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A Study on Recognizing the Value of Chestnut (*Castanea sativa*) Blossom Waste

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ABSTRACT
Chestnut (*Castanea sativa*) blossoms are natural resources that are not put to economic use. They are completely mixed with soil as waste. Thus, this extensive study was designed and remarkable results were found showing the potential usefulness of chestnut blossoms. In addition to the phenolic capacity and antioxidant capacity of the aqueous and ethanolic extracts of dried chestnut flowers, the anti-urease activity of these extracts was studied to demonstrate their therapeutic value. The binding interaction of phenolic substances present in chestnut blossom with urease was shown using molecular docking research. The aqueous extract, with most effect, had total phenolic content of 46.67 ± 0.37 mg GAE/g and total flavonoid content of 6.14 ± 0.40 mg QUE/g. The antioxidant activity was determined by FRAP (648.47 ± 5.27 µmol $FeSO_4.7H_2O$/g for aqueous extract and 347.53 ± 2.09 µmol $FeSO_4.7H_2O$/g for ethanolic extract) and DPPH (0.05 ± 0.01 mg/mL for SC_{50} of aqueous extract and 0.11 ± 0.01 mg/mL for SC_{50} of ethanolic extract) assays, and rutin was found to be the dominant phenolic compound according to HPLC. IC_{50} values for urease in aqueous and ethanolic extracts were 2.55 ± 0.09 mg/mL and 4.57 ± 0.24 mg/mL, respectively. According to the docking experiments, which were important to support the hypothesis of anti-urease activity, myricetin and luteolin showed different and effective bonding degrees to the target protein when compared with the reference molecule acetohydroxamic acid. In summary, chestnut flowers are rich in phenolic compounds which are responsible for a wide range of biological activities including antioxidant features and urease inhibition. These blossoms could be evaluated as potentially important raw materials for food.

Keywords: Chestnut, Blossom, Antioxidant, Phenolics, Molecular docking, Anti-urease

1. Introduction

Chestnut trees are highly valuable forest plants providing many benefits. In addition to wood and lumber, they also provide important non-wood products such as fruit and blossoms. Honeybees also directly benefit from these natural products (Carocho et al. 2015; Kolayli et al. 2016; Caleja et al. 2019; Rodrigues et al. 2020). The high antioxidant and other biologically active properties of chestnut honey, pollen, and propolis are due to high amounts of polyphenols in their composition (Comandini et al. 2014; Sahin et al. 2019; Karkar et al. 2021). Türkiye, a country rich in chestnut forests, is also the world's largest producer of chestnut honey. This type of honey, also known as medicinal honey, is one of the most valuable honeys in the world, with dark color, non-crystalline structure, and high antimicrobial and antiviral value. It is frequently used for asymptomatic treatments and wound healing therapies. Chestnut bee pollen and propolis are reported to be rich in polyphenols and tannins (Comandini et al. 2014; Carocho et al. 2015; Rodríguez-Flores et al. 2023). Tannins are complex polyphenols produced by a variety of plants, including chestnut trees (Aimone et al. 2023). Recent studies showed that chestnut blossoms should be evaluated as functional foods. The studies also indicated that in addition to chestnut honey and bee pollen, chestnut blossoms contain high levels of polyphenols (Barreira et al. 2008; Peng et al. 2022).

Comparisons of chestnut honey, blossoms, barks, leaves, and fruits showed that each product exhibits different antioxidant activities, with the highest levels being observed in blossoms (Barreira et al. 2008). Moreover, coumarins, flavonoids and their derivatives, proanthocyanidins, and sterols were found in horse chestnut seeds (Dudek-Makuch & Studzińska-Sroka 2015). Dried chestnut blossoms are also rich in oil, protein, sugar, antioxidants, polyphenols, and mineral and fiber substances, and they are used as natural ingredients in the bakery industry (Carocho et al. 2015).

Although there have been many studies about the chemical and biochemical properties of chestnut fruit, the studies involving chestnut blossoms are limited. The purpose of this study was to assess the usefulness of chestnut blossom extracts for the food industry, identify their biologically active properties, and describe their potential benefits to the economy. In brief, aqueous and ethanolic chestnut blossom extracts were tested for phenolic content, antioxidant activity, and anti-urease activity.

