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A Comparative Investigation of Synthesized Coumarin-Chalcone with Vitamin C for the Inhibition of Enzymatic Browning in Potato

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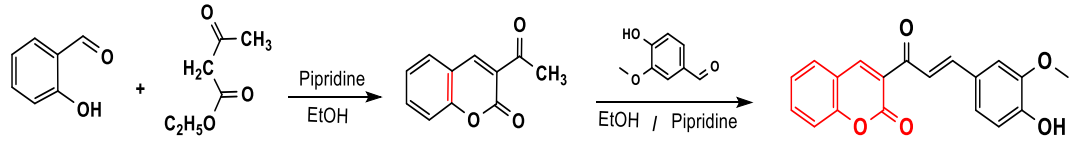
ABSTRACT

The enzymatic browning reaction is one of the primary factors contributing to the deterioration of food products, such as fruits and vegetables, after harvest. This phenomenon is primarily caused by the enzyme polyphenol oxidase (PPO), which alters the organoleptic properties and nutritional value, ultimately leading to economic losses worldwide. The inhibition of PPO prevents this reaction and preserves food products. Coumarin chalcone bearing a hydroxyl group has shown promising antioxidant and enzyme-inhibitory activities. Here, a novel coumarin chalcone, 3-(3-(4-hydroxy-3-methoxyphenyl) acryloyl)-2H-chromen-2-one (CCh), was synthesized, characterized, and tested for its ability to inhibit enzymatic browning in potatoes. The compound was produced through a condensation reaction between 3-acetylcoumarin and vanillin. Its structure was confirmed using 1H, 13C-NMR, and FT-IR spectroscopy. The anti-browning effect of CCh, compared to the known antioxidant, vitamin C, was evaluated over five days of frozen storage. Results showed that CCh effectively inhibited PPO activity and slowed the browning process. Molecular docking simulations with PPO (PDB ID: 2P3X) confirmed CCh's binding to amino acid residues GLN A331, LYS A61, LEU A53, PRO A89, and THR A136 through hydrogen bonds and hydrophobic interactions. The calculated binding energy of CCh was −4.71 kcal/mol, similar to vitamin C (−5.2 kcal/mol). These findings highlight CCh as a potential natural food preservative and capable of inhibiting enzymatic browning in potatoes, and could extend the shelf life of fruits and vegetables.

1-Introduction

Coumarins are polyphenolic compounds belonging to colorless and crystalline oxygenated heterocyclic compounds [1]. Coumarin (1,2-benzopyrone or 2H-1-benzopyran-2-one) and coumarin derivatives are extensively dispersed in nature and can be found in both naturally occurring and synthetic active compounds [2, 3]. Nowadays, coumarin and its derivatives, especially chalcones derived from coumarins, belong to the most active classes of compounds and possess a wide spectrum of biological activities [4, 5]. They act as antioxidants, enzyme inhibitors, and precursors of toxic substances [6, 7]. In the food systems, antioxidants act as natural or synthetic food preservatives added to products. They mitigate oxidative deterioration and prolong the shelf life of products [8]. One of the most important problems that some food products, such as fruits and vegetables, suffer from is the phenomenon of browning [9]. Browning is an important chemical reaction during food processing and storage [10]. Fruits and vegetables lose their nutritional value and sensory appeal due to browning; consumers may even be put off buying fresh products [11]. Browning occurs either non-enzymatically or mainly enzymatically [12, 13]. Enzymatic browning is a main process involving the oxidation by polyphenol oxidase (PPO) [14]. PPO (EC 1.10.3.1) is an enzyme that contains copper as a cofactor and can convert phenols to quinones, which combine with other substances to produce melanoid, a brown pigment[15]. The control of this enzyme can help to reduce or prevent this phenomenon, thus maintain the nutritional value, natural appearance of food products, and their acceptance by consumers [16]. Therefore, this study aimed to prepare and identify novel coumarin chalcone and investigate its ability to prevent or reduce enzymatic browning.



o-Hydroxy benzaldehyde | Ethyl acetoacetate | 3-Acetylcoumarin | Coumarin Chalcone (**CCh**)

2- Materials and methods

All chemicals and solvents were obtained from commercial suppliers. The product was examined for purity on TLC plates coated with silica gel 60F254 aluminum sheets (TLC, Darmstadt, Germany) and purified by column chromatography in an EtOAc: hexane: CH_2Cl_2 system. The ^{1}H and ^{13}C NMR spectra were acquired using a Bruker Bio Spin GmbH (400 MHz) in DMSO-d_6. An infrared spectrum was obtained using a Bruker Alpha (Platinum ATR, Germany). The melting point was determined using the Stuart SMP3 apparatus (Staffordshire, UK).

