*Article*

# Evaluation of the Anti-Inflammatory Activity of Microwave Extracts of *Thymus algeriensis*: *In Vitro*, *In Vivo*, and *In Silico* Studies

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**Abstract:** The objective of this work is to study the anti-inflammatory effect *in vitro* and *in vivo* of microwave (MW) extracts of *Thymus algeriensis*. The *in vitro* study was performed by the human red blood cell protection test, while the *in vivo* study used the carrageenan-induced rat paw edema model. The experimental results were confirmed by a molecular docking calculation. The results indicated that all the microwave extracts have a moderate anti-inflammatory effect, depending on their richness in phenolic compounds. Among the extracts studied, the one obtained at 100 °C for 15 min exhibited the most pronounced anti-inflammatory effect, with an inhibition of 78.52%, which is attributed to its high flavonoid content. In particular, the flavonoids naringin and catechin showed the best affinity for the target protein, with values of −10.3 kcal/mol and −9.2 kcal/mol, respectively, as well as low inhibition constants of 0.028 μM and 0.18 μM. These results indicate that these flavonoids generate interactions that enhance the stability of the target ligand–protein complex, thus contributing to the observed anti-inflammatory effect.

**Keywords:** *Thymus algeriensis*; anti-inflammatory activity; phenolic compounds; microwave-assisted extraction; molecular docking

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## 1. Introduction

Medicinal plants contain various types of antioxidants, mostly carotenoids, phenolic compounds, benzoic acid derivatives, flavonoids, proanthocyanidins, stilbenes, coumarins, lignans, and lignins, which exhibit high antioxidant activity [1,2]. Recommendations based on epidemiological studies assessed that fruits, vegetables, and less processed staple foods can ensure the best protection against the development of diseases caused by oxidative stress, such as cancer, coronary heart disease, obesity, type 2 diabetes, hypertension, and cataracts [1]. The explanation for this relies on beneficial health effects, due to their

antioxidant agents. Indeed, among 50 analyzed food products with high antioxidant content, 13 were spices, 8 were fruits and vegetables, 5 were berries, 5 were chocolate-based food, 5 were breakfast cereals, and 4 were nuts or seeds [3].

Inflammation is a complex biological response of vascular tissue to harmful stimuli and pathogens, characterized by redness, warmth, swelling, and pain [4]. Prolonged inflammation leads to rheumatoid arthritis, atherosclerosis, hay fever, and ischemic heart diseases, and it is also a common manifestation of infectious diseases [5,6].

In the plant kingdom, the genus Thymus belongs to the Lamiaceae family which comprises about 215 to 400 species according to previous studies [7]. Generally, this genus consists of perennial plants and subshrubs native mainly in Europe, Western Asia, and Mediterranean countries [8]. Historically, the volatile constituent-rich aerial parts of the Thymus species have been commonly used as herbal teas, condiments, and spices. In addition, they have shown many ethnomedicinal properties such as tonic, carminative, digestive, antispasmodic, antimicrobial, antioxidant, and anti-inflammatory ones [9–15]. *T. algeriensis* is the most pervasive North African species, endemic to Morocco, Tunisia, Algeria, and Libya [16–20]. Its essential oil was proposed as being antimicrobial, acridicidal, antioxidant, anti-inflammatory, and neuroprotective after extensive knowledge of its safety, chemical composition, and stability [21–31]. Recent investigations were focused on the polyphenolic and flavonoid contents isolated by classical extraction from the aerial parts of this species as alternative components to exert a valuable pharmacological activity [32–37].

To continue our investigations into the biological activities of Thymus [38], this study was designed to investigate the anti-inflammatory activity of microwave-assisted extracts of Algerian *T. algeriensis* aerial parts. Microwave-assisted (MW) extraction was applied to recover a high-benefit composition from this endemic species.

Furthermore, to support the in vivo results and gain deeper insights into the molecular mechanisms behind the anti-inflammatory activity of the extracts, we have employed in silico studies, specifically molecular docking simulations. Molecular docking allows for the prediction of the binding affinity and interaction of bioactive compounds with specific proteins or receptors involved in inflammatory pathways. This computational technique can provide valuable information regarding the potential mechanisms of action of the compounds extracted from *T. algeriensis* and help identify key targets involved in their anti-inflammatory effects. Recent studies have shown that in silico approaches, including molecular docking, are highly effective in understanding the bioactivity of plant-derived compounds, aiding the drug discovery process by identifying promising candidates for further experimental validation [39,40]. By simulating the binding interactions of compounds with key receptors or enzymes involved in inflammation, molecular docking can provide insights into the mechanisms underlying their therapeutic effects [41,42]. Moreover, this method has been widely used in drug discovery to streamline the identification of promising candidates for further experimental validation.

## 2. Materials and Methods

### 2.1. Plant Material and Preparation of Extracts

*Thymus algeriensis* was collected from the Biskra region (coordinates: 34°51′01″ N/5°43′40″ E), Algeria. The plant was identified by Prof. M. Kaabeche (Biology Department, University of Setif 1, Algeria). A relative specimen voucher has been registered in the Herbarium of Frères Mentouri University, Algeria. The microwave-assisted (MW) extraction of the aerial part of *T. algeriensis* was performed using a single-mode microwave reactor: the automatic Biotage Initiator™ 2.0. (Biotage AB, Uppsala, Sweden) characterized by a power range of 0–300 W and 2.45 GHz microwaves. The MW extraction was performed

at discrete temperatures (T = 40, 60, 80, 100, or 120 °C) for 5, 10, or 15 min in water as the solvent as previously reported.

*2.2. Profile of Bioactive Compounds*

We measured the total amount of phenolic acid and flavonoids in *T. algeriensis* extracts that had been microwave-irradiated. We also looked for chlorophylls and carotenoids. We wrote more about the exact steps we took in our previous paper [38]. High-quality standard chemicals for the chromatographic analyses (gallic acid, catechin, chlorogenic acid, epicatechin, 4-hydroxybenzoic acid, *t*-ferulic acid, naringin, vanillic acid, *p*-coumaric acid, rutin, syringic acid, benzoic acid, *o*-coumaric acid, 3-hydroxybenzoic acid, sinapinic acid, 2,3-dimethoxybenzoic acid, quercetin, isovanillin) were purchased from Merck (Milan, Italy). The HPLC-PDA method was used for the quantitative characterization of the compounds in these extracts (Supplementary Table S1).

*2.3. In Vitro Anti-Inflammatory Activity*

The anti-inflammatory activity of MW extracts was evaluated using the human red blood cell (RBC) membrane stabilization method [43]. The blood was collected from a healthy human volunteer who had not taken any NSAIDs for 2 weeks prior to the experiment. The collected blood was then mixed with an equal volume of Alsever solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid, and 0.42% NaCl) and centrifuged at 1730 g. The packed cells were washed with isosaline, and a 10% suspension was made. Different concentrations of extracts were prepared (250, 500, and 1000 μg/mL) using distilled water. To each concentration, 1 mL of phosphate buffer (0.15 M, pH 7.4), 2 mL of hyposaline (0.36%), and 0.5 mL of HRBC (human red blood cell) suspension (10%) were added. Each sample was incubated at 37 °C for a duration of 30 min, followed by centrifugation at 1730 g for 20 min. The supernatant solution was then analyzed spectrophotometrically at a wavelength of 560 nm to estimate the hemoglobin content.

$$\textbf{Percentage inhibition}(\%) = \left[\mathbf{100} - \frac{\mathbf{A_{test}}}{\mathbf{A_{control}}}\right] \times \mathbf{100} \quad (1)$$

where $A_{test}$ is the absorbance of the sample containing the test extract, and $A_{control}$ is the absorbance of the simple without the extract.

