 7. A DCF stock solution was prepared in DMSO and diluted in the assay buffer to achieve the desired concentration of 100 µM. The worms were then incubated at 24 ± 2 °C for 2 h with the DCF and solution, allowing DCF to penetrate and react with ROS. Fluorescence intensity was measured using a Tecan microplate reader with excitation and emission wavelengths set at 485 nm and 535 nm, respectively.

**2.2.5.3 ROS production by fluorescence microscopy:**

To verify ROS production, adult nematodes were synchronized, and eggs were placed on new NGM plates with *E. coli* OP50 bacteria and samples (100 µg/mL) and incubated at 22 ± 2 °C until reaching the L3 stage. Upon reaching the L3 stage, 15 nematodes were transferred to new NGM plates with 5-fluoro-2'-deoxyuridine (FUdR; Sigma-Aldrich, Canada) 100 µM to prevent offspring and juglone 150 µM to induce oxidative stress and incubated at 22 ± 2 °C for 12 h. Upon reaching adulthood, nematodes were transferred to 200 µL of M9 solution containing 2',7'-dichlorofluorescin diacetate (DCF; Sigma-Aldrich, Mexico) and incubated in the dark at room temperature for 1.5 h. Subsequently, 50 µL of M9 solution containing 10 mM sodium azide ($NaN_3$; Sigma-Aldrich, Canada) as an anesthetic was added, and they were transferred to an agar slide mounted on a petri plate. The slides were placed on a fluorescence microscope using the Axio Imager 2 microscope equipped with an Axiocam 506 camera (Carl Zeiss, Germany). Zen 2.3 pro software (Carl Zeiss, Germany) to capture the fluorescence of the oxidized DCF product.

**2.2.6 Statistical Analysis:**

All experiments were conducted in triplicate. The results were reported as mean values ± standard deviation and analyzed by one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test. The graphs were plotted, and analyses were carried out using GraphPad Prism 9

(GraphPad Software, La Jolla CA). All differences were considered statistically significant when the p-values were below 0.05 ($P < 0.05$).

## 2.3 RESULTS & DISCUSSION:

### 2.3.1 Free Sulfhydryl group of the Peptide-quercetin Interactions:

The SH content is a key indicator of tertiary and quaternary structural changes in proteins (Zhang et al., 2023). It is also a redox active group and major antioxidant functional agent of bioactive peptides. Therefore, the SH content can be used to assess the reactivity of cysteine-containing peptides. Unexpectedly, the content of free SH of the samples increased after the addition of quercetin to the various dipeptide concentrations (**Figure 2.1**). This shows that the polyphenol may have acted in reducing the oxidized peptide, although reduced GSH was used in the experiments. This shows the dipeptide may have been pre-oxidized, thereby allowing quercetin to donate electrons increasing the SH content. Pi et al. previously reported that the cleavage of the S–S bond and the exposure of free -SH group in the protein results in an increase in the free SH content, indicating the unfolding of the proteins (Pi et al., 2023). Thus, the breakage of the S–S bonds and exposure of free SH observed in the peptide-quercetin complexes signify the reduction and structural changes in the dipeptide.

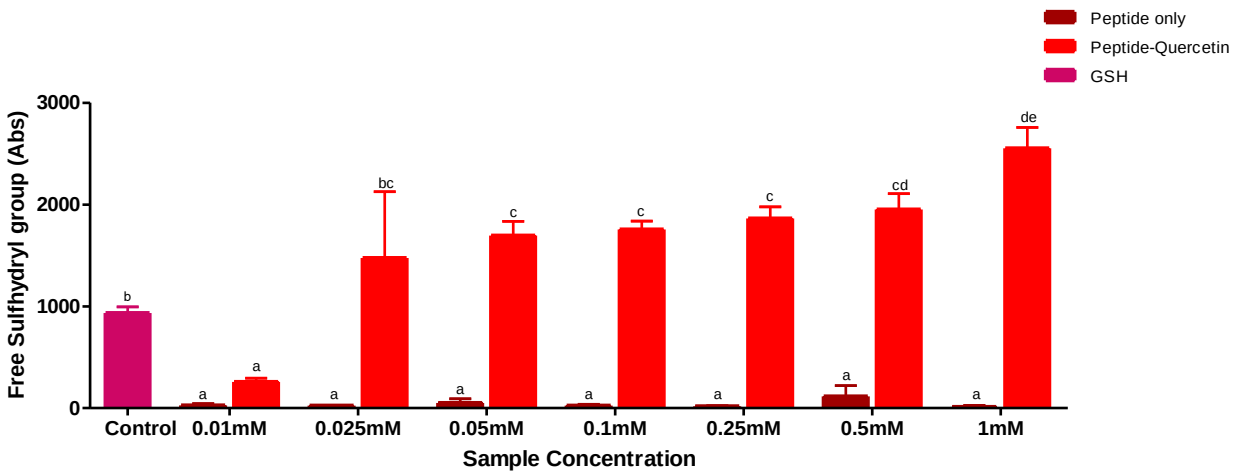



**Figure 2.1.** Sulfhydryl content activities of the RuBisCo dipeptide and peptide-quercetin mixtures. Data are given as the mean ± SD (n=3). Different superscript letters on the bars in each graph represent differences between means ($P < 0.05$).

