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RESEARCH ARTICLE

Anti-inflammatory Activity of Polyphenols from *Labisia pumila* Leaves Extract

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Abstract Non-Steroidal Anti-inflammatory Drugs (NSAIDs) represent a class of pharmaceutical agents that are frequently misused and misconstrued within the medical landscape. While demonstrably efficacious in providing transient pain relief, NSAIDs do not effectively address the fundamental etiology of pain and are associated with a spectrum of potential adverse effects. *Labisia pumila* var alata also known as "Kacip Fatimah" has been traditionally used which is attributed to its antioxidant properties. Nonetheless, little attempt has been made to examine its antioxidant and anti-inflammatory characteristics. This study determined the molecular interactions and inhibitory activity profiles of *L. pumila* methanolic extract (LPE) against the corresponding enzymes via in chemico and in silico approaches. In chemico analysis was done on antioxidant activity and anti-inflammatory properties of *L. pumila*. The findings indicated that LPE exhibit the potent anti-inflammatory activity. Through high-performance liquid chromatography, it was shown that LPE had the highest gallic acid concentration at 10.74 ± 2.23 mg/mL. LPE did not exhibit cytotoxicity up to 100 µg/mL and displayed optimal protective against UVB-irradiation at 50 µg/mL towards HSF1184 Fibroblast cell line. LPE exhibited potent anti-inflammatory activity as it inhibited elastase and COX-2. Molecular docking studies indicated that gallic acid has a good affinity for collagenase (-5.68 kcal/mol), elastase (-4.88 kcal/mol) and COX 2 (-4.91 kcal/mol). These findings collectively suggested that *L. pumila* extract has significant potential for the formulation of the natural anti-inflammatory remedies which offer a safer alternative treatment for pain relief, especially for long-term use replacing the conventional NSAIDs medicines.

Keywords: *Labisia pumila*, Anti-inflammatory, Polyphenols, Natural pain reliever, Plant extract

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Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are frequently employed to reduce pain and inflammation. Aspirin, ibuprofen and celecoxib are among the commonly used NSAIDs. However, the prolonged and frequent use of these drugs can cause serious side effects such as peptic ulcer, perforation and gastrointestinal bleeding [1]. Although aspirin prevents platelet aggregation, which protects the heart, it also induces gastrointestinal bleeding [2]. Meanwhile, the administration of celecoxib has been found to potentially elevate the likelihood of cardiovascular thrombotic events, myocardial infarction, and stroke [3, 4]. Consequently, there has been a substantial increase in the utilization of complementary and alternative medicines as either a substitute for or adjunct to conventional pharmaceutical interventions within human society over the preceding decades.

Fortunately, several naturally occurring substances exhibit anti-inflammatory properties. For instance, it has been observed that omega-3 fatty acids possess the ability to impede the activity of pro-inflammatory molecules, including eicosanoids, cytokines, and reactive oxygen species (ROS) [5]. Besides that, medicinal plants and natural bioactive compounds have been explored due to its potent anti-inflammatory properties. Among many bioactive phytochemicals, phenolic acids such as punicalagin and curcumin has exhibited strong antioxidant and anti-inflammatory [6, 7] as these compounds serve as fundamental constituents for several substances that contribute to plant defence mechanisms against diseases, animals, and insects.

Labisia pumila var alata, popularly known as "Kacip Fatimah," has a long history of use by Malaysian women to facilitate childbirth and treat postpartum problems and is hence renowned as the "queen of plants" among Malaysian medicinal plants [8, 9]. *L. pumila* extract is rich in polyphenolic chemicals with anticancer and weight management benefits [10]. *L. pumila*'s value as a disease preventative is mostly attributable to its phytoestrogen, anti-inflammatory, and antioxidant properties [11, 12]. According to literature, gallic acid constitutes the predominant compound, constituting approximately 40% of *L. pumila* extracts [13] and has been observed to manifest distinct pharmacological effects, notably encompassing antioxidant, anti-inflammatory, anti-tumor, and anti-bacterial properties [14]. Thus, the aim of this research is to examine the potential of *L. pumila* var alata extract as a potent anti-inflammatory agent by investigating its inhibitory effect towards inflammation mediators MMPs (collagenase and elastase) and cyclooxygenase-2 (COX-2) activity through bio-enzyme assay and molecular docking interactions.

Materials and Methods

Materials

Labisia pumila (var alata) leaves was supplied by Delima Jelita Enterprise (Malaysia) (Batch No: KF031212). All solvents used in this study were HPLC grade and were purchased from Merck (Germany). Tris-HCl buffer and DMSO for in vitro assay were brought from Sigma-Aldrich (USA). COX-2 (human) Inhibitor Screening Assay Kit (Catalogue No: 701080) was supplied by Cayman Chemical Company (USA). Human elastase and N-Succinyl-Ala-Ala-Ala-p-nitroanilide (SANA) were procured from Sigma-Aldrich (USA). Sircol Collagen Assay Kit was purchased from Biocolor Ltd. (Northern Ireland). Standards in this study were ascorbic acid, oleanolic acid (≥97.0%), gallic acid (≥97.9%), catechin (≥97.0%), cinnamic acid (≥97.0%) and caffeic acid (≥97.0%) were provided by Sigma-Aldrich (USA).

Preparation of Polyphenol Rich Extracts

The extraction of polyphenols from *L. pumila* was performed according to Kammerer *et al*. [15] with slight modification. Initially, 8 g of grounded leaves of *Labisia pumila* was extracted using 200 ml of methanol while continuous stirring using magnetic stirrer at 30°C for 1 hour. The extract was filtered and the residue was extracted again using fresh 200 ml of methanol for another 30 minutes. This step was repeated 3 times. All the supernatants were combined, concentrated using a rotary evaporator at 40°C, dried in an oven at 50°C. The dried crude extract of *L. pumila* (LPE) was stored at -20°C until further analysis.

Quantification of Phytocompounds using HPLC

The HPLC method was carried out according to Wittenauer *et al*. [16] by using Waters, e2695 Separation Module. The detailed parameters of HPLC are presented in Table 1. Identification of polyphenols was accomplished through UV/Vis spectra and/or comparison with literature data and calibration curves of the reference compounds: gallic acid, catechin, caffeic acid and cinnamic acid. All determinations were carried out in triplicates.

