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# Anti-inflammatory, Antioxidant, and Antibacterial Activities with Molecular Docking Studies of *Vitex Trifolia* L. Targeting Human COX-2 and Peroxiredoxin-5

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*Vitex trifolia* L. is widely used as a traditional medicine owing to its secondary metabolites. This study aimed to determine the phytochemical profiling of the ethanolic extract of *V. trifolia* leaves (EEVL). Additionally, biological activities were investigated, supported by molecular docking studies. The total flavonoid and phenolic contents of the EEVL were 35.25 ± 0.13 mg QE/g extract and 39.55 ± 1.43 mg GAE/g extract, respectively. The EEVL exhibits anti-inflammatory activity with a half-maximal inhibitory concentration ($IC_{50}$) value of 153.59 µg/mL. The antioxidant activity of the EEVL exhibited an $IC_{50}$ value of 20.41 ± 0.08 µg/mL with the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method, a half-maximal effective concentration value of 80.85 ± 0.39 µg/mL with the cupric-reducing antioxidant capacity (CUPRAC) method, and a capacity of 180.58 mg QE/g extract with the ferric-reducing antioxidant power (FRAP) method. At 16% concentration, the EEVL exhibited an antibacterial activity against some pathogenic bacteria. Molecular docking studies reported that casticin interacts with sodium diclofenac similarly toward 1PXX. However, artemitin exhibited better affinity toward 1HD2 than ascorbic acid. Thus, the EEVL has significant potential as a natural source for treating various diseases related to inflammation, oxidation, and infection. Moreover, screening of its active compounds is necessary for further exploration.

## 1. Introduction

Inflammation is a natural process that occurs in response to harmful agents, such as microbial infections, physical injuries, oxidative stress, and other phenomena.[1] This process involves the development of inflammatory mediators by the enzyme cyclooxygenase-2 (COX-2), which converts free arachidonic acid to prostaglandins. These prostaglandins are associated with acute and chronic inflammatory disorders.[2] An inflammation caused by bacterial infection can be treated with antibiotics. However, their irrational use often leads to bacterial resistance. Previous research has found that phytochemical components from plant extracts can act synergistically to inhibit bacterial growth and prevent the development of drug resistance.[3]

Polyphenols are plant-based chemical compounds that have been shown to alleviate different types of inflammation. These compounds are effective owing to their antioxidant properties and ability to inhibit enzymes involved in the inflammatory process, such as the COX-2 enzyme.[4] Previous research indicates that polyphenolic compounds possess antioxidant,[5] anti-inflammatory, anticancer, and antibacterial properties.[6]

*Vitex trifolia* L. is widely distributed in Southeast Asia, Micronesia, Australia, and East Africa.[7] Its fruits have long been used in folk medicine to cure headaches, colds, migraines, eye pain,[8] inflammation, infections, and cancer.[9] These benefits are believed to come from secondary metabolites, such as alkaloids, saponins, flavonoids, phenols, tannins, and terpenoids, in *V. trifolia* fruits.[10] As the utilization of *V. trifolia* fruit is well-recognized, it has been the subject of numerous studies. However, the phytochemical profile and biological activity of *V. trifolia* leaves are not completely understood. A previous study reported that the aqueous extract of *V. trifolia* leaves exhibits an anti-inflammatory activity.[11] Thus, this study aimed to thoroughly evaluate the chemical profile, biological activities, and molecular docking of the ethanolic extract of *V. trifolia* leaves (EEVL).

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**Table 1.** TFC and TPC results of EEVL.

| Absorbance | | Linearity Regression | Initial Concentration (mg/L) | TFC[a] or TPC[b] | Average |
|---|---|---|---|---|---|
| TFC | 0.44 | $y = 0.0228x - 0.002$ ($r = 0.998$) | 19.39 | 35.25 | 35.25 ± 0.13 |
| | 0.43 | | 19.12 | 35.41 | |
| | 0.45 | | 19.65 | 35.09 | |
| TPC | 0.25 | $y = 0.0093x + 0.0651$ ($r = 0.999$) | 19.88 | 37.87 | 39.55 ± 1.43 |
| | 0.25 | | 20.09 | 39.40 | |
| | 0.27 | | 21.92 | 41.37 | |

[a] Calculated as mg QE/g extract.
[b] Calculated as mg GAE/g extract.

