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Original Research Article

In vitro and *In silico* study of antioxidants and anti-inflammatory activity of bitter leaves (*Vernonia amygdalina* del.) Extract

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ABSTRACT

Bitter leaves (*Vernonia amygdalina*) are known by the people of Southern Nigeria as a traditional medicine that can be used for various diseases. Bitter leaves have compounds that act as antioxidants to neutralize free radicals and have anti-inflammatory effects, which regulate arachidonic acid metabolism by inhibiting the activity of cyclooxygenase (COX) and lipoxygenase. The study aims to determine the secondary metabolite content of bitter leaf extract and antioxidant and anti-inflammatory activity using *in vitro* and *in silico* assays. Antioxidant activity was evaluated using the DPPH method, while anti-inflammatory was carried out using the protein denaturation method and spectrophotometry. Meanwhile, *an in silico* study was conducted using computational molecular docking tests. The study results showed the methanol extract of Bitter Leaves contained secondary metabolites in the form of alkaloids, flavonoids, tannins, saponins, and steroids with total phenolics of 112.067 ± 0.157135 (mgGAE/g) and total flavonoids of 32.808 ± 1.473139 (mgQE/g). The bitter leaf extract exhibits potent antioxidant activity with an IC_{50} = 6.10 ppm and an anti-inflammatory IC_{50} = 25.33 ppm. The docking results showed that the bioactive compounds of bitter leaves have potential anti-inflammatory activity based on the binding energy value, inhibition constant, and average bond distance. Bitter leaves have compounds acting as antioxidants and anti-inflammatory effects, as shown in *in vitro* and *in silico* studies.

Keywords: *Vernonia amygdalina*, Secondary metabolite, Antioxidant, Anti-inflammatory.

Introduction

Most of the world's population relies on plants because of their medicinal value and rarity.[1] Medicinal plants have been used to treat diseases for years.[2] Despite rapid technological advances in modern medicine, 75% of Africa still relies on traditional medicinal plants for daily healthcare. Medicinal plants could be used alone or with other plants to treat diseases.[1] Free radicals and inflammation are two factors that play a major role in degenerative diseases such as cancer, diabetes, and cardiovascular-related diseases. Those diseases can be treated with antioxidant and anti-inflammatory agents.[3]*Vernonia amygdalina* Del. is a shrub or perennial woody plant belonging to the Asteraceae family. This plant is found in many parts of Africa and is characterised by the bitter sap from its leaves, which has been widely explored for medicinal use. *Vernonia amygdalina* Del. could grow up to 10 m in height with leaf stalks about 6 mm in diameter and elliptical. Traditionally, *Vernonia amygdalina* could treat diseases such as diabetes and antihelminthic, antimalarial, laxative, digestive tonic, and antipyretic.

In several African countries, including Ethiopia, *Vernonia amygdalina* Del. is a medicine for malaria, worm infections, digestive disorders, and fever. This plant is also used to accelerate wound healing and treat microbial infections.[4]Bitter leaves contain various bioactive compounds, such as saponins, alkaloids, terpenes, steroids, coumarins, flavonoids, phenolic acids, lignans, xanthones, anthraquinones, and sesquiterpenes. The phenolic and flavonoids of this plant have antioxidant properties, free radicals scavenging potential, singlet oxygen quenchers, and metal chelators. Therefore, plants' phytochemicals content can support the human body's health as natural antioxidants. These phytoconstituents' activity depends on the geographic location where the plant material is collected, the weather, and the maturity of the leaves.[5]Today, anti-inflammatory products are available as synthetic drugs, however, with undesired effects. Besides, natural phytochemicals are commonly used in medicine because they have minimal side effects compared to synthetic drugs. Therefore, alternative anti-inflammatory agents from natural compounds become essential. One medicinal plant widely known to have various benefits is bitter leaves. *Vernonia amygdalina* leaf extract can be formulated as medicine because these leaves' phytochemical components, such as flavonoid and phenolic content, are very beneficial in treating various diseases.[6] Natural medicine can be manufactured in Indonesia, considering the technology needed for natural medicinal product development in local pharmaceutical industries. Therefore, the research studied the potential of methanol extract from *Vernonia amygdalina* leaves for antioxidant and anti-inflammatory effects using *in vitro* and *in silico* protocols.

