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Molecular Docking Polifenol Solution of 96% Ethanol Extract of Stevia (*Stevia Rebaudiana*) Leaves with Four Antiaging Enzymes

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Abstract

Natural plant-derived compounds and herbal-derived formulations are now widely used in line with the Indonesian Government's Domestic Component Level program. The various active ingredients are safe and work through different mechanisms of activity on skin aging signalling pathways. Stevia leaf gel contains potent constituents that can reduce wrinkles and mild acne when worn for 21 days due to its antioxidant and antimicrobial properties. The purpose of this study was to predict the anti-aging effect of 96% ethanol extract compound of Stevia Rebaudiana leaves through inhibitory pathway of Enzyme elastase, Enzyme Collagenase, Hyaluronidase and Enzyme Tyrosinase. To determine the content of polyphenolic compounds in 96% Stevia ethanol extract, the analysis was carried out using *High-Performance Liquid Chromatography* (HPLC) of Stevia Rebaudiana 96% ethanol extract, then physicochemical properties were analysed using PKCSM and toxicity using ProTox II online tool.

The results of the selection of 10 compounds were prepared using ChemDraw application using Chem 3D application then molecular docking was carried out with Molegro application. The results of in silico studies showed the strongest binding of Rozmarinic Acid compounds (one of the stevia polyphenols) to Elastase and Collagenase Enzymes with RMSD ≤ 2Å and the best Rerank score. Absorption of stevia compounds through the skin -2.725 logKp (<-2.5) indicates this compound has low permeability in the skin. So if used for skin-applied preparations, preparations that improve permeation must be selected, thus requiring the development of Transdermal Drug Delivery using stevia raw materials. It has a low VDss value of 0.113 log L/Kg so that in small doses it can be distributed into blood plasma. The LD50 of stevia is 1190mg/kg which means its acute toxicity is high. So that the dose, metabolism, excretion and toxicity are still being researched.

Keywords: Stevia, Antiaging, Molecular Docking.

1. Introduction

The skin is formed by the epidermis, dermis, and subcutaneous tissue which can be affected by the aging process. However, the effects of skin ageing are most pronounced in the dermis, which, when atrophied by intrinsic and extrinsic factors, causes wrinkles and reduces skin elasticity. Skin atrophy is closely related to the reduction in the amount of extracellular matrix (ECM), including collagen, elastin, proteoglycans, glycosaminoglycans, fibronectin, and other glycoproteins [1]. Skin has three defence functions: UV protection, anti-oxidant, and antibacterial [5]. Plant extracts can have moisturising, nourishing, capillary stabilising, cleansing, anti-inflammatory, antimicrobial, emollient, melanin inhibiting, antimutagenic, astringent, regenerating, and UV protective properties. Topical application of antioxidants derived from plant extracts, support the skin's endogenous defence mechanisms, help reduce Ultra Violet Radiation (UVR)-mediated oxidative damage and prevent OxS-mediated diseases [6].

Collagen and elastin in the dermal layer of the skin are susceptible to the proteolytic action of metalloproteinases, which are secreted by keratinocytes and dermal fibroblasts stimulated by oxidative stress, UV radiation, and other cytokines. Figure 1 illustrates the main components of the ECM, including its major proteins collagen, elastin, and hyaluronic acid, and the enzymes responsible for their degradation. The following process image illustrates this process.

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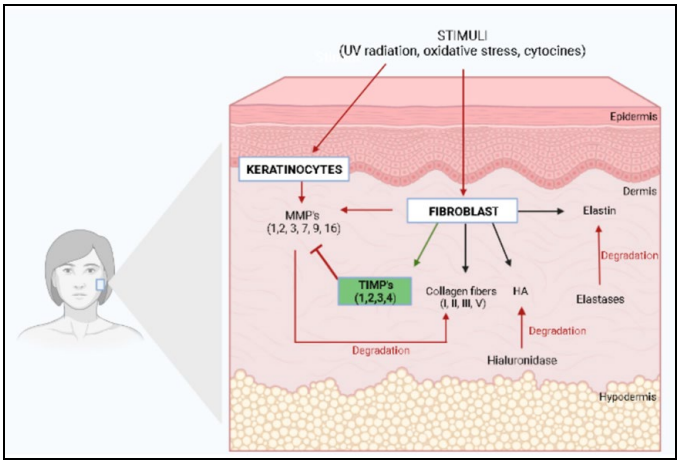


Fig 1: Extracellular matrix (ECM) components and skin elasticity. HA, Hyaluronic acid; MMP, Metalloproteinase; TIMP, Tissue Inhibitor of Metalloproteinase. Illustrations were drawn using BioRender software [1].