2. Material and Methods

2.1. Chemicals

Phenolic standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). Daidzein was supplied by Cayman Chemical (Michigan, USA), and other chemicals required for current assays were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

2.2. Samples

Chestnut blossoms were collected from natural chestnut trees (*Castanea sativa*) on private land in Samsun, Türkiye, in June 2019. The fresh blossoms were collected, dried at room temperature, and powdered in a grinder. Then, the samples were extracted with distilled water and ethanol. For the aqueous extract, 6 g of dry sample was mixed with 60 mL of distilled water and brewed at 100 °C for 10 minutes. This extraction method is also known as infusion. The extract was filtered and stored at -20 °C until use. The second extract was prepared with 70% ethanol. For this purpose, 6 g of sample was added to 60 mL of 70% ethanol, shaken for 24 h, and filtered and evaporated under a vacuum evaporator (Heidolph, Schwabach, Germany) at 40 ºC, after which the extract was finally dissolved in a small amount of 70% ethanol.

2.3. Determination of total phenolic content (TPC)

The TPC of the extracts was determined using the Folin-Ciocalteu method (Singleton & Rossi 1965) with gallic acid as the standard. TPC is expressed as mg gallic acid equivalent (GAE)/g dry sample using a standard curve.

2.4. Determination of total flavonoid content (TFC)

TFC of both extracts was measured using a spectrophotometric method with quercetin as standard (Fukumoto & Mazza 2000). TFC is expressed as mg quercetin equivalent (QUE)/g base on the curve.

2.5. Analysis of ferric reducing/antioxidant power (FRAP)

The ferric reducing/antioxidant power assay (FRAP) method described by Benzie & Strain (1999) was used to calculate the total antioxidant capacity of the extracts. For FRAP values, the results are given as µmol $FeSO_4.7H_2O$ equivalents/ g dry matter.

2.6. DPPH•-free radical scavenging assay

The DPPH• assay was developed using a spectrophotometric method described previously by Brand-Williams *et al.* (1995). All DPPH• assay results are given with SC_{50}, which is the sample concentration that causes 50% radical scavenging.

2.7. Analyses of phenolic composition by HPLC-UV

To prepare the extract for chromatographic analysis, 10 mL of blossom extract was evaporated (Heidolph, Schwabach, Germany) at 40 °C to dryness. The residue was then dissolved in 10 mL distilled water (pH: 2), and the resulting aqueous solution was extracted three times with 5 mL diethyl ether (15 min, 200 rpm, room temperature). Following each extraction, the upper organic phase was collected. After that, the aqueous solution was extracted three times with 5 mL ethyl acetate (15 min, 200 rpm, room temperature). After these extractions, the organic phases were mixed and evaporated (Heidolph, Schwabach, Germany) at 30 ºC to dryness. The residue was dissolved in 2 mL methanol, filtered through 0.45 µm filters, and analyzed using an HPLC device.

Calibrations were also performed for HPLC using 19 standard phenolic compounds at 280 and 340 nm (Elite La Chrome; Hitachi, Tokyo, Japan) on a device fitted with a reverse phase C_{18} column (150 mm, 4.6 mm, 5 µm; Fortis). The R^2 values for each compound were between 0.998-1.000. The program employed was described in previous studies, with acetic acid, water, and acetonitrile being used as the mobile phase (Malkoç et al. 2019). The mobile phase was composed of (A) 2% acetic acid in water and (B) acetonitrile: water (70:30). Finally, 20 µL of the sample was injected individually at 25 °C, and the flow rate was set to 0.75 mL/min.

2.8. Anti-urease activity

The anti-urease activity test is based on urease inhibition of the indophenol method (Weatherburn 1967), and jack bean urease is used for the test. The absorbance was recorded at 625 nm (Thermo Scientific Spectrophotometer, Waltham, MA). The results for the samples and for acetohydroxamic acid (AA), which was used as a standard inhibitor compound, are expressed as IC_{50}, the level producing 50% inhibition of maximal activity.

2.9. In silico methods (Protocol for molecular docking study)

To analyze the interactions of four ligands, molecular docking experiments were performed using Autodock 4.2 software. The crystal structure of jack bean urease (*Canavalia ensiformis*) (PDB ID: 4GY7, Res: 1.49 Å) was downloaded from RCSB Protein Data Bank (https://www.rcsb.org/). In general, binding free energies (ΔG) for crystal structures and docking models are determined to assess the accuracy of binding affinity prediction between ligands and target proteins. The structures of the ligands were obtained from the Pubchem Database (pubchem.ncbi.nlm.nih.gov) and converted to a pdf file with BIOVIA Discovery Studio Visualizer 2018. The prepared ligands and proteins were used as input files for AutoDock 4.2 software (Morris et al. 2009). With the help of the software, a Lamarckian genetic algorithm technique was used. After minimizing the energy, the water molecules were removed, and a rigid protein and a flexible ligand were docked using the standard docking method with 100 independent runs for each ligand's torsion angle. In the catalytic site of the protein, Autodock 4.2 was used for all docking experiments. With a grid spacing of 0.375 Å, a grid was constructed with 126, 126, and 126 points in the x, y, and z directions. All other parameters were left at their default settings. The ligand-protein docked complexes were analyzed based on minimum binding energy values and ligand interaction (hydrogen/hydrophobic) patterns to predict the binding strength of four ligands. BIOVIA Discovery Studio Visualizer 2018 (Dassault Systèmes BIOVIA 2016) was used for the final visualization of the docked structures.