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Materials and Methods

Materials
Bitter leaves (*Vernonia amygdalina* Del.), methanol, hydrochloric acid (2N HCl), distilled water, Reagents (Mayer, Bouchardat, and Dragendorrf), magnesium powder, concentrated HCl, amyl alcohol, iron (III) chloride reagent, n-hexane, Liebermann Burchard reagent, 96% ethanol, Folin-Ciocalteu reagent, 1 M Na_2CO_3 solution, aluminum (III) chloride, sodium acetate, quercetin, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Bovine serum albumin (BSA) solution.
Tools used in this research were a drying oven (DHG-9053A, YiHeng, China), sonicator (511, Branson, USA), glassware, UV-Vis Spectrophotometer (Genesys 1XX, Thermo Fisher Scientific, USA), Water bath (WTB11, Memmert, Germany), Intel Core i3-1115G4 computer with 8 GB of RAM.

Methods
Simplicia preparation
Bitter leaves (*Vernonia amygdalina* Del.). were collected in February 2024 at Carang Pulang Village, Bogor, West Java, Indonesia (Figure 1). Simplicia was prepared by sorting out fresh bitter leaves. Then, the fresh leaves were washed and dried using an oven at 40°C.
Extraction
Ultrasonic-assisted extraction (UAE) was used. The extraction method uses a 20-40 kHz low-frequency ultrasonic and the solvent at 25˚C temperature.[7] Dried leaves of simplicia were grounded and weighed. Simplicia powder was put into a beaker then 80% methanol solvent was added in a ratio of 1:6.[8] The solution was homogenized by stirring and placed into a sonicator with a power of 200 W and a frequency of 20 kHz for 30 minutes. During extraction, aluminium foil was used to cover the beaker to prevent solvent evaporation. The filtrate was obtained and evaporated over a water bath at 65°C.

Phytochemicals Screening of Simplicia and Extract
The crude extract was subjected to phytochemical screening to test for the presence of various plant secondary metabolites (alkaloids, flavonoids, saponins, tannins, steroids, and triterpenes) using standard protocols.[9-10]

Quantitative Extract Test
Determination of total phenolic content
The extract (20 mg) was dissolved in 10 mL of 96% ethanol. 5 mL of the mixture was transferred into a 25 mL volumetric flask and filled with distilled water to the mark. To 0.5 mL of this was added 5 mL of Folin-Ciocalteu (1:10) and 4 mL 1 M Na_2CO_3 solution. The mixture was incubated for 15 min. The absorbance was measured at a wavelength of 765 nm using a UV-Vis spectrophotometer.[11]

Determination of total flavonoid in extracts
The sample (20 mg) was dissolved in 10 mL of ethanol p.a and vortexed. Then, to 0.5 mL of the sample, 0.1 mL aluminum (III) chloride 10%, 0.1 mL sodium acetate 1 M, and 2.8 mL distilled water was added and incubated for 30 min. Quercetin was used to generate a standard measured at a wavelength of 412 nm using a UV-Vis spectrophotometer. Total flavonoid content (mgQE/g) was calculated using a linear regression equation with a calibration curve.[12]
Determination of antioxidant activity using the DPPH method
The extract (2 mL) was pipetted and transferred into a vial, and 2 mL of a 40 ppm DPPH solution was added to each test sample and incubated for 30 min in the dark at 25°C. The absorbance was measured using a UV-Vis spectrophotometer at 515 nm. The absorbance data was calculated as IC_{50} % inhibition.[13]

Determination of Anti-inflammation activity
The sample (500 µL) of each concentration was pipetted, then 0.2% BSA solution was added into the volumetric flask to make up to 5 mL. The mixture was incubated for 30 minutes at 25°C and heated for 5 min at 70°C in a water bath, then cooled at 25°C for 10 min. The mixtures were vortexed, and the absorbance was measured at a wavelength of 660 nm using a UV-Vis spectrophotometer. The absorption data was calculated for % inhibition.[14]

In silico Analysis
Molecular docking used MarvinSketch 15.5.11, PyMol 2.3.3, Chimera 1.10.2, Autodock 4.2., Discovery studio V21.1.0.20298, SwissAdme software, and way2drug.com database. The plant phytochemicals were retrieved from the PubChem database.[15] Anti-inflammatory macromolecular targets 6OP0 (crystal structure of Tumor necrosis factor-alpha (TNF-α)) and 1T49 (crystal structure of Protein tyrosine phosphatases (PTPs)1B) with the PDB IDs were downloaded with www.rcsb.org. The molecular docking simulation stages include the preparation of the target macromolecular structure, which includes searching, optimizing, the preparation of downloading, and verifying nonstandard residues, and the addition of Gasteiger energy and hydrogen atoms. Then, the Gridbox coordinates are set as a place of interaction, creating a Grid Parameter File (GFP) and Docking Parameter File (DPF). The final steps involve the analysis and visualisation of a docking simulation.[16]