### 2.3.2 Particle characteristics of the Peptide-quercetin Interactions:

Dynamic light scattering (DLS) was used to investigate the interactions between the peptide and polyphenol samples. DLS is a technique that employs Brownian motion to determine the particle size distribution, which is mostly characterized by the average size and polydispersity (Stetefeld et al., 2016). The average particle size and polydispersity index from DLS analysis are presented in **Figure 2.6.** The mean particle size of the peptide-quercetin complex was found to be comparable to that of quercetin alone, suggesting that the peptide-quercetin interaction did not cause any apparent change in the particle characteristics. This observation explains the *in vitro* and *in vivo* results where the antioxidant effects of the peptide-quercetin complex were lower than that of quercetin alone.

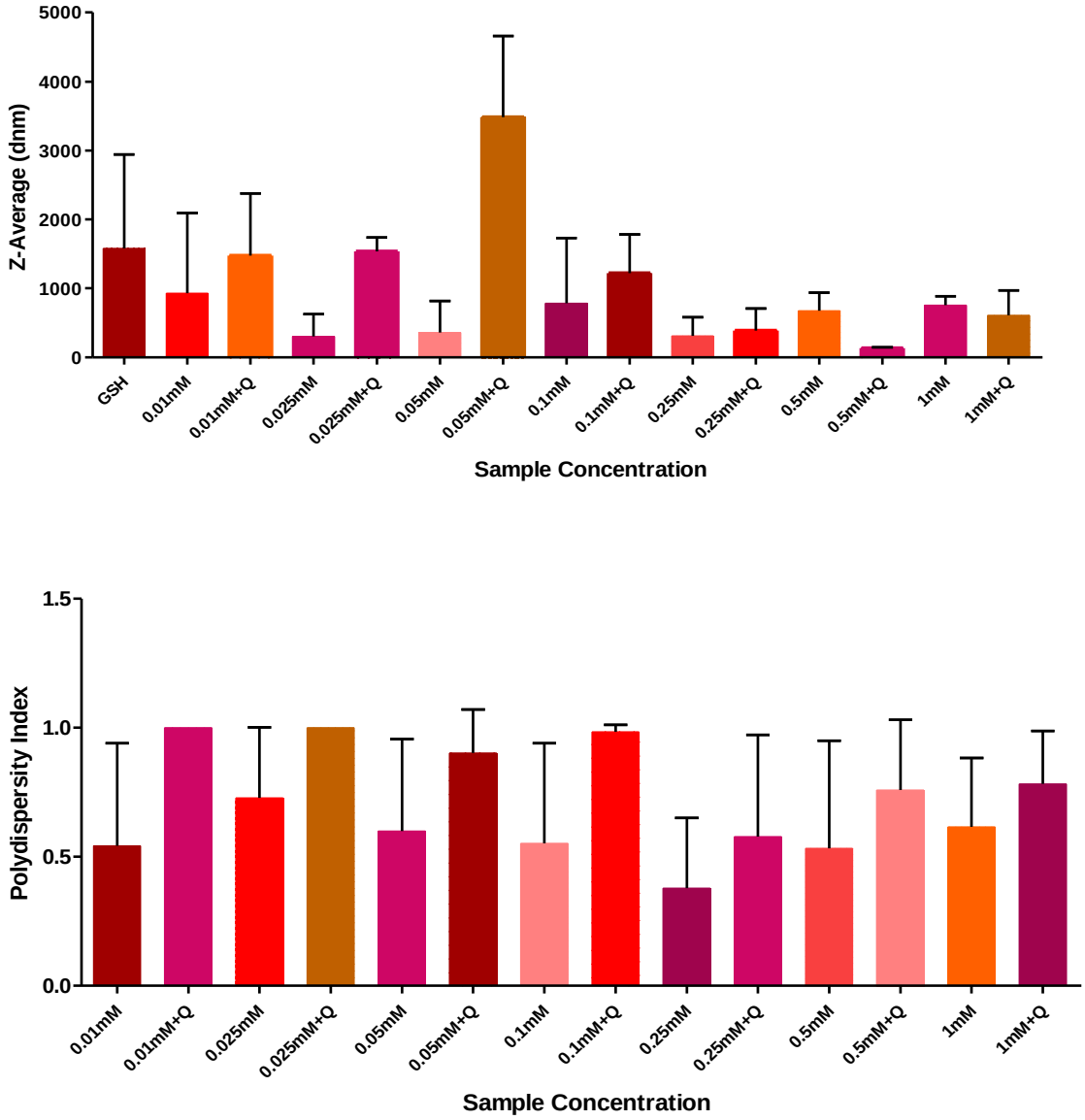



**Figure 2.2. (a)** Average particle size diameter and **(b)** Polydispersity index (PDI) of the dipeptide (Phe-Cys), Quercetin, and peptide-quercetin mixtures. Data are given as the mean ± SD (n=3).

Su et al. however reported that covalent interaction between myofibrillar proteins and quercetin resulted in quercetin-protein aggregates that exhibited a synergistic antioxidant effect compared to free quercetin or protein (Su et al., 2022b). These findings demonstrate the un-uniformity in the

size distribution of the samples which suggests the presence of multiple populations of dominant particles with different sizes and aggregation occurring within the peptide and peptide-quercetin.

### 2.3.3 *In silico* of Peptide-quercetin Interactions:

To further explain the absorption spectra, molecular docking was performed using quercetin and dipeptide. As shown in **Figure 2.3**, the interaction between the Phe side chain and the A-ring of quercetin resulted in *pi-pi* stacking. The stacking affected neither the structure of the dipeptide nor the A-ring of the flavonoid since the spectra still showed the unchanged peak at 380 nm. Also, H-bonding occurred with the side chains of cysteine and phenylalanine. This result showed non-covalent interactions occurred between the benzene rings when the quercetin was added to the dipeptide.