2.10. Statistical analyses

SPSS version 11.5 software was used for statistical analysis (IBM SPSS Statistics, Armonk, New York, USA). Mean and standard deviation are used to express descriptive statistics. Correlation analysis was performed using the Mann–Whitney U test. The significance level was set at $P<0.05$.

3. Results and Discussion

3.1. TPC and TFC results for chestnut blossoms

The relevant values are shown in Table 1. The total amount of phenolic substance was 46.67 ± 0.37 mg GAE/g in the aqueous extract and 25.78 ± 0.15 mg GAE/g in the ethanolic extract. These data were statistically significantly different ($P<0.05$). The higher amount of polyphenols in the aqueous phase indicates that the polyphenols found in chestnut blossom are rich in polar or hydrophilic compounds. Almost all phenolic acids are such compounds and are soluble in water. Flavonoids, the largest member of the polyphenol family, were determined at higher levels in the aqueous extract (6.14 ± 0.40 mg QE/g) than in the ethanolic extract (mg QE/g), although the statistical difference was significant ($P<0.05$). Even though there was a statistically significant difference between the present values, the coefficient difference between the extracts was not as high as the difference for the total phenolic substance findings. The principal reason for this is that flavonoids are relatively non-polar in character, because ethanol has a lower polarity than water and, conversely, are more non-polar in character. Similar to the results of the present research, a previous study using the heat-assisted extraction method to extract total phenolic substances from chestnut blossoms reported rich water-soluble tannin (hydrolyzed tannin) and flavonoid contents (Caleja et al. 2019). When TPC and TFC values were compared with those of previous studies, this study clearly illustrates the bio-efficiency of chestnut blossoms. In a study using fresh chestnut flowers, TPC and TFC values were confirmed as 298 mg GAE/g and 160 mg catechin equivalent (CE)/g, respectively (Barreira et al. 2008). Another comprehensive study in the literature analyzed different parts of chestnut except the blossom in terms of some bioactivity assays (Silva et al. 2020). In this study, each part of the chestnut separately had value in terms of total phenolic substances. Although the study used a different unit than the current study, it was emphasized that the leaves (385.4 g of epicatechin equivalents per mg of residue) had higher total phenolic content than the inner and outer shells and burs of chestnut (Silva et al. 2020). In addition to this study, other indirect studies were carried out related to chestnut honey made from chestnut blossom nectar. Kolayli et al. (2016) determined the range of TPC and TFC in chestnut honey was 76.20-94.05 mg GAE/ 100 g and 4.20-6.50 mg QUE/ 100 g, and Can et al. (2015) found the mean value of TPC and TFC in chestnut honeys was 98.26 mg GAE/ 100 g and 8.10 mg QUE/100 g, respectively.

Table 1- Antioxidant properties of the chestnut blossom extracts

Analysis Parameters	*Aqueous Extract*	*Ethanolic Extract*
Total Phenolic Content (mg GAE/g)	46.67 ± 0.37^{a}	25.78 ± 0.15^{b}
Total Flavonoid Content (mg QE/g)	6.14 ± 0.40^{a}	5.02 ± 0.30^{b}
Total Antioxidant Capacity- FRAP (µmol $FeSO_4.7H_2O$/g)	648.47 ± 5.27^{a}	347.53 ± 2.09^{b}
DPPH Radical Scavenging-SC_{50} (mg/mL)	0.05 ± 0.01^{b}	0.11 ± 0.01^{a}

a, b: letters in the same lines are significantly different at the 5% level (P<0.05).

3.2. Antioxidant activity of chestnut blossoms

FRAP and DPPH• radical scavenging activities were utilized to evaluate the antioxidant properties of the chestnut blossoms. The FRAP method, which is based on the reduction of Fe (III)-complex in the presence of antioxidants, is used to calculate total antioxidant capacity. In general terms, a high FRAP value indicates high antioxidant capacity, and these values were approximately two times higher in aqueous extract than in ethanolic extract (648.47 ± 5.27 µmol $FeSO_4.7H_2O$/g for aqueous extract and 347.53 ± 2.09 µmol $FeSO_4.7H_2O$/g for ethanolic extract; P<0.005).