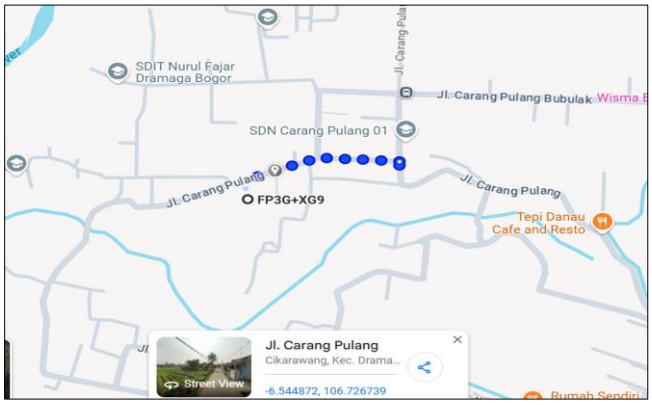

Figure 1: Sampling Location

Results and Discussions

Phytochemical screening of simplicia and extracts was performed as an initial qualitative analysis of the plant's secondary metabolite content. The principle of the qualitative analysis test is based on the colourimetric test using specific reagents/solvents, which can provide information regarding the content of secondary metabolites, including alkaloids, saponins, flavonoids, steroids, and tannins. The results of the phytochemical screening of bitter leaves (*Vernonia amygdalina* Del.) are presented in Table 1. The testing aims to confirm that the simplicia's chemicals can be removed during the extraction procedure. The extraction results show the same results as those of Simplicia, which means that the existing secondary metabolite compounds can be completely extracted by the solvent (methanol) used in this process.

Table 1: Secondary metabolite content of Simplicia and extracts

Secondary Metabolite	Results	
	Simplicia	Extract
Alkaloids	+	+
Flavonoids	+	+
Saponins	+	+
Tannins	+	+
Steroids	+	+

Note: (+) = positive; (-) = negative

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Methanol is a versatile polar solvent that is very good for preliminary extraction. It has the property of penetrating cell wall materials to extract bioactive compounds quickly.[18] In plants, phenolic chemicals play a crucial role as antioxidants.[19] Gallic acid was used as a standard in this study to determine the total phenolic components in bitter leaves. Gallic acid is a naturally occurring phenolic compound produced from hydroxybenzoic acid. When gallic acid and folin-ciocalteu react in an alkaline environment, the result is a blackish-green hue, suggesting phenol or something positive. Alkaline environments cause phenolic compounds to react, dissociating protons to produce phenolic ions.[20] The total phenolic content was determined using the UV-Vis spectrophotometer with a maximum wavelength of 765 nm. Gallic acid as a standard with a concentration of 10-70 ppm was used to generate a calibration curve y= 0.009x + 0.1067, with R^2=0.9974 to calculate the total phenolic content in the plant extract (Figure 2). The gallic acid equation obtained a linear relationship between absorbance and concentration. An R^2 close to one indicates that the regression equation is linear. This calculation is based on the Lambert-Beer law, which shows the linear relationship between increasing analyte levels and increasing absorbance. The phenolic content is expressed as GAE (gallic acid equivalent), that is, the equivalent number of milligrams of gallic acid (3,4,5-trihydroxybenzoic acid) in one gram of sample.

Flavonoids are phenolic compounds that function as antioxidants in the body, so they are suitable for protecting cell structure as an anti-inflammatory and antibiotic agent.[21] The UV-Vis spectrophotometer was used to measure the total flavonoid levels. Quercetin was used as a standard because this flavonoid is mainly found in plants.[22] Measurement of the absorbance value of quercetin as a standard with a concentration varying from 40-100 ppm was carried out at the optimum wavelength of 412 nm to generate a regression equation y = 0.0048x + 0.0648, R^2 = 0.9999 to compute the total flavonoid content (Figure 3).