Preparation of coumarin chalconeSynthesis of 3-(3-(4-hydroxy-3-methoxyphenyl)acryloyl)-2H-chromen-2-one (CCh).

In a two-neck round-bottom flask, a magnetic stirrer was used to dissolve (0.01 mol) of 3-acetylcoumarin in (25 mL) of abs. (EtOH). Then, (0.01 mol) of vanillin was added, with continuous stirring for 30 minutes. Following this, (0.5 mL) of piperidine was added, with heating and stirring at a temperature of 75-80 °C for 8-10 hours **[17, 18]**. After confirming the completion of the reaction via TLC, the mixture was poured into a beaker containing crushed ice (20 g), stirred to complete the precipitation process, the resulting product was filtered, washed thoroughly with cold water and recrystallized from absolute ethyl alcohol to give the target compound (CCh), M.P. (°C) 212–214, Yield % 77, Colour, Yellow, R_f: Hex: EtOAc: DCM (3.5:1:0.5), which showed δ_H (400 MHz, DMSO-d_6, δ/ppm): 10.62 (s, 1H), 8.66 (s, 1H, coumarin), 8.02 – 7.97 (m, 2H), 7.97 – 7.90 (m, 1H), 7.86 (1H, d, J = 13.3 Hz), 7.75 – 7.59 (m, 3H), 7.33 (1H, d, J = 7.2 Hz,), 7.15 (1H, d, J = 6.1 Hz), 3.87 (s, 3H); δ_C (100 MHz, DMSO-d_6,, δ/ppm): 180.0, 157.6, 154.0, 149.1, 147.7, 129.7, 128.6, 127.5, 126.3, 125.0, 123.4, 122.0, 121.5, 118.7, 117.8, 116.1, 115.2, 112.4, 55.2; IR (ATR, cm^{-1}):3220, 3050, 2980, 1702, 1661, 1624, 1564, 1486, 1454, 1200, 1094.

Sample collection
In this study, one of the most important food products that is used almost daily, which is potatoes, was used. It was bought from the local market and did not exhibit mechanical damage resulting from packaging and transportation.

Preparation of solutions:
A- Dissolve 0.5 and 1 g of compound **CCh** separately in 1 liter of ethanol.
B- Dissolve 1 g of vitamin C in 1 liter of distilled water as a standard antioxidant.
Sample treatment: The samples were immediately immersed in water for 2 minutes after being brought in to remove dust and suspended matter. Then, they were removed and placed in a basin containing 0.001% sodium hypochlorite for 5 minutes. They were lifted out and dried thoroughly by wiping with a piece of cotton cloth, then cut into slices of approximately equal size, and divided into two parts.
Part I: Potato slices were placed in special dishes in an open-ventilated area. The slices were sprayed with the prepared solution of **CCh** at a concentration of 0.5 g per liter. The color changing to brown was monitored over 12 hours, and photographs of the samples were taken. This experiment served as a preliminary test to evaluate the ability of this compound and its effectiveness in preventing or delaying browning in the slices. Slices treated with distilled water were used as a negative control, while slices treated with vitamin C served as a positive control group.

Part II: The slices were immersed in the prepared compound solution for 4 minutes at room temperature. The slices were then removed and placed in special food storage bags made of polyvinyl (15 x 9 cm). The bags were subsequently stored at -20°C for five days. Afterward, they were divided into the following groups:
1. Negative control group (C-): treated with distilled water only.
2. Positive control group (C+): treated with vitamin C.
3. Group treated with **CCh** compound at a concentration of 0.5 g/L
4. Group treated with **CCh** compound at a concentration of 1 g/L
Before freezing at zero time, 80 g of each group was taken, and 100 mL of distilled water was added to it. It was crushed for 5 minutes using a Blender, taking into account stopping every 30 seconds. The mixture was filtered using several layers of gauze and then separated using a refrigerated centrifuge at 3000 rpm for 5 minutes. After separation, the precipitate was discarded, and the filtrate was taken for necessary measurements. This sample was used for comparison with the frozen samples, which were subjected to the same process every 24 hours. [19, 20].