**Table 2.1.** Molecular docking properties and binding energy score

| S/N | Ligands (Compounds) | PHE-CYS peptide binding affinity (kcal.mol$^{-1}$) |
|---|---|---|
| 1 | Quercetin (Pubchem CID: 5280343) | -2.536 |

Taken together the molecular docking, and antioxidant assay results suggest the formation of peptide-quercetin complexes; thus, $\pi$-$\pi$ stacking interaction between the molecules may have decreased the radical scavenging activity of the polyphenol. Recent studies have demonstrated that $\pi$-$\pi$ stacking creates an extended conjugated system between two structures to form a more effective radical scavenging species (Li et al., 2018; Mazzone et al., 2013). Moreover, Ding et al. noted that the number and the position of the hydroxyl groups affects the antioxidant capacity of the polyphenol. In more recent studies, it was denoted that $\pi$-$\pi$ stacking between the phenylalanine

and the polyphenol decreased the radical scavenging activity, likely because of the changes in particle characteristics and reduced accessibility to antioxidant functional groups, when compared to the polyphenol alone (Ding et al., 2012). The higher the negative binding affinity values, the stronger the bond and the more stable the complex formed between the ligand and the molecule (Uzzaman et al., 2019). Based on the result, it was demonstrated that the dipeptide exhibited a high binding affinity (-2.536 kcal.mol$^{-1}$).

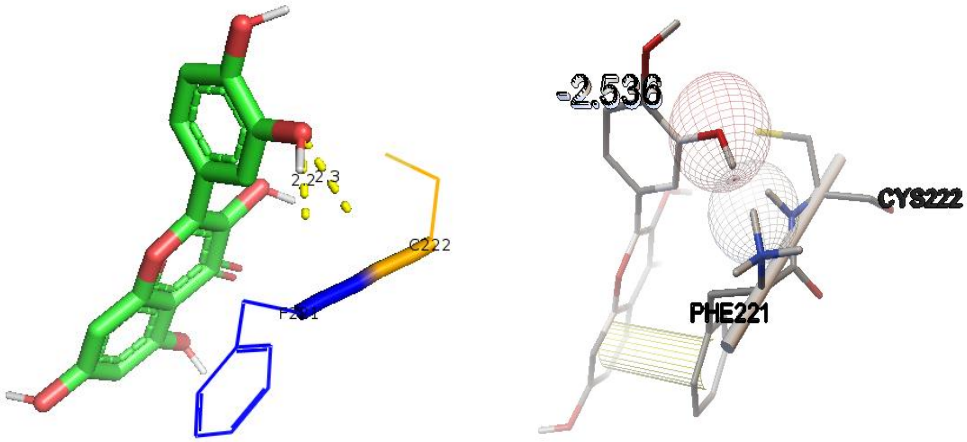



**Figure 2.3:** 3D Interaction of the dipeptide Phe-Cys with quercetin.

### 2.3.4 *In vitro* Antioxidant Capacity of the Peptide-quercetin Mixtures:

Antioxidants can act at different levels in an oxidative sequence, protecting the human body from free radicals and retarding the progress of many chronic diseases. These antioxidants can act as inhibitors of lipid peroxidation, as direct scavengers of free radicals, and as agents to chelate the transition metal ions that catalase the generation of radical species (Zhang et al. 2011). The antioxidant properties of bioactive compounds are crucial for supporting the body's regulatory systems against oxidative stress and relative cellular damages (Zaky et al., 2022). The *in vitro* assays in this study were done to determine the ability of the peptide-quercetin mixture to scavenge

the DPPH and ABTS radicals, or for ferric reducing and metal chelation activities, to assess potential synergistic, additive, or antagonistic effects of the combined treatment. Protein-derived bioactive peptide Phe-Cys was selected as a model because of its phenylalanine (F) and cysteine (C) residues, which can potentially form H-bonding and π-stacking with the polyphenols, respectively (Du et al., 2019).

**Figure 2.4** shows the *in vitro* radical scavenging antioxidant activities of the pea peptide, polyphenols, and peptide-quercetin mixtures. Overall, from all the samples, both DPPH and ABTS showed an increasing effect as the concentration increased for peptide only but when quercetin is added to the mixture, an antagonist effect in the activity was observed. Quercetin has a free catechol group on their B ring, 2,3-double bond in conjugation with the 4-oxofunction of the carbonyl group in their C ring, and hydroxyl groups at the 3 and 5 positions, which are mostly responsible for their free radical scavenging activities (Shrivastava et al., 2012). For the DPPH assay, reduced glutathione (GSH) had lower activity compared to the dipeptide at concentrations 0.1, 0.25, 0.5 and 1 mM. This is plausible because the higher the concentration, the more Phe-Cys can donate single electron or hydrogen atom to quench the free radical. For the Phe-Cys dipeptide, the cysteine residue and aromatic ring of the phenylalanine residue may be responsible for its antioxidant activity (Xu et al., 2017). Nonetheless, the peptide alone exhibited the least radical scavenging activities and no detectable DPPH radical scavenging activity at the lowest concentrations. For the ABTS assay, the radical scavenging activity of the peptide stayed the same with increasing concentration when compared to GSH. This is possibly due to molecular crowding effect because of increased peptide-peptide interaction (Du et al., 2019).