Table 1. HPLC parameters for detection of polyphenol in crude extract of *L. pumila* leaves

Parameters	Specification
Injection volume	10 µL
HPLC column	Synergy-Hydro column 150 mm × 3.0 mm × 4 µm
Mobile phase	A= 2 % acetic acid in water
	B= 0.5 % acetic acid in 1:1 ratio of water and acetonitrile
Gradient system	At 0 min 0-5% B
	At 36 min 5-20% B
	At 81 min 20-100% B
	At 111 min 100% B
	At 114 min 100-0% B
Flow rate	0.8 mL/min
UV detector	280 nm

Total Phenolic Content

The quantification of the total phenolic content (TPC) in the LPE was conducted using the Folin–Ciocalteau (FC) colorimetric method in a 96-wells plate, as outlined by Idris *et al*. [17] with some modifications. Approximately 100 µL of LPE was added with 500 µL of FC reagent (10% v/v) and 1.5 mL of sodium bicarbonate (60 g/L). The mixture was incubated for 2 h, before its absorbance was recorded at 720 nm using microplate reader (BioTek ELx808). Gallic acid was employed to calibrate a standard curve. The TPC of the LPE was quantified in mg of gallic acid equivalents (GAE) per gram of sample.

Cytotoxicity Assay

The cytotoxicity of LPE was evaluated using the MTT (3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide) assay on human skin fibroblast cells (HSF 1184) with some modifications [18]. The cells were inoculated into each of 96-wells at a concentration of 5×10^3 cells and incubated for 24 h to allow the cells to achieve 80% confluence. The experimental procedure commenced by introducing LPE at various concentrations spanning from 0 to 1000 µg/mL. After 24 h treatment, the MTT solution was administered and subjected to a 4 h incubation period. Subsequently, the formazan derived from the developed MTT assay was solubilized in dimethyl sulfoxide and subjected to spectrophotometric analysis at a wavelength of 570 nm.

Bio-enzyme Assay

Elastase Assay

Inhibition of human neutrophil elastase (HNE) by LPE was assayed spectrophotometrically based on the release of p-nitroaniline chromophore from SANA substrate and in accordance with the method described by Satardekar and Deodhar [19] with some modifications. The experiment was performed according to the following steps: 50 µl of Tris-HCl buffer were dispensed in each well of 96 well microplate, 25 µl of enzyme solution (HNE) were added, 25 µl of LPE (6.25, 12.5, 25 and 50 µg/ml) were added and homogenously mixed, incubated for 30 min, 100 µl of substrate solution (SANA) were dispensed in the well, and finally the absorbance is monitored in a microplate reader for 60 min at 405 nm. Oleanolic acid was used as positive control. The blank sample was prepared without the addition of enzyme solution (HNE). The percentage of inhibition of HNE activity by LPE and oleanolic acid were calculated according to the Eq. 1.

$$Inhibition\ of\ enzyme\ (\%) = \frac{(A-B)}{A}\ x\ 100 \qquad \text{(Eq. 1)}$$

Where A indicates the enzyme activity without inhibitor (negative control) and B is the activity in the presence of inhibitor.

Cyclooxygenase-2 (COX-2) Assay

The inhibition of COX-2 activity by LPE was performed according to manufacturer manuals. The sequence of the experiment was as followed: 160 µl of RB1X were pipetted to all of the well, 10 µl of Heme were added, 10 µl of COX-2 were pipetted to all of the wells except for Blank wells (for Blank wells inactivated COX-2 was used instead), 10 µl of sample inhibitor were added to the well accordingly (for Blank and negative control, 10 µl of dH_2O was used instead), the reaction mixture was incubated for 10 min at 37 °C, 10 µl of arachidonic acid substrate were added to all wells, incubated for 2 min at 37°C, 30 µl of stannous chloride ($SnCl_2$) were pipetted to all of the wells, incubated for 5 min and finally, the absorbance reading was taken at 412 nm using microplate reader for 60 min. Aspirin was used as positive control. The blank sample was prepared without the addition of the active enzyme. The percentage of inhibition of COX-2 activity by LPE and aspirin were calculated using Eq.1.

Sircol Collagen Assay (SCA)

The SCA procedure was performed according to manufacturer manuals. The experimental manual is summarized into: collagen-extracted sample from 1 mL of LPE-treated HSF 1884 was added with 1 mL of Sircol Dye Reagent in microcentrifuge tube, mixed using vortex for 30 min, centrifuged at 13000g for 10 min, supernatant was carefully drained, washed with ice cold diluted acid-salt wash reagent, centrifuged, added with 250 µl of alkali reagent and vortexed to recover the dye-bound collagen. The spectrophotometric readings were observed at 555 nm on a microplate reader. Collagen content of the sample was calculated based on the standard calibrated curve generated from collagen type I standard in the range 5–100 µg per 0.1 ml.

Molecular Docking

The preparation of ligands and proteins was conducted following a procedure similar to that outlined by Rabiu *et al*. [20]. The 3-dimensional chemical structures of gallic acid (CID: 370), ascorbic acid (CID: 54670067), oleanolic acid (CID: 10494), and aspirin (CID: 2244) were obtained from the PubChem database. The X-ray crystal structures of COX-2, elastase, and collagenase with PDB ID of 3LN1, 1BRU and 2TCL, respectively, were acquired from the RCSB Protein Data Bank. These structures were then processed using AutoDockTools 1.5.6. Grid maps were generated with a grid spacing of 0.375Å in the x, y, and z-dimensions, consisting of 40×40×40 points. The grid box parameters employed for all target proteins were listed in Table 2 which were inspired by Rabiu *et al*. [20] and Mechqoq *et al*. [21] with some modifications. The Lamarckian genetic algorithm was employed and the parameters were defined according to the Othman *et al*. [22]. The docking process was conducted by utilizing Autodock 4.2.6. The clustering of the output was performed using a root-mean-square deviation (RMSD) tolerance of 2.0 Å. The outcomes of hydrogen bonding, hydrophobic interactions, and electrostatic interactions were acquired through the utilization of BIOVIA Discovery Studio Visualizer v20.1.0.19295. For self-validation, the inhibitor compound which was present in the crystallized enzyme, was extracted independently and docked again to its enzyme. Self-validation level was measured based on the RMSD value of the docked ligand and original crystal ligand, where below than 2 Å indicates good, between 2 and 3 Å is considered acceptable while more than 3 Å is bad and not acceptable [23, 24].