The DPPH• radical is an unnatural, synthetic radical, and the method based on it is a very sensitive, reliable, and simple test measuring the radical-scavenging ability of natural products. Any antioxidant scavenging this radical has high potential to eliminate dietary radicals, hydroxyl, superoxide, and nitric oxide formed by oxidative stress in the body. The amount of substance that cleanses half of this radical is defined as SC_{50} (scavenging activity); the lower this value, the higher the activity. The DPPH• scavenging ability of the aqueous extract in the present study was approximately twice as high as for the ethanolic extract (0.05 ± 0.01 mg/mL SC_{50} for aqueous extract and 0.11± 0.01 mg/mL SC_{50} for ethanolic extract; P<0.005). In short, the results of both antioxidant tests showed that the aqueous extract had significant antioxidant capacity. This is mostly because the aqueous extract contains many phenolic compounds, which was also confirmed by HPLC-UV in this study. Some previous studies also confirmed that aqueous extracts of chestnut blossoms have high antioxidant value (Tuyen et al. 2017; Caleja et al. 2019).

3.3. Evaluation of the phenolic profile of chestnut blossoms

According to studies in the literature, the profusion of phenolic compounds present in the composition of chestnut flowers allows for combination of their remarkable bioactive properties. Moreover, these studies highlighted that the phenolic compounds in chestnut flowers are promising agents as natural food preservative in the food industry (Carocho et al. 2014; Caleja et al. 2019; Alaya et al. 2021).

The phenolic compositions of the current samples, extracted with two different extract polarities, are summarized in Table 2. The primary phenolic compounds in the aqueous extract, which analyzed 19 phenolic standards using HPLC-UV, were rutin, gallic acid, and myricetin, and the main phenolic compounds in the ethanolic extract were rutin, luteolin, and resveratrol. The ethanolic extract contained more rutin (1228.93 ± 2.76 µg/g) than the aqueous extract (1117.72 ± 2.92 µg/g) (P<0.005). Also, gallic acid values were approximately 10 times higher in the aqueous extract (979.47 ± 2.01 µg/g) than in the ethanolic extract (60.61 ± 0.88 µg/g) (P<0.005). A previous study also reported that chestnut blossoms are very rich in gallic acid (Tuyen et al. 2017). Luteolin, a flavonoid derivative and a flavone exhibiting wide biological activity, was detected at the highest level in the ethanolic extracts but not in the aqueous extracts. Rutin, also known as quercetin-3-O-rutinoside (α-L-rhamnopyranosyl- (1→6) -β-D-glucopyranose), is a product of glycosylation of the flavanol quercetin with a disaccharide and was detected at similar amounts in both extracts since it is amphipathic in character (Gullón et al. 2017).

Table 2- HPLC-UV analyses of the chestnut blossom extracts

Phenolic Compounds (µg/g)	*Aqueous Extract*	*Ethanolic Extract*
Gallic acid	979.47 ± 2.01[b]	60.61 ± 0.88[a]
Protocatechuic acid	77.60 ± 0.51[b]	35.75 ± 0.44[a]
***p*-OH benzoic acid**	N.D.	4.85 ± 0.09
Catechin	104.22 ± 0.78[b]	42.65 ± 0.31[a]
Caffeic Acid	N.D.	N.D.
Syringic Acid	3.38 ± 0.07[b]	2.03 ± 0.06[a]
Epicatechin	N.D.	N.D.
***p*-coumaric acid**	45.01 ± 0.39[b]	37.86 ± 0.27[a]
Ferulic acid	N.D.	N.D.
Rutin	1117.72 ± 2.92[a]	1228.93 ± 2.76[b]
Myricetin	334.59 ± 1.01[b]	51.97 ± 0.56[a]
Resveratrol	14.91 ± 0.16[a]	104.42 ± 0.96[b]
Daidzein	6.82 ± 0.17[b]	2.42 ± 0.08[a]
Luteolin	N.D.	918.24 ± 1.31
***t*-cinnamic acid**	1.79 ± 0.09	N.D.
Hesperetin	N.D.	15.04 ± 0.38
Chrysin	N.D.	N.D.
Pinocembrin	N.D.	N.D.
CAPE	N.D.	N.D.