Diclofenac was used as the reference drug (Sigma-Aldrich, St. Louis, MO, USA) and tested at the same concentrations as the extracts (250, 500, and 1000 μg/mL) for comparison.

*2.4. Inflammatory Paw Edema Test in Rats*

In this part of the experiments, the anti-inflammatory activity of the microwave-assisted extract was investigated on the carrageenan-induced inflammatory paw edema [44]. Female Wistar rats (80–125 g) were obtained from the Pasteur Institute (Algeria). Rats were housed in standard polypropylene cages, with four per cage. The extracts were dissolved and dispersed in physiological saline (0.9%) and administered orally to a pre-treated group of rats at a dosage of 50 mg/kg. The control group received physiological saline (0.9%) at the same volume as the vehicle used for administering the extracts. One hour after administration, 0.1 mL of 1% carrageenan solution was injected into the footpad of the hind paws of each rat in all groups. Prior to the carrageenan (Sigma-Aldrich) injection, the rat paw volume was measured. The increase in the carrageenan-induced inflammatory paw volume was measured at 1, 2, and 3 h over the injection. The anti-inflammatory activity of

the *T. algeriensis* extracts was compared with that of 50 mg/kg diclofenac. The percentages of inhibition were calculated according to the following formula:

$$\mathbf{Inhibition}(\%) = \frac{(\mathbf{V_T} - \mathbf{V_0})_{\mathbf{control}} - (\mathbf{V_T} - \mathbf{V_0})_{\mathbf{treated\ group}}}{(\mathbf{V_T} - \mathbf{V_0})_{\mathbf{control}}} \times \mathbf{100} \quad (2)$$

where V is the diameter of injected paw, $V_0$ is the average inflammation (hind paw edema) of the control group at a given time 0, and $V_T$ is the average of diameters of the hind paw edema of the drug-treated rat at the same time.

This test was approved by the ethics committee (the committee of the Algerian Association of Sciences in Animal Experimentation (N°. 8808/1988), associated with veterinary medical activities and animal health protection N° JORA: 004/1988). All experimental procedures were conducted in accordance with established ethical principles to ensure respect for the participants' rights and well-being.

### 2.5. Molecular Docking Study

The study of the molecular docking of the organic molecules that had been extracted from the plant was performed to complement and confirm the results in the experimental section for the in vitro and in vivo activities using the AutoDock Vina program. The protein used to simulate this activity, named COX2, was obtained from the RCSB database with the code [3LN0] [45]. The active site of the protein used was delimited by a box of dimensions as follows: a volume of $40 \times 40 \times 40$ Å$^3$ and centers x, y, z of 41, 35, and 47, respectively.

To ensure an effective simulation, the ligands (19 molecules: gallic acid, catechin, chlorogenic acid, epicatechin, 4-hydroxy-benzoic acid, *t*-ferulic acid, naringin, vanillic acid, *p*-coumaric acid, rutin, syringic acid, benzoic acid, *o*-coumaric acid, 3-hydroxy-benzoic acid, sinapinic acid, 2,3-dimethoxy-benzoic acid, quercetin, isovanillin, and dichlofenac) were initially prepared by optimizing them using Chem3D 16.0 to achieve a stable geometry with minimum energy. Next, the receptors (proteins) for the two activities were prepared using Discovery Studio. This involved removing water molecules and heteroatoms and adding polar hydrogen atoms. Afterwards, molecular docking simulations were conducted using the AutoDock Vina program [46]

Also, the molecular interactions between the protein targets and compounds were viewed using BIOVIA Discovery Studio. Amino acids engaging with the ligand, hydrogen bonds (H-bonds), hydrophobic interactions, and individual atoms involved were examined in each ligand cluster (Supplementary Table S2).

### 2.6. Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 27. Comparisons were made using one-way analysis of variance (ANOVA), followed by Tukey's test. The results of both the in vitro and in vivo experiments are presented as mean $\pm$ standard deviation (SD) with (n = 3), when the $p$-value was less than 0.05.

## 3. Results and Discussion

### 3.1. In Vitro Anti-Inflammatory Activity in Human RBCs (HBRCs)

The microwave-assisted extracts of the *T. algeriensis* were then studied for in vitro anti-inflammatory activity by the HRBC membrane stabilization method. The results are reported in Table 1. The in vitro anti-inflammatory activity of the extracts was concentration-dependent, and again, the higher protection of HRBCs in the hypotonic solution was attributed to extracts obtained at a higher temperature (120 °C) or time of extraction (15 min). All the results were compared with standard diclofenac, which showed 96.67 $\pm$ 0.15% protection at the highest concentration.

**Table 1.** In vitro anti-inflammatory activity of the MW extract of *Thymus algeriensis* using the HRBC method.

| | Percentage Inhibition (%) | | |
|---|---|---|---|
| | 250 µg/mL | 500 µg/mL | 1000 µg/mL |
| **MW 40 °C** | 77.18 ± 0.17 *** | 84.92 ± 0.30 *** | 89.28 ± 0.20 *** |
| **MW 60 °C** | 78.59 ± 0.18 *** | 87.43 ± 0.22 *** | 90.67 ± 0.10 *** |
| **MW 80 °C** | 80.53 ± 0.20 *** | 88.12 ± 0.13 *** | 91.97 ± 0.15 *** |
| **MW 100 °C** | 77.06 ± 3.04 *** | 88.99 ± 0.31 *** | 94.94 ± 0.25 *** |
| **MW 120 °C** | 86.25 ± 0.12 ns | 91.13 ± 0.25 ns | 96.88 ± 0.15 ns |
| **MW 100 °C, 5 min** | 76.92 ± 0.20 *** | 85.09 ± 0.22 *** | 88.64 ± 0.18 *** |
| **MW 100 °C, 15 min** | 88.96± 0.17 ns | 90.95 ± 0.15 ns | 96.85 ± 0.17 ns |
| **Diclofenac** | 89.91 ± 0.17 | 91.91 ± 0.2 | 96.67 ± 0.15 |

Results are presented as mean ± SD (n = 3): ns: no significant difference; *** $p < 0.001$ compared with diclofenac.

The results show that the in vitro anti-inflammatory activity increases with the treatment temperature, reaching its maximum at 120 °C for all tested concentrations, with more pronounced effects at 0.5 mg/mL and 1 mg/mL. The treatment at 100 °C for 15 min also shows a significant improvement compared to a 5 min exposure. Although the effectiveness of the tested treatments is close to that of diclofenac, it remains slightly lower, but the differences are not always statistically significant. These results suggest that combining high temperature with extended treatment time optimizes the anti-inflammatory activity, offering potential comparable to that of reference anti-inflammatory drugs.

The microwave-assisted extracts exhibited a membrane stabilization effect by inhibiting the hypotonicity-induced lysis of the erythrocyte membrane. The erythrocyte membrane is analogous to the lysosomal membrane [47], and its stabilization implies that the extract may also stabilize the lysosomal membranes. Stabilization of the lysosomal membrane is important in limiting the inflammatory response by preventing the release of the lysosomal constituents of activated neutrophils such as bactericidal enzymes and proteases, which cause further tissue inflammation and damage upon extracellular release [48].