Analysis
Colorless intermediate and browning intensity measurement:
To evaluate the colorless intermediate and browning intensity, absorbance was measured at 294 and 420 nm, respectively [21].

Total phenolic compound content estimation
By using the Folin-Ciocalteu reagent, total phenolic compounds were spectrophotometrically evaluated at 765nm [22]. For the standard curve, gallic acid (10-100 µg/mL) was used.
The PPO assay
PPO activity was measured using a spectrophotometer. One milliliter of 0.01M substrate, catechol, 0.9 mL of buffer (pH 7.2), and 0.1 mL of enzyme were combined. The absorbance was recorded at 420 nm every ten seconds for five minutes [23].

Molecular docking study
To clarify the mechanism by which the compound CCh reduces the intensity of browning, a molecular docking study of the compound was conducted in comparison with the enzyme polyphenol oxidase, which is responsible for enzymatic browning reactions. The enzyme coded (2P3X) was used, isolated from American grapes (*Vitis vinifera* L., cv), which were obtained from the protein bank from the website (www.rcsb.org) [24]. This study was based on the compound **CCh,** which has antioxidant properties through the presence of two phenolic hydroxyl groups and a coumarin moiety. The docking study was investigated using (Auto Dock Vina), and (Discovery Studio software). Vital interactions and binding conformations were revealed.

3- Results and discussion
Studies have shown that coumarin derivatives have many applications, including food, pharmaceuticals, and cosmetics [25]. In the United States, coumarin compounds extracted

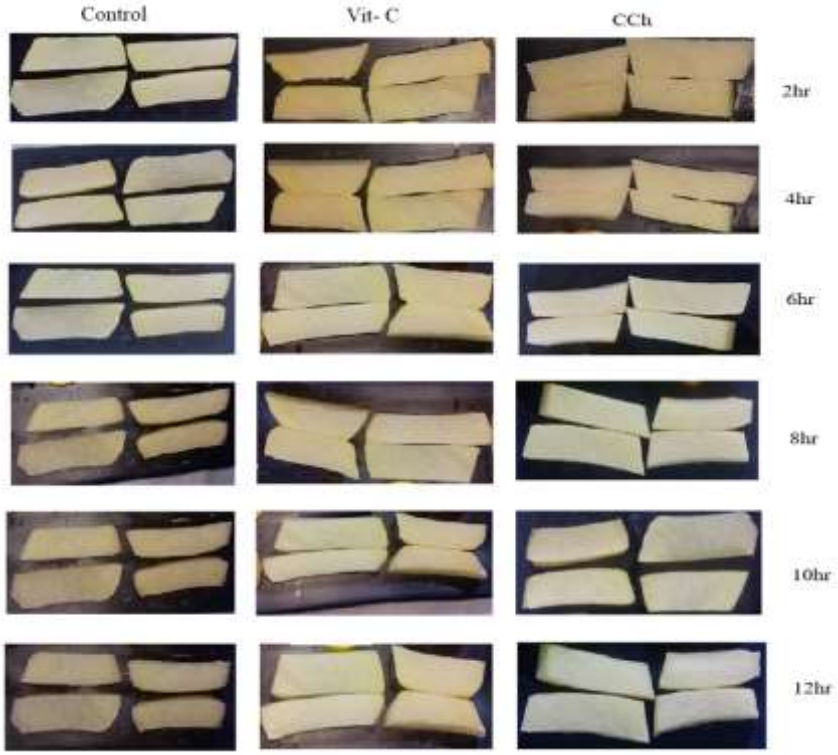
from tonka beans (rich in coumarins) have been used as additives to some foods [26]. In addition, they are antioxidant compounds because they contain hydroxyl (phenolic) groups [27].