N.D. Not Detected; a, b letters in the same line are significantly different at the 5% level (P< 0.05)

3.4. Urease inhibition activity of chestnut blossoms

The other biological activity test for both samples was urease inhibition. Urease enzyme inhibitors are particularly important for *Helicobacter pylori* inhibition, and bacteria survive with this enzyme secreted into the extracellular environment. Both chestnut blossom extracts were found to inhibit the enzyme, but the aqueous extracts (IC_{50}: 2.55 ± 0.09 mg/mL) caused greater inhibition than the ethanolic extracts (IC_{50}: 4.57 ± 0.24 mg/mL). The statistical difference was confirmed at P<0.005. (Table 3). Urease enzyme inhibition is thought to be caused by polyphenols; even so, it was shown that natural products with high phenolic acid and flavonoid contents exhibit higher activity in some urease inhibition studies (Al-Rooqi et al. 2023; Kataria & Khatkar 2019a). Aside from that, previous reports about natural compounds defended a close synergistic effect between urease inhibition activity and phenolic agents, which is similar to our claim (Uddin et al. 2011; Paun et al. 2014; Can et al. 2022). Moreover, in this study, the molecular docking properties of some compounds abundantly found in chestnut blossom extracts are presented to show the binding interaction of these compounds as a reason for urease inhibition.

Table 3- Urease inhibitions of the chestnut blossom extracts

Samples	*Inhibition IC_{50} (mg/mL)*
Aqueous Extract	2.55 ± 0.09[b]
Ethanolic Extract	4.57 ± 0.24[a]
Acetohydroxamic acid (AA) (µg/mL)	25.09 ± 0.02[c]

a, b, c: letters in the same column are significantly different at the 5% level (P<0.05)

3.4. Molecular docking assessment of major phenolics found in chestnut blossoms

The results for successful docking of all ligands used in these docking experiments revealed significant interactions of the ligands with the target receptors. The target protein was more effectively bonded by four ligands (myricetin, gallic acid, rutin, and luteolin) than by the reference molecule. The ligand myricetin was strongly bound to jack bean urease with a binding energy of -7.30 kcal/mol. With a binding energy of -7.21 kcal/mol, the ligand luteolin also effectively docked with the target receptors. The ligands rutin and gallic acid were bound to the target protein with binding energies of -6.75 and -5.48 kcal/mol, respectively. Table 4 contains additional information. Figures 1-4 depict docked position in the target receptor for each ligand, as well as the residues with which each ligand interacts and the interactions. The molecular binding of some ligands, including phenolic compounds, with the urease enzyme was reported in the literature. In one study, some synthesized ligands showed significantly higher binding result to the active cavity of jack bean protein, and there was consistency between *in silico* and *in vitro* results (Kataria & Khatkar 2019a). Kataria & Khatkar (2019b) studied molecular docking with natural phenolic compounds as possible urease inhibitors. According to that study, five compounds -diosmin, morin, chlorogenic acid, capsaicin, and resveratrol- showed remarkable affinity towards the receptor.

Table 4- Summary of ligands against Urease enzyme from Jack Bean with binding energy, K_i and interacted residues in the binding site

No	*Receptor Name*	*Receptor PDB ID*	*Ligand Name*	*Binding Energy (kcal/mol)*	*K_i*	*Interacted Residues with Ligand*
1	*Urease from Jack bean (Canavalia ensiformis)* EC: 3.5.1.5	4GY7	Myricetin (3,3′,4′,5,5′,7-Hexahydroxyflavone)	-7.30	4.44 µM	Thr740, Val81, Ala80, Val36, Tyr32, Val744, Asp730, Glu718, Phe712, Lys716, Glu742
2			*Luteolin (2-(3,4-Dihydroxyphenyl)- 5,7-dihydroxy-4-chromenone)*	-7.21	5.18 µM	Arg835, Phe840, Glu34, Arg29
3			*Rutin (Quercetin-3-rutinoside hydrate)*	-6.75	11.28 µM	Val744, Phe840, Phe838, Glu418, Lys745, Met746, Pro717, Ser421, Lys716
4			Gallic acid (3,4,5-trihydroxybenzoic acid)	-5.48	95.88 µM	Lys709, Glu742, Gln82, Ala80, Leu77
5			*Acetohydroxamic acid (N-hydroxyacetamide)	-4.51	491.58 µM	Leu833, Asn836, Val831, Ser834, Asp295