b

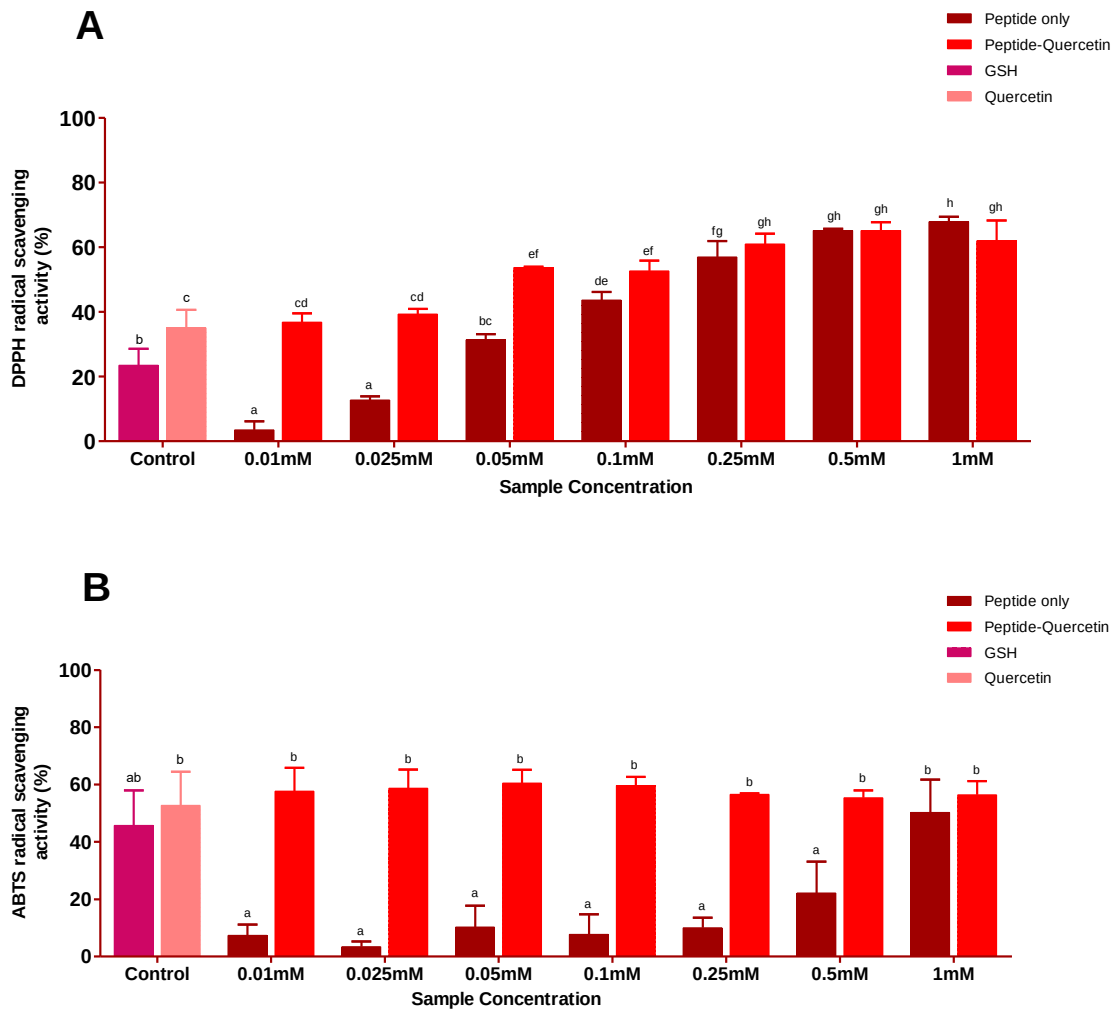



**Figure 2.4. (a)** DPPH and **(b)** ABTS radical scavenging activities of the RuBisCo dipeptide, quercetin, and peptide-quercetin mixtures. Data are given as the mean ± SD (n=3). Different superscript letters on the bars in each graph represent differences between means ($P < 0.05$).

The peptide-quercetin mixtures had significantly lower DPPH and ABTS radical scavenging activities than quercetin. This result depicts an antagonistic effect of the peptide. Considering the high antioxidant activity of the polyphenol when compared to the peptide, it is apparent that quercetin is the main compound responsible for electron donation within the mixtures. The

antagonistic effect suggests that the peptide interacted with the polyphenols, which influenced the hydroxyl groups of the polyphenols to become less accessible for reaction with the free radical (Dai et al., 2019; Du et al., 2019). Moreover, antioxidant activity of the peptide-quercetin mixtures was dose-dependent, i.e., there were some patterns in the antagonistic effect observed from 0.01 mM to 1 mM peptide concentrations.

As shown in **Figure 2.4b**, ABTS was more sensitive to the samples, leading to significantly higher scavenging activities ($P < 0.05$) when compared to DPPH. These findings are consistent with the previous report by Lee et al. (2015) that, owing to a more rapid reaction kinetics, the ABTS assay has a higher sensitivity in determining radical scavenging activity.

The reducing power of a compound provides a measure of its antioxidant activity. The ferric reducing antioxidant potential (FRAP) assay measures the conversion of $Fe^{3+}$ to $Fe^{2+}$ by an antioxidant (Parolia et al., 2022). In contrast, metal chelation assay evaluates the ability of a compound to bind prooxidant divalent metals, thus preventing them from participation in oxidative reactions in the body.

**Figure 2.5a** and **2.5b** present the FRAP values and metal chelation activity of the dipeptide, quercetin, and peptide-quercetin mixture. Reducing power for all the different samples were observed in the following order: quercetin > peptide > peptide-quercetin mixture > GSH. Similar to DPPH and ABTS results, both FRAP and metal chelation depict antagonistic effects.