Chronic UV exposure to human skin leads to solar elastosis, extracellular matrix (ECM) degradation and wrinkle formation. The dermal matrix contains ECM proteins such as collagen, elastin and proteoglycans that are responsible for providing strength and resilience to the skin. UV exposure can trigger the formation of Reactive Oxygen Species (ROS) which will then be followed by the expression of genes and proteins that trigger skin damage and skin cancer. Intracellular Reactive Oxygen Species (ROS) contribute greatly to skin ageing, where intracellular ROS activate the mitogen-activated protein kinase (MAPK) pathway, which forms the transcription factor activator protein-1 (AP-1). This AP-1 transcription factor is important in the regulation of matrix metalloproteinases (MMPS) transcription enzymes, especially MMP-1 (collagenase type-1) that degrade type I collagen in the skin. Inhibition of MMPS enzymes can degrade skin collagen so that skin photoaging problems can be resolved [7].

The biological activity in scavenging free radicals and the antioxidant properties of plants are related to the active components contained in herbs such as polyphenols, tocopherols, carotenoids, ascorbic acid, macro molecules namely polysaccharides and peptides and essential oil components. Polyphenols are a very large and important group of natural compounds commonly found in the plant world. Polyphenols contain two or more hydroxyl groups attached to an aromatic ring. Depending on their chemical structure, they can be classified as flavonoids, phenolic acids, tannins, or stilbenes. Flavonoids are the best-known group of polyphenols. They can be present as free molecules, known as aglycones, or in a form bound to sugars, as glycosides [8]. Plant phenolic compounds are widely used in pharmaceutical and cosmetic production, while flavonoid compounds are the largest group of plant phenols with significant therapeutic activity. Some flavonoids, such as flavones and flavonols are also used in cosmetic formulations due to their anti-aging properties and therapeutic potential against skin inflammation [9].

In Stevia Rebaudiana leaves, the mass content of steviol glycosides is about 8% to 13% of dry leaves, while the mass content of Stevia Polyphenols (PPS) is about 2% to 5% of dry leaves. Currently most Steviol Glycoside factories still leave PPS in Stevia solid waste generated from steviol glycoside production [10]. In the research of Gawel *et al*. the high content of flavonoids, phenols, and peptides in stevia extracts after being analysed found that the antioxidant activity of stevia leaf extracts was significant. Stevia becomes a source of bioactive substances for the production of dietary supplements if extracted with water and 96% ethanol solvents (E). E and Glycolic-Air (GA) solvents are used for skin care products. Stevia with GA solvent had the highest toxicity test results compared to ethanol and water so this study used 96% ethanol as a solvent. The phenolic compounds obtained in ethanol extract of Stevia are Benzoic Acid, Ferulic Acid, (0.86 mg/g), Rozmaric Acid derivative (0.42 mg/g), Rozmaric Acid (0.36 mg/g) and Chlorogenic Acid (0.30 mg/g) [9].

The following is the data of Gawel's research which looked for Stevia Polyphenol content with 3 kinds of solvents:

Table 1: Composition of Polyphenol Content in Stevia Rebaudiana in Various Solvents [9].