***:** Reference compound

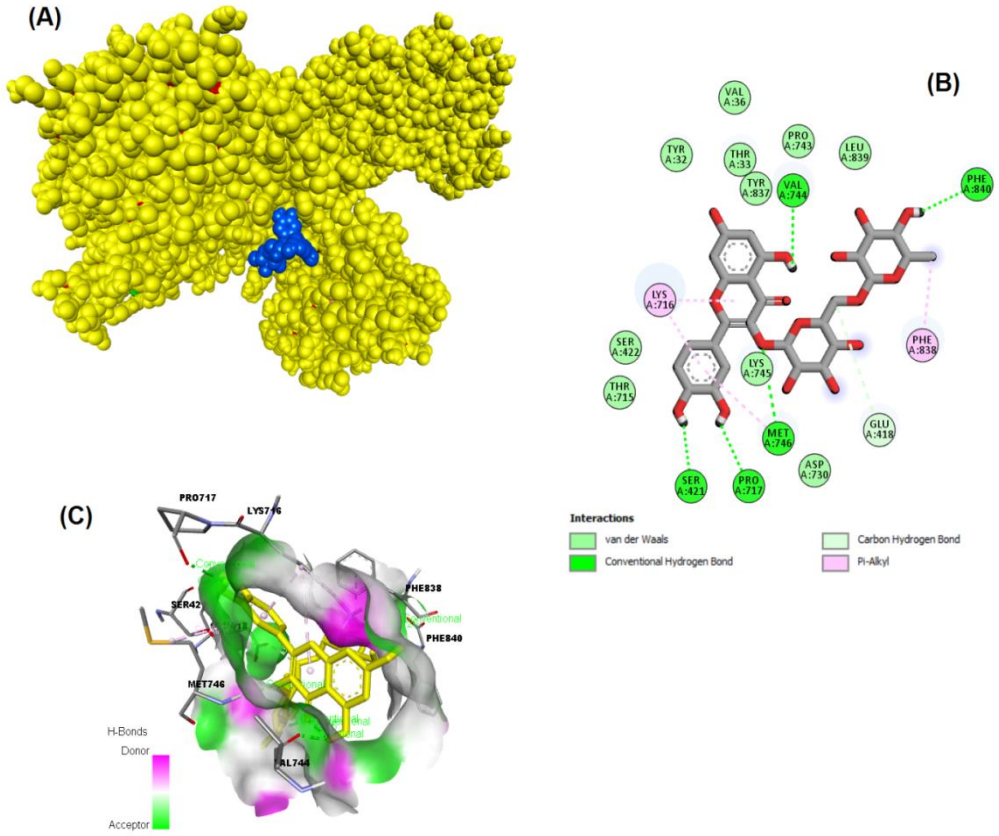

Figure 1- Binding pose profile of Rutin (Quercetin-3-rutinoside hydrate) in the target protein (A), blue shaped molecule represents the ligand and yellow shaped molecule indicates the receptor. The two-dimension (2D) (B) and three-dimension (3D) (C) interactions analysis of Urease from Jack bean with compound Rutin. (Representation of docked structures with BIOVIA Discovery Studio Visualizer software)

