Toxicity and α-Amylase Inhibitory Potential of *Tagetes erecta* Leaf Extract: *In Vitro* and *In Silico* Approaches

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Abstract: Tagetes erecta *is one of traditional herbs with a variety of pharmacological actions. This study attempted to assess the toxicity and antidiabetic activity of* T. erecta *leaf extract. The extraction was carried out by maceration, then continued with phytochemical analysis. Toxicity of the extract was conducted using the brine shrimp lethality test. The antidiabetic activity was evaluated by α-amylase inhibitory using the 3,5-dinitrosalicylic acid method. The phytochemical of the most active extract was identified using GC-MS and subjected to bind the α-amylase (PDB ID: 2QV4) employing molecular docking. The* LC_{50} *values of* n-*hexane, EtOAc, and MeOH extracts were 33.41, 14.00, and 35.03 ppm, respectively, indicating high toxicity. The antidiabetic activity showed that EtOAc extract has the lowest* IC_{50} *value (1053.95 mg/L). Molecular docking analysis revealed the compounds* ***1****–****5*** *has range of binding energy at −4.07 to −4.83 kcal/mol. Acarbose as a positive control showed the lower binding energy at −5.03 kcal/mol, indicated more effective α-amylase inhibitory. This study revealed that* T. erecta *leaf extract has significant cytotoxic potential, which may warrant further exploration for anticancer applications. However, the relatively weak α-amylase inhibitory and lower binding affinity compared to acarbose imply limited utility as an antidiabetic agent.*

Keywords: *antidiabetic; molecular docking;* Tagetes erecta*; toxicity*

■ INTRODUCTION

Tagetes erecta is classified into the Asteraceae and the Tagetes genus, a traditional medicinal plant native to North and South America. This plant is known as Mexican marigold or Aztec marigold [1]. In Indonesia, *T. erecta* is known as kenikir [2]. This plant is used as an ornamental plant because it can thrive throughout the season [3]. In addition, *T. erecta* has a phytoremediation

ability to accumulate heavy metals such as zinc, cadmium, and lead in the environment [4].

Various studies have used parts of this plant (flowers, leaves, stems, and roots) as traditional medicine to treat different types of diseases such as haemorrhoids, kidney disorders, muscle pain, ulcers, wounds, earache, cough, scabies, and respiratory infections [5-6]. The plant exhibits a range of significant pharmacological activities, such as antidepressant, antioxidant, antipyretic, antidiabetic, hypolipidemic agent [7], antimicrobial, antifungal, antibacterial, cytotoxic, insecticidal, larvacidal, and mosquitocidal [8]. Therefore, with time, this plant has developed into a valuable resource in modern medicinal applications [9].

According to the research of George [10], the development of natural ingredients as medicinal plants needs to consider various aspects, especially in terms of safety. Therefore, it is necessary to conduct preclinical tests to determine the safety of *T. erecta* plants as medicinal plants. Toxicity testing is one type of test that can be performed. Toxicity tests are critical to this evaluation, employing biological activity tests with model organisms such as fish, mosquito larvae, and shrimp larvae to monitor mortality responses [11]. According to Olmedo et al. [12], one widely recognized method for assessing toxicity is the brine shrimp lethality test (BSLT), a technique noted for its simplicity, rapid execution, and cost-effectiveness. Initially developed by Meyer, this method employs *Artemia salina* larvae as the model organism. The toxicity level of the sample is expressed as the lethal concentration (LC_{50}) value through probit analysis [13].

Plant extracts with toxic effects are therapeutically valuable for developing chemopreventive therapy and treating diabetes [14-15]. Research conducted by Abdiwijoyo et al. [16] reported that the chemical contents contained in *T. erecta* leaf extract are alkaloids, flavonoids, glycosides, phenolics, terpenoids, and tannins. According to Masaenah et al. [17], plants containing phytochemical constituents such as glycosides, flavonoids, alkaloids, steroids, and terpenoids have the potential to reduce blood glucose levels. The antidiabetic activity of the chemical constituents of *T. erecta* leaf extract is determined by various mechanisms, such as inhibition of α-amylase enzyme activity. Inhibition of α-amylase enzyme activity can inhibit glucose metabolism, thus preventing an increase in glucose in the blood due to carbohydrate consumption [18-19].