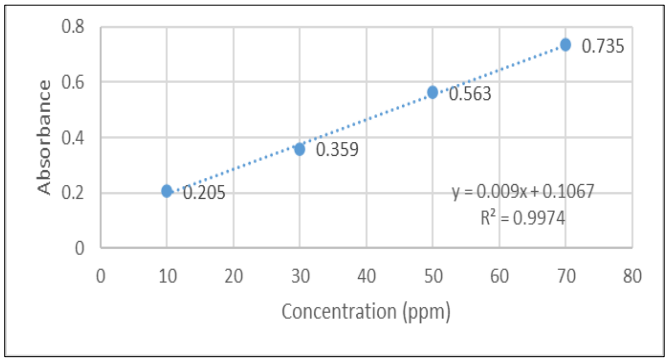

Figure 2: Standard gallic acid calibration curve

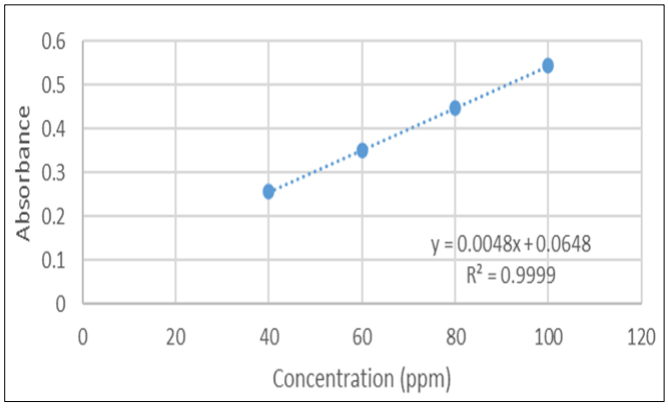

Figure 3: Standard quercetin calibration curve

The absorbance of quercetin increases with concentration due to the reaction of the keto group on C4 and the hydroxyl group on C3 or C5 of flavones/flavanols reacting with $AlCl_3$ to form a complex with the ortho dihydroxy group of flavonoids.[23] The higher the concentration of quercetin, the greater the possibility of complex formation shown in the intensity of the reaction colour, resulting in a greater absorbance value. Apart from that, potassium acetate stabilizes the complex compound that has been formed.

From the study, the computed phenolic and flavonoid content of the methanol extract of bitter leaves were 112.067 ± 0.157 (mgGAE/g) and 32.808 ± 1.473 (mgQE/g), as shown in Figure 4. In previous research, phenolic and flavonoid compounds were known to have various biological effects, such as antioxidants, anti-inflammatory, and antiseptic properties, which can also protect cell structure.[24]

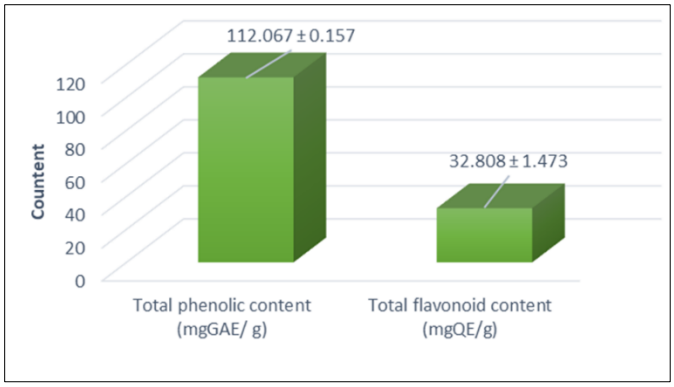

Figure 4: Values of Total Phenolic and Flavonoid Contents of Extract of Bitter leaf