The exact mechanism for the membrane-stabilizing effect of these extracts of *T. algeriensis* and the chemical constituents responsible for this effect is hitherto not known. However, a number of studies have shown that flavonoids exhibit analgesic and anti-inflammatory effects as a result of their membrane stabilizing ability in various experimental models [49]. The extract may inhibit the processes, which may stimulate or enhance the efflux of these intracellular components [50]. It has also been shown that the microwave-assisted extracts of *T. algeriensis* contain flavonoids such as rutin, catechin, and epicatechin [38]. Thus, these extracts showed significant in vitro anti-inflammatory activity as compared to that of the standard. On the basis of the above results, it can be concluded that these MW extracts of *T. algeriensis* have a potential anti-inflammatory activity.

### *3.2. Carrageenan-Induced Paw Oedema*

The paw oedema thickness was measured at 1, 2, and 3 h post-inflammation induction in animals pre-treated with the MW extract. The percentage of oedema inhibition by the MW extract was measured. The inflammation induced by carrageenan develops into two distinct phases. The initial phase, occurring within one hour of exposure, is identified by the release of histamine, serotonin, and bradykinin. The late phase, occurring after more than an hour, results from the release of prostaglandin-like substances [51,52]. Research has

demonstrated the involvement of free radicals in the initial phase of acute inflammation induced by carrageenan [53].

Table 2 shows the effect of microwave-assisted extracts of *T. algeriensis* on the carrageenin-induced rat paw edema test. After carrageenan induction, there was a significant ($p < 0.001$) increase in edema formation in normal rats. Carrageenan-induced hind paw edema is the standard experimental model of acute inflammation. Moreover, the experimental model exhibits a high degree of reproducibility [54].

**Table 2.** Effect of microwave-assisted *T. algeriensis* extracts on carrageenan-induced paw edema in rats.

| | Mean Increase in Paw Thickness (mm) ± SD | | |
|---|---|---|---|
| **Group** | 1 h | 2 h | 3 h |
| **Control** | 1.92 ± 0.04 | 1.74 ± 0.09 | 1.49 ± 0.07 |
| **Diclofenac** | 1.48 ± 0.04 *** | 0.72 ± 0.04 *** | 0.24 ± 0.04 *** |
| **MW 40 °C** | 1.45 ± 0.04 *** | 1.05 ± 0.04 *** | 0.73 ± 0.01 *** |
| **MW 60 °C** | 1.28 ± 0.02 *** | 1 ± 0.02 *** | 0.49 ± 0.04 *** |
| **MW 80 °C** | 1.41 ± 0.04 *** | 0.79 ± 0.04 *** | 0.49 ± 0.05 *** |
| **MW 100 °C** | 1.4 ± 0.03 *** | 0.96 ± 0.05 *** | 0.53 ± 0.02 *** |
| **MW 120 °C** | 1.36 ± 0.02 *** | 0.99 ± 0.01 *** | 0.46 ± 0.04 *** |
| **MW 100 °C 5 min** | 1.44 ± 0.01 *** | 1.17 ± 0.02 *** | 0.77 ± 0.02 *** |
| **MW 100 °C 15 min** | 1.37 ± 0.04 *** | 0.76 ± 0.02 *** | 0.32 ± 0.02 *** |

Results are presented as mean ± SD (n = 3); *** $p < 0.001$ compared with control.

Figure 1 shows the inhibition percentages of the microwave-treated extracts of *Thymus algeriensis* and diclofenac. One hour after the carrageenan injection, all the microwave extracts showed a moderate anti-inflammatory effect comparable to that of the reference drug (diclofenac), with inhibition percentages ranging from 24% to 29% for extracts and 23% for diclofenac. These results suggest that microwave-treated extracts of *Thymus algeriensis* have similar anti-inflammatory activity to that of diclofenac, although moderate.

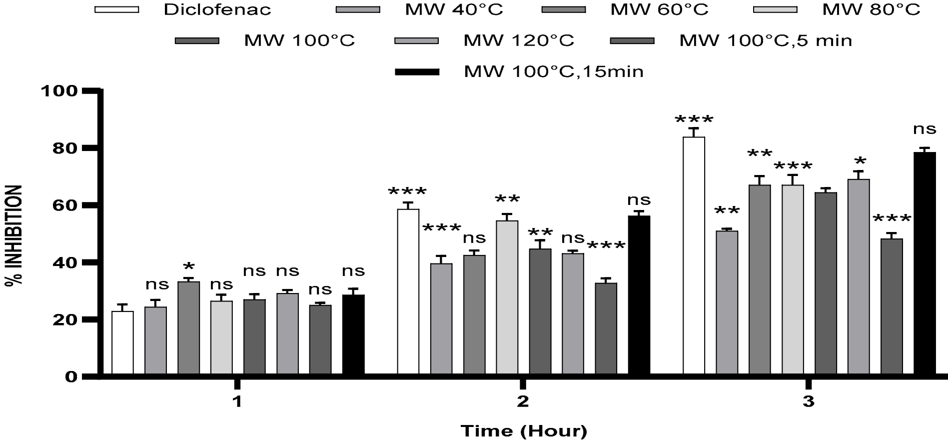



**Figure 1.** Percentage of inhibition inflammation of MW extracts in rats with carrageenan-induced hind paw edema (values presented as means ± SD (n = 3): ns: not significant difference; * $p < 0.05$ ** $p < 0.01$, and *** $p < 0.001$ considered significant when compared with diclofenac.

After three hours of experimentation, diclofenac showed a marked anti-inflammatory effect, reaching 83.74%, which confirms the efficacy of non-steroidal anti-inflammatories, which act by the inhibition of the COX-1 and COX-2 enzymes [55]. On the other hand,

the microwave-treated extracts showed significant inhibition, although lower than that of diclofenac, with percentages ranging between 51% and 70%. However, the extract treated at 100 °C for 15 min showed an anti-inflammatory effect similar to that of diclofenac, with a percentage of 78.52%, suggesting that this treatment could be an interesting alternative with efficacy comparable to that of the reference drug.

Some studies have indicated that the flavonoids contained in plant extracts have anti-inflammatory properties and that they are able to modulate the functioning of the immune system by inhibiting the activity of enzymes which are responsible for inflammation; they may also modulate monocyte adhesion during atherosclerotic inflammation by inhibiting the expression of inflammatory mediators [50]. Our previous phytochemical study of *T. algeriensis* aerial parts revealed the presence of some of these compounds (epicatechin, catechin, rutin, quercetin and naringin), which could be responsible for this anti-inflammatory activity of the extracts. It has been also demonstrated that various biologically active flavonoids (such as rutin and quercetin) produced significant antinociceptive and/or anti-inflammatory activities [56,57].

The extract treated at 100 °C for 15 min (MW 100 °C 15 min) had the most significant anti-inflammatory effects compared to the other extracts tested. The higher efficacy is due to the extract's availability of flavonoids, including quercetin, rutin, catechin, epicatechin, and naringin [38], which are compounds known for their ability to reduce inflammation. These flavonoids, by their action on pro-inflammatory enzymes and their ability to modulate inflammatory mediators, play a key role in reducing inflammatory processes.