Although *T. erecta* plant extracts, particularly those from the flowers, have been shown in numerous studies to have antidiabetic properties [20], the leaves of the plant extract have not been the subject of any research. In light of this knowledge gap, the present study aims to evaluate the toxicity level and potential antidiabetic effects of the extract from *T. erecta* leaves.

■ EXPERIMENTAL SECTION

Materials

The materials used in this study, including *T. erecta* leaf, *n*-hexane (technical grade), ethyl acetate (EtOAc, technical grade), methanol (MeOH, technical grade), α-amylase enzyme (Merck), hydrochloric acid (HCl, Merck), Mayer reagent (Merck), magnesium powder (Mg, Merck), iron(III) chloride ($FeCl_3$, Pudak), chloroform ($CHCl_3$, Merck), acetic acid (CH_3COOH, Merck), sulfuric acid (H_2SO_4, Merck), sodium chloride (NaCl, Merck), *A. salina* shrimp larvae, amylum 1% (Merck), 3,5-dinitrosalicylic acid powder (DNSA, Sigma Aldrich), dimethyl sulfoxide (DMSO, Merck), acarbose (Dexa Medica), distilled water, and 3D structure of protein that was downloaded from Protein Data Bank (http://www.rcsb.org.pdb) PDB ID 2QV4.

Instrumentation

Analysis bioactive compound of the promising extracts was carried out using gas chromatography-mass spectroscopy (GC-MS, Ultra Shimadzu GCMS-QP2010), UV-vis spectrophotometer (Shimadzu) used in the antidiabetic activity test, a set of computers for molecular docking analysis equipped with AutoDockTools 1.5.7 [21], Chimera [22], and Discovery Studio Visualizer programs [23].

Procedure

Extraction

The leaves of *T. erecta* were collected and allowed to dry at room temperature. The dried *T. erecta* leaves were ground into a powder and weighed. After the powdered

foliage was allowed to macerate gradually for 3×24 h in three different solvents, namely *n*-hexane, EtOAc, and MeOH, respectively. The crude extract was then obtained by evaporating the filtrate in a rotary evaporator [24].

Phytochemical test

Phytochemical tests of *T. erecta* leaf extracts were conducted to determine the class of secondary metabolites such as flavonoids, tannins, alkaloids, steroids/triterpenoids, and tannins. The test was carried out using Harborne's standard methods [25].

Toxicity test using the BSLT method

To prepare artificial seawater, 38 g of NaCl were dissolved in 1 L of distilled water [26]. The hatching apparatus consisted of a dual-compartment container with distinct dark and light sections featuring a 2 mm diameter hole to permit the passage of hatched *A. salina* larvae. Initially, the eggs of *A. salina* were placed in the dark compartment. After 48 h, larvae were collected from the light compartment for the BSLT experiment.

T. erecta leaf extract stock solutions were made by dissolving 50 mg of extract into 0.5 mL of DMSO, then diluted with distilled water until a concentration of 10,000 µg/mL was obtained. The stock solution (2 mL) was then diluted to make a final series concentration of 1 to 1,000 µg/mL after adding 2 mL of artificial seawater containing 15 *A. salina* shrimp larvae. The samples were incubated for 24 h, and toxicity was assessed based on the number of dead larvae [27-28]. Each concentration was tested in triplicate.

In vitro *α-amylase inhibitory studies*

The antidiabetic test of *T. erecta* leaf extracts was conducted using the DNSA method [29]. The *T. erecta* leaf extracts were treated in DMSO and then added to a pH 6.9 phosphate buffer solution to provide concentrations ranging from 1 to 1,000 mg/L. A 500 µL extract sample was combined with 500 µL of α-amylase enzyme solution, and the mixture was incubated for 10 min at 37 °C. Then 500 µL of 1% (w/v) amylum solution was added, and the mixture was then incubated once more. The extract was then reacted with 500 µL of DNSA reagent, heated for 5 min, cooled at room temperature, and added 1 mL of distilled water, then, the absorbance of the solution was measured using a UV-vis spectrophotometer (540 nm). Acarbose was used as a positive control in a similar technique, with 500 µL of phosphate buffer solution pH 6.9 used in place of the extract solution.