Herein, the colorimetric method was employed, with quercetin and gallic acid used as references, to determine the total flavonoid and phenolic contents of the EEVL. Anti-inflammatory evaluation was conducted in vitro, modulated by protein denaturation protection. Antioxidant activity was carried out using the 1,1-diphenyl-2-picryhydrazil (DPPH), cupric-reducing antioxidant capacity (CUPRAC), and ferric-reducing antioxidant power (FRAP) methods. Antibacterial activity was evaluated using the agar disc diffusion method against various pathogenic bacteria. Molecular docking studies of the major chemical compound from *V. trifolia* were conducted targeting human cyclooxygenase-2 (COX-2) and peroxiredoxin-5.

## 2. Results and Discussion

### 2.1. Total Flavonoid (TFC) and Phenolic Content (TPC)

The qualitative analysis was conducted using a previously described method. Approximately 3–4 drops of 10% ferric chloride ($FeCl_3$) solution were added to the EEVL solution to conduct the phenolic test. If the solution turned dark blue or black, it indicated the presence of phenolic compounds. Magnesium (Mg) powder and a few drops of concentrated hydrochloric acid (HCl) were added to the EEVL solution to conduct the flavonoid test. If the solution turned pink, green, or dark red after a few minutes, it indicated the presence of the flavonoid compound.[12] Based on the color reaction obtained, the results indicated that the EEVL contained flavonoids and phenolic compounds. The presence of flavonoids was indicated by the red color produced when the EEVL was reacted with Mg and HCl. Meanwhile, the presence of phenolic compound was indicated by the dark black color produced when the EEVL was reacted with $FeCl_3$.

The TFC and TPC of the EEVL were examined using aluminum chloride ($AlCl_3$) colorimetric method and the Folin–Ciocalteu assay, respectively, as previously described.[13] A regression equation for the standard curves of quercetin and gallic acid was obtained with a correlation coefficient ($r$) value, as shown in Table 1. To ensure accuracy, the TFC and TPC of the EEVL were measured three times; the results are presented in Table 1. The TFC and TPC evaluation indicated that each gram of EEVL contained 35.25 ± 0.13 mg of flavonoids and 39.55 ± 1.43 mg of phenolic compound, which were equivalent to those of quercetin and gallic acid, respectively. These results exhibited lower levels than the previous study, which had TFC (57.41 mg QE/g) and TPC (77.20 mg GAE/g).[10] This difference in levels was likely caused by the differences in the environment where the samples were grown, thereby influencing the formation of secondary metabolites in plants.

Flavonoids are the primary active chemicals found in *V. rotundifolia* and *V. trifolia*.[14] Flavonoids are a group of chemicals with the basic nucleus 2-phenylchromone, the majority of which have the basic skeleton C6-C3-C6. Flavonoids are molecules that have an oxygen-free group at the 3-position, whereas flavonols have hydroxyl or other oxygen-containing groups at the 3-position. The hydrogenated structure of the 2, 3 double bonds in the basic nucleus of flavonoids is dihydroflavonoids, and that of the 2-, 3-positions of flavonols is dihydroflavonol. The basic nucleus of isoflavones is 3-phenylchromone, which connects the B ring to the 3-position of the C ring.[14]

Numerous flavonoids, such as casticin, chrysosplenol D, luteolin, vitexin, and apigenin, have been isolated from different parts of *V. rotundifolia* and *V. trifolia* including 19 flavones, 10 dihydroflavones, 4 flavonols, 1 dihydroflavonols, 1 isoflavone, 13 flavone glycosides, and 3 xanthones. Vitexicarpin, also referred to as casticin, is the primary chemical isolated from the fruits of *V. trifolia* and *V. rotundifolia*. Diterpenes, lignans,[15] and various flavonoids have also been isolated from *V. trifolia*.[7] The isolated compounds include persicogenin, artemitin, luteolin, penduletin, chrysosplenol-D, vitexilactone, rotundifuran, and vitetrifoline.[15] However, research on the flavonoids of *V. trifolia* is limited, and flavonoids constitute a small proportion of the total compounds. Hence, it is possible to isolate new compounds.