Polifenol Compound (mg/g Stevia)	Water	Etanol 96%	Glycolic-Water
Derivat Benzoic acid	$0,10^{b} \pm 0,02$	$0,05^{d} \pm 0,01$	Nd
Caffeic acid	$0,29^{a} + 0,08$	$0,06^{d} \pm 0,02$	$0,19^{d} \pm 0,07$
Derivat Caffeic acid	$0,06^{c} \pm 0,02$	$0,03^{d} \pm 0,01$	$0,36^{c} \pm 0,06$
Chlorogenic acid	Nd	$0,30^{b} \pm 0,10$	Nd
Derivat Chlorogenic acid	Nd	$0,14^{c} \pm 0,04$	Nd
Derivat Ferulic acid	Nd	$0,86^{c} \pm 0,08$	$5,50^{a} \pm 0,23$
Protocatechuic acid	$0,12^{b} \pm 0,05$	Nd	Nd
Rozmaric acid	Nd	$0,36^{b} \pm 0,04$	Nd
Derivat Rozmaric acid	Nd	$0,42^{b} \pm 0,06$	$4,95^{a} \pm 0,66$
Derivat Salicylic acid	$0,06^{c} \pm 0,02$	Nd	Nd
Derivat Campherol	Nd	$0,15^{c} \pm 0,05$	$0,23^{d} \pm 0,08$
Catechin	$0,24^{a} \pm 0,04$	Nd	Nd
Derivat Catechin	$0,29^{a} \pm 0,05$	$0,12^{c} \pm 0,02$	Nd
Epicatechin	Nd	$0,11^{c} \pm 0,05$	Nd
Luteolin	Nd	0,03c ± 0,01	Nd
Derivat Luteolin	Nd	0,01c ± 0,01	$0,86^{a} \pm 0,08$
Rutin	Nd	Nd	$0,17^{a} \pm 0,07$
Derivat Rutin	Nd	0,12c ± 0,04	$1,05^{a} \pm 0,09$
Total	1,16	2,96	13,35

Note: nd-not detected; means, within columns, for each component followed by different lowercase letters a-e are significantly different at $\alpha = 0.05$ [9]

This study conducted molecular docking to assess the interaction of polyphenolic compounds identified in ethanol extract of stevia leaves with four major enzyme targets associated with skin aging process namely Elastase (1Y93), Tyrosinase (2Y9X), Hyaluronidase (2PE4), Collagenase (966C). Molecular dynamic simulation was used to monitor the binding affinity of some selected compounds to the target enzymes. Researchers want to perform molecular docking to predict and confirm ligand binding at the target site. Molecular docking can be applied at several levels. The drug development process has three main objectives: predicting the binding model of known active ligands, searching for new ligands, using virtal screening and predicting the binding affinity of several series of active compounds. Ligands are active compounds that are bound to the amino acids of a protein. A receptor is a specific cellular macromolecule to which a ligand binds to trigger a chemical signal in the cell and cause an effect. Receptors generally have larger bonds, such as enzymes or proteins.

2. Tools and Methods

i). Tools: This research uses Windows 11 Home Single Language 64 bit laptop (10.0 Build 22631.13th Gen Intel Core TM i7-1355U (12 CPUs) Memory 16384RAMM Using Pubchem Software, Chemdraw, Chem 3D, Molegro PkCSM and Protox II.

ii). Materials: 2D ten structures of Stevia Polyphenols from the research of Gawel *et al*. And the four enzyme proteins obtained from PDB ID: 1Y93, 2Y9X, 2PE4, and 966C

iii). Research Procedure

a) Protein and Ligand Preparation

The structure of the test compound that has been drawn in 2D with the ChemDraw application is converted into 3D with energy minimisation in the Chem3D application, then saved in smile format.

This is an example of one of the Stevia Polyphenol Compounds from the preparation:

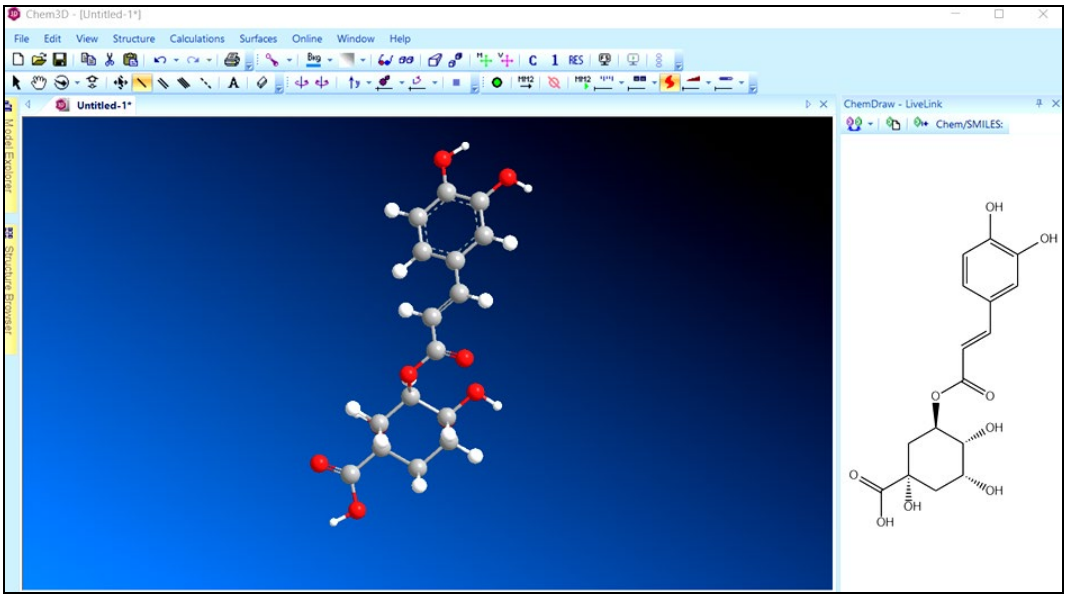


Fig 2: Chlorogenic Acid Compound Preparation Results.

The proteins of four antiaging enzymes were downloaded from the Protein Data Bank database (https://www.rscb.org/) using the codes 1Y93, 2Y9X, 2PE4, and 966C. The 3-dimensional structures of the enzymes were saved as PDB. files and then loaded on the Molegro worksheet, the original proteins and ligands were separated and saved under the file names protein.mol2 and ref_ligand.mol2. Protein preparation aims to separate the native ligand from the target protein, thus providing a pocket or binding site that will be used during the docking process.

This is an example of 3D Crystal Structure of Fibroblast Collagenase-1 Crystal Structure Complexed with Diphenyl-Eter Sulfate Based Hydroxamic Acid

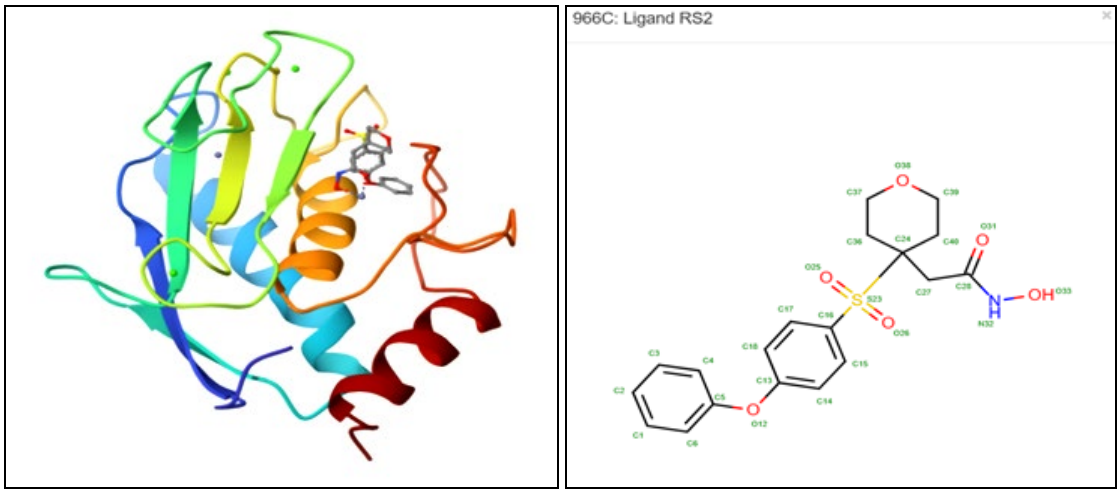


Fig 3: 2D and 3D Structure of MMP1 Molecular Structure.