The microwave extraction process at 100 °C for 15 min seems to have optimized the extraction of these compounds, which explains the higher efficiency of this extract. Indeed, this method allows a faster and more complete release of flavonoids while preserving their structure and bioactive activity. These results reinforce the idea that microwave extraction can be an effective method to obtain plant extracts rich in anti-inflammatory compounds.

Several researchers have also demonstrated the anti-inflammatory effect of *Thymus algeriensis* extracts, showing that they can reduce inflammation markers and attenuate inflammatory responses in various experimental models.

According to Mensour et al. (2020) [22] and Mahdi et al. (2022) [32], *Thymus algeriensis* has significant anti-inflammatory activities. Extracts from the leaves of *Thymus algeriensis* were evaluated for their ability to inhibit the enzymes cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), as well as lipoxygenase (5-LOX). The results indicated that the extract of *Thymus algeriensis* is more selective for COX-2 than for COX-1, with an index of selectivity similar to that of celecoxib, a selective inhibitor of COX-2. The extract also showed anti-inflammatory effects in living things, like rats with leg swelling caused by carrageenan and mice with white blood cells moving into the peritoneal cavity [22,32].

### *3.3. Molecular Docking*

The docking of molecules is a modern bioinformatics method that predicts the probable experimental orientation and the binding affinity needed to produce a stable complex structure between a ligand and a target [58]. The AutoDock Vina tool was used to conduct molecular docking tests to see if each of the identified compounds had an anti-inflammatory effect compared to the standard drug, dichlofenac.

The findings allowed the assessment of the binding energy between the protein (Figure 2) and different ligand positions (Table 3). A negative binding energy indicates a potential for binding between the ligand and the receptor. The inhibition constant (Ki) was calculated using the formula as follows: Ki = exp(ΔG/RT), where ΔG indicates the binding energy, R is the gas constant ($1.9872036 \times 10^{-3}$ kcal. $Mol^{-1}$), and T represents the

ambient temperature (298.15 K) [59]. A smaller inhibition constant signifies a more effective drug derived from the title molecule.

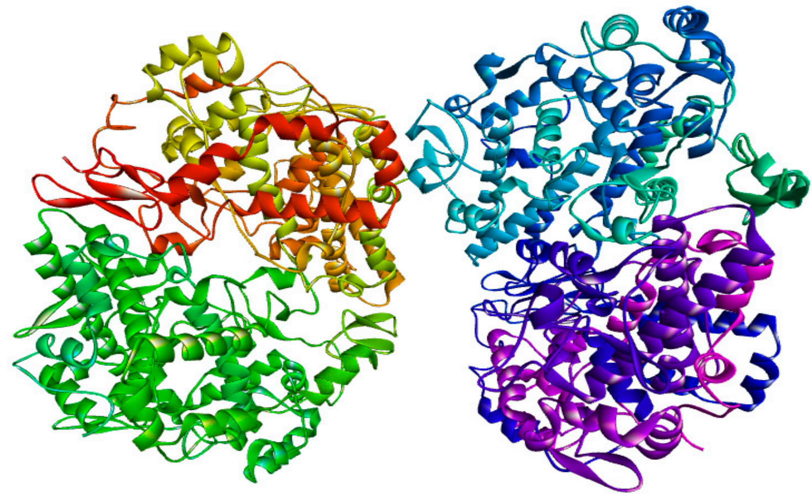


**Figure 2.** The 3D structure of the inflammatory protein COX-2 [PDB ID: 3LN0].

**Table 3.** Results for affinity and inhibition constant of ligands with the same protein.

| **Biomolecules** | **Anti-Inflammatory Activity** | |
|---|---|---|
| | **Protein: COX-2 (3LN0)** | |
| | **ΔG (Kcal/mol)** | **Ki (μmol)** |
| 2,3-dimethoxybenzoic acid | −6.0 | 39.95 |
| 3-hydroxybenzoic acid | −6.1 | 33.74 |
| 4-hydroxybenzoic acid | −5.7 | 66.28 |
| syringic acid | −5.9 | 47.29 |
| gallic acid | −6.4 | 20.33 |
| benzoic acid | −5.5 | 92.90 |
| catechin | −9.2 | 0.18 |
| chlorogenic acid | −8.5 | 0.58 |
| epicatechin | −8.9 | 0.29 |
| isovanillin | −5.9 | 47.29 |
| naringin | −10.3 | 0.028 |
| ***o***-coumaric acid | −6.3 | 24.07 |
| ***p***-coumaric acid | −6.1 | 33.74 |
| quercetin | −7.9 | 1.61 |
| rutin | −8.8 | 0.35 |
| sinapinic acid | −5.8 | 55.99 |
| t-ferulic acid | −6.1 | 33.74 |
| vanillic acid | −6.2 | 28.50 |
| dichlofenac | −7.1 | 6.23 |

It was found that naringin and catechin have the best affinity, with values of −10.3 kcal/ Mol and −9.2 kcal/ Mol, respectively, as well as a low inhibition constants of 0.028 μM and 0.18 μM, respectively. Additionally, we performed molecular docking

using a commercialized drug named diclofenac. According to Table 2, catechin, d acid, epicatechin, naringin, quercetin, and rutin have shown favorable results compared to diclofenac (Table 3).

The binding affinity of the fixed ligands to the target proteins in this investigation was affected by non-covalent interactions, including hydrogen bonds and hydrophobic interactions. Several investigations have demonstrated that binding affinity increases when ligands can participate in hydrophobic interactions with a significant amount of hydrophobic residues of amino acids within the binding region of a target protein [60,61]. Stojanovi and Zari [62] emphasized the significance of hydrophobic interactions in various systems characterized by powerful intermolecular forces [63]. This mechanism could be responsible for the significant binding affinity of the active chemicals associated with the proteins selected in this study. The significance of hydrogen bonds in stabilizing molecular interactions between ligands and proteins must not be ignored, because they play a crucial role in enzymatic catalysis and the formation of protein–substrate and protein–inhibitor complexes, contributing to the overall stability of multiple biological molecules [62,63].

For this purpose, the analysis of the molecular interactions of the active compounds, carried out on the basis of their binding energy, hydrogen bonds, and hydrophobic interactions with the surrounding amino acids in the selected protein targets, revealed a significant impact of the ligand–protein complex status. In particular, it was seen that flavonoids taken from *Thymus algeriensis* have incredibly low-free-binding-energy values compared to those of the other polyphenolic compounds being studied. Low free energy indicates a more stable interaction and the strong affinity of flavonoids for their protein targets.

Also, the inhibition constant values (Ki) for these flavonoids were lower than those for other polyphenolic compounds. This means that they had a very strong affinity for binding to the target proteins. Low Ki values are synonymous with a better ability of these flavonoids to effectively inhibit the activity of target enzymes, thus enhancing their role in the modulation of inflammatory pathways. This evidence suggests that flavonoids, including quercetin, rutin, catechin, epicatechin, and naringin, are able to bind stably to the active sites of proteins, which may be the main reason for their anti-inflammatory symptoms noted in this study.