### 2.2. Anti-inflammatory Activity

Several studies have demonstrated that *V. trifolia* exhibits interesting activity regarding the modulation of inflammatory processes. A previous study reported that the aqueous extract of *V. trifolia* has the potential to stimulate the production of the anti-inflammatory cytokine interleukin (IL)-10, which is dependent on lipopolysaccharide.[11] These findings were confirmed by conducting an enzyme-linked immunosorbent assay (ELISA) with


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**Table 2.** Anti-inflammatory activity of sodium diclofenac and EEVL.

| Sample | Concentration (μg/mL) | Absorbance (660 nm) | Inhibition (%) | Linearity Regression | $IC_{50}$ (μg/mL) |
|---|---|---|---|---|---|
| Sodium diclofenac | 0 | 0 | 0 | | 20.37 |
| | 100 | 0.81 | 3.12 | $y = 2.4197x + 0.7289$ | |
| | 200 | 0.79 | 5.76 | ($r = 0.999$) | |
| | 400 | 0.75 | 10.67 | | |
| | 800 | 0.67 | 19.42 | | |
| | 1600 | 0.50 | 39.69 | | |
| EEVL | 0 | 0 | 0 | | 153.59 |
| | 1000 | 0.65 | 21.94 | $y = 0.1916x + 20.663$ | |
| | 2000 | 0.63 | 24.94 | ($r = 0998$) | |
| | 4000 | 0.60 | 28.06 | | |
| | 8000 | 0.53 | 36.81 | | |
| | 16000 | 0.41 | 50.96 | | |

specific antibodies for mouse IL-6, IL-10, and tumor necrosis factor (TNF)-$\alpha$.[11] Thus, the anti-inflammatory activity of *V. trifolia* leaves was tested herein using ethanolic extract and employing different methods.

The positive control used diclofenac sodium, which is a well-known NSAID and is commonly used to treat pain and inflammatory diseases, such as rheumatoid arthritis, postoperative traumatic pain, migraines, and fever,[16] to inhibit prostaglandin synthesis by blocking COX-1 and COX-2.[17] The results of the inhibition of protein denaturation of the positive control sodium diclofenac and EEVL are presented in Table 2.

The results of the linear equation between concentration ($x$) and % inhibition ($y$) indicate that the regression $y = 2.4197x + 0.7289$ was obtained with a correlation coefficient of $r = 0.999$. Diclofenac sodium has a half-maximal inhibitory concentration ($IC_{50}$) value of 20.37 μg/mL, which can inhibit protein denaturation by up to 50% at that concentration. Furthermore, EEVL inhibited protein denaturation at all concentrations, with an $IC_{50}$ value of 153.59 μg/mL. Flavonoids are often associated with potential anti-inflammatory effects of natural resources.[18] *V. trifolia* exhibits substantial anti-inflammatory effects, possibly due to casticin and terpenoids. It inhibits pro-inflammatory factors, chemokines, and NO while boosting anti-inflammatory ones, possibly through NF$\kappa$B signaling pathway control.[14]

Although in vivo anti-inflammatory research has been conducted, the principles used were different from in vitro methods. Thus, the mechanism of action of the chemical components will be extremely important. In our study, anti-inflammatory testing using the approach of protein denaturation inhibition was not conducted on EEVL. As a result, the in vitro assay results that we use can supplement previously available data, enhancing the anti-inflammatory potential of *V. trifolia* leaves. Animal studies conducted to examine the anti-inflammatory activities of *V. trifolia* leaf extract via carrageenan-induced rat paw edema assay reported that the extract (200 mg/kg body weight) exhibited dose-dependent anti-inflammatory properties. At this dosage, both aqueous and ethanolic extracts exhibited considerable effectiveness ($p < 0.0001$) against acute inflammatory response, with decreases of 46.91% and 60.49%, respectively.[19]