Table 2. Grid box coordinates for molecular docking of ligands and macromolecules (elastase, COX-2 and MMP-1 collagenase)

Enzymes (PDB ID)	Coordinates		
	X	Y	Z
Elastase (1BRU)	23.204	47.660	17.090
COX-2 (3LN1)	31.724	−22.006	17.132
MMP-1 collagenase (2TCL)	73.818	8.730	9.114

Statistical Analysis

The assay was performed three times with triplicate parallel samples. All values were expressed as mean ± standard deviations. The IC_{50} values (which is the concentration of LPE that required for 50% inhibition of elastase/ COX-2 *in vitro*) were obtained from dose-response curves by logarithm regression. The quantitative results were analyzed by Microsoft Office Excel and GraphPad Prism 7.0 software.

Results and Discussion

Phytochemical Characterization

Prior to the anti-inflammatory assays, the identification and quantification of the polyphenolic compounds by HPLC was prerequisite to delineate the phytocompounds present in the LPE which potentially be responsible for the anti-inflammatory effects. Phenolic compounds are secondary metabolites which are ubiquitously distributed in the terrestrial plants [25]. The considerable interest in phenolics stems from their perceived potential benefits to human health. The antioxidative mechanism inherent in phenolics predominantly arises from their redox properties, facilitating their role as effective reducing agents. Karimi *et al*. [12] reported the presence of gallic acid and caffeic acid in all of the components from the three species of *L. pumila*. In concordance with previous literatures, the most prominent and major polyphenols of the *L. pumila* in this present study is gallic acid with concentration of 11.54 ± 2.45 µg/mL as shown in Table 2. Meanwhile, catechin, caffeic acid and cinnamic acid were found to be minor compounds (Figure 1). The results align to the other studies where leaf extract of *L. pumila* exhibited high phenolic content, comprising of gallic acid as the major phytochemical compound [26,27]. Phenolic compounds present in *L. pumila* have been reported to be responsible for the anti-inflammatory, antimicrobial and antioxidative properties of this plant [11, 12, 26, 28].

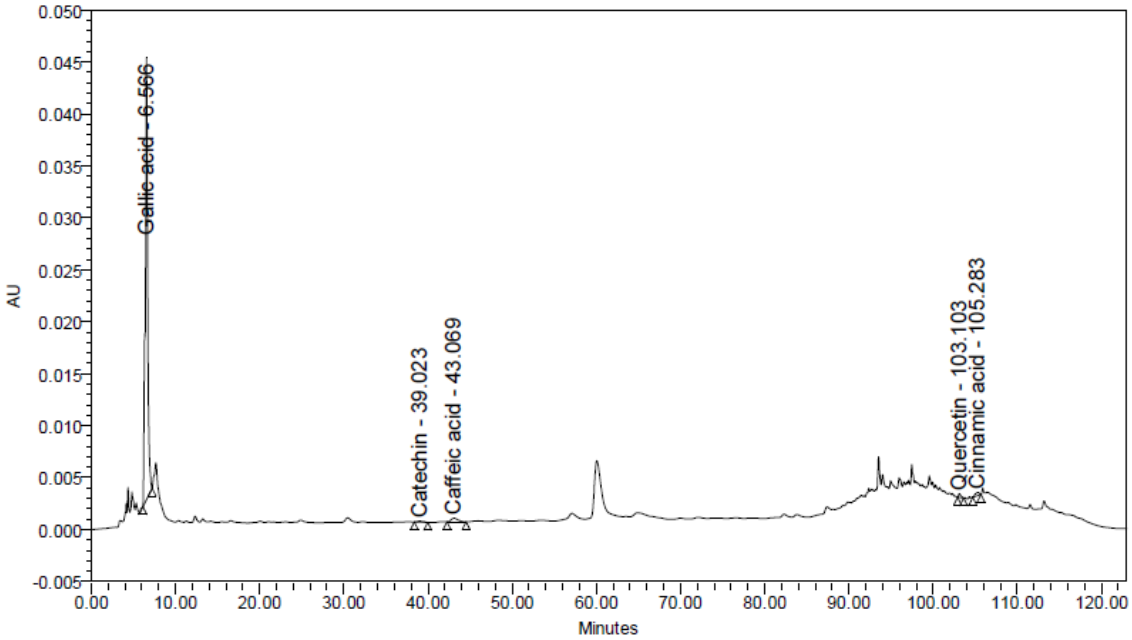

Figure 1. HPLC chromatogram of polyphenols present in crude *L. pumila* extract, LPE (280 nm). Peak assignment: (1) gallic acid, (2) catechin, (3) caffeic acid, (4) cinnamic acid were determined using reference compounds acquired from supplier

Table 3. HPLC parameters for detection of polyphenol in crude extract of *L. pumila* leaves

No.	Retention time (min)	Identity	Concentration (µg/mL) of *L. pumila* crude extract (LPE)
1	6.6	Gallic acid	11.54 ± 2.45
2	38.1	Catechin	3.98 ± 0.11
3	46.1	Caffeic acid	0.91 ± 0.82
4	103.2	Cinnamic acid	0.63 ± 0.04
		Total amount	17.06 ± 3.25

Results displayed in mean value of triplicates measurements ± standard deviation.

The HPLC result was further corroborated by the high total phenolic content (TPC) of LPE which was 148.69 ± 26.49 mg GAE/g. The TPC result demonstrated that the *L. pumila* exhibited a substantial content of (poly)phenolic compounds [11, 26]. Phenolic compounds are a class of cyclic organic compounds that possess one phenol unit while polyphenols possess multiple phenol unit. Gallic acid is an example of simple phenolic compound that has multiple hydroxyl groups. Catechin, caffeic acid and cinnamic acid are polyphenols. The high total phenolic contents of *L. pumila* leaves were also observed in the study by Awang *et al*. [28] which were 120 and 90 mg GAE/g from water and ethanol, respectively as solvent extraction. Hence, the HPLC and TPC results delineated that LPE has high phenolic content where the major compound of LPE is gallic acid.