In the *in vivo* test, it was found that the extract treated by the microwave at 100 °C for 15 min (MW 100 °C 15 min) revealed the most powerful anti-inflammatory effect. This result is directly related to the presence of a maximum level of flavonoids in this extract. Extracts with the highest concentrations of flavonoids showed the significant inhibition of inflammatory markers, which confirms that the richness in flavonoids plays a decisive role in the anti-inflammatory efficacy observed. These results are consistent with data from the study of molecular interaction between the different ligands and their target proteins, where flavonoids showed a strong affinity for the active sites of the target proteins, due to low Ki values and particularly low bonding energies.

Figures 3 and 4 show numerous hydrogen bonds between each ligand and the target protein, illustrating the stability and efficacy of these interactions. In addition, non-covalent interactions such as hydrophobic and electrostatic interactions were also observed, further enhancing the stability of ligand–protein complexes. These non-covalent interactions are crucial for the stability of complexes and increase their effectiveness by inhibiting the activity of proteins responsible for inflammation.

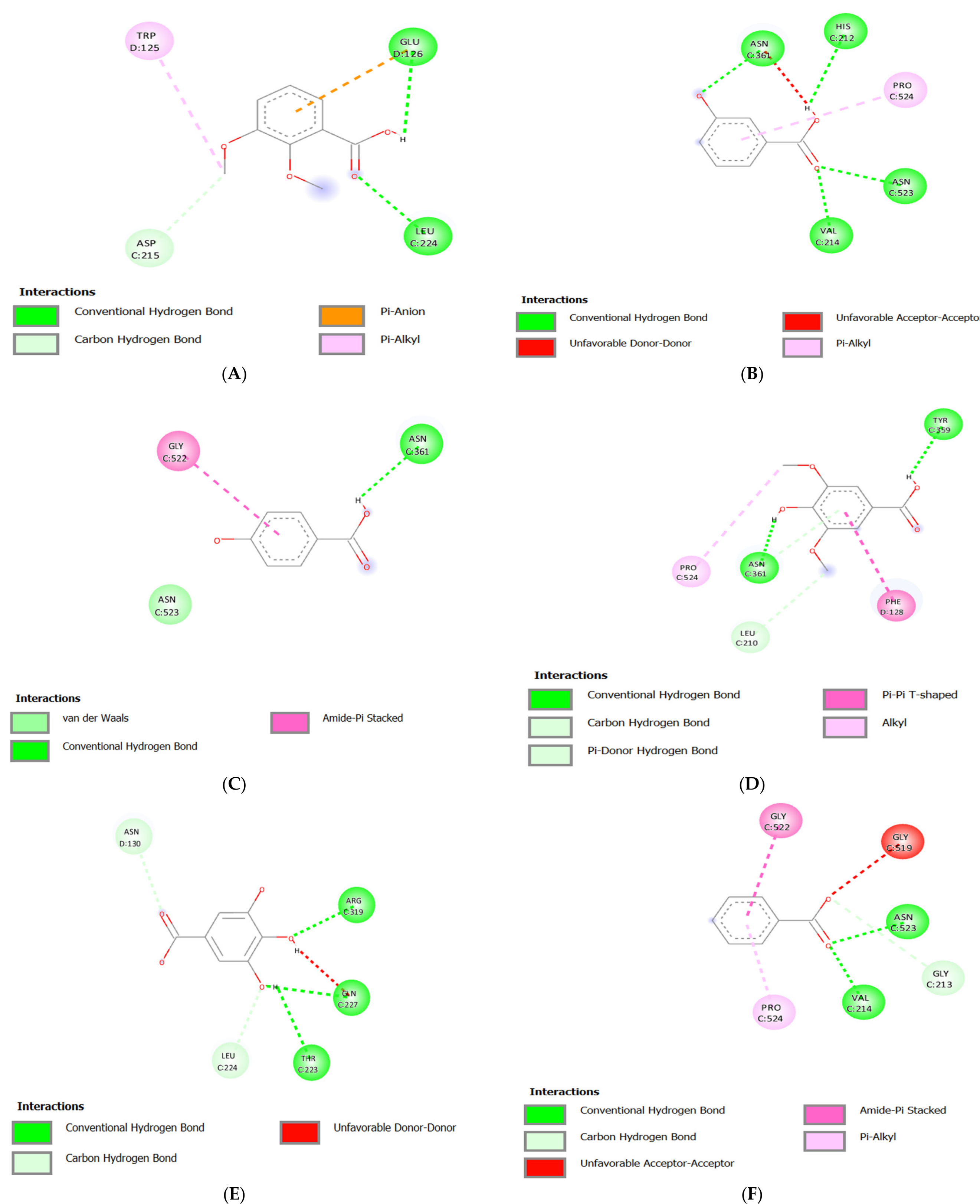



**Figure 3.** *Cont.*

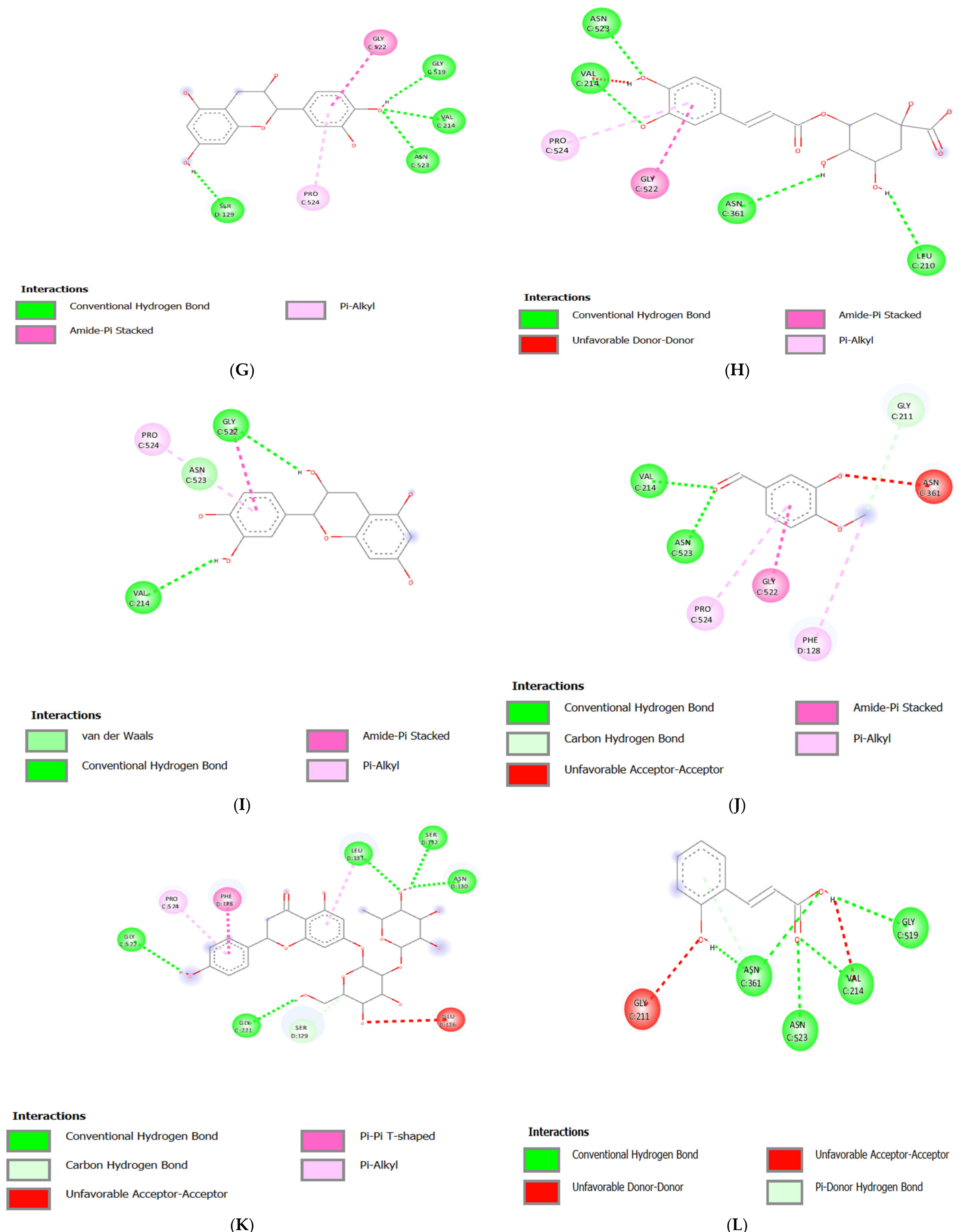



**Figure 3.** *Cont.*

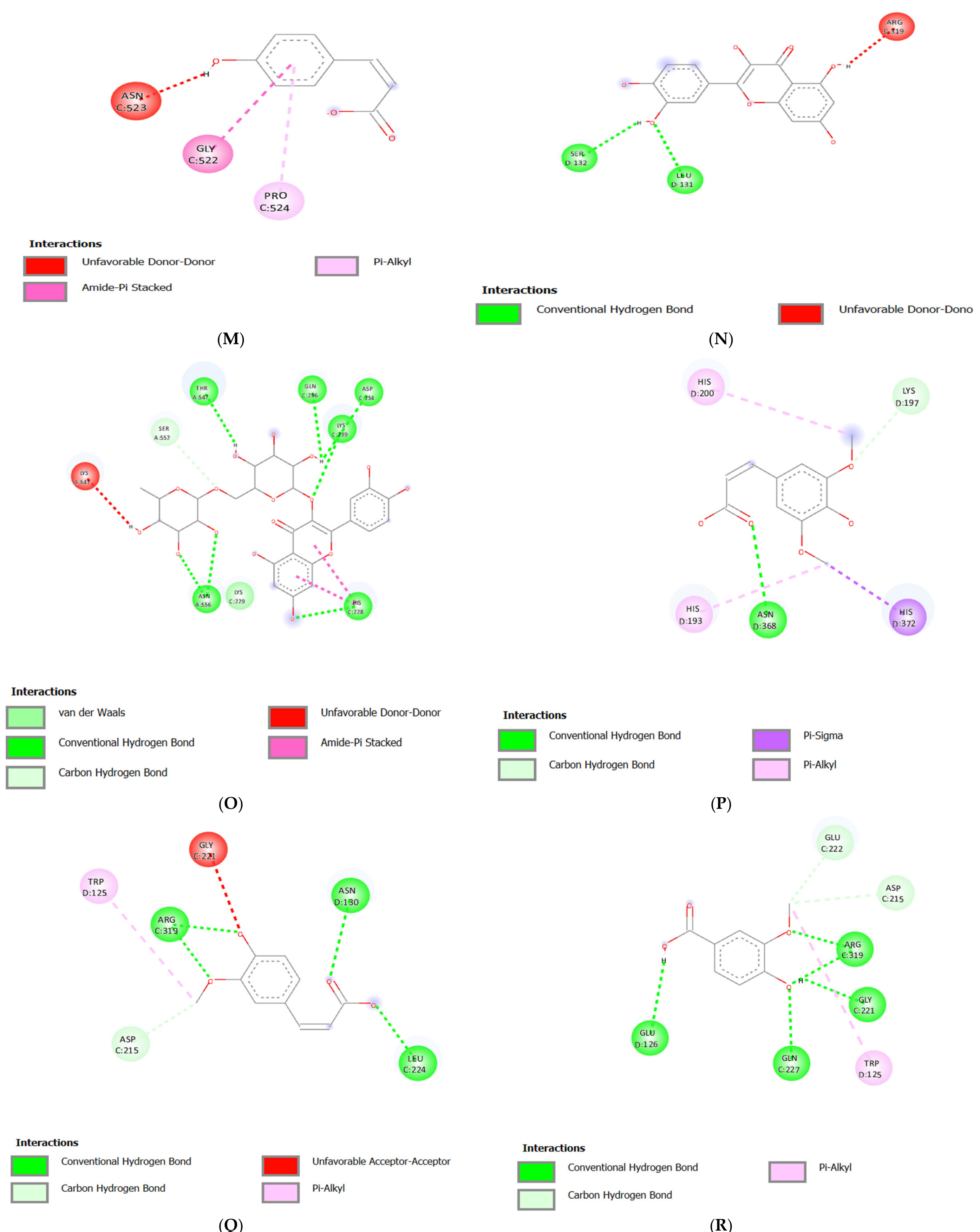
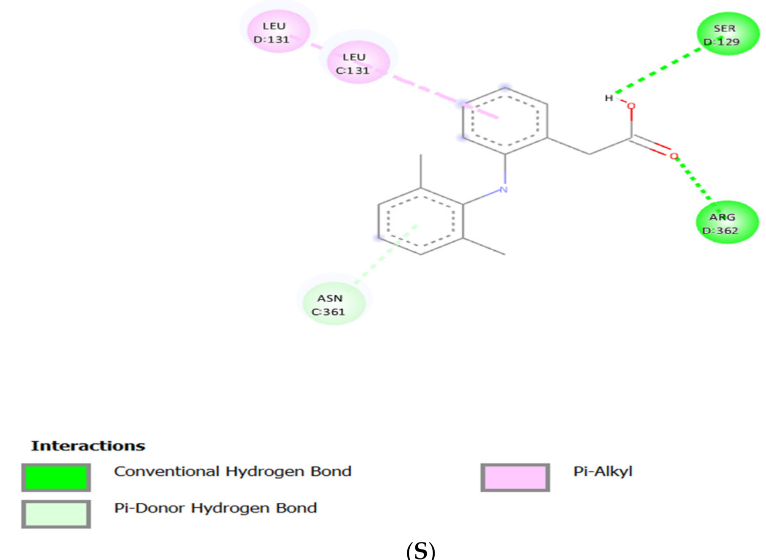



**Figure 3.** *Cont.*




(**S**)

**Figure 3.** Two-dimensional detailed binding sites of each ligand into the receptor [PDB ID: 3LN0]. (**A**): 2,3-dimethoxybenzoic acid; (**B**): 3-hydroxybenzoic acid; (**C**): 4-hydrobenzoic acid; (**D**): syringic acid; (**E**): gallic acid; (**F**): benzoic acid; (**G**): catechin; (**H**): chlorogenic acid; (**I**): epicatechin; (**J**): isovanillin; (**K**): naringin; (**L**): o-coumaric acid; (**M**): p-coumaric acid; (**N**): quercetin; (**O**): rutin; (**P**): sinapinic acid; (**Q**): t-ferulic acid; (**R**): vanillic acid; (**S**): diclofenac.