The inhibition (%) value was used to assess each extract's antidiabetic potential. To determine the IC_{50} value, the percentage inhibition value was plotted against the extract concentration value. Eq. (1) can be used to calculate the percentage inhibition value:

$$\alpha-\text{amylase inhib. (\%)} = \frac{\text{Abs}_{100\%\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{100\%\text{control}}} \times 100\% \quad (1)$$

GC-MS analysis of T. erecta *leaf extract*

Analysis of *T. erecta* leaf extract was performed by GC-MS. The compound components contained in the extracts were compared with retention times and mass spectra in the National Institute of Standard and Technology (NIST) database.

Molecular docking analysis

Preparation of the protein structure. Protein (receptor) structure was prepared employing AutoDockTools 1.5.7 by removing heteroatoms and water molecules, then adding polar hydrogen and Kollman charges. The structure was imported from the Protein Data Bank (https://www.rcsb.org/). At a resolution of 1.97 Å, the protein represents the 3D structure of human pancreatic α-amylase complexed with acarbose [30].

Preparation of ligands and molecular docking analysis. The ligand structure was prepared and optimized by Amber force field 14SB (FF14SB) using Chimera and saved in (.pdb) format. Then, Gasteiger partial atomic charges were added and saved in (.pdbqt) format for analysis using AutoDockTools 1.5.7. The grid centers used in docking were x = 12.631, y = 47.341, z = 26.244 (grid box: 50 × 50 × 50). The 2D and 3D structures were visualized using Discovery Studio Visualizer.

■ RESULTS AND DISCUSSION

Extraction

T. erecta leaves were extracted using three solvents: *n*-hexane (nonpolar), EtOAc (semipolar), and MeOH (polar). The highest percentage yield was produced by

the MeOH extract of *T. erecta* leaf compared to EtOAc (1.92%), and *n*-hexane (1.59%) extracts (Table 1). Differences in solvent polarity can cause differences in the amount of yield obtained [31-32]. The low yield obtained with *n*-hexane solvent is due to its non-polarity, so the attraction between molecules is weak, especially for compounds with different polarity than *n*-hexane solvent [33]. The compounds contained in *T. erecta* leaf extract are more easily extracted with solvents of the same polarity. Based on the extraction results, the highest yield is obtained with MeOH extract, indicating that the compounds contained in *T. erecta* leaf are mostly polar compounds. In addition, MeOH solvents with high polarity are able to attract nonpolar to polar compounds, thereby increasing the amount of extract yield produced [34]. The results are in line with Siddiqa et al. [35], where the content of compounds contained in the flower extract of *T. erecta* is soluble in polar solvents. In this study, water (polar) solvent produced the highest yield (13.86%) compared to *n*-hexane (13.12%) and EtOAc (9.64%) solvents.

Phytochemical Test

To determine the class of secondary metabolite in the extracts, phytochemical assays were carried out [36]. Table 2 presents the findings from the phytochemical analyses conducted on the three extracts. The *n*-hexane extract of *T. erecta* leaf showed mildly positive results for tannin, alkaloid, steroid, and triterpenoid components. Furthermore, the EtOAc extract of *T. erecta* leaf showed negative results for tannin, triterpenoid, and saponin compounds. MeOH extract showed the content of tannin and saponin compounds (highly positive), steroids (moderately positive), and alkaloids (mildly positive). Referring to the research of Abdiwijoyo et al. [16], the MeOH extract of *T. erecta* leaf contains alkaloid, flavonoid, steroid, terpenoid, saponin, and tannin compounds. Another research by Rajvanshi and Dwivedi [37], reported that *T. erecta* leaf extract contains phytochemical constituents such as flavonoids, terpenoids, alkaloids, and tannins.