MTT Bioassay

LPE with concentration ranging from 10 to 100 µg/ml were applied to the treated and untreated HSF cells with 5 µg/ml ascorbic acid was used as positive control and serum free media (SFM) act as control guidelines. All concentrations of the samples show the capability of increasing the cells viability upon extract treatments as compared with control (SFM) as shown in Figure 2(a). This is a good indicator of showing that LPE did not cause any toxicity activity towards HSF 1184. It also can be seen that the increased in cell viability upon treatment with extract is comparable with positive control ascorbic acid activity however the most prominent optimum extract was found to be at the concentration of 50 µg/ml. Test on binucleated cultured human peripheral blood lymphocytes revealed no cytotoxicity from exposure to *L. pumila* extract at high concentration of 250 µg/mL [27]. This result is supported by acute and subacute toxicity on rats where minimal toxicity was observed at high dose of 1000 mg/kg body weight per day [29]. Meanwhile, to study the restorative and proliferative activity of the extract, the HSF cells were irradiated with UVB before treated with the respective treatments and controls. Figure 1(b)

shows the UV-treated HSF cell's viability when treated with LPE with concentration ranging from 10 to 100 µg/ml. The control (SFM) cell's viability decreased by approximately 60% when irradiated with UV light. The exposure to the crude extract of *L. pumila* at all tested concentrations managed to restore and decrease the effect of the UV irradiation towards the cell viability. The cell viability remained high at more than 75%. At 50 µg/ml of crude extract treatment, the cell viability was the highest than other concentrations and comparable to the positive standard of ascorbic acid. The UV-protective capability of the extract can be attributed by its major compound, gallic acid. Gallic acid has been proven to be UV-protective for the cells as it inhibits melanogenesis and overcomes pigmentation [30]. In a similar study, *Labisia pumila* (Myrsinaceae) exhibits anti-photoaging capability by inhibiting TNF-α production, reducing COX-2 expression and downregulating MMP-1 expression [11]. Since the most optimal potent concentration is at 50 µg/ml, this concentration was selected to be used in the following assays. to evaluate the anti-inflammatory activity of the extract.

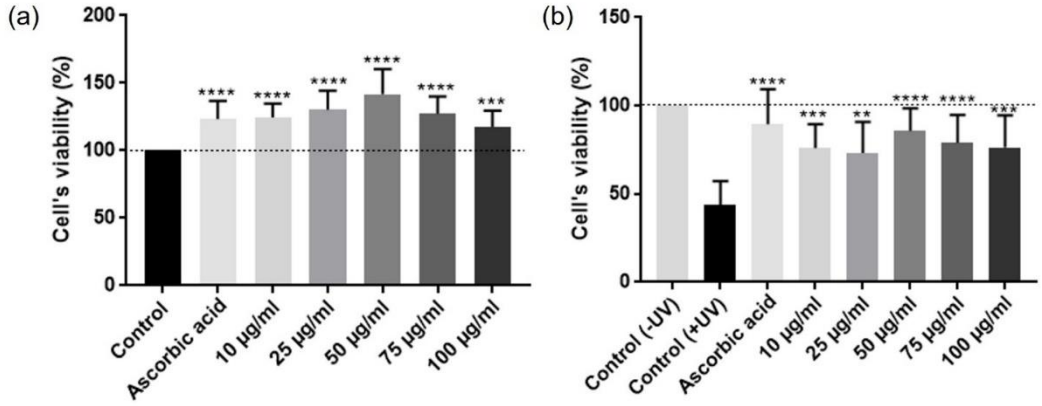

Figure 2. HSF cells viability percentage when treated with positive control, (ascorbic acid, 5µg/ml), *L. pumila* crude extract and its fractions with concentration ranging from 10 to 100 µg/ml (a) without treatment of UV and (b) with treatment of UV. Control indicates control serum free media (SFM). Values are expressed as mean cell viability (%) ± standard deviation. Asterix (*) mean the results were significantly different compared to (a) Control and (b) Control (+UV) indicated by ** P ≤ 0.01 *** P ≤ 0.001 **** P ≤ 0.0001

Anti-inflammatory Activity of *L. pumila* Extracts

For elastase inhibition, LPE with an increasing concentration of 6.25, 12.5, 25 and 50 µg/ml were used to further investigate their inhibitory effect, establish dose-dependent relationships and calculate the half maximal inhibitory concentration (IC_{50}). The elastase inhibition assay was measured according to the quantity of p-nitroaniline (chromophore) products based on elastase enzyme activity [31]. The inhibition effect of human neutrophil elastase activity by oleanolic acid and LPE is presented in Figure 3(a). Both LPE and oleanolic acid exerted an elastase inhibitory effect in a dose-dependent manner. Oleanolic acid demonstrated higher elastase inhibition activity than LPE. Relative inhibition of elastase activity of LPE was 24.29% at a concentration of 6.25 µg/ml, and the inhibition percentage further increased at higher concentration to 75.23% at concentration of 50 µg/ml. However, the value was slightly lower than oleanolic acid at concentration of 50 µg/ml which was 80.74%. Nevertheless, based on the results, LPE was observed to inhibit human elastase activity by more than 70% at concentration of 50 µg/ml is comparable to the positive control in this study. As shown in Table 2, the IC_{50} of oleanolic acid was similar to a report by Vanjare *et al*. [32] while the IC_{50} value for LPE of 19.90 ± 1.00 µg/mL was slightly higher than the oleanolic values. Both values were obtained by logarithm curve fitting indicated the concentration of the samples at which 50% of elastase activity was inhibited. The lower the IC_{50} value indicates better efficacy of the extract to inhibit elastase activity. This result was coherent with preceding studies reporting the ability of the natural plant extracts to inhibit elastase activity was due to the presence of polyphenols in it [16, 33]. Elastase inhibitory effect of 22 Korean traditional herbal medicine was conducted by Kim *et al*. [34] and they found out that among those herbal medicines tested, Rhus vernciflua stokes (RVS) inhibit significant elastase and tyrosinase activity. In the present study, it demonstrates human elastase inhibition activity by *L. pumila*, a traditional Malaysian medicinal plant for the first time.

Table 4. Inhibition concentration (IC_{50}) of positive controls (oleanolic acid and aspirin) and crude extract of *L. pumila* leaves (LPE) on human elastase and cyclooxygenase-2 (COX-2) inhibitory activity

Samples	Inhibition concentration (IC_{50}) (µg/mL)
Elastase	
Oleanolic acid*	13.03 ± 0.13[a]
Crude extract	19.90 ± 1.00[b]
COX-2	
Aspirin*	19.18 ± 3.22[a]
Crude extract	22.18 ± 5.16[a]

Results displayed in mean value of triplicates measurements ± standard deviation. Different letters mean results were significantly different among the samples at $p \leq 0.05$. *Oleanolic acid and aspirin were the positive controls for elastase and COX-2 inhibition assays, respectively.