From this standpoint, coumarin chalcone (**CCh**) was prepared, which is characterized by containing important chemical functional groups such as phenolic hydroxyl and the unsaturated alpha-beta chain in which the phenomenon of succession occurs with benzaldehyde (vanillin), in addition to the coumarin group. This compound was tested in terms of its ability to control the browning reactions that occur widely in foods, especially those handled almost daily, considering that these compounds are slightly toxic and are found in plant extracts. They have many applications in the food industry [5, 28]. To determine the effectiveness of the compound, it was first sprayed on potato slices and then tested during the freezing period.

Anti-browning reaction

After preparing the **CCh** solution (1g/L) and spraying it on the potato slides arranged on the plates, the samples were monitored every two hours for a period of up to 12 hours. Photographs were taken to observe the changes that occurred in the samples and to identify the effect of these compounds and their effectiveness in stopping the browning reactions.

Picture (1) shows the results of spraying potato samples with **CCh**. It was noted that there was a difference between the untreated and treated samples, which gave results close to vitamin C as antioxidants and their control over the development of browning in potato slices. This is consistent with what the researcher [29] reached when treating cut potato samples with vitamin C. It is clear that the untreated samples started to brown from the fifth hour, and their intensity increased to its maximum at that hour (11). As for the group treated with vitamin C, it showed a slight browning at the edges of the slices in the last hours starting from the ninth hour until the end of the hour (11), and this was also observed in the samples treated with **CCh**, which was closest to vitamin C in its antioxidant efficiency as a compound in controlling the browning of potato slices, considering that the compound is chemically an antioxidant.



Pictures (1): The effect of CCh compounds compared to the effect of vitamin C in preventing browning in potato slices.

Measuring intermediate compounds and browning intensity spectrophotometrically: Monitoring intermediates formed during browning is very important to determine the extent of development and continuation of this phenomenon. Figure 1 shows that there is a gradual increase in absorbance at 294 nm proportional to the storage time of the untreated group.

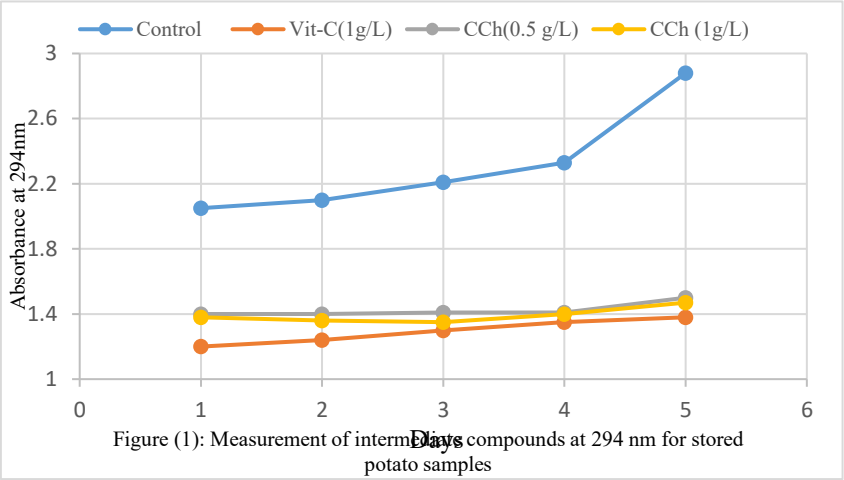


Figure (1): Measurement of intermediate compounds at 294 nm for stored potato samples

It was also observed through the study that the intensity of browning of the control samples increased directly with the storage time (Figure 2), as an increase in absorbance was observed at 420 nm. This increase has been found in many systems as it depends on the nature of the sugar, intermediate compounds, and colored polymers that are produced [30].