Docking Validation Protocol is performed by calculating the Root Mean Square Deviation (RMSD) value between the native pdb.id ligand and the docked ligand conformation. RMSD is an important parameter used to evaluate the conformity between the ligand position generated from the docking simulation and the native ligand position obtained from the crystallographic structure. The RMSD value indicates how well the docking model can reproduce the ligand interaction with the target receptor. The parameter of the validation result is Root Mean Square Deviation (RMSD) value. The test is declared valid when the RMSD result is ≤ 2 Å. If the RMSD value > 2 Å indicates that the deviation from the calculation results is greater so that the results of ligand and receptor interactions in silico cannot be used as a reference [1, 12].

b) Molecular Docking

Target Protein Preparation Target proteins used for molecular docking analysis are 1Y93, 2Y9X, 2PE4, and 966C obtained from the Protein Data Bank (PDB) website address were validated and then prepared as input data Separated proteins between receptors in the form of macromolecules and ligands from unused molecules such as water molecules. From the docking process, the docking score is obtained as output data that shows the energy of the ligand in binding to the target protein. The more negative the docking core value, the stronger the affinity of ligand binding to protein.

c) Toxicity Screening

Toxicity screening was performed using the ProTox webserver, test compounds were prepared with the SMILES file type. It was then entered into the ProTox webserver and run the toxicity calculation programme. The toxicity level was done in units of LD50, mg/kg. Toxicity screening was carried out using the ProTox webserver, test compounds were prepared with the SMILES file type then entered into the ProTox webserver and ran the toxicity calculation programme. The toxicity level was carried out in units of LD50, mg/kg.

iv). Analysis of Ligand-Protein Interaction by Molecular Docking

Analysis of the interaction between protein and ligand through molecular binding was performed using MVD on all 3D structures of the Stevia Polyphenol ligand or compound. Previously, a re-docking process was performed on the 3D molecules of Stevia Polyphenols in the protein binding site region to validate the docking method used. The validity requirement of the method was set with Root Mean Square Deviation (RMSD) value < 2 Å. Afterwards, molecular docking was applied to the 3D structure of the ligand against the crystal structure of the protein In this docking process, the parameters measured include the energy values involved, such as MolDock Score, Rerank Score, and Hbond. To evaluate the strength of the bond between the ligand and the receptor protein, Rerank Score is often a commonly used parameter.

v). In silico analysis of ADMET using pkCSM.

Absorption, Distribution, Metabolism and Excretion (ADMET) analysis was performed on stevia compounds obtained from pubchem as candidate inhibitors of Elastase, Tyrosinase, Hyaluronidase and Collagenase enzymes.

3. Results and Discussion

The RMSD values obtained from the docking protocol validation process are as the following table.

Table 2: RMSD 4 Antiaging Enzyme Receptor

Name of Enzyme and Protein Code	Nilai RMSD
Elastase (1Y93)	1.52783
Tyrosinase (2Y9X)	2.89363
Hyaluronidase (2PE4)	1,18354
Collagenase (966C)	0,954043

From the Root Mean Square Deviation (RMSD) analysis, the molecular docking study showed that the docking score of ligands paired with antiaging enzymes was only 1 more than 2, namely Tyrosinase Enzyme. Stevia-Collagenase Polyphenol Complex with 966C Enzyme was identified as the lead compound with the lowest RMSD value and qualified for the protocol to be used for further docking process. The alignment between the reference ligand and the docked ligand conformation is very good. The docking process of stevia polyphenol compounds was carried out on 10 stevia polyphenol compounds that have been tested in silico by QSAR method which have better antioxidant activity than (Table 1).

Here are the results of protein docking of 4 antiaging enzymes with 10 Stevia polyphenol compounds.

Table 3: Rerank score of Stevia Polyphenol Molecular Docking Results with Antiaging Enzyme Receptor.