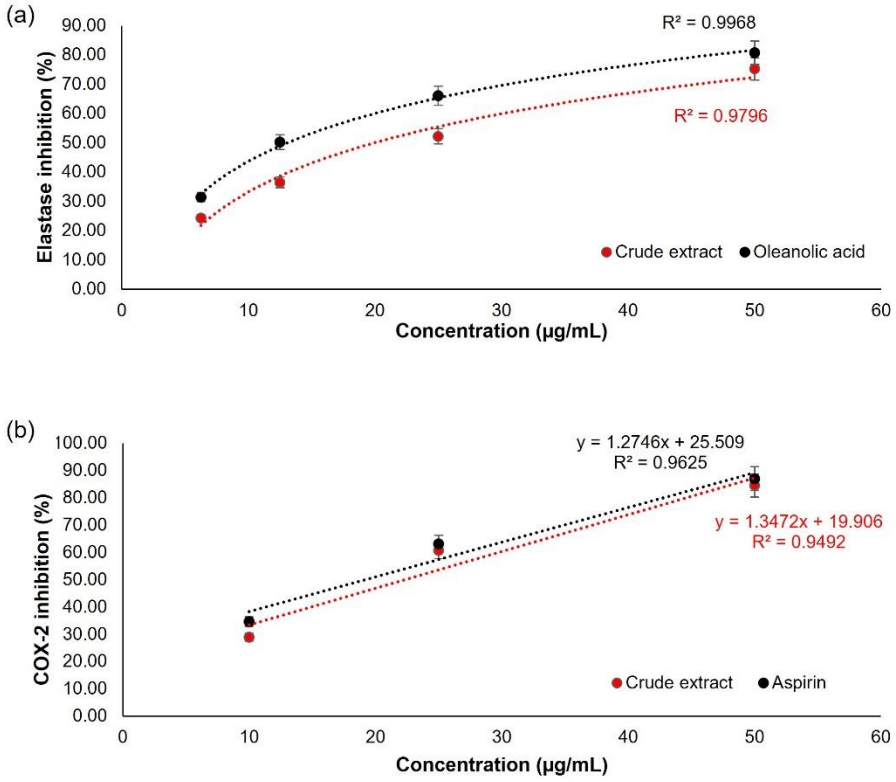

Figure 3. The inhibitory effect of crude extract of *L. pumila* leaves (LPE) and positive control (oleanolic acid or aspirin) on (a) elastase and (b) cyclooxygenase-2 (COX-2) activity. Each point represents the mean value of triplicates measurement

Cyclooxygenase (COX) is a bifunctional enzyme that exhibits both COX and peroxidase activities that catalyzes the conversion of arachidonic acid to prostaglandin (PGH2), the precursor of PGs and thromboxanes. COX-2 is primarily associated with inflammatory mechanisms as it works only under inflammatory conditions. Phenolic compounds e.g. benzoic acid have been proposed among the effective natural inhibitors [20]. In the present analysis, the anti-inflammatory activities of LPE was evaluated based on COX-2 inhibition. The peroxidase activity of COX-2 is assayed by monitoring the release of oxidized chromophore substance which is N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) at 412 nm [35]. LPE and aspirin were evaluated at increasing concentration of 10, 25 and 50 µg/ml in order to plot the dose-response curve and to determine the half maximal inhibitory concentration (IC_{50}) of the samples [36]. LPE and aspirin inhibited the COX-2 enzymatic activity in dose-dependency manner

as shown in Figure 3(b) as higher concentration of the samples induced more inhibition of COX-2. In term of IC_{50}, LPE (22.18 ± 5.16 µg/mL) showed comparable COX-2 inhibitory effect to the aspirin which showed only slightly better performance (19.18 ± 3.22 µg/mL). Choi *et al*. [11] revealed that the treatment of *L. pumila* extract to UVB-exposed human skin keratinocytes significantly decreased the expression of COX-2 and inhibited the production of TNF-α. Overall, the findings of this study presented enough evidence indicating that the LPE possessed potent anti-inflammatory activity via the inhibition of elastase and COX-2 enzymatic activity, demonstrating comparable performance to their respective positive controls.

Total Collagen Content

To examine the effect of LPE on the collagenase inhibition, indirect assay was done by measuring the soluble collagen content in human dermal fibroblasts which were irradiated with UVB at 20 mJ/cm^2, followed by treatment with LPE for 24 h. UVB induces increase in ROS, subsequently leading to higher expression of MMPs e.g. MMP-1 [37], breaking down collagen [38] and thus lower the detection of soluble collagen content by the Sircol collagen assay. In this study, UVB irradiation affected the collagen synthesis of HSF cells as its collagen content reduced by 51% compared to the non-UVB irradiated cells, thus highlighting the adverse impact of UVB on the human skin cells. Based on Figure 4, LPE significantly increased the level of collagen content compared to UV-irradiated cells control to a comparable value as non-UV-irradiated cells level. The LPE treatment showed slightly higher collagen level than ascorbic acid. The results indicated that the treatment of LPE and ascorbic acid were able to retain the collagen synthesis activity in UV-irradiated cells to a similar level prior to UVB treatment. Both samples might act as MMP-1 collagenase inhibitors, thus preventing the subsequent breakdown of collagen. It might also attributed to the antioxidant activity of polyphenols in LPE and ascorbic acid that scavenged the UVB-induced ROS, thus reducing damages to the TGF-β signaling pathway [38]. Ascorbic acid has been identified to increase collagen synthesis, reduce collagen degradation and inhibit collagenase. [39]. As proven previously through the HPLC analysis, gallic acid as the main/major bioactive compound in the LPE may act as UV-protective for collagen synthesis pathways via collagenase inhibition or antioxidant to scavenge the ROS in the UV-irradiated cells [16]. Thus, the LPE reduces UV-induced inflammation by exhibiting anti-collagenase activity. Further study on the possible inhibition mechanism of collagenase was performed using molecular docking approach.