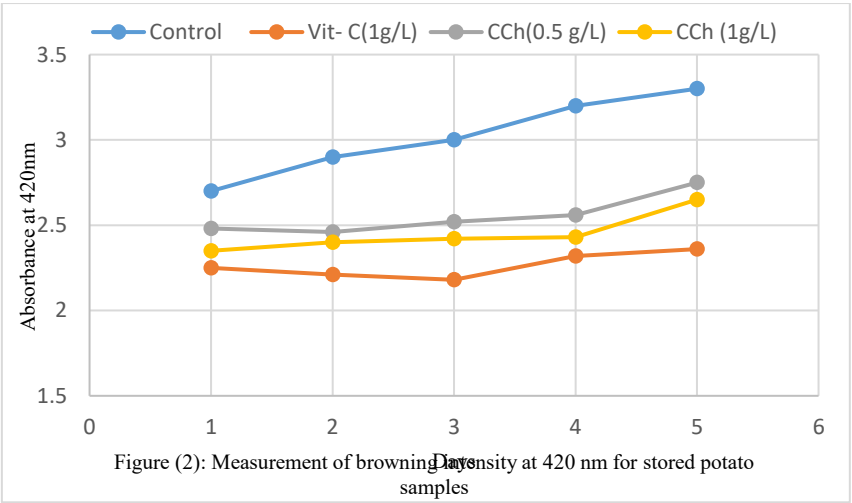


Figure (2): Measurement of browning intensity at 420 nm for stored potato samples

As for the samples treated with vitamin C, a significant decrease in absorbance was observed compared to the control group over 5 days. On the other hand, a decrease in absorbance was observed at 294 and 420 nm for the groups treated with two different concentrations of the **CCh** throughout 5 days compared to the control group. It was shown by monitoring the absorbance that the compound has an efficiency similar to vitamin C, which confirms its ability to prevent the formation of intermediate compounds and thus control the development of browning.

Absorbance in the untreated samples (control) gradually increased from the first day and continued to rise until the fifth day, when it reached its highest level. In samples treated with vitamin C, the absorbance was lower from the first day and increased slightly over time until the fifth day. Samples treated with CCh at a concentration of one g/ml showed absorbance levels similar to vitamin C, starting from the first day and remaining so through the fifth day

for the potato samples, as shown in Figures 1 and 2.

Change in PPO and total phenolic content

The phenolic components found in many edible plants, such as fresh vegetables and fruits, are oxidized by the enzyme polyphenol oxidase in the presence of oxygen, forming quinones. These compounds then polymerize with each other or with other molecules like proteins or amino acids, creating brown pigments [31]. The quinones produced in the initial browning reaction can also participate in oxidative coupling reactions, enabling them to oxidize additional polyphenolic compounds that cannot be directly enzymatically oxidized. Changes in the levels of phenols indicate the capacity and efficiency of phenol oxidase enzymes to oxidize these compounds. When the total phenols in a food sample decrease, it suggests an increased activity of the enzyme PPO, and vice versa [32].

The results showed that the activity of the polyphenol oxidase enzyme was directly proportional to the storage period for the untreated samples. Meanwhile, the samples treated with vitamin C showed a noticeable decrease in the activity of the enzyme. This decrease was also observed for the samples treated with the two concentrations used for the CCh compound compared to the untreated samples (Figure 3). The effect of this compound was less than vitamin C, which is considered one of the strongest, most widely used, and safest antioxidants

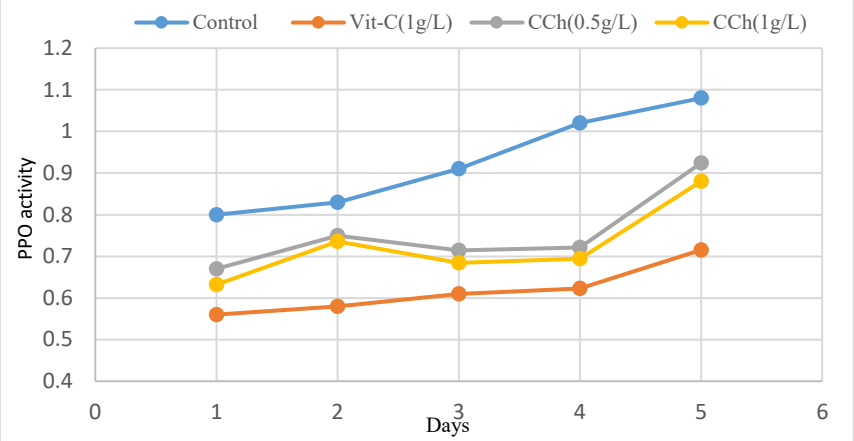


Figure (3): Measuring the PPO activity of stored potato samples.