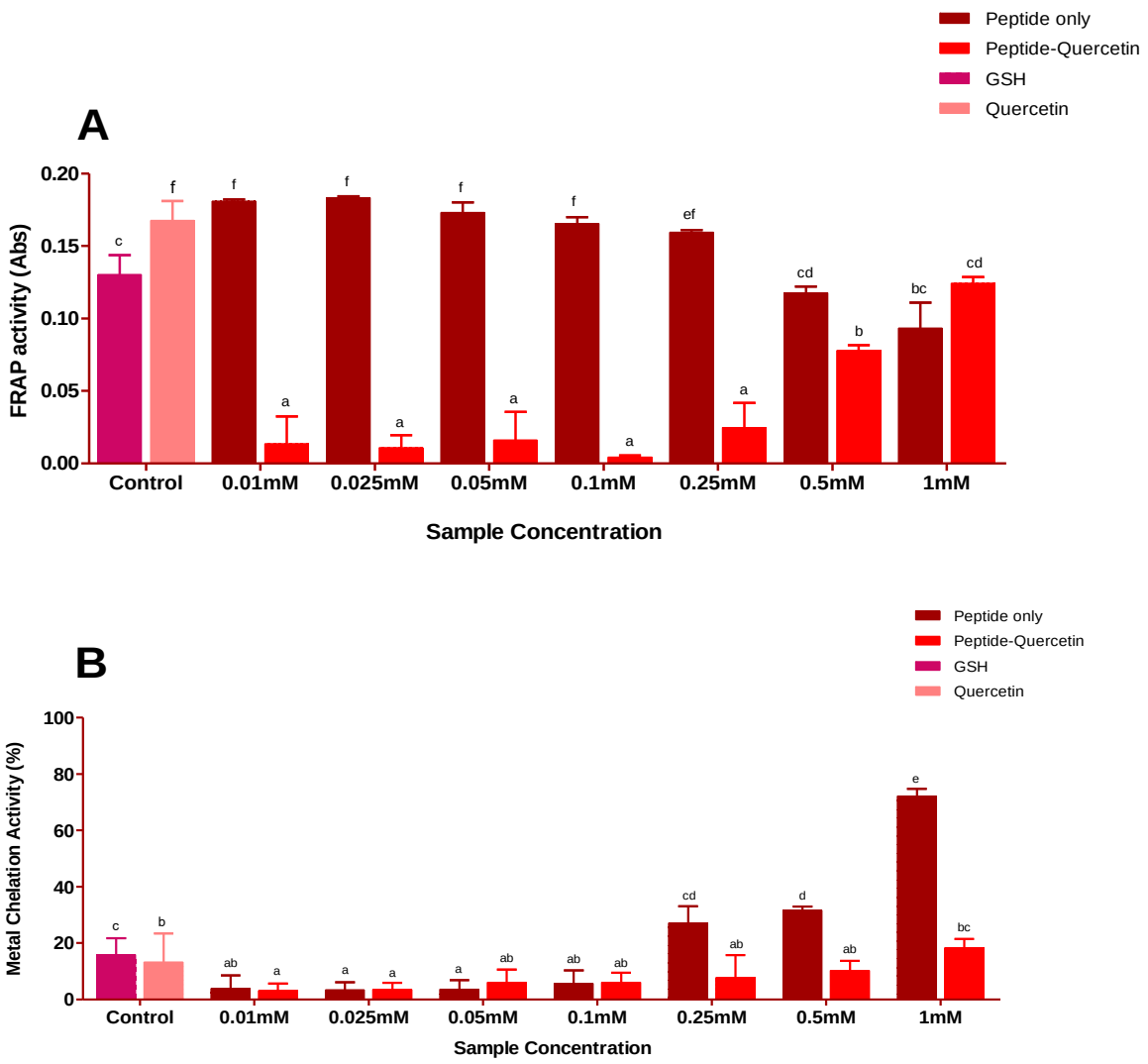



**Figure 2.5.** **(a)** FRAP and **(b)** Metal chelation activities of the RuBisCo dipeptide, quercetin, and peptide-quercetin mixtures. Data are given as the mean ± SD (n=3). Different superscript letters on the bars in each graph represent differences between means ($P < 0.05$).

The interaction of Phe-Cys with quercetin resulted in a significant decrease in activity, which indicates that intermolecular interactions between the nutraceuticals prevented their reactive residues from efficiently associating with the targets. Furthermore, due to the acidic pH of the

reagent used, quercetin can exist in its neutral form rather than as phenolate ion, thus the reduced possibility to transfer electrons, which explains the lower antioxidant activity (Dueñas et al., 2011).

### 2.3.5 *In vivo* Antioxidant Activity of the Peptide-quercetin Mixture in *C. elegans* Model:

The endogenous antioxidant system is an important barrier against oxidative stress, scavenging intracellular ROS in organisms. Many metabolic pathways and processes are evolutionarily conserved between *C. elegans* and humans. For example, key pathways involved in energy metabolism (such as glycolysis, the tricarboxylic acid cycle, and oxidative phosphorylation) are similar in both organisms. Studies have shown that *C. elegans* utilizes these pathways to generate ATP and metabolize nutrients, much like humans (Braeckman et al., 2016). Although the peptide-quercetin compounds exhibited antagonistic antioxidant capability *in vitro*, potential *in vivo* antioxidant effects were further assessed using the *C. elegans* survival model against juglone. Juglone was used as a control. Juglone is a prooxidant redox cycler that causes the intracellular generation of superoxide radical ($O^{2\bullet-}$) (De Castro et al., 2004).

While several studies have evaluated the antioxidant potential of peptide-quercetin mixtures *in vitro*, limited research has been conducted in vivo to confirm the implications of the biomolecular interactions on radical scavenging effects. To determine the in vivo antioxidant effects, the mean life (lifespan) and survival rate of *C. elegans* exposed to juglone and then treated with samples (peptide, polyphenol, and peptide-quercetin at 1:1 molar ratio) were assessed. *C. elegans* lifespan is a significant physiological model employed in evaluating the effects of a compound, whether the results are positive or negative, leading to longevity or toxicity and decreased lifespan respectively.