### 2.3. Antioxidant Activity

It is crucial to evaluate the antioxidant activity of plant extracts using three different methods for several reasons: 1) Diverse mechanisms of action. Antioxidants can work through various mechanisms, such as scavenging free radicals, chelating metal ions, or inhibiting oxidative enzymes. The use of different methods allows for a more comprehensive understanding of the antioxidant capacity of a plant extract. 2) Method-specific sensitivities. Other assays can vary in sensitivity and specificity to certain antioxidants. Using multiple methods ensures that a wider range of antioxidant compounds is assessed, leading to more reliable conclusions. 3) Validation and reliability. Reliance on a single method can lead to biased results or misinterpretations. By employing three different approaches, the findings can be cross-validated, enhancing the reliability and robustness of the data. This multifaceted evaluation confirms the effectiveness of the extracts and provides a more accurate picture of their antioxidant properties. Therefore, the use of multiple methods improves the understanding of antioxidant activity in EEVL and strengthens the scientific evidence for their potential health benefits.

In the antioxidant activity assay, different concentrations were used to determine the $IC_{50}$ value, which represents the concentration needed to inhibit 50% of the radicals. This value provides a quantitative measure of the antioxidant strength, allowing for the comparison of various samples and standards. Furthermore, using a range of concentrations allows for a direct comparison between the sample and the standard, which is essential for evaluating the effectiveness of the plant extract compared with that of established antioxidants and providing context to the findings. Testing multiple concentrations helps reduce variability and ensures that the observed effects are not simply caused by chance fluctuations, thereby increasing the reliability of the data and supporting more robust conclusions.

### 2.4. DPPH Radical Scavenging Activity

The DPPH method is widely employed to measure the free radical-scavenging activity of general antioxidants, such as


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**Table 3.** Antioxidant activity of ascorbic acid and EEVL by DPPH method.

| Sample | Concentration (μg/mL) | Absorbance (515 nm) | Inhibition (%) | $IC_{50}$ (μg/mL) |
|---|---|---|---|---|
| Ascorbic acid | 1 | 0.86 ± 0.03 | 40.37 ± 0.03 | 2.75 ± 0.03 |
| | 3 | 0.73 ± 0.01 | 49.12 ± 0.02 | |
| | 5 | 0.49 ± 0.01 | 65.37 ± 0.02 | |
| | 7 | 0.38 ± 0.03 | 73.70 ± 0.03 | |
| | 9 | 0.25 ± 0.02 | 82.57 ± 0.01 | |
| EEVL | 17.5 | 0.72 ± 0.01 | 41.03 ± 0.47 | 20.41 ± 0.08 |
| | 20 | 0.64 ± 0.01 | 48.29 ± 0.87 | |
| | 22.5 | 0.50 ± 0.02 | 57.82 ± 1.26 | |
| | 25 | 0.42 ± 0.03 | 62.45 ± 2.42 | |
| | 27.5 | 0.33 ± 0.01 | 73.90 ± 0.47 | |

ascorbic acid, flavonoids, peptides, and phenolic acids.[20] DPPH is classified as a stable free radical and has many advantages, such as simplicity, low cost, and faster results than other tests. In its initial radical form, DPPH appears dark purple but changes to yellow when it is reduced by an antioxidant agent.[21] This color change indicates the presence of either the hydrogen or electron donation.[22] Spectrophotometry is an easy method for measuring color changes and determining the antioxidant activity of samples.[23] The $IC_{50}$ value was calculated to determine the sample concentration required to inhibit DPPH radicals by 50%. The lower the $IC_{50}$ value, the stronger the antioxidant activity.[24] In this study, EEVL was compared with ascorbic acid; the results are presented in Table 3.