Accordingly, it is clear from Figure 4 that a significant decrease in the phenolic content occurred during storage for the untreated samples. This decrease may be due to the oxidation of these compounds by PPO with the advancement of storage time. In contrast, the samples treated with vitamin C showed a gradual increase in the phenolic content, which was also observed by [33]. This gradual increase was also observed for the samples treated with **CCh**.

The effectiveness of this compound may be due to the presence of two (OH) phenolic groups in addition to the coumarin ring, with a good reductive nature that gives it antioxidant properties. Through this feature, the compound may work to inhibit the polyphenol oxidase enzyme, which leads to an increase in the phenolic content or at least maintains its level compared to the untreated control group.

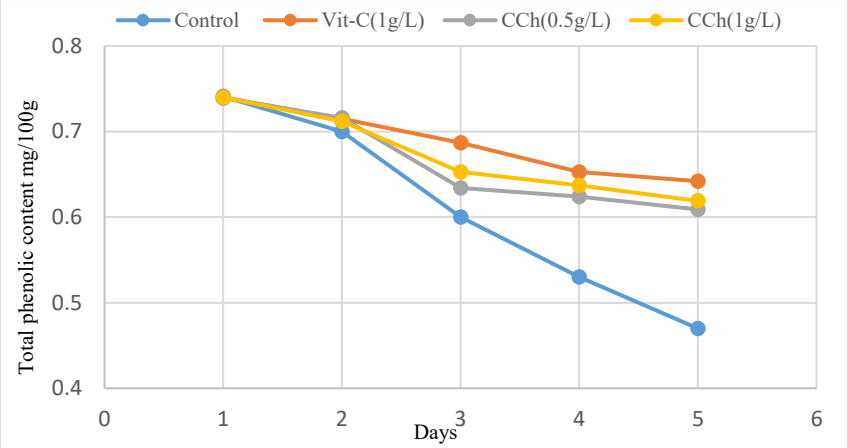


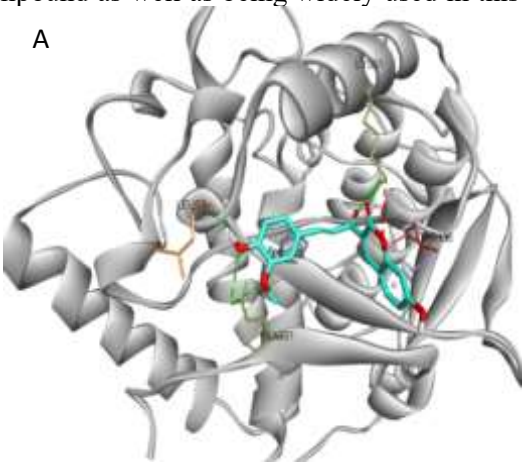
Figure (4): Measuring the total phenolic content of stored potato samples

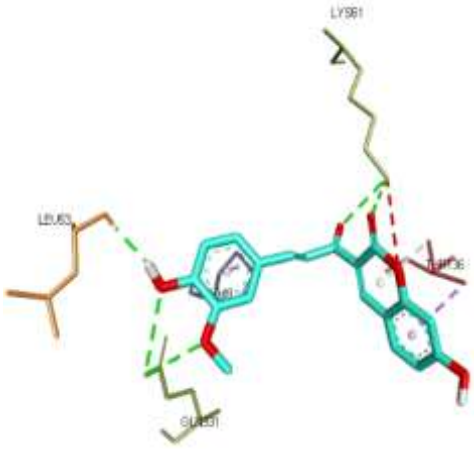
Molecular docking

One of the mechanisms by which browning reactions can be controlled during the storage period may be by inhibiting the activity of PPO. Therefore, we deemed it necessary to conduct a molecular docking study between the effective compound **CCh** and the polyphenol oxidase enzyme (ID: 2p3x), extracted from American grapefruit [34]. This study aims to know the connections and bonds that occur between the compound and the enzyme theoretically and thus know how the **CCh** affects the enzyme. To make the study more objective, vitamin C was also used for comparison as it is an antioxidant compound as well as being widely used in this field due to its non-toxicity and safe use [35, 36].