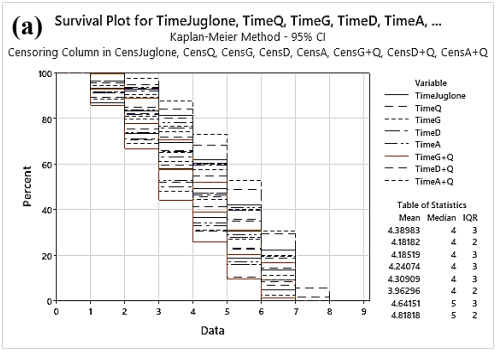
**Table 2.2**. Effects of the dipeptide (Phe-Cys), quercetin, and peptide-quercetin mixtures on the mean life of juglone-treated *Caenorhabditis elegans* at three different generations (G1, G2 and G3).

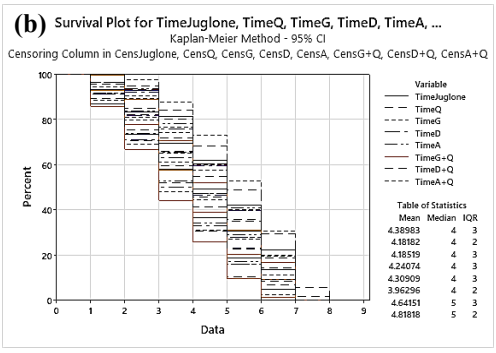
| Sample | Mean life (h) | | |
|---|---|---|---|
| | **G1** | **G2** | **G3** |
| Juglone | 4.14 ± 0.023 $^{f}$ | 3.5 ± 0.05 $^{b}$ | 4.24 ± 0.048 $^{d}$ |
| Q | 4.18 ± 0.006 $^{e}$ | 3.6 ± 0 $^{b}$ | 5.14 ± 0.009 $^{a}$ |
| G | 4.19 ± 0.006 $^{e}$ | 3.6 ± 0 $^{b}$ | 4.1 ± 0.007 $^{e}$ |
| D | 4.24 ± 0.001 $^{d}$ | 3.5 ± 0 $^{b}$ | 4.18 ± 0.011 $^{de}$ |
| A | 4.31 ± 0.005 $^{c}$ | 3.5 ± 0.07 $^{b}$ | 4.52 ± 0.047 $^{c}$ |
| G+Q | 3.96 ± 0.01 $^{g}$ | 3.2 ± 0.07 $^{c}$ | 4.19 ± 0.015 $^{de}$ |
| D+Q | 4.64 ± 0.007 $^{b}$ | 3.2 ± 0 $^{c}$ | 4.58 ± 0.086 $^{c}$ |
| A+Q | 4.82 ± 0.043 $^{a}$ | 4.4 ± 0.01$^{a}$ | 4.96 ± 0.041 $^{b}$ |

Q = quercetin, G = 0.01mM, D = 0.1mM, A = 1mM. Results expressed as mean ± SD (n=3). Different letters in each column indicate mean values with significant differences ($P < 0.05$). The control group is treated with juglone only.

As shown in **Figure 2.6 and Table 2.2**, the survival rate results suggest that *C. elegans* from Generations 2 and 3 exhibited better survival rates than Generation 1. Indeed, nematodes from Generation 1 experienced a 50% survival rate after 4 h whereas nematodes from Generations 2 and 3 experienced a 50% survival rate after 6 h. This finding, coupled with the data on the mean life across the three generations, further indicated that the nematodes have achieved a change in their genetic traits through their life cycle to improve their healthiness (Braeckman et al., 2016). Upon assessing the first generation of nematodes, i.e., the first generation being exposed to

juglone, it was evident that treatments with the peptide had no apparent effect on the survival rate of the nematodes (G2). Indeed, nematodes treated with and without the peptide exhibited the same fate, i.e., a 0% survival rate after 7 h. However, when data from all the three generations were analyzed together, it can be concluded that treatments with quercetin, alone or in combination with the peptide, significantly increased ($P < 0.05$) the survival rate of the nematodes. Both the mean life and survival rate of nematodes followed the same pattern. This supports the hypothesis of (Grünz et al., 2012) who suggested that the lifespan of nematodes could be increased depending on the number of hydroxyl groups in the flavonoids (Grünz et al., 2012). Interestingly, the authors further highlighted that the impact on mean life would be more pronounced depending on the structural properties of the C-ring and the number of hydroxyl groups attached to the B-ring of the flavonoids (Grünz et al., 2012).



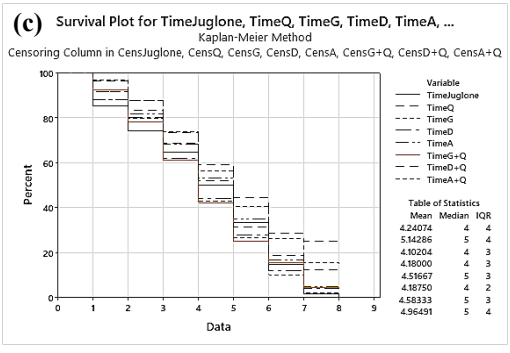



**Figure 2.6.** Effect of the dipeptide, quercetin, and peptide-quercetin mixtures on the survival rate of *Caenorhabditis elegans* at different life stages under oxidative stress condition: **(a)** Generation 1, **(b)** Generation 2, and **(c)** Generation 3. The juglone-treated nematodes were treated without (control) or with samples at 0.01, 0.1, 1 mM concentration. The survival rate was counted every hour, and the results are shown in a Kaplan-Meier curve. Q = quercetin, G = 0.01mM, D = 0.1mM, A = 1mM.