Toxicity Test Using BSLT Method

The LC_{50} is used to measure the toxicity of the extracts [38]. The LC_{50} value category refers to the toxicity according to Nguta and Mbaria [39], namely 0–100 (highly toxic), 100–500 (moderately toxic), 500–1000 (low toxic), and > 1000 ppm (non-toxic). The toxicity of *T. erecta* leaf extracts is shown in Table 3. MeOH and *n*-hexane *T. erecta* leaf extracts were able to cause more than 40% larval mortality at extract concentrations of 31.25–500.00 ppm. In contrast, the EtOAc extract of *T. erecta* leaf showed more than 15% mortality of shrimp larvae at lower extract concentrations of 0.3125–80.00 ppm. The three extracts showed LC_{50} values of 33.41, 14.00, and 35.03 ppm, which are classified as highly toxic. Referring to the research of Chaniad et al. [40], who tested the toxicity of the *T. erecta* flowers extract, which showed less toxic effect. In this study, the leaf part of *T. erecta* was shown to be more toxic than the flower. The toxicity properties shown by the three extracts indicate that *T. erecta* leaf extract has potential as an anticancer agent. BSLT is a quick and economical initial screening method to prioritize plant extracts for further research on their anticancer properties. Its strong correlation with the

Table 1. Yield of *T. erecta* leaf extract

Extract	Extract weight (g)	Yield (%)
n-Hexane	10.80	1.59
EtOAc	12.83	1.92
MeOH	41.08	6.96

Table 2. Phytochemical test result of *T. erecta* leaf extract

Secondary metabolites	Test *T. erecta* leaf extract		
	n-hexane	EtOAc	MeOH
Flavonoids	–	+	–
Tannins	+	–	+++
Alkaloids	+	++	+
Steroids	+	+++	++
Triterpenoids	+	–	–
Saponins	–	–	+++

Note: (–) negative; (+) mildly positive; (++) moderately positive; (+++) highly positive

Table 3. Biological properties of *T. erecta* leaf extract

Extract	Toxicity (LC_{50} in ppm)	α-Amylase inhibitory (IC_{50} in mg/L)
n-Hexane	33.41	1656.76
EtOAc	14.00	1053.95
MeOH	35.03	7326.73

cytotoxic effects on human cancer cells (validated at a 95% confidence level in various studies) highlights its importance in natural product drug discovery. However, it cannot replace human cell-based testing to confirm therapeutic potential.

In Vitro α-Amylase Inhibitory Studies

The research aimed to investigate *T. erecta* leaf extract as an antidiabetic agent by inhibiting the activity of the α-amylase enzyme. The findings of phytochemical analyses showed that the extracts of *T. erecta* leaf each included varying secondary metabolites. Most plants with secondary metabolites, such as alkaloids, terpenoids, flavonoids, carotenoids, etc., have antidiabetic activity [41]. In according to Wang et al. [20], quercetagetin isolated purified from *T. erecta* flower can inhibit α-amylase activity with an IC_{50} value of 137.71 μmol/L. Research on the antidiabetic activity of the extract has been reported on another species of the same genus, *Tagetes minuta*. The results showed IC_{50} values of 7.8–26.9 μM [42].

The α-amylase inhibitory activity of *T. erecta* leaf extract is shown in Table 3. The EtOAc extract showed better inhibitory effect with an IC_{50} value of 1053.95 mg/L compared to *n*-hexane and MeOH extracts with IC_{50} values of 1656.76 and 7326.73 mg/L, respectively. Acarbose, as a positive control significantly had higher inhibitory effect than the three extracts with an IC_{50} value of 13.31 mg/L. The IC_{50} values are classified into several types, namely very strong (< 11 mg/L), strong (11–100 mg/L), and weak (> 100 mg/L) [43]. According to the IC_{50} value category, the three extracts of *T. erecta* leaf have antidiabetic activity, which is classified as weak. Previous studies revealed that the α-amylase inhibitory activity of *T. erecta* flower extract was comparatively low. Among the nine varieties of *T. erecta* evaluated, Yellow Queen 002 (YQ2) and Nata 001 (NT1) displayed the most substantial α-amylase inhibition, only exhibiting IC_{50} values of 2370 and 2570 mg/L, respectively. In contrast, the Sara Orange (SO) variety demonstrated the weakest inhibitory effect, with an IC_{50} value of 4870 mg/L [44].