The antioxidant category is divided into four subcategories, namely, powerful, strong, moderate, and weak, based on their $IC_{50}$ values.[20] An $IC_{50}$ value of <50 μg/mL is considered to be powerful, whereas values between 50 and 100, 100 and 250, and 250 and 500 μg/mL are considered to be strong, moderate, and weak, respectively.[25] The $IC_{50}$ of the EEVL was found to be 20.41 ± 0.08 μg/mL, which is considered to be powerful. However, the antioxidant activity of ascorbic acid (2.75 ± 0.03 μg/mL) is 10-fold stronger than that of the EEVL. This is because ascorbic acid is a pure compound with a powerful antioxidant activity.[26] The $IC_{50}$ value of EEVL in our study was greater than those reported in previous research, which were 81.72 μg/mL[10] and 64.5 μg/mL.[27] Interestingly, the TFC and TPC, which are often associated with antioxidant activity, were higher in the previous study than in ours. This suggests that factors other than quercetin and gallic acid compounds contribute to the antioxidant activity of EEVL. Therefore, further studies are warranted to identify other flavonoid and phenolic compounds present in the EEVL and their contribution to its antioxidant activity.

According to Scherer and Godoy, antioxidant activity can be classified based on the antioxidant activity index (AAI) value.[28] Ascorbic acid and EEVL had AAI values of 18.18 and 2.45, respectively. Both were considered to be very strong antioxidants as their AAI values were >2.0.[29] A compound is classified as a weak antioxidant if its AAI value is <0.5, a moderate antioxidant if the AAI value is 0.5–1.0, a strong antioxidant if the AAI value is 1.0–2.0, and a very strong antioxidant if the value is >2.0.[30]

**Table 4.** Antioxidant activity of ascorbic acid and EEVL by CUPRAC method.

| Sample | Concentration (μg/mL) | Absorbance (450 nm) | Capacity (%) | $EC_{50}$ (μg/mL) |
|---|---|---|---|---|
| Ascorbic acid | 350 | 0.59 ± 0.002 | 72.96 ± 0.13 | 20.36 ± 0.06 |
| | 300 | 0.49 ± 0.003 | 65.64 ± 0.23 | |
| | 200 | 0.32 ± 0.000 | 49.03 ± 0.06 | |
| | 150 | 0.26 ± 0.000 | 41.83 ± 0.06 | |
| | 100 | 0.20 ± 0.002 | 33.47 ± 0.33 | |
| EEVL | 20 | 0.11 ± 0.001 | 18.86 ± 0.28 | 80.85 ± 0.39 |
| | 30 | 0.16 ± 0.002 | 27.76 ± 0.41 | |
| | 50 | 0.27 ± 0.001 | 43.08 ± 0.11 | |
| | 100 | 0.51 ± 0.003 | 67.55 ± 0.23 | |
| | 150 | 0.74 ± 0.001 | 81.00 ± 0.06 | |
| | 200 | 0.88 ± 0.008 | 86.22 ± 0.26 | |

### 2.5. CUPRAC Antioxidant Capacity

CUPRAC was used to evaluate the antioxidant capacity by assessing its effectiveness in reducing $Cu^{2+}$ to $Cu^{+}$ complexes, based on the principle of free radical scavenging activity.[31] It has various advantages, such as the selectivity of the CUPRAC reagent, low cost, and stability, compared with other chromogenic reagents. In addition, the response of the CUPRAC is not influenced by environmental factors, such as air, humidity, and sunlight.[32] In this study, ascorbic acid was used as a reference standard. The antioxidant activities of EEVL and ascorbic acid are presented in Table 4.

The EEVL exhibited a half-maximal effective concentration ($EC_{50}$) value of 80.49 μg/mL and was considered to be a strong antioxidant as its $EC_{50}$ value was 50–100 μg/mL. Meanwhile, ascorbic acid was classified as a very strong antioxidant as its $EC_{50}$ value was <50 μg/mL. The AAI values of ascorbic acid and EEVL were 4.91 and 1.24, respectively, which were consistent with the $EC_{50}$ values, indicating that EEVL is a strong antioxidant. In the same genus, the water and methanolic extract of *V. rotundifolia* fruit exhibit much stronger antioxidant activity than trolox antioxidant (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), attributed to its phenolic content.[33]

### 2.6. FRAP Antioxidant Capacity

The polyphenol content of the plant extract is positively associated with its antioxidant activity.[34] To assess the ability of the extract to counteract free radicals, either in vitro or in vivo evaluations need to be conducted. Although antioxidant activity has been investigated, the FRAP approach has not yet been discovered in the EEVL. Thus, the application of the FRAP method in assessing the antioxidant activity of the EEVL is critical to the supplementation of previously available data.