A major challenge in establishing the exact function of ROS in metabolism and aging is their accurate detection. Because redox signaling acts through small, local, and transient changes, redox detection ideally be selective, sensitive, instantaneous, reversible, compartment-specific, noninvasive, and applicable *in vivo* (Back et al., 2012). As a result, the fluorescent properties of the molecules are a direct measure of the levels of the ROS or metabolite *in vivo*. The transparency of *C. elegans* along their embryonic and postembryonic stages offers an exceptional advantage for assessing the *in vivo* function of ROS in the context of a complete organism, while its size allows for analysis by both microplate fluorometry and confocal microscopy (Back et al., 2012; Miranda-Vizuete & Veal, 2017). Thus, expressing the $H_2O_2$ redox sensor (HyPer) under the control of a ubiquitous promoter, has demonstrated that $H_2O_2$ levels are higher during larval development but upon transition to reproductive stage peroxide levels considerably decrease, remaining low during the reproductive period and then increasing again as the animals age (Miranda-Vizuete & Veal, 2017).

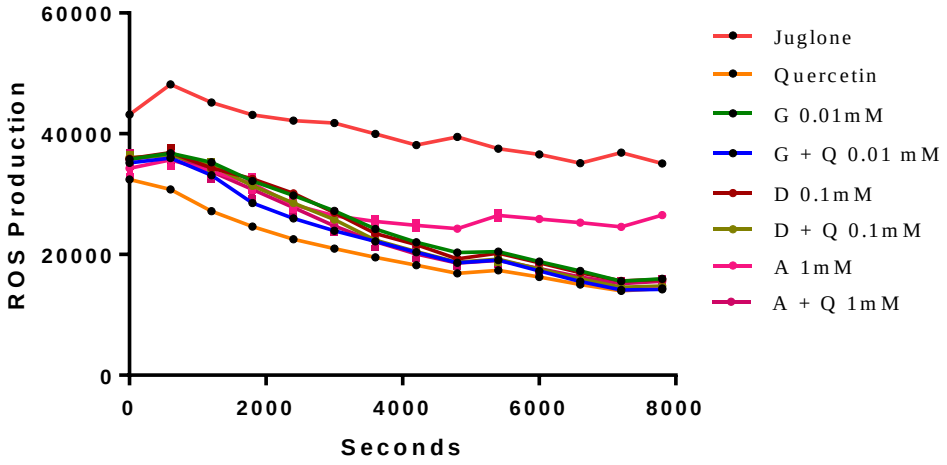



**Figure 2.7.** Effect of the dipeptide, quercetin, and peptide-quercetin mixtures on the ROS production activities of *Caenorhabditis elegans* under oxidative stress condition. The juglone-treated nematodes were treated without (control) or with samples at 0.01, 0.1, 1 mM concentration. The survival rate was counted every hour, and the results are shown in a Kaplan-Meier curve. Q = quercetin, G = 0.01mM, D = 0.1mM, A = 1mM.

Exposure to low doses of juglone activates oxidative stress and the antioxidant defense, which is expected to lower the survival and lifespan of the organism on prolonged exposure. Therefore, the presence of exogenous antioxidants, such as the peptide, quercetin, and peptide-quercetin mixture in this study, is required for the lifespan extension in worms exposed to low concentration of juglone. The peptide, polyphenol and combined treatments all reduced the ROS production level induced by juglone. These results indicate that the decreased ROS production was mediated by peptides (Phe-Cys), quercetin, or peptide-quercetin mixtures, thereby extending the survival and mean lifespan of the nematodes. As shown in **Figure 2.4**, the peptide-quercetin mixture resulted in antagonistic effects across all the concentrations. Notably, 1 mM peptide sample had a reduced activity compared to the lower concentrations, which is possibly due to molecular crowding which

limits the bio accessibility of reactive groups. Taken together, the peptide-quercetin mixtures could reduce the ROS levels in the oxidatively-damaged worms. This supports previous findings suggesting that antioxidant peptides can play an important role in attenuating oxidative stress in *C. elegans* (Yu et al., 2020).

## 2.4. CONCLUSION:

Interaction of these components generally affects the functional attributes of food products and eating quality. The study highlights that peptide-quercetin mixtures may exhibit antagonistic effects on antioxidant capacity *in vitro*, particularly reducing quercetin's efficiency. Consistently, *in vivo* results in *C. elegans* suggest that the peptide-quercetin mixtures still confer antioxidant benefits by reducing ROS and extending lifespan. This indicates that while interactions between peptides and quercetin may lower immediate antioxidant activity, they still play a role in reducing oxidative stress in living organisms. Protein-quercetin complex exhibit strong antioxidant activities and better emulsifying properties, thus increasing their efficacy for uses in food products. Further research is needed to understand the molecular mechanisms of these interactions and their potential therapeutic applications.

**Funding:**

This research was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant (RGPIN-2018-06839) and the University Research Chairs Program of the University of Ottawa, Canada.

**Conflict of interest:**

Authors declare that there are no conflict of interest.

**Authors' contributions:**

**Oluwaseyi A. Ogunrinola:** Conceptualization, Investigation, Data Curation, Methodology, Writing – Original Draft, Writing – Review & Editing, Visualization. **Samanta S. Reyes:** Investigation, Data Curation, Writing – Review & Editing, Visualization. **Chibuike C. Udenigwe:** Conceptualization, Resources, Writing – Review & Editing, Supervision, Funding Acquisition.