Although the bioactivity data obtained are better than those reported previously, these results do not necessarily indicate that the EtOAc extract of *T. erecta* has promising pharmacological potential. One possible factor contributing to the observed bioactivity is the interaction between bioactive molecules within the extract. Based on this, it is necessary to analyze the bioactive compounds through molecular simulation.

GC-MS Analysis of *T. erecta* Leaf Extract

Analysis of chemical compound content using GC-MS was carried out on extracts that showed the best pharmacological activity. The EtOAc extract of *T. erecta* obtained the lowest LC_{50} and IC_{50} values of 14.00 ppm and 1053.65 mg/L, respectively, among the three extracts tested for toxicity and antidiabetic properties. The chromatogram of chemicals found in the EtOAc extract of *T. erecta* leaf is shown in Fig. 1. The chromatogram shows that there are 42 types of compounds with retention times ranging from 7.141 to 44.100 min. Table 4 shows information on the five main substances found in the EtOAc extract of *T. erecta* leaf. Referring to the research of Bahroi et al. [45], most of the chemicals found in *T. erecta* leaf extract were compounds **2** and **3**.

Compounds **3** and **5** have antiarthritic, anticancer, antimicrobial, and antioxidant activities [46-47]. According to several studies, there are anti-inflammatory, antioxidant, and anticancer pharmacological activities in

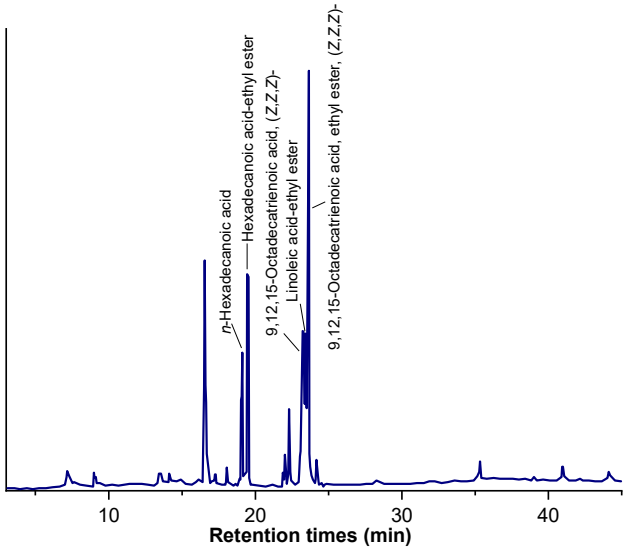


Fig 1. GC-MS chromatogram of EtOAc extract

Table 4. GC-MS data of five mayor compounds in EtOAc leaf extract of *T. erecta*

Compounds Name	Area (%)	Time (min)
n-Hexadecanoic acid	10.320	19.046
Hexadecanoic acid-ethyl ester	9.770	19.458
9,12,15-Octadecatrienoic acid (*Z*,*Z*,*Z*)	13.860	23.273
Linoleic acid-ethyl ester	7.690	23.423
9,12,15-Octadecatrienoic acid-ethyl ester	23.850	23.608

compound **1** [48-49]. Compound **2** also has anticancer activity [50], and compound **4** has anti-inflammatory activity [51]. The major components in the *T. erecta* leaf EtOAc extract exhibits anticancer activity. This is consistent with the results of the extract toxicity test, where *n*-hexane, EtOAc, and MeOH extracts of *T. erecta* leaf showed highly toxic results.