The FRAP method, which is employed to test antioxidants, involves reducing $Fe^{3+}$ complexes to $Fe^{2+}$.[35] Free radicals are compounds or molecules containing one or more unpaired electrons in their outermost orbital. These unpaired electrons make


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**Table 5.** Antioxidant capacity of EEVL by FRAP method.

| Quercetin | | EEVL | | Antioxidant Capacity (mg QE/g) | Average (mg QE/g) |
|---|---|---|---|---|---|
| Concentration (μg/mL) | Absorbance (721 nm) | Concentration (μg/mL) | Absorbance (721 nm) | | |
| 15 | 0.28 | 500 | 0.864 | 180.81 | 180.58 |
| 20 | 0.38 | 500 | 0.862 | 180.61 | |
| 25 | 0.50 | 500 | 0.857 | 180.13 | |
| 30 | 0.61 | | | | |
| 35 | 0.73 | | | | |
| 40 | 0.86 | | | | |

**Table 6.** Antibacterial activity of EEVL by agar disc diffusion.

| Concentrations (%) | Screening Antibacterial Activity | | | | |
|---|---|---|---|---|---|
| | SA | ST | PA | BS | EC |
| 0.1 | – | – | – | + | + |
| 0.5 | + | ++ | – | + | ++ |
| 1 | ++ | ++ | ++ | ++ | ++ |
| | Inhibition Zone (mm) on Antibacterial Activity | | | | |
| 0.5 | – | – | – | – | – |
| 1 | 6.11 | 6.21 | 6.10 | 6.05 | 6.05 |
| 2 | 6.21 | 6.25 | 6.50 | 6.40 | 7.05 |
| 4 | 6.80 | 6.58 | 7.48 | 6.60 | 8.27 |
| 8 | 7.31 | 7.55 | 8.48 | 9.09 | 10.10 |
| 16 | 10.41 | 10.67 | 11.68 | 9.65 | 12.06 |

the compounds highly reactive, causing them to attack and bond with surrounding electrons, such as those in cell membrane macromolecules, DNA, and proteins.[36] Free radicals are produced during normal cellular metabolic processes and in response to external factors, such as air pollution and UV light.[37]

The absorbance measurement of quercetin served as a basis for constructing the linear regression equation $y = 0.0206x - 0.9983$ ($r = 0.9991$). The linearity data indicates a correlation coefficient closely aligned with the optimal value, suggesting that concentration and absorbance have a robust association. Furthermore, this data verifies the precision and accuracy of the spectrophotometer used in the preparation of the quercetin standard solution. The results of antioxidant capacity assessment, as depicted in Table 5, indicate that EEVL exhibits an antioxidant capacity equivalent to that of quercetin.

### 2.7. Antibacterial Activity

The antibacterial properties of the EEVL were determined using the standard agar disc diffusion technique as previously described, with slight modifications.[38] The screening tests for the antibacterial activity of the EEVL against several pathogenic bacteria are presented in Table 6. The screening tests conducted on multiple bacteria indicated that the EEVL exerted inhibitory effects on the growth of the *Bacillus subtilis* (BS) and *Escherichia coli* (EC) bacteria at a concentration of 0.1%. At a concentration of 0.5%, it inhibited the growth of the *Staphylococcus aureus* (SA) and BS bacteria while leading to the demise of *Salmonella typhi* (ST) and EC. Ultimately, at a concentration of 1%, EEVL proved to be a lethal agent against all test bacteria, including Pseudomonas aeruginosa (PA). The antibacterial activity of EEVL was evaluated against pathogenic bacteria.