The results showed that the stability energy of the complex **CCh** with the enzyme is equal to (ΔG binding energy = -4.71 kcal/mol). Figure (5) shows that the amino acids that formed interactions and hydrogen bonds between the surface of the enzyme and the complex are GLN: A331, GLN: A331, LYS: A61, LYS: A61, LEU: A53, while the acid PRO: A89 formed π-alkyl bonds and the amino acid THR: A136 formed a pi-sigma bond, in addition to other forces such as (van der Waals, pi-donor H-B).

A





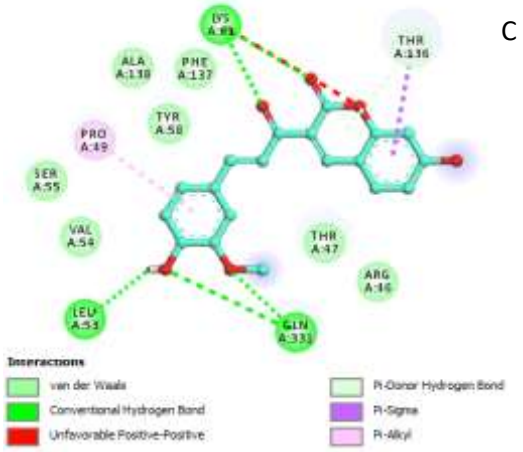
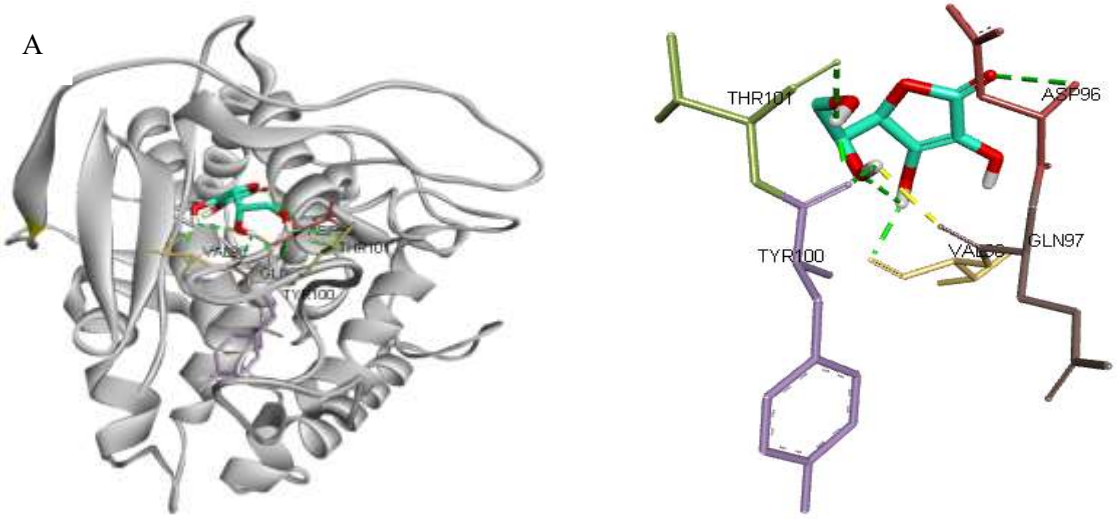


Figure (5): A shows the location of CCh within the protein cavity ID:2p3x)) of the enzyme polyphenol oxidase in a three-dimensional (3D) form. B shows the most important interactions between the groups of the complex (CCh and amino acids ID:2p3x) in a three-dimensional (3D) form. C shows the most important interactions between the functional groups (CCh and the type of amino acids ID:2p3x)) in a two-dimensional (2D) form

When the simulation was conducted for the same enzyme with vitamin C, it was found that there was an interference with some amino acids present on the surface of the enzyme, as the interference was by hydrogen bonds for the amino acids TYRA:100, THRA:101, GLNA:97, VALA:98, ASPA:96 (Figure 6), in addition to the van der Waals forces for several amino acids surrounding the functional groups in the complex. It was found that the complex formed by (enzyme + vitamin C) has stabilization energy equal to (ΔG binding energy = -5.2 kcal/mol).