The extract obtained was also evaluated for its antioxidant capacity. Antioxidant determination of bitter leaf extract was carried out using the DPPH method because it is easy and quick. The change in colour of the solution from purple to yellow indicated the ability of the sample to neutralise DPPH free radicals, measured using a UV-Vis spectrophotometer.[25] This change of colour occurs because compounds that donate hydrogen atoms to the DPPH radical are reduced to more stable forms such as DPPH-H.[26] The spectrum area used in measurements ranges between 380 – 780 nm wavelengths. Colour reaction measurements were carried out at varied extract concentrations to determine the level of colour attenuation due to the presence of antioxidant compounds that can reduce the purple colour intensity of DPPH. The higher the concentration of the extract, the more significant the colour reduction, which is indicated by the formation of a yellow colour, indicating potent antioxidant activity.[27] The antioxidant activity is indicated by the IC_{50} value. The IC_{50} value is the value of the sample that can neutralise 50% of DPPH radicals. In the linear regression equation for the methanol extract of bitter leaves, R^2 = 0.9604 (Figure 5a). From the equation, the IC_{50} for the methanol extract of bitter leaves was 6.10 ppm. In the linear regression equation for vitamin C, the value of R^2 = 0.9908 (Figure 5b), and the IC_{50} value of vitamin C was 2.34 ppm (Figure 6). The antioxidant activity is inversely proportional to the IC_{50} value. The smaller the IC_{50}, the stronger the antioxidant activity. The antioxidant category is very strong if the IC_{50} is less than 50 ppm, strong if the IC_{50} is 50-100 ppm, moderate if the IC_{50} is 100-150 ppm, low if the IC_{50} is 151-200 ppm, and very low if the IC_{50} is greater than 200 ppm.[28] Based on the results, both vitamin C and bitter leaf extract are in the very strong category

V. amygdalina leaf simplicia extract contains various secondary metabolites, such as saponins, glycosides, flavonoids, sesquiterpene lactones (vernodalin, vernolepin, vernoladol, vernolide, and vernomygdin), alkaloids, tannins, polyphenol, steroids, terpenoids, monoterpenes, quinines, and luteolin.[6] The presence of these metabolite compounds also contributes to the antioxidant activity. Flavonoid compounds, such as flavones and flavonols, can act as antioxidants. The antioxidant activity of flavonoids depends on the number and location of –OH groups, which act as free radical neutralizers and are related to

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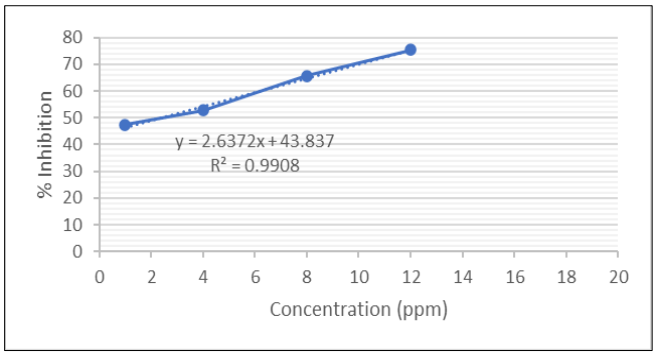

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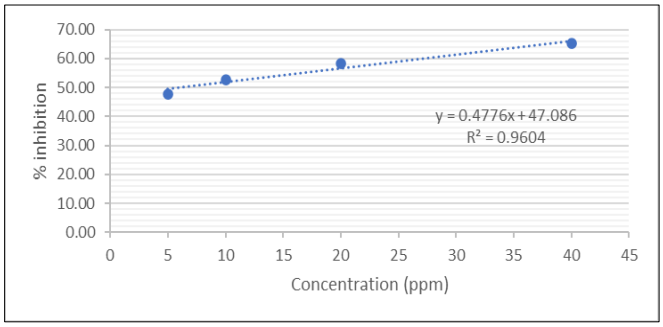


b

Figure 5: (a) Standard Curve of Vitamin C (b) Antioxidant Curve of Bitter Leaves Extract

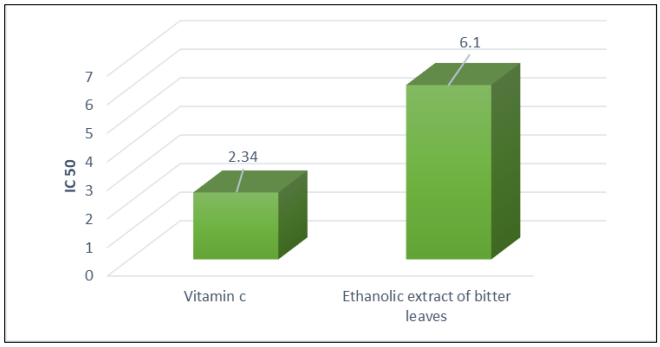
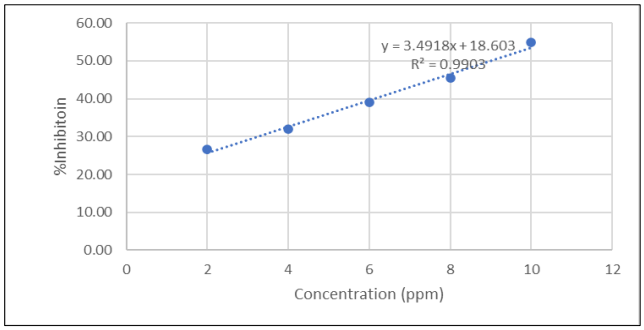