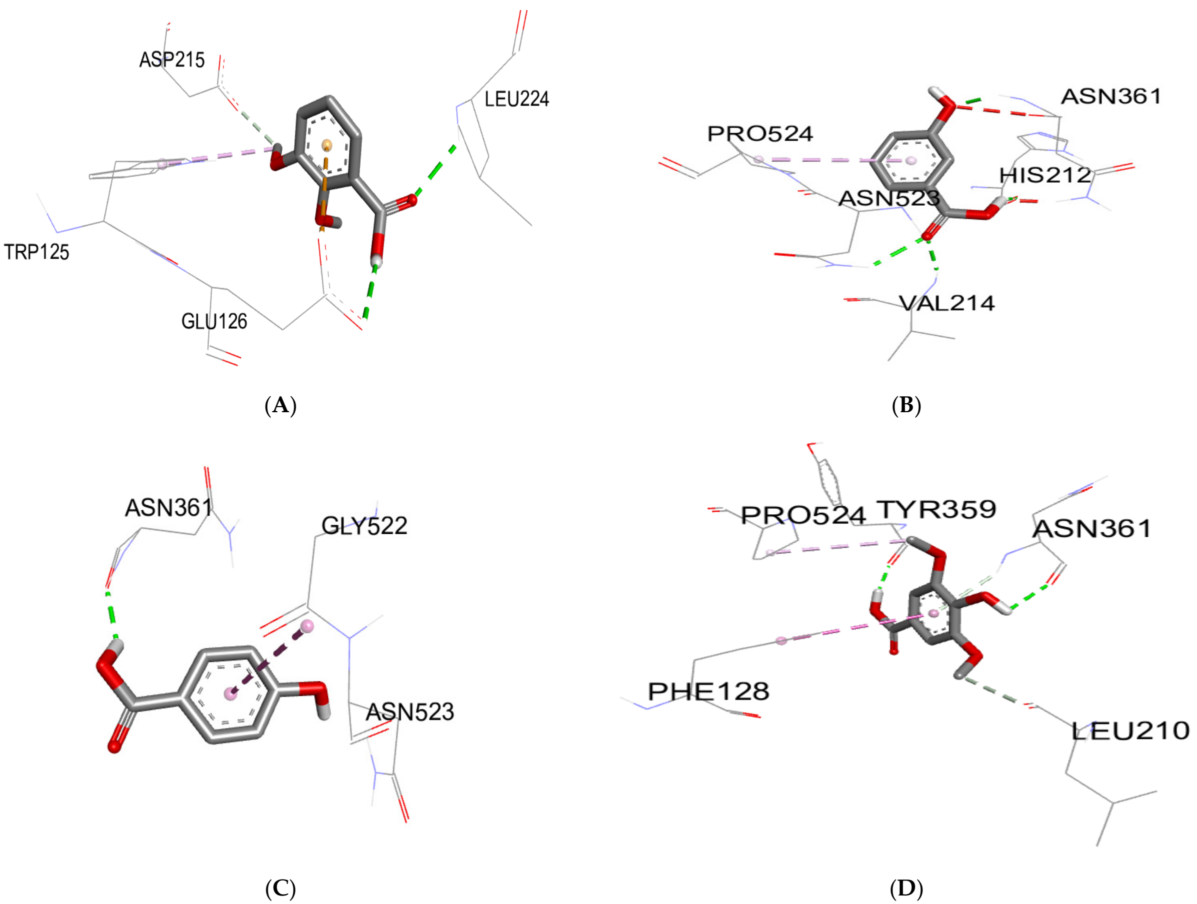



**Figure 4.** *Cont.*

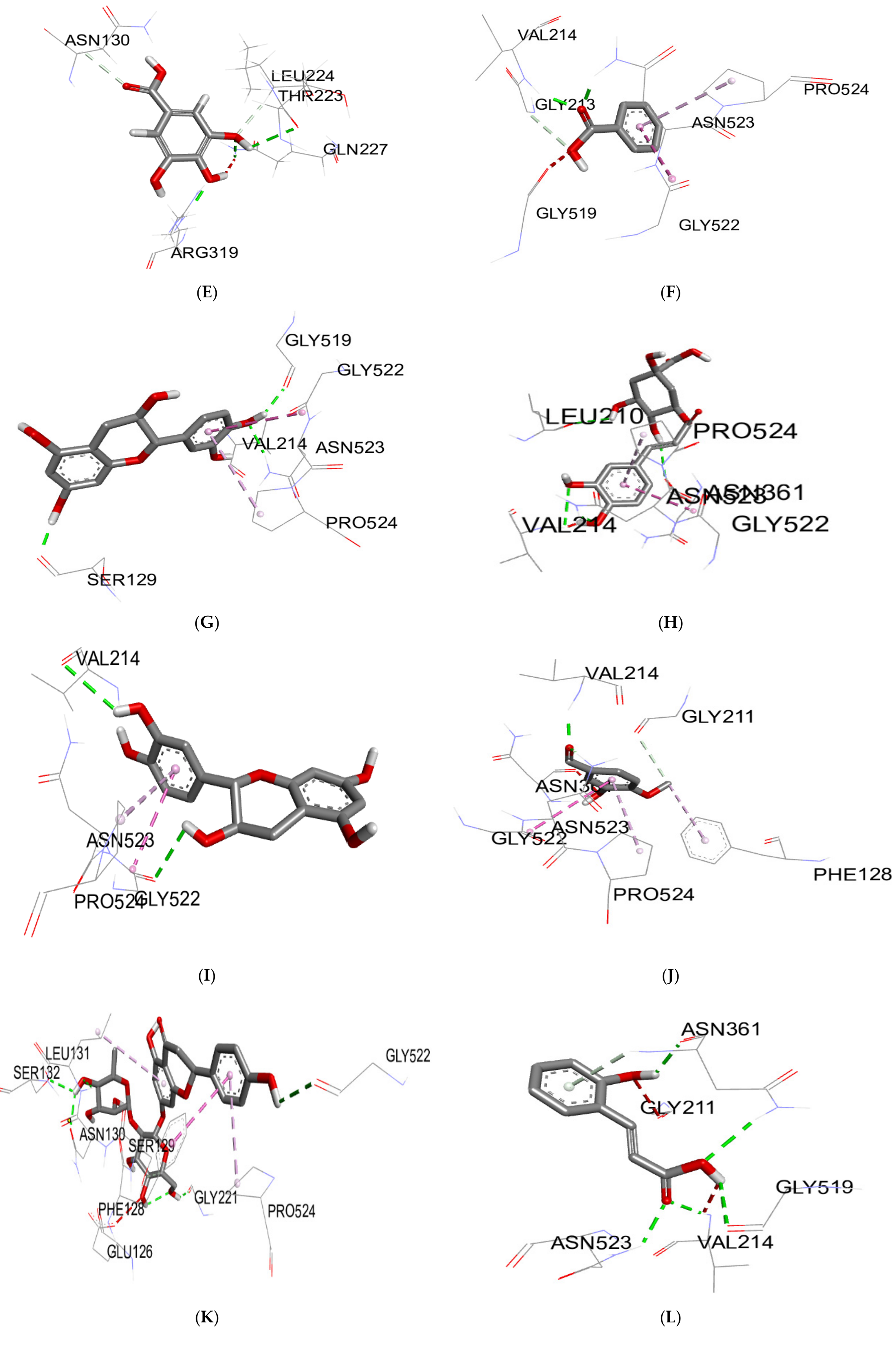
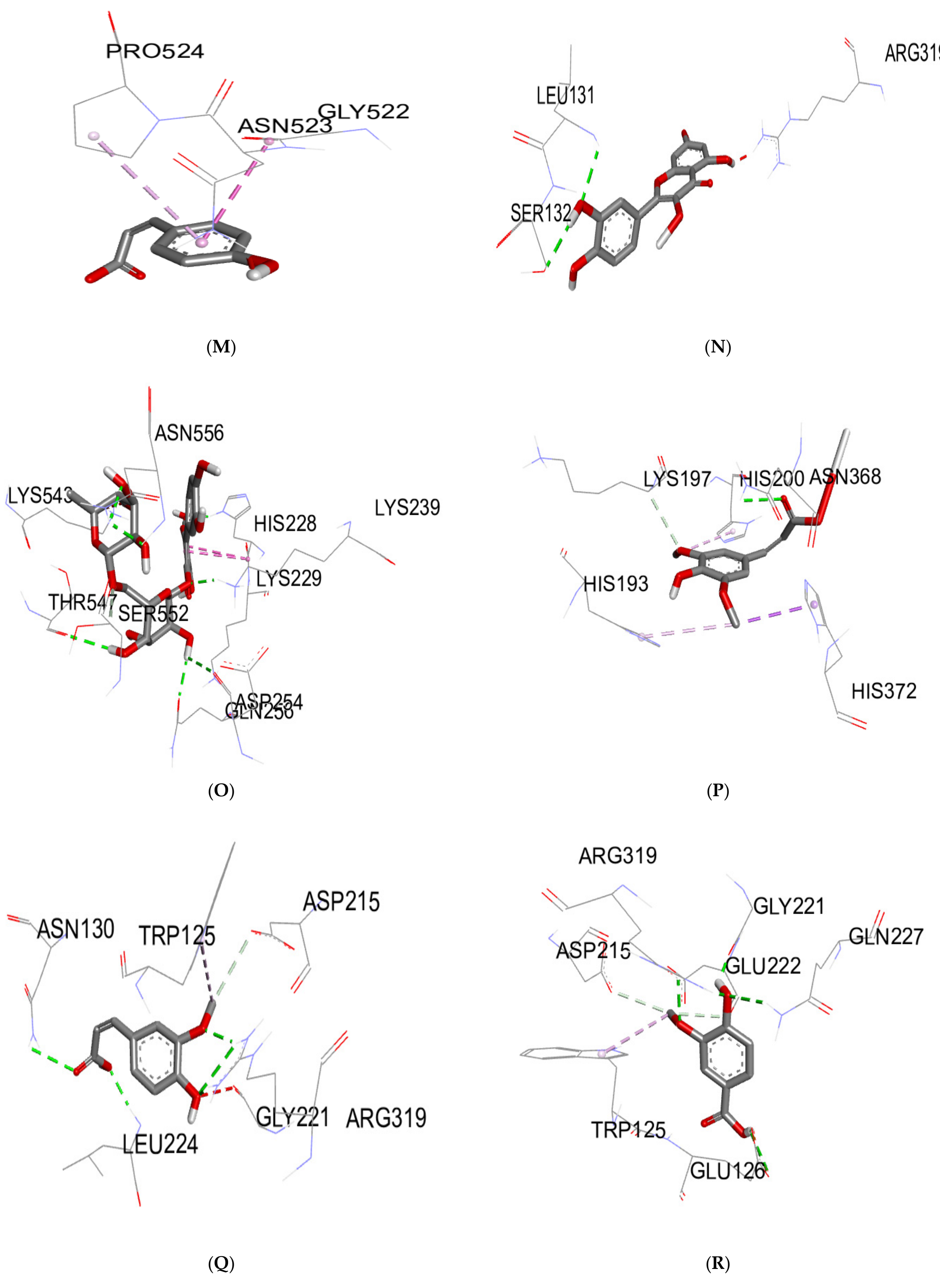