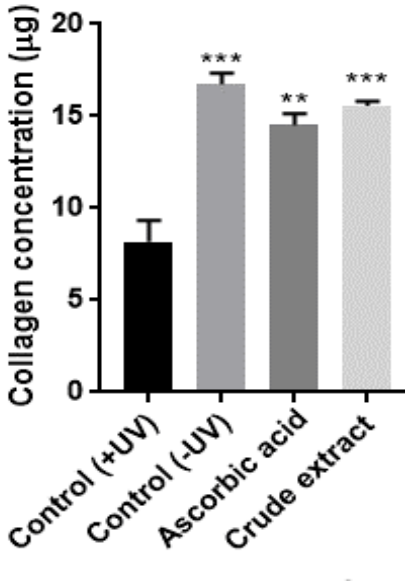

Figure 4. HSF cells viability percentage when treated with positive control (ascorbic acid, 5µg/ml), *L. pumila* crude extract (LPE) with concentration of 50 µg/ml after treated with UV at 20 mJ/cm^2. Values are expressed as mean cell viability (%) ± standard deviation. Asterix (*) mean the results were significantly different compared to Control (+UV) indicated by ** P ≤ 0.01 *** P ≤ 0.001

Molecular Docking

Molecular docking was performed to predict anti-inflammatory activity of gallic acid through inhibition of COX-2, elastase and collagenase enzymes. Gallic acid was selected as the ligand as it constitutes the

predominant compound of LPE as evidenced by the HPLC result and preceded study (Yeop *et al*., 2021). Table 5 represents the molecular docking results which include lowest and binding energy, frequency of the clusters out of 100 runs, hydrogen and pi bond interaction formed between the chemical compounds and the target enzymes. As per the docking results acquired, it is discerned that gallic acid established a stable ligand-protein complex with the target enzymes, as evidenced by its negative binding energy. In addition, the conformation of the GA for each enzyme represents the major cluster making up of at least 59 runs (elastase) out of 100 runs simulation. It means that similar conformation in similar position was frequent and highly probable to happen.

Table 5. Binding affinity of gallic acid and its bonding interaction towards collagenase, elastase and cyclooxygenase 2 (COX-2)

Compound	LowestBE (Kcal/mol)	Mean BE (Kcal/mol)	Number in cluster	Hydrogen bond	Pi bond
Elastase (1BRU)					
Oleanolic acid	-5.52	-5.49	100	His57, Ser217	-
Gallic acid	-4.88	-4.45	59	Ser190,Ser195, Ser226	Ser190,Gly216, Cys220
COX-2 (3LN1)					
Aspirin	-4.79	-4.64	96	Ser516	Tyr371, Phe504, Met508, Gly512
Gallic acid	-4.91	-4.53	61	Gln178,Leu338, Tyr341, Arg499	Ala502, Val509
MMP-1 Collagenase (2TCL)					
Ascorbic acid	-4.74	-3.74	38	Leu81, Ala82, His118,Glu119, Tyr137	-
Gallic acid	-5.68	-5.19	86	Gly79, Leu81, Ala82, Tyr137, Tyr140	-

BE: binding energy

For elastase 1BRU, superimposition of docked GR143783 on the crystal inhibitor ligand within the protein validated the grid with RMSD of 2.34 Å. The RMSD value was acceptable solution, although conformation deviated from the reference but the compound retained the desired orientation [23]. Docked complex of elastase-oleanolic acid showed the lowest docking energy of −5.52 kcal/mol while −4.88 kcal/mol in the elastase-gallic acid complex. The binding energy of oleanolic acid is contributed by its one conventional hydrogen bond (Ser217) and its van der walls interaction. Meanwhile, gallic acid formed four hydrogen bonds with three amino acid residues of Ser190, Ser195 and Ser226 as well as polar interactions with Ser190, Gly216 and Cys220 as shown in Figure 5. These residual interactions have also been observed by Desmiaty *et al*. [40] as they docked *Rubus rosifolius* leaves extract and its constituents. Binding to Ser195 residue plays an important role as it belongs to the conserved catalytic triad of elastase besides His57 and Asp102 [41]. Additionally, His57, Ser189, Cys191, Asn192, Gly193, Asp194, Val213, Phe215, Ser217, and Val227 residues showed hydrophobic affinities for gallic acid which similarly to the report by binding formation of caffeine towards elastase (Eun Lee *et al*., 2019).