Figure 6: Antioxidant screening result

their ability to donate electrons. The stability of phenoxy flavonoid radicals (reactive oxygen) will reduce the speed of propagation of auto-oxidation chain reactions.[29] The anti-inflammatory activity against protein denaturation was determined by adding bovine serum albumin (BSA) solution as a substitute for live specimens. When BSA is heated, denaturation occurs. According to Nasution *et al*. [(29)], compounds that can stabilize proteins from the protein denaturation process are compounds that have the potential to be anti-inflammatory.[29] An interaction between BSA and the active compound results in the active substance bonding with tyrosine and lysine. When bonded to tyrosin, the active substance will not prevent BSA denaturation. This test used methanol extract of bitter leaves as a test sample and diclofenac sodium as a standard sample. Diclofenac sodium was chosen because it can block the COX-2 isoenzyme ten times greater than other NSAID drugs. The results of the anti-inflammatory activity are shown in Figures 7 and 8 for the extract and diclofenac sodium, respectively.

The inhibitory activity of protein denaturation of the bitter leaves' methanol extract was carried out at concentrations of 25 ppm, 50 ppm, 100 ppm, 200 ppm, and 400 ppm. Based on the linear regression equation between concentration (X) and % inhibition (Y), the IC_{50} value for bitter leaf methanol extract was 25.33 ppm and 8.99 ppm for diclofenac sodium. Both bitter leaf extract and diclofenac sodium have anti-inflammatory activity in the very strong category because the IC_{50} value was less than 50 ppm. However, the IC_{50} value of bitter leaf extract was not comparable to that of diclofenac sodium because the methanol extract of bitter leaf still has many compounds that can affect its activity.[31] This inhibition of protein denaturation is due to the presence of secondary metabolites in bitter leaf extract, which have the potential to act as anti-inflammatory because they can inhibit protein denaturation in the body caused by the formation of free radicals, which cause inflammatory mechanisms by stimulating the release of inflammatory mediators.



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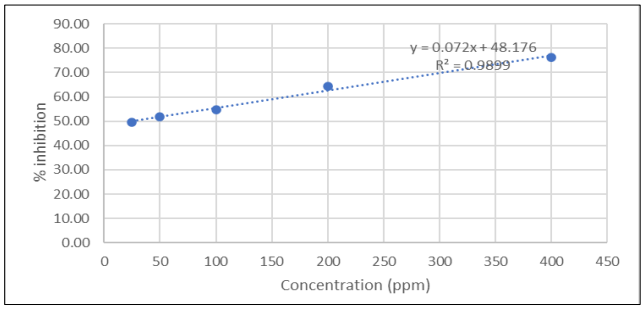

b

Figure 7: (a) Standard Curve of Diclofenac Sodium (b) Anti-inflammation Curve of Bitter Leaves Extract

According to research by Tian *et al*. [(31)], the interaction between phenolic compounds and glycine in the BSA protein triggered the increase in protein thermal stability.[31] The interaction of phenol with protein binding sites dramatically influences the secondary structure of proteins by creating a series of hydrophobic interactions further complemented by hydrogen bonds.[33] According to Vezza *et al*. [(33)], flavonoids directly inhibit the lipoxygenase pathway, inhibiting eicosanoid biosynthesis and activating free radicals that can attract various inflammatory mediators.[33] Tannins can influence the inflammatory response with their activity as free radical scavengers. Steroids can inhibit the release of prostaglandins from the source cells so that the formation of histamine, prostaglandins, and other chemical mediators that cause inflammation can be inhibited.[35] Secondary metabolite compounds that act as anti-inflammatory agents have hydroxyl groups (OH) that can protect membranes, inhibit mediators' release, and inactivate free radicals.