**Figure 4.** *Cont.*

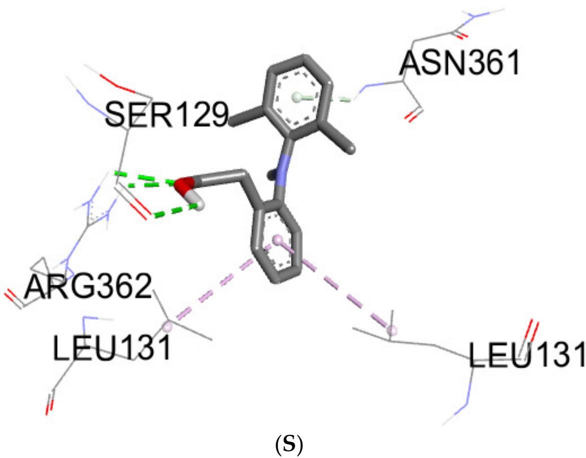



(M) (N)

(O) (P)

(Q) (R)

**Figure 4.** *Cont.*




(**S**)

**Figure 4.** The 3D detailed binding sites of each ligand into the receptor [PDB ID: 3LN0]. (**A**): 2,3-dimethoxybenzoic acid; (**B**): 3-hydroxybenzoic acid; (**C**): 4-hydrobenzoic acid; (**D**): syringic acid; (**E**): gallic acid; (**F**): benzoic acid; (**G**): catechin; (**H**): chlorogenic acid; (**I**): epicatechin; (**J**): isovanillin; (**K**): naringin; (**L**): o-coumaric acid; (**M**): p-coumaric acid; (**N**): quercetin; (**O**): rutin; (**P**): sinapinic acid; (**Q**): t-ferulic acid; (**R**): vanillic acid; (**S**): dichlofenac.

Based on the results obtained, it appears that each flavonoid (ligand) has the potential to be an effective molecule in the fight against inflammation, thanks to its ability to interact stably with the target proteins. The combined effect of the 18 ligands tested could still produce improved results, suggesting that the use of these flavonoids in combination therapies may have a stronger impact on inflammatory processes.

In addition, the *in silico* results, which were obtained by simulating ligand–protein interactions, fully corroborate the experimental results obtained during in vivo tests. This concordance between the molecular analyses and the experimental results reinforces the validity of the data and highlights the reducing action of the inflammation of the extracts of *Thymus algeriensis*, validating the efficacy of flavonoids as potential therapeutic agents.

## 4. Conclusions

After the characterization of the *Thymus algeriensis* extracts obtained by the microwave, we focused our study on the anti-inflammatory activity of these extracts (*in vitro* and *in vivo*), evaluating the inhibition of inflammation according to their composition in secondary metabolites. All the extracts showed moderate results, but the extract treated at 100 °C for 15 min had the most pronounced anti-inflammatory effect, effectively reducing inflammatory edema in rats due to its high content of flavonoids, compared to other extracts. These results were corroborated by an *in silico* analysis, which revealed that the flavonoids present in the microwave-processed extracts have a strong affinity for the target protein, thus explaining the efficacy of the extract in reducing inflammation.

**Supplementary Materials:** The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/analytica6020016/s1. Table S1: (HPLC-PDA) analysis of the phenolic profile of Thymus algeriensis microwave-assisted extracts. Table S2: Binding interaction.

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**Conflicts of Interest:** The authors declare no conflicts of interest.

## Abbreviations

The following abbreviations are used in this manuscript:

| | |
|---|---|
| MW | Microwave extracts |
| RBC | Red blood cell |
| NSAIDs | Non-steroidal anti-inflammatory drugs |

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