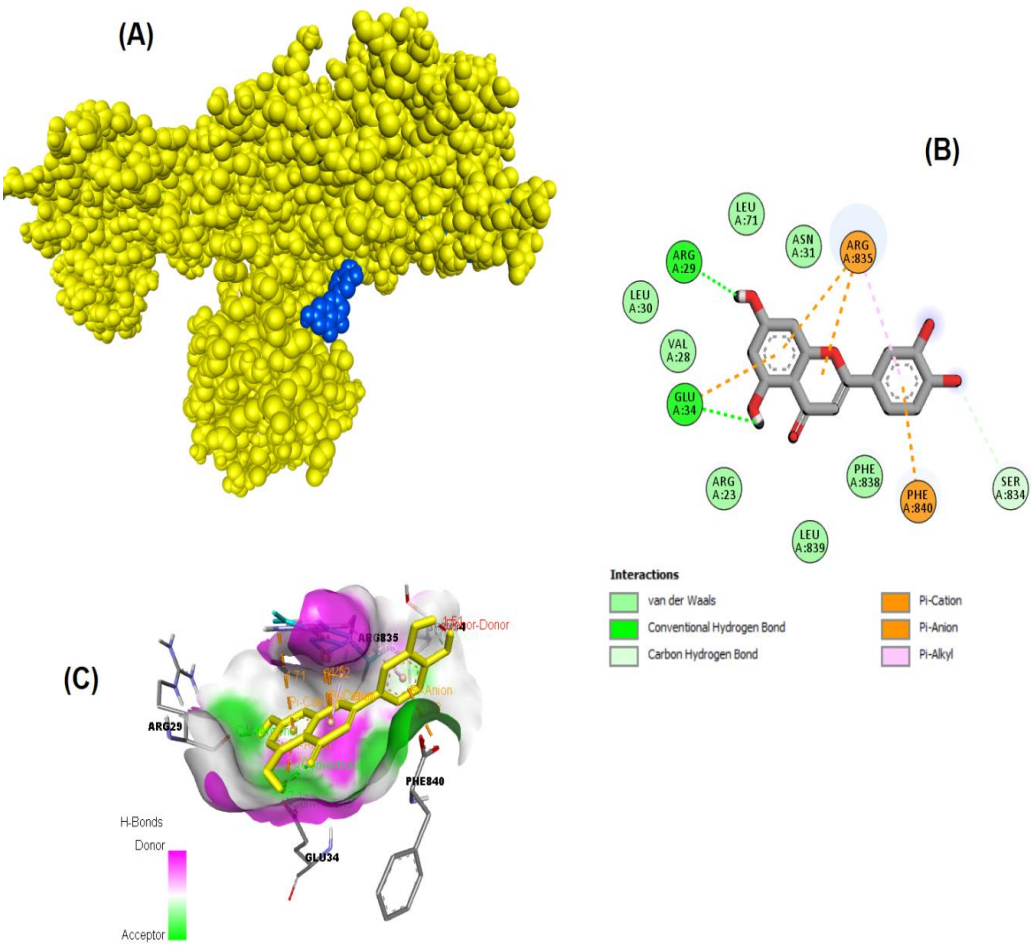

Figure 2- Binding pose profile of Luteolin (2-(3,4-Dihydroxyphenyl)- 5,7-dihydroxy-4-chromenone) in the target protein (A), blue shaped molecule represents the ligand and yellow shaped molecule indicates the receptor. The two-dimension (2D) (B) and three-dimension (3D) (C) interactions analysis of Urease from Jack bean with compound Luteolin. (Representation of docked structures with BIOVIA Discovery Studio Visualizer software)

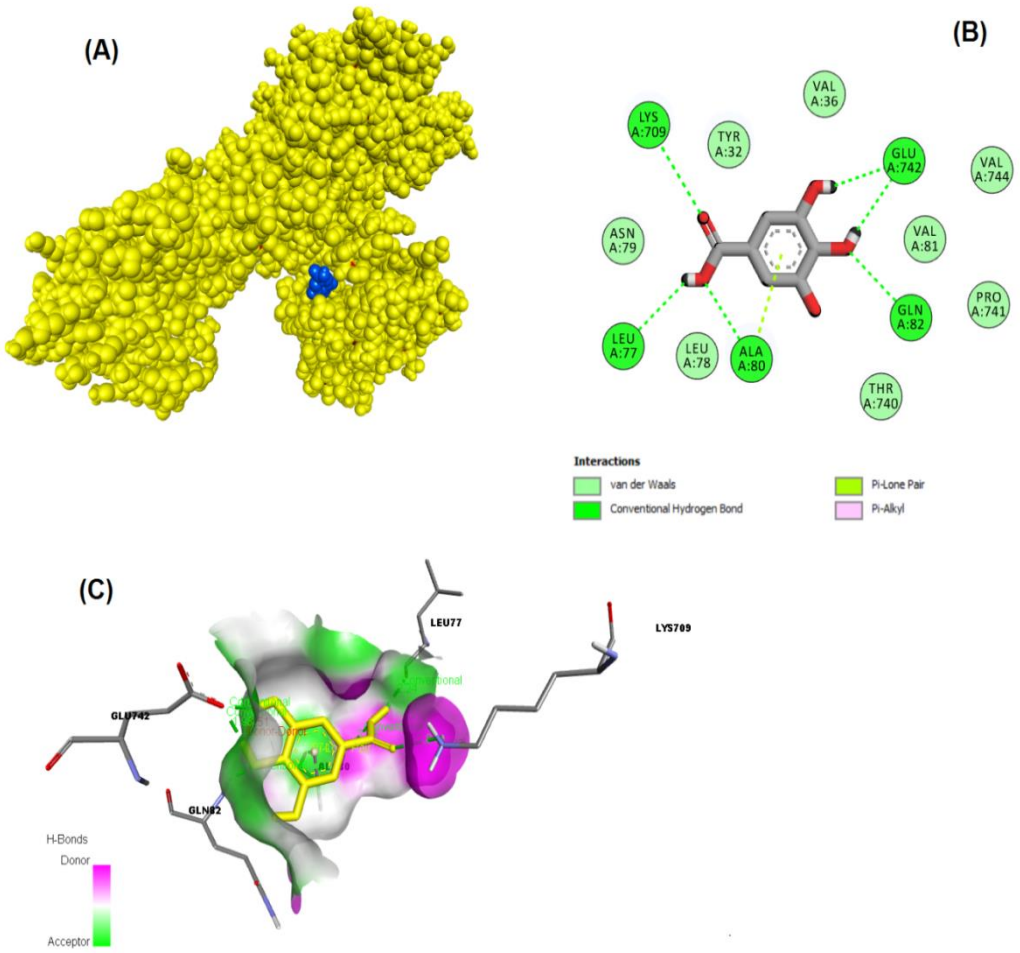

Figure 3- Binding pose profile of Gallic Acid (3,4,5-trihydroxybenzoic acid) in the target protein (A), blue shaped molecule represents the ligand and yellow shaped molecule indicates the receptor. The two-dimension (2D) (B) and three-dimension (3D) (C) interactions analysis of Urease from Jack bean with compound Gallic Acid. (Representation of docked structures with BIOVIA Discovery Studio Visualizer software)