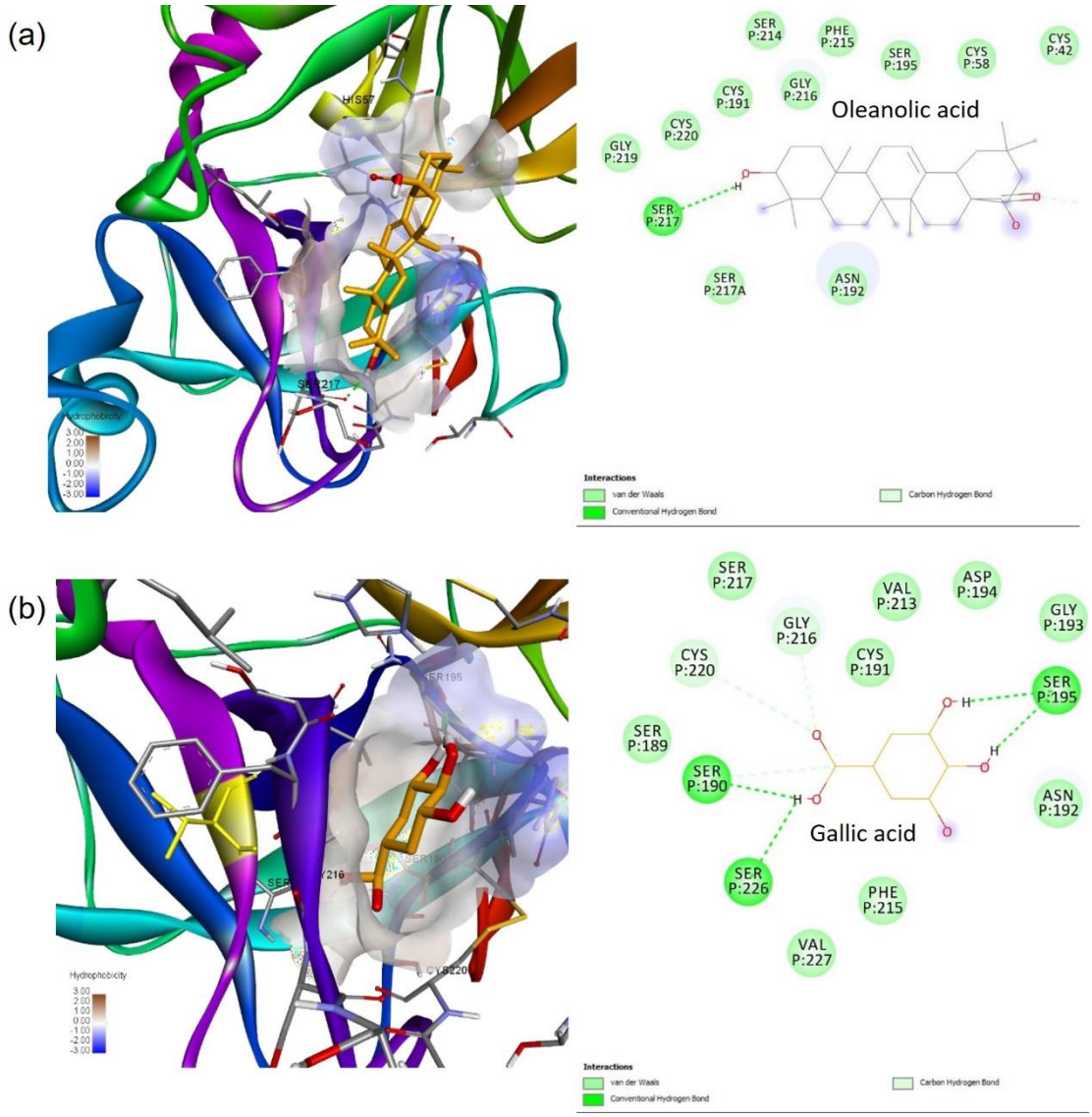

Figure 5. 3D and 2D molecular docking poses for elastase with (a) oleanolic acid (b) gallic acid and their intermolecular interactions as simulated by Autodock software

For COX-2, the binding of aspirin to Ser516 is supported with finding by Vane and Botting [42] and Rabiu *et al*. [20] which subsequently leads to irreversible COX inhibition. Although, gallic acid was observed to not bind to the Ser516 residue but it still occupied the active site of COX-2 as it formed hydrogen bonding to Gln178, Leu338, Tyr341 and Arg499 and hydrophobic interaction with Ala502 and Val509 [20, 43] as shown in Figure 6. These binding residues have been observed in celecoxib and other inhibitors binding interaction as reported by [44]. The lowest binding energy of gallic acid (-4.91 kcal/mol) was slightly better than aspirin (-4.79 kcal/mol) towards COX-2, though the mean binding energy of gallic acid showed slightly lower -4.53 kcal/mol. Overall, the result might show that the gallic acid can exhibit inhibitory activity comparable to aspirin, though it might not cause irreversible inhibition as aspirin.

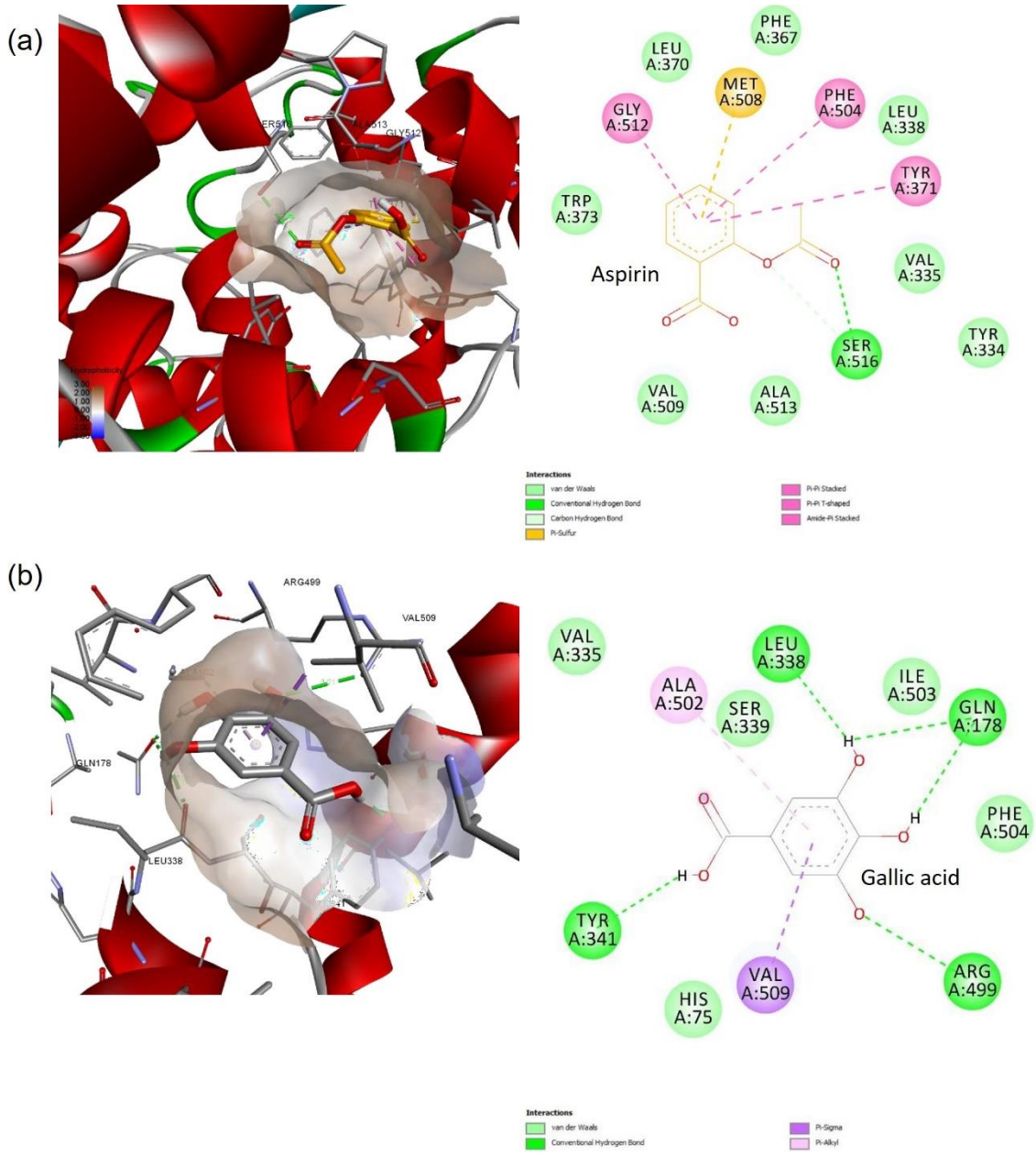

Figure 6. 3D and 2D molecular docking poses for Cox-2 and (a) aspirin (b) gallic acid and their intermolecular interactions