Komposisi Polifenol Stevia	Elastase (1Y93)	Tyrosinase (2Y9X)	Hyaluronidase (2PE4)	Collagenase (966C)
Benzoic Acid	-48.157	-60.1364	-52.0421	-51.6093
Caffeic Acid	-72.1086	-25.182	-75.9208	-67,.8567
Chlorogenic Acid	-145.776	1571.15	-60.3695	-2.14405
Ferulic Acid	-68.6842	-20.2643	-67.2297	-76.1045
Rozmarinic Acid	-159,046	9.56187	-66.4557	-119.948
Kaempferol	-116.836	1686.97	-71.2472	-107.806
Epicatechin	-134.666	2758.56	-60.4613	-101.707
Luteolin	-128.869	975.713	-71.3101	-111.017
Rutin	-128.842	1318.74	-41.5802	-107.806
Catechin	-134.66	818.539	-37.5065	-110.413

Rerank Score (RS) is one of the molecular docking result parameters used to evaluate the interaction between ligand and receptor. In the context of using Molegro Virtual Docker (MVD) software, RS gives an indication of the stability and strength of the bond between the ligand and the target protein. A low RS value signifies higher potential biological activity and better bonding stability. Therefore, Rerank Score is a critical evaluation tool in drug development and biomedical research.

i). Rerank Score Interpretation

a) Negative RS Value: The more negative the RS value, the stronger and more stable the interaction between the ligand and the receptor. For example, the compound Rozmarinic Acid with an RS value of -159.046 kcal/mol showed the best interaction compared to the other 9 compounds against Receptor 1Y93.

b) Stability Criteria: Generally, RS values below -50 kcal/mol are considered to indicate good interaction potential, while values above -50 kcal/mol may indicate less stable interactions. In the table above of the 10 Stevia Polyphenol compounds that bind to the Elastase receptor (1Y93) whose rerank value is >-50 is Benzoic Acid with an RS value of -48.157kcal/mol.

c) Comparison between Compounds: Rerank Score can also be used to compare the effectiveness of various compounds against the target receptor. Compounds with lower RS values than controls or other compounds can be considered to have better potential activity.(13) From the table above, the best interaction of 10 phenol compounds

against Elastase is Rozmarinic Acid with RS value of -159.046 kcal/mol, against Tyrosinase is Benzoic Acid compound with RS -60.1364 kcal/mol, against the best Hyaluronidase compound Cafeic Acid with RS value -75.9208, against the best Collagenase enzyme compound Rozmarinic Acid with RS value -119.948 kcal/mol.

From the above conclusions, the Rozmarinic compound has the best bond to the Elastase and Collagenase Enzymes. Where if you look at the results of research by Gawel *et al*. Rozmarinic Acid is only found in Stevia leaf extract with 96% ethanol solvent, in water and Glycolic-Water.

This Figures will showed The Interaction of Polifenol Stevia with Elastase Enzym and Collagenase Enzyme which The RMSD under 2.