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# CHAPTER THREE

## CONCLUSION

The pathophysiology of many chronic diseases, including cancer, neurodegeneration, cardiovascular problems, and aging, is significantly influenced by oxidative stress. As a result, the biomedical and food sciences have paid close attention to the hunt for potent natural antioxidants. Although polyphenols, such as quercetin, are known to possess potent antioxidant properties, their poor stability, solubility, and bioavailability frequently impede their practical use. Their medicinal efficacy in nutraceutical formulations and functional foods is diminished by these restrictions. However, bioactive peptides made from dietary proteins have demonstrated encouraging health benefits like anti-inflammatory, antihypertensive, and antioxidant properties. Their relationship with polyphenols and the possibility of a synergistic increase in biological activity, however, are yet little understood. Considering its short lifespan, well-characterized genetics, and conservation of oxidative stress mechanisms, the *Caenorhabditis elegans* worm model provides a potent *in vivo* system for assessing antioxidant activity, redox balance, and lifespan consequences. By using this approach, the functional effects of peptide-quercetin complexes can be evaluated in a way that is both economical and biologically meaningful. This study fills a major knowledge gap regarding the mechanistic and functional effects of peptide-polyphenol interactions and investigates their possible use in nutraceuticals and food products that promote health.

The findings of this study provide significant insights into the antioxidant interactions between peptides and polyphenols, specifically focusing on the dipeptide Phe-Cys and quercetin. While the *in vitro* assays, including DPPH, ABTS, FRAP, and metal chelation, demonstrated an overall reduction in antioxidant capacity when these compounds were combined, this antagonistic effect

did not entirely diminish their biological relevance. The reduction in antioxidant activity was largely attributed to the interactions between the peptide and quercetin, particularly through π-π stacking and hydrogen bonding, which influenced the availability of reactive groups necessary for free radical scavenging.

Despite the observed antagonistic interactions *in vitro*, the *in vivo* analysis using *C. elegans* demonstrated a more promising outcome. The peptide-quercetin mixtures, even with reduced *in vitro* antioxidant activity, significantly reduced ROS levels and extended the survival and mean lifespan of the nematodes exposed to oxidative stress induced by juglone. These findings suggest that, while *in vitro* assays provide valuable mechanistic insights, they may not fully capture the biological complexity of antioxidant interactions within living organisms. The *C. elegans* model highlighted the potential of these mixtures to confer protective effects against oxidative stress, indicating that the peptide-quercetin interactions may play a beneficial role in modulating oxidative damage *in vivo*.

The study emphasizes the importance of understanding the molecular mechanisms underpinning peptide-quercetin interactions. The antagonistic effect observed *in vitro* and *in vivo* may stem from the formation of complexes that limit the accessibility of polyphenol hydroxyl groups, thus reducing their effectiveness as radical scavengers. Molecular docking analysis supported this, revealing π-π stacking interactions between the phenylalanine side chain of the peptide and the aromatic rings of quercetin. These interactions may create steric hindrances, reducing the ability of quercetin to donate electrons or hydrogen atoms to neutralize free radicals. Additionally, the altered particle characteristics, as revealed by dynamic light scattering, suggest that peptide-quercetin complexes may undergo aggregation, further influencing their bioactivity.

The implications of these findings are broad, as they provide a deeper understanding of how peptide-quercetin interactions affect antioxidant capacity. The study indicates that while such interactions may reduce immediate *in vitro* effectiveness, they do not necessarily preclude beneficial antioxidant effects *in vivo*. This expands new avenues for developing peptide-quercetin complexes as functional food applications by including some additional processing techniques (such as; heating, sonication, ultrasound, microwave, etc.), particularly for applications aimed at reducing oxidative stress and promoting bioavailability and bio-accessibility.

Further research is needed to elucidate the precise molecular pathways through which these interactions affect biological systems. Additionally, exploring different peptide-quercetin combinations, varying concentrations, and environmental conditions could help optimize their antioxidant potential. By refining our understanding of these interactions, there is potential to develop more effective formulations that harness the strengths of both peptides and polyphenols, overcoming the antagonistic effects seen in vitro while maximizing their *in vivo* benefits.

In order to better mimic human physiology, future study should build on this work by confirming the effects shown in mammalian systems, such as mouse models. Mechanistic understanding will be enhanced by employing transcriptome or proteomic analysis to examine biological pathways implicated in lifespan and the oxidative stress response. Formulation options will also be guided by the peptide-polyphenol complex's advanced structural characterisation. Moreover, to enhance stability, solubility, and targeted release in the gastrointestinal tract, future studies should investigate the encapsulation of these complexes in food-grade delivery vehicles as hydrogels or nanoparticles.

Extending the focus to encompass other bioactive peptides and polyphenols may reveal combinations with heightened bioactivity, which might be customized to particular health outcomes like neuroprotection, metabolic control, or anti-inflammatory effects. Additionally, assessing the level of synergy with techniques such as the Chou-Talalay combination index would aid in maximizing the functional effect of these interactions. When taken as a whole, these potential paths will not only improve the scientific basis of peptide-polyphenol research but also hasten its use in the creation of novel, health-promoting foods and nutraceuticals.

In conclusion, this study provides a foundation for future research into peptide-quercetin interactions, demonstrating that despite the complexities of their interactions, these compounds can play a critical role in combating oxidative stress in living organisms. Understanding the balance between antagonistic and synergistic effects in different biological contexts will be key to unlocking their full potential for therapeutic and nutraceutical applications.