For validation of the prepared grid, RO314724 was simulated docking with the protein MMP-1 collagenase 2TCL. The resulted ligand was superimposed on the original conformation of RO314724, yielding RMSD of 1.99 Å, inferring the quality of the selected grid was good and maintaining high positional similarity to the original inhibitor [24]. Re-docking of the inhibitor ligand produced the lowest binding energy of -6.7 kcal/mol. Molecular docking analysis for the gallic acid and ascorbic acid with MMP-1 collagenase showed weaker than the RO314724 but good binding affinity with lowest binding energy of −5.68 kcal/mol and −4.74 kcal/mol, respectively. The same trend where gallic acid exhibited stronger binding affinity to the active site of the collagenase than ascorbic acid was also observed from the mean binding energy for both compounds as gallic acid (-5.19 kcal/mol) was lower than ascorbic acid (-3.74 kcal/mol). Both compounds docked in similar location of RO314724 in the active site of the collagenase as they formed hydrogen bonds with amino acid residues of Gly79, Leu81, Ala82, His118, Glu119, Tyr137 and Tyr140 [45–47] as shown in Figure 7. This docking result aligned to the experimental result in which the collagen concentration was higher when treated with gallic acid compared to ascorbic acid.

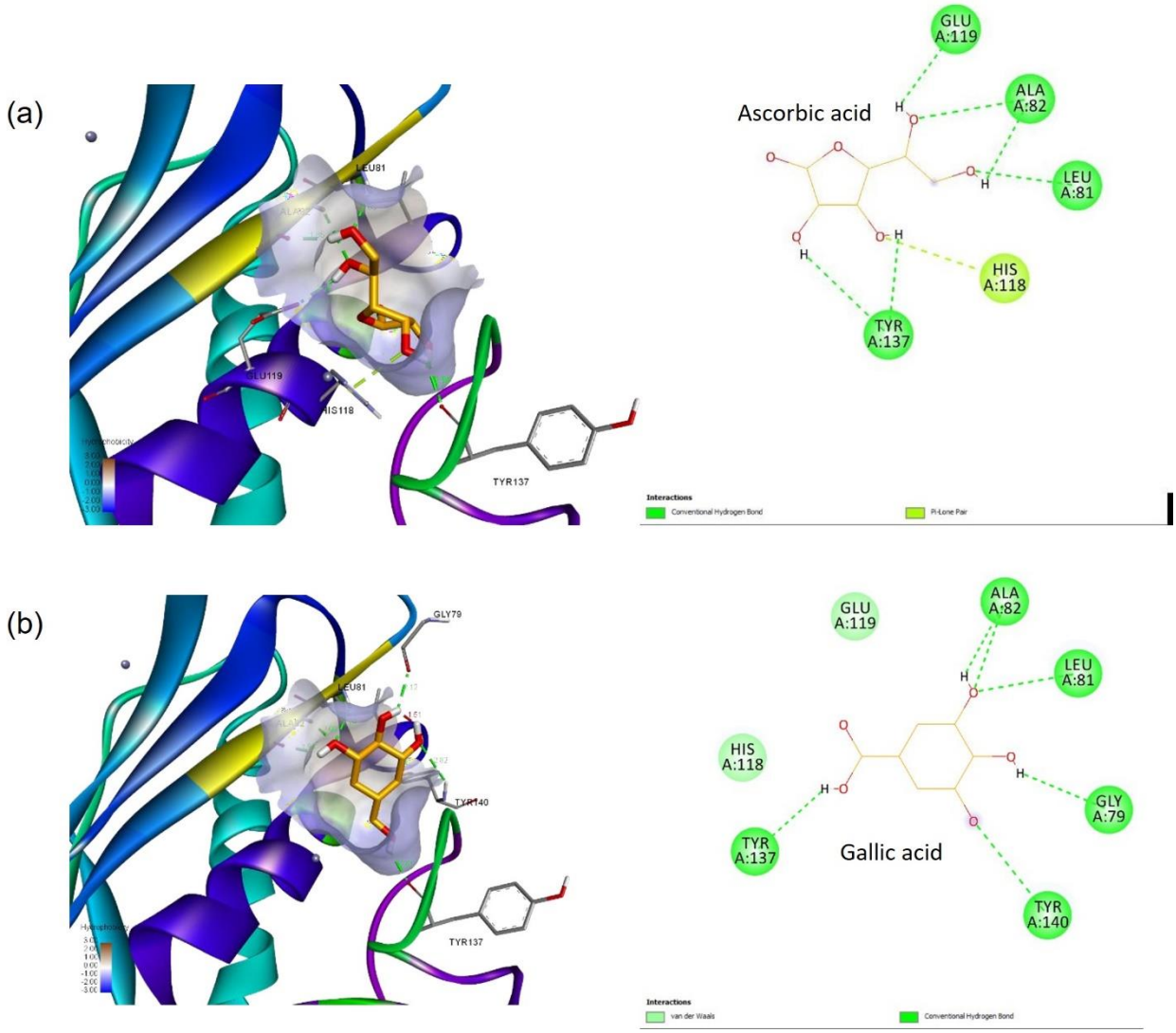

Figure 7. 3D and 2D molecular docking poses for MMP-1 collagenase enzyme with (a) ascorbic acid (b) gallic acid.

Conclusions

In conclusion, the results in this study showed that *Labisia pumila* methanolic extract (LPE) which consisted of gallic acid as major compound exhibited photoprotective against the damaging effect of the UVB irradiation through antioxidant and anti-inflammatory mechanisms. LPE was proven to directly inhibit elastase and COX-2 while indirectly inhibit MMP-1 collagenase. Molecular docking simulation also proved the ability of gallic acid compounds to inhibit these enzymes. This characteristic would be very beneficial in the development of natural NSAIDs. Further study is required to effect of fractionation process on the antioxidant and anti-inflammatory of the LPE fractions. It is evident that *Labisia pumila* exhibits numerous applications and harbors substantial potential for exploration in the future.

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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