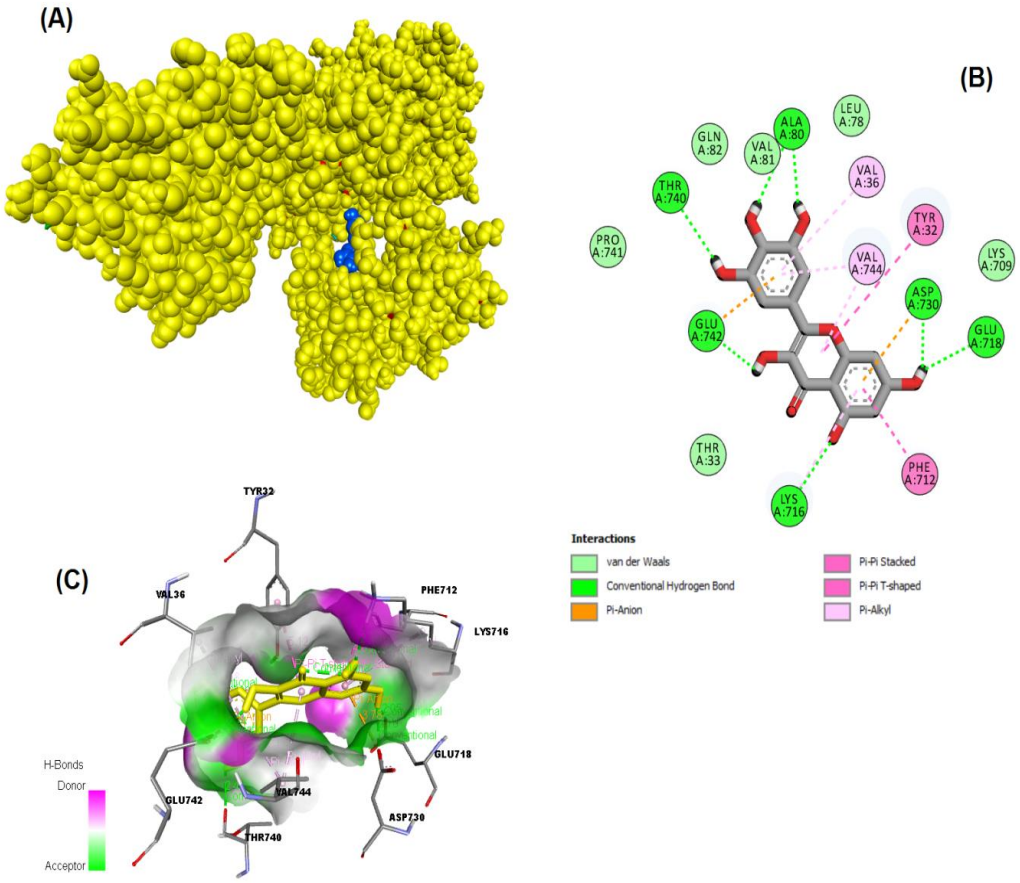

Figure 4- Binding pose profile of Myricetin (3,3′,4′,5,5′,7-Hexahydroxyflavone) in the target protein (A), blue shaped molecule represents the ligand and yellow shaped molecule indicates the receptor. The two-dimension (2D) (B) and three-dimension (3D) (C) interactions analysis of Urease from Jack bean with compound Myricetin. (Representation of docked structures with BIOVIA Discovery Studio Visualizer software)

4. Conclusions

Thousands of tons of chestnut blossoms fall into the soil every year, and unfortunately, they rot spontaneously. However, it is well known that chestnut blossoms are a natural product with high biological activity that can be used as a natural food additive with numerous useful bio-properties. The current findings corroborate this assertion, especially the analysis of enzyme inhibition and antioxidant effects of the aqueous extract, which showed a nearly two-fold effect based on quantitative data. Furthermore, attempts were made to explain both antioxidants and urease inhibition by the phenolics found in each extract. Especially, rutin, which was the dominant phenolic in both chestnut blossom extract types, was a significant reason for the level of antioxidants; moreover, myricetin and luteolin were evaluated as excellent urease inhibitors due to having an effective response on molecular docking analysis at micromolar (µM) concentrations of 4.44 and 5.18, respectively. Even though this study explains some of the bioactive properties of chestnut blossoms, it is obvious that further research is needed to learn more about them.

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