It was found that a concentration of 0.5% was not able to inhibit all the test bacteria. However, starting at a concentration of 1%, it was found that all bacteria had a comparable inhibitory zone, particularly in the 6-mm range. The diameter of the inhibitory zone increases with the concentration of the EEVL. The greatest zone of inhibition was seen at a concentration of 16% in EC. BS, conversely, had the narrowest zone of inhibition, even at a concentration of 16%. Based on these observations, it is plausible to conclude that EEVL has a very strong antibacterial power against EC compared to other bacteria.

A previous study reported that *V. trifolia* leaf extract was tested on 12 bacteria strains, including *Escherichia coli* and *Staphylococcus aureus*, using the in vitro disk diffusion method. All extracts demonstrated effective antibacterial activity, with methanol extract outperforming ethanol and ethyl acetate extracts.[39] While volatile oil and terpenoids may be the primary antibacterial active components, in vitro research has been conducted, and no in vivo studies have been published.[14]

Furthermore, a previous study reported that vitrifolin A in nanoparticle formulations containing *V. trifolia* leaf extract plays a pivotal role in increasing the antibacterial properties of the extract. It accomplishes this role by adhering to nanoparticle surfaces, resulting in more efficient and targeted antimicrobial agent delivery. This method causes vitrifolin A to strongly influence germs, adding to the overall increased activity of the leaf extract against several ailments.[40]

### 2.8. Molecular Docking Studies

The antioxidant and anti-inflammatory activities of the *V. trifolia* fruit extract have been thoroughly investigated, demonstrating significant potential. However, the mechanism of action and description of molecular interactions with target receptors remains unknown. Thus, molecular docking study is warranted to strengthen the potential anti-inflammatory and antioxidant


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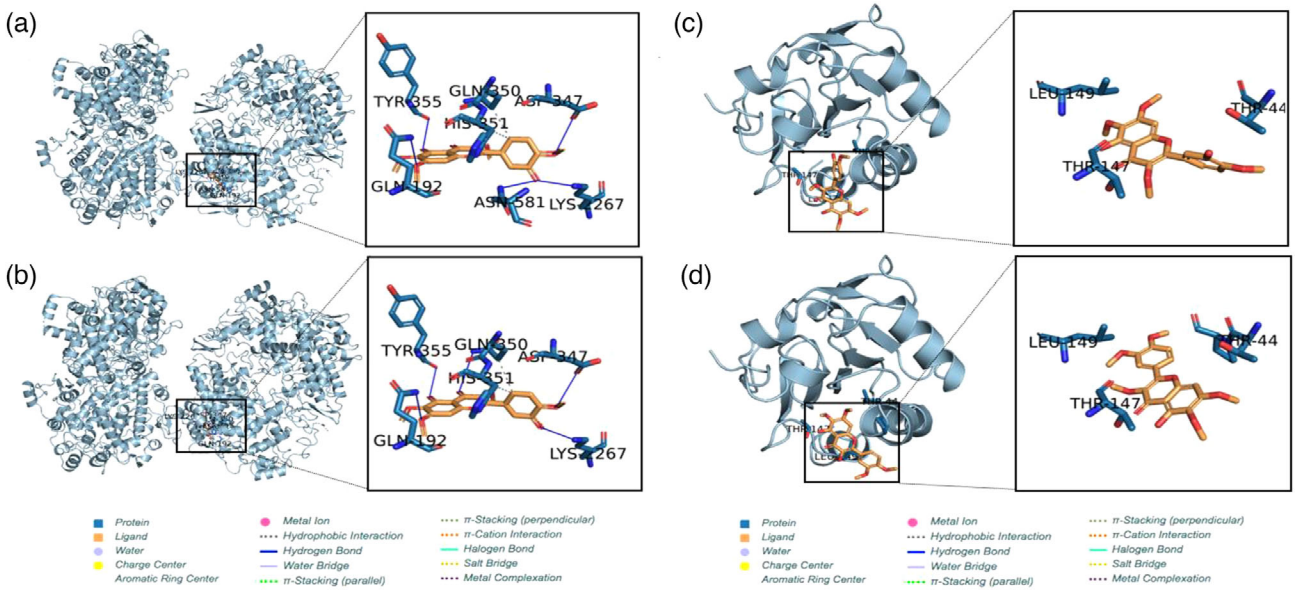


**Figure 1.** Conformational posture of ligands in the catalytic site of COX-2: (a) Casticin, (b) artemitin, and peroxiredoxin-5, (c) casticin, (d) artemitin. Protein and ligand are presented by the cartoon and stick models visualized by web server PLIP (https://plip-tool.biotec.tu-dresden.de/plip-web/plip/index) gathered with PyMOL software (https://pymol.org/2/).