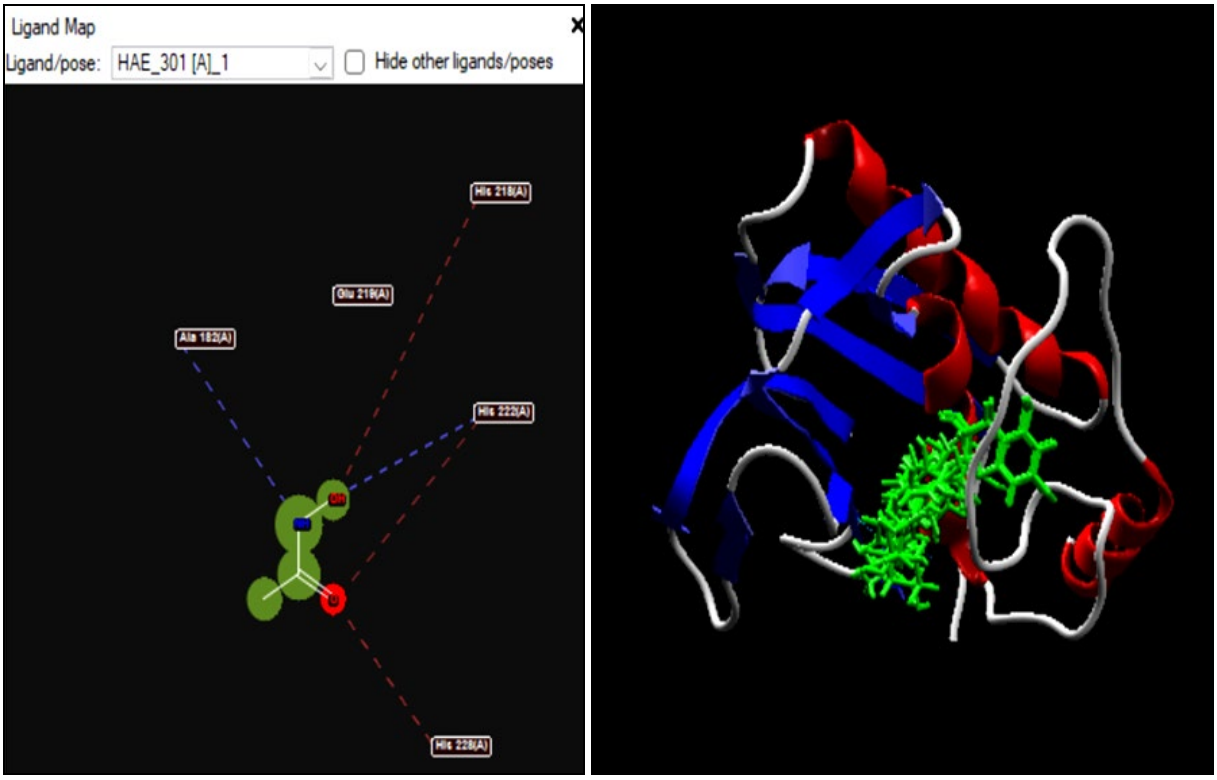


Fig 4: 2D and 3 D images of Stevia polyphenols bonding with Elastase enzyme

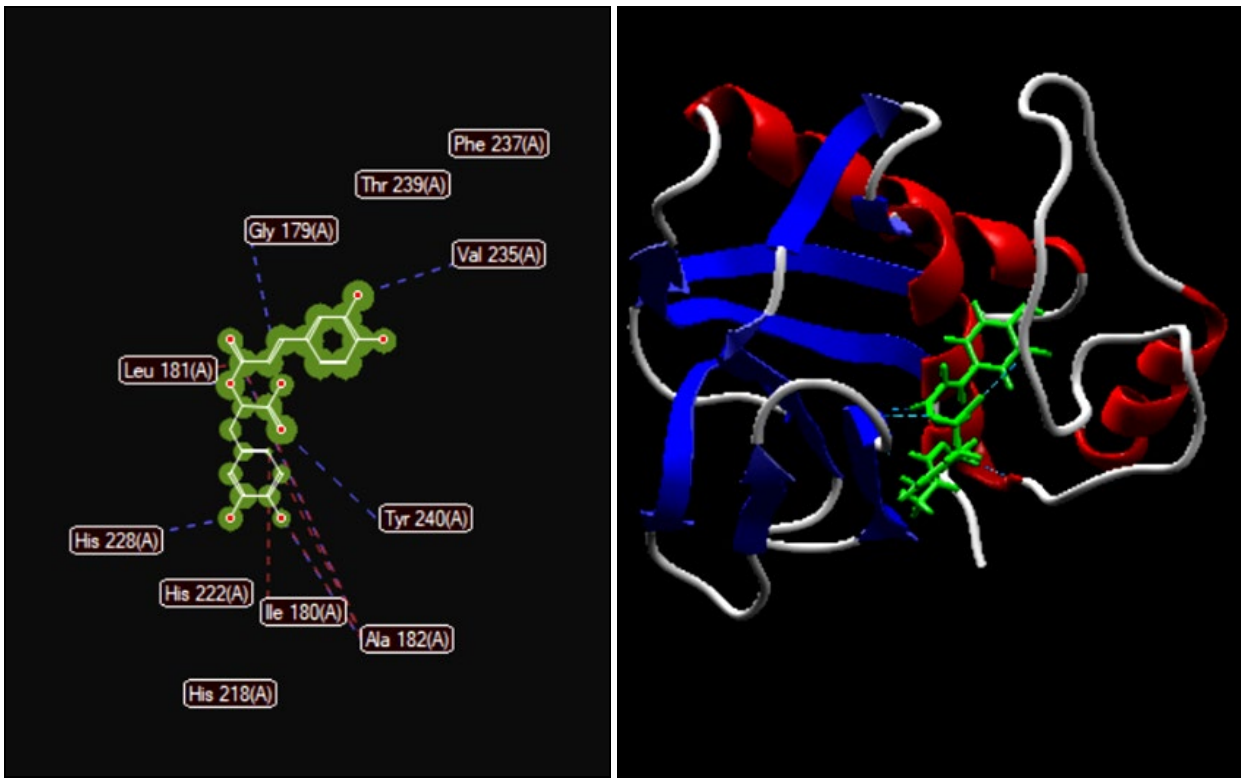