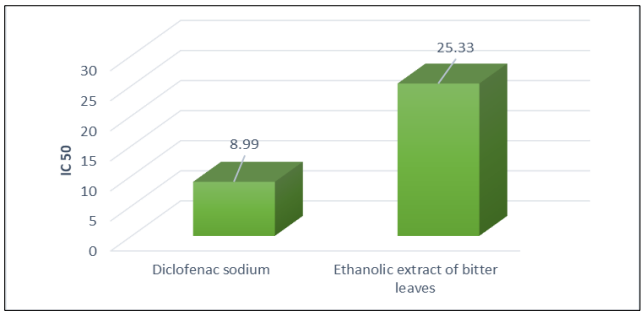

Figure 8: Anti-inflammatory Result

Protein denaturation is when proteins lose their tertiary and secondary structure by external compounds, such as strong acids or bases, organic salts, organic solvents, and heating.[31]

The compounds successfully isolated from bitter leaves include vernolide and vernodalol, which are sesquiterpene lactones in the phenolic group and belong to the terpenoid subfamily.[15] The anti-inflammatory mechanisms of sesquiterpene lactones include inhibition of the production of cytokines, lipid mediators, and other related molecules, modulation of pro-and antioxidant contents, and regulation of intracellular signalling pathways. Molecular docking was performed to determine and analyze the interaction between bioactive compounds as ligands and target proteins. The docking results showed that the bioactive compounds of bitter leaves have potential activity as anti-inflammatory agents based on the binding energy value, inhibition constant, and average bond distance at -10.15 kcal/mol, 0.036 µM, and 2.59 Å for vernolide and -9.97 kcal/mol, 0.076 µM and 2.11 Å for vernodalol against TNF-α protein. The activities against PTPs 1B are -8.14 kcal/mol, 1.09 µM, and 2.92 Å for vernolide; -6.80 kcal/mol, 10.53 µM and 2.52 for vernadalol. In addition, the inhibition constant (Ki) value is directly proportional to its energy; the lower the binding energy value, the smaller the Ki value, which means that the interaction between the ligand-protein complex will be more stable, which has a major impact on its activity. According to Dolgonosov (2017), the lower the binding energy, the higher the stability of the complex. The bonds formed by bioactive compounds with amino acids are hydrogen bonds with an average bond distance of <3.0 Å because the compound is hydrophilic, so it tends to increase its solubility. A good hydrogen bond interaction has a bond distance for both hydrogen donors and acceptors of <3.5 Å.[37] The bioactive compounds of bitter leaves exhibit anti-inflammatory potentials, as shown by in silico and in vitro studies, due to their rich antioxidant capacity. They act by inhibiting the cytokine TNF-*α*, a major regulator of the inflammatory response and involved in the pathogenesis of autoimmune diseases.[38] Overexpression of TNF-*α* can cause several diseases, including rheumatoid arthritis, colitis, psoriatic arthritis, psoriasis, and others.[39-40] In line with the TNF-*α* protein, PTPs 1B are also involved in various, a family of tyrosine phosphatase proteins involved in phosphorylation and disruption of multifactorial metabolic pathways.[44] diseases, including cancer, diabetes, autoimmune, and neurological diseases due to dysregulation of protein phosphorylation.[41] In the TNF-*α* protein, the bioactive compounds of bitter leaves form strong hydrogen bonds with the amino acid TYR119 (Figure 9). For PTPs 1B, it binds strongly to the amino acids LYS197, ASN193, and GLY277 (Figure 10). Xu *et al*. [41] *in silico* study showed that the active compounds curcumin glucoside, curcumin monoglucoside, and sophoricoside have the potential to inhibit TNF-*α* which forms hydrogen bonds and interacts strongly with the amino acids LYS11, LEU120, GLY121, and TYR151. Furthermore, Ganeshpurkar *et al*. [42] reported that the rutin compound forms strong interactions with the amino acids GLY121 and TYR121. In other studies, inhibition occurs in the PTPs 1B proteinSeveral molecular docking studies explain that inhibition of PTPs 1B is an essential target in influencing various signal transduction pathways,