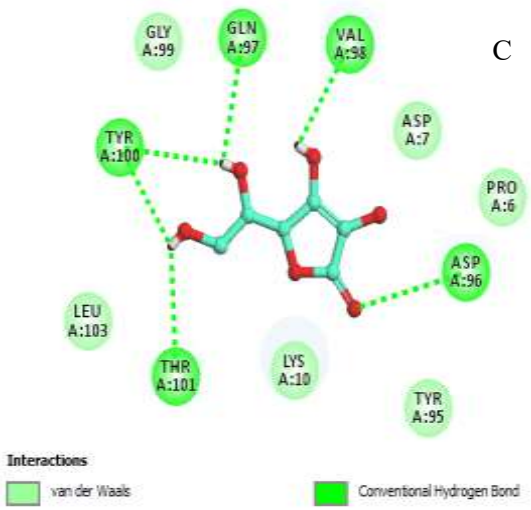


Figure (6): A shows the location of vitamin C within the protein cavity (ID:2p3x)) of the enzyme polyphenol oxidase in a three-dimensional (3D) form. B shows the most important interactions between the groups of the (C) compound and the amino acids (ID:2p3x)) in a three-dimensional (3D) form. C shows the most important interactions between the functional groups (C) and the type of amino acids (ID:2p3x)) in a two-dimensional (2D) form.

When comparing compound CCh with vitamin C, we notice that there is a great convergence in the number of hydrogen bonds, as compound CCh has five hydrogen bonds and three different amino acids for the enzyme. In comparison, vitamin C has six hydrogen bonds and five amino acids. Therefore, the strongest inhibition is when vitamin C interferes with the enzyme, and the least is compound CCh with the enzyme, and the laboratory study confirms this, as vitamin C is a clear antioxidant in all the previously mentioned biometric methods. The researcher [37] found that the molecular docking process of vitamin C with polyphenol phenol oxidase, which had been extracted from the lotus rhizome *Nelumbo nucifera*, where the study showed the presence of 6 hydrogen bonds between vitamin C and the targeted enzyme in that study.

In a recent study, it was shown that the binding of blueberry PPO (*Vitis vinifera*) (2Y9W) with glutamic acid was (-2.00 kcal mol) through simulation in the three-dimensional structure. Glutamic acid formed five hydrogen bonds with the amino acids Lys 70, Gln 74, Tyr 78, and Met 325 of the enzyme. The inhibition of the enzyme was competitive [38]. In vitro study, 4-hydroxycinnamic acid and naringin showed an inhibitory effect on PPO, with the greatest effect observed for 4-hydroxycinnamic acid. Molecular docking also revealed that 4-hydroxycinnamic acid and naringin interacted with PPO through hydrogen bonding and hydrophobic interaction, and more interactions were observed near the carboxylic group [39]. The inhibitory effect of ascorbic acid, L-cysteine, glutathione, and citric acid on PPO from Granny Smith apples was investigated using molecular docking. The amino acid residues His 180, His 201, His 366, Cys 184, Glu 328, and Asn 333 were the important binding sites in the active site. The inhibitors were bound to PPO through hydrogen bonds and ion contacts [40].

4-Conclusion

This study revealed that the newly synthesized coumarin chalcone (CCh) compound has a significant antioxidant and anti-browning activities, suggesting it could serve as a natural preservative with the potential to replace synthetic options like vitamin C in foods. The structure of the synthesized compound was confirmed through various spectroscopic techniques, verifying its purity. When applied to potato slices, CCh markedly delayed enzymatic browning, evidenced by reducing the absorbance at 294 and 420 nm compared to untreated controls. The treated samples also retained higher total phenolic content at the end of storage, indicating that the compound could inhibit PPO activity. Furthermore, molecular docking study confirmed that CCh interacts with PPO via diverse hydrogen bonds and hydrophobic interactions, supporting the hypothesis of enzyme inhibition at the molecular level. Although its binding affinity was lower than that of vitamin C, it was still comparable, and the compound demonstrated similar efficacy in inhibiting browning and preserving phenolic content. Overall, CCh

could be a canditate as a promising antioxidant for manufacturing use in preventing enzymatic browning in foods.

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Conflict of interest

The authors have no conflicts of interest to disclose that are compatible with the subject matter of this article.

Consent to participate

All authors have expressed their authorization to engage in this publication.

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Ethical Statement

Except for sensory evaluation, we have not conducted any human or animal investigations during this study. Regarding the sensory case.

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