Fig 5: 2D and 3D Images of Stevia Polyphenol Compound Bonds

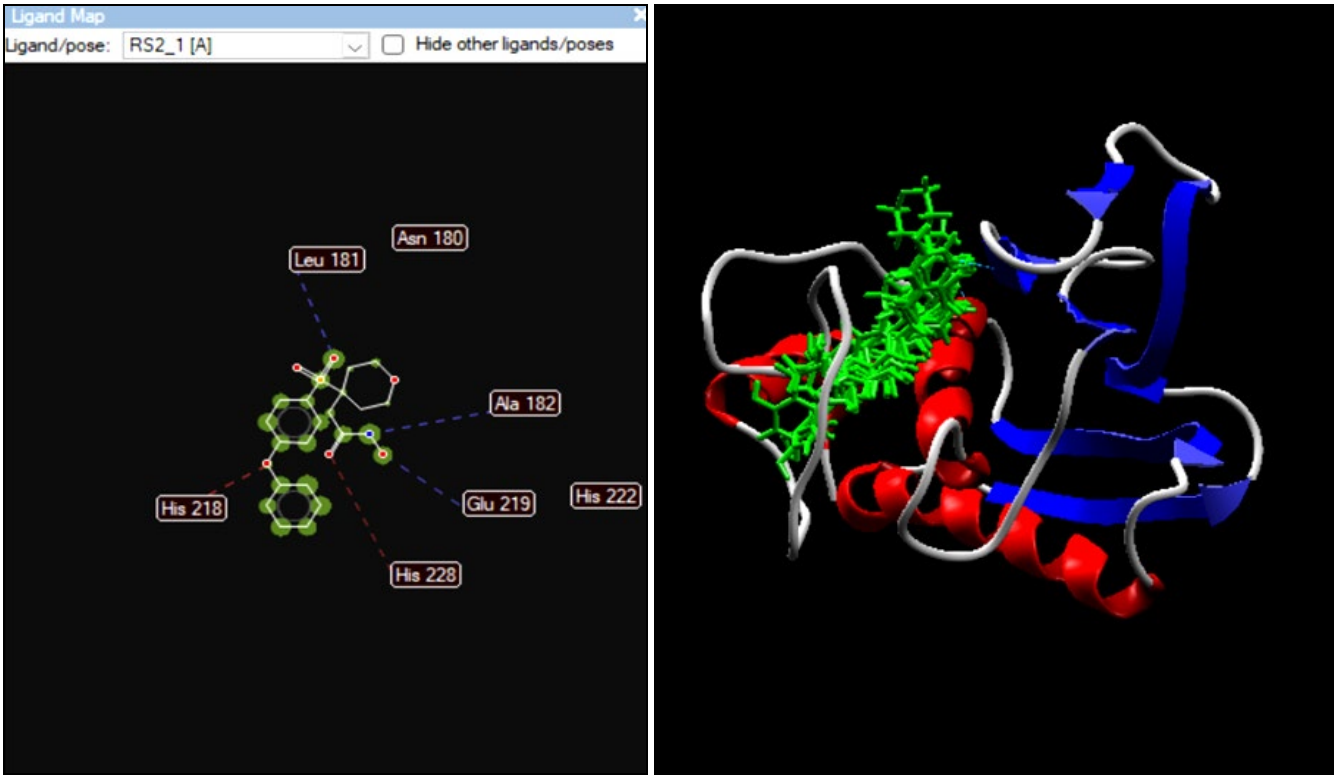


Fig 6: 2D and 3D Images of Stevia Polyphenols Bonding to Collagenase Enzyme