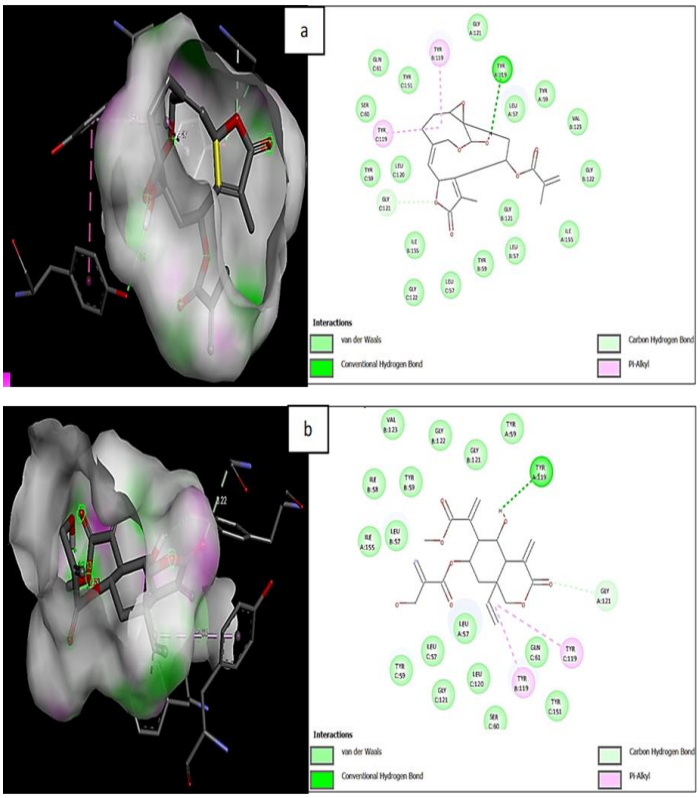

Figure 9: Interaction of Bitter Leaf Bioactive Compounds with TNF-α Protein; a) Vernolide and b) Vernodalol

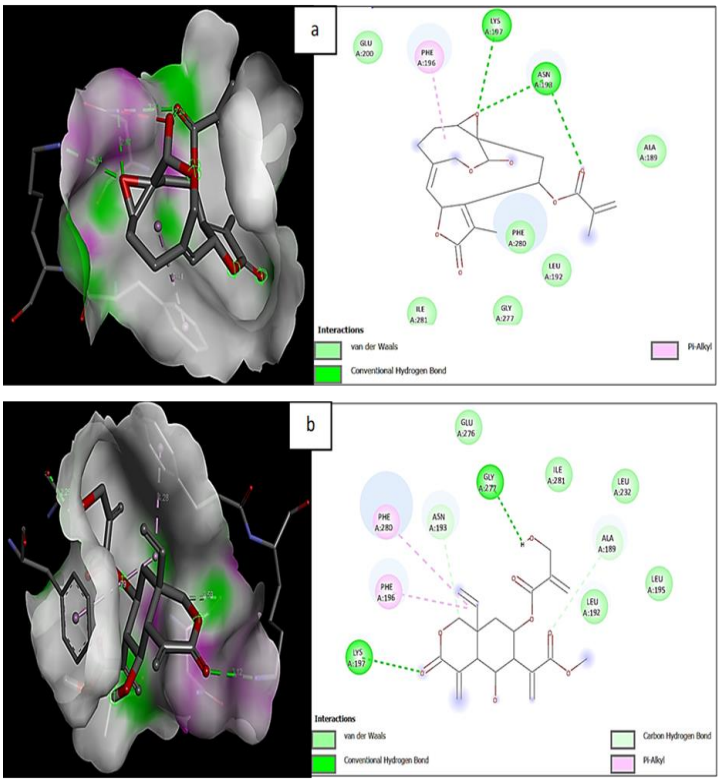

Figure 10: Interaction of Bitter Leaf Bioactive Compounds with PTPs Proteins; a) Vernolide and b) Vernodalol

including insulin and leptin receptors.[45] Hesperidin inhibits PTPs 1B activity by interacting with amino acids, LYS120, ARG221, PHE182, PRO180, GLN266, and GLY183[46,] and allosteric inhibitor compounds have the potential to inhibit PTPs 1B through hydrogen bond interactions with amino acids GLY183, SER216, GLN266.[47]

Conclusion

The methanol extract of bitter leaves contains secondary metabolites such as alkaloids, flavonoids, tannins, saponins, and steroids. This study also established that the extract has copious amounts of total phenolic and flavonoid compounds, which may have contributed to its biological activity. The extract also possesses potent antioxidants and very strong anti-inflammatory properties. *In silico* studies show that bioactive compounds have excellent anti-inflammatory activity with different binding energies, inhibition constants, and protein-ligand interactions related to antioxidant activity. The results showed that bitter leaf extract has the potential to be formulated into medicinal products.

Conflict of Interest

The authors declare no conflict of interest.

Author's Declaration

The authors hereby declare that the work presented in this article is original and that my liability for claims relating to the content of this article will be borne by the authors.

Acknowledgment

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