**Table 7.** Binding energy of ligands in complex with the receptors.

| Compound | 1PXX | 1HD2 |
|---|---|---|
| Sodium diclofenac | −8.4 | – |
| Ascorbic acid | – | −4.5 |
| Casticin | −7.2 | −4.3 |
| Artemitin | −6.9 | −5.0 |
| Vitexilactone | −6.6 | −3.9 |

activities of EEVL against COX-2 and peroxiredoxin-5 receptors. The interaction between the ligand and receptor complexes was estimated using the AutoDock Vina package[41] as previously described.[42] Casticin, artemitin, and vitexilactone (Figure S1) were used as ligands in molecular docking assays. Figure 1 presents the conformational posture of ligands in the catalytic site of COX-2 and peroxiredoxin-5. Our results exhibited that the free energy in the interaction of test ligands and target receptor has a similar potential to the native ligand, as presented in Table 7. Molecular docking studies toward 1PXX and 1HD2 correspond to COX-2 and peroxiredoxin-5 receptors, respectively, showing that casticin interacts with sodium diclofenac similarly toward 1PXX. Notably, artemitin exhibited a better affinity towards 1HD2 than ascorbic acid. Tables S1–S4 in the Supporting Information summarizes the detailed information on the ligand–receptor interactions by hydrogen and hydrophobic bonds.

The selection of receptors for docking studies depends on their biological relevance and their direct involvement in the biological pathways of interest. In this study, the PDB ID 1PXX and 1HD2 were used, which correspond to the COX-2 and peroxiredoxin-5 receptors, respectively. These receptors have specific binding sites that are relevant for interactions with the ligand. Both 1PXX[43] and 1HD2[44] have been used in previous studies for their roles in inflammatory or antioxidant responses, thereby providing a solid foundation for correlating docking results with biological activity.

The interpretation of docking results can be in the form of binding affinity or docking scores, which indicate how strongly the ligand (e.g., a plant extract or its components) interacts with the receptor. A lower binding energy typically indicates stronger interactions, which can correlate with greater biological activity. Interaction profiles could also be analyzed by the types of interactions, such as hydrogen bonds and hydrophobic interactions, that the ligand forms with the receptor.[45] These details can provide insights into how the ligand may exert its antioxidant or anti-inflammatory effects.

## 3. Conclusion

This study investigated the potential health benefits of the leaf extract of *V. trifolia*, demonstrating its significant anti-inflammatory, antioxidant, and antibacterial properties across various concentrations. The extract effectively reduced protein denaturation, indicating strong anti-inflammatory effects, and demonstrated robust antioxidant activity in DPPH, CUPRAC, and FRAP assays. Additionally, it exerted potent antibacterial effects against EC. These beneficial biological activities were attributed to the presence of compounds, such as flavonoids and phenolics, in the extract. Notably, casticin and artemitin were identified as having remarkable anti-inflammatory and antioxidant properties against specific target receptors. These findings strongly support the traditional use of *V. trifolia* leaves and highlight the importance of preserving medicinal plants for potential drug discovery. The study emphasizes the need for further exploration of *V. trifolia* and its constituents to develop innovative treatment approaches for immune-mediated inflammatory conditions.


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## Supporting Information

Experimental materials, methods, and molecular docking results are provided in the Supporting Information.[5,12,13,28,38,41,46]

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## *Conflict of Interests*

The authors declare no conflict of interest.

## *Data Availability Statement*

The data that support the findings of this study are available in the supplementary material of this article.

**Keywords:** Antioxidants · Inflammation · Molecular modeling · Oxidation · Radical reactions

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