Molecular Docking Analysis

As detected by GC-MS, all major compounds were used as ligands in the docking study to decrease the activity of the α-amylase enzyme and were subjected to molecular docking analysis. Redocking the target receptor (2QV4) with its native ligand (AAO) is the first step in the docking procedure, which verifies the docking parameters that will be applied to the target molecule. Following redocking, the root mean square deviation (RMSD) value was 1.83 Å, with an inhibition constant of 605.35 μM and a binding energy value of −4.39 kcal/mol. The RMSD of redocking the native ligand was lower than 2 Å, an indication of the validity of the docking procedure [52]. The threshold ensures that the ligand's binding mode closely matches the experimentally determined structure, ensuring critical protein-ligand interactions are retained [53].

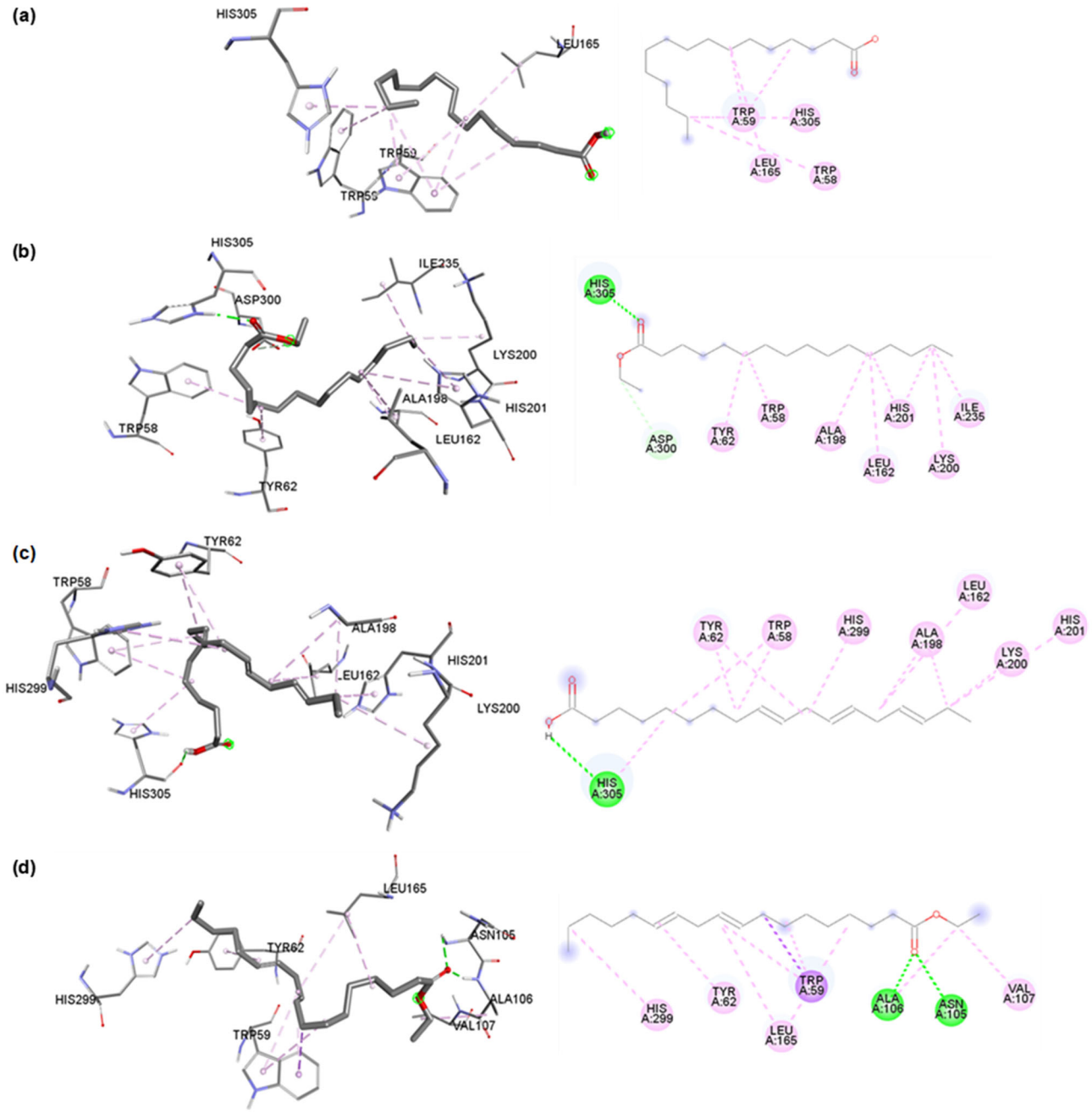
The docking results are shown in Table 5. Referring to the research of Thomas et al. [54], docking of compound **3** against α-amylase enzyme with different receptors has been reported. In this study, compounds **3**, **4**, and **5** have better binding energy and inhibition constant value against the 2QV4 receptor compared to the native ligand (AAO). More negative binding energy values indicate more stable interactions between the receptor and ligand. The small value of the inhibition constant indicates a stronger binding [55]. As a positive control, acarbose demonstrated a lower binding energy value (−5.03 kcal/mol) than the five compounds in the EtOAc extract, which indicates that acarbose is more effective in inhibiting α-amylase enzyme activity. This aligns with the outcomes of the *in vitro* analysis to evaluate the inhibitory potential of *T. erecta* leaf extract to inhibit the α-amylase enzyme.

According to Aksatha et al. [30], acarbose as a positive control has 13 hydrogen bonds with the α-amylase enzyme, namely Trp59, Tyr62, Gln63, Asn105, Ala106, Val107, His101, Thr163, Gly164, Arg195, Glu233, His299, and Asp300. In this study, docking against acarbose showed 6 similar hydrogen bonds, namely Gln63, Thr163, Arg195, Glu233, His299, and Asp300. In

Table 5. Binding affinities and potential interaction of 2QV4 receptor with five major compounds in EtOAc leaf extract, native ligand, and acarbose

Ligands	PubChem ID	Binding energy (kcal/mol)	Inhibition constants (μM)	Hydrogen bond interaction
1	985	−4.23	795.23	-
2	12366	−4.07	1030.00	**His305**
3	5280934	−4.83	290.24	**His305**
4	5282184	−4.62	412.35	**Asn105**, Ala106
5	5367460	−4.70	365.80	-
Native (AAO)	24755467	−4.39	605.35	**Asn105, Thr163**, **Glu233**, **His305**
Acarbose	41774	−5.03	204.21	Gln63, Thr163, Arg195, Asp197, **Glu233**, His299, Asp300

addition, the docking results of compound **4** also showed the same hydrogen bond interactions, namely Asn105 and Ala106. According to Chen et al. [56], the same type of hydrogen bonding between the native ligand and the test ligand against the target receptor indicates the ability to inhibit protein (receptor) activity by replacing the native ligand position with the test ligand. This is also evidenced by the binding energy value of the compound **4**, which is lower than that of the native ligand. The binding affinity of compound **4** toward the target protein does not significantly differ from that with acarbose. Despite the minimal difference in docking scores, the two compounds share overlapping binding sites, which may suggest a potential functional similarity. However, such an inference should be interpreted cautiously and supported by further experimental validation. The 2D and 3D interaction between acarbose and five major compounds as ligands with the 2QV4 receptor is shown in Fig. 2.



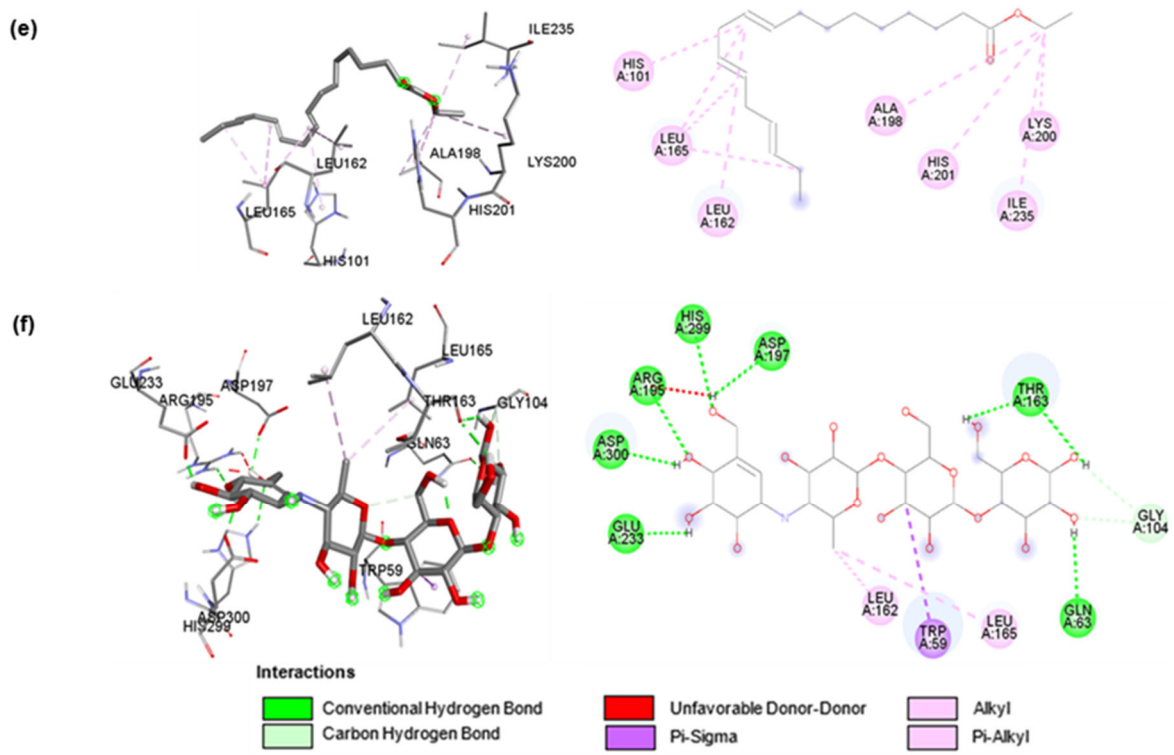


Fig 2. The 2D and 3D interaction between α-amylase enzyme and (a) compound **1**, (b) compound **2**, (c) compound **3**, (d) compound **4**, (e) compound **5**, and (f) acarbose

■ CONCLUSION

Tests on *T. erecta* leaf extract's toxicity and antidiabetic properties have been carried out. The percentage yield of each leaf extract in this study was 1.59% (*n*-hexane), 1.92% (EtOAc), and 6.96% (MeOH). The IC_{50} values shown by leaf extracts include 33.41 (*n*-hexane), 14.00 (EtOAc), and 35.03 ppm (MeOH), which indicates that the three extracts are very toxic. The antidiabetic activity test of the extracts showed IC_{50} values of 1656.76 (*n*-hexane), 1053.95 (EtOAc), and 7326.73 mg/L (MeOH), these values indicate that the antidiabetic activity of the extracts is classified as weak. This study is in line with the results of molecular docking analysis, where the binding energy value of major compounds contained in the extract is lower than the binding energy value of acarbose as a positive control. According to the investigation's findings, it is necessary to purify the compounds to reveal the prospects of *T. erecta* leaf as α-amylase enzyme inhibitors. Furthermore, because of its good toxicity, the *T. erecta* leaf extract can be used to determine another bioactivity like anticancer.

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■ CONFLICT OF INTEREST

The authors have no conflict of interest.

■ AUTHOR CONTRIBUTIONS

Herlina Rasyid conducted the research design, experimental, writing and overall supervision. Nunuk Hariani Soekamto, Bulkis Musa, and Siswanto contributed to literature search and data processing. Arniati Labanni, Artania Adnin Tri Suma and Nur Hilal A Syahrir contributed to involved in field works/analysis and critical review. Bahrun, Kadek Susi Badrawati, Mohammad Taufik Yusuf contributed to writing the manuscript, involved in field works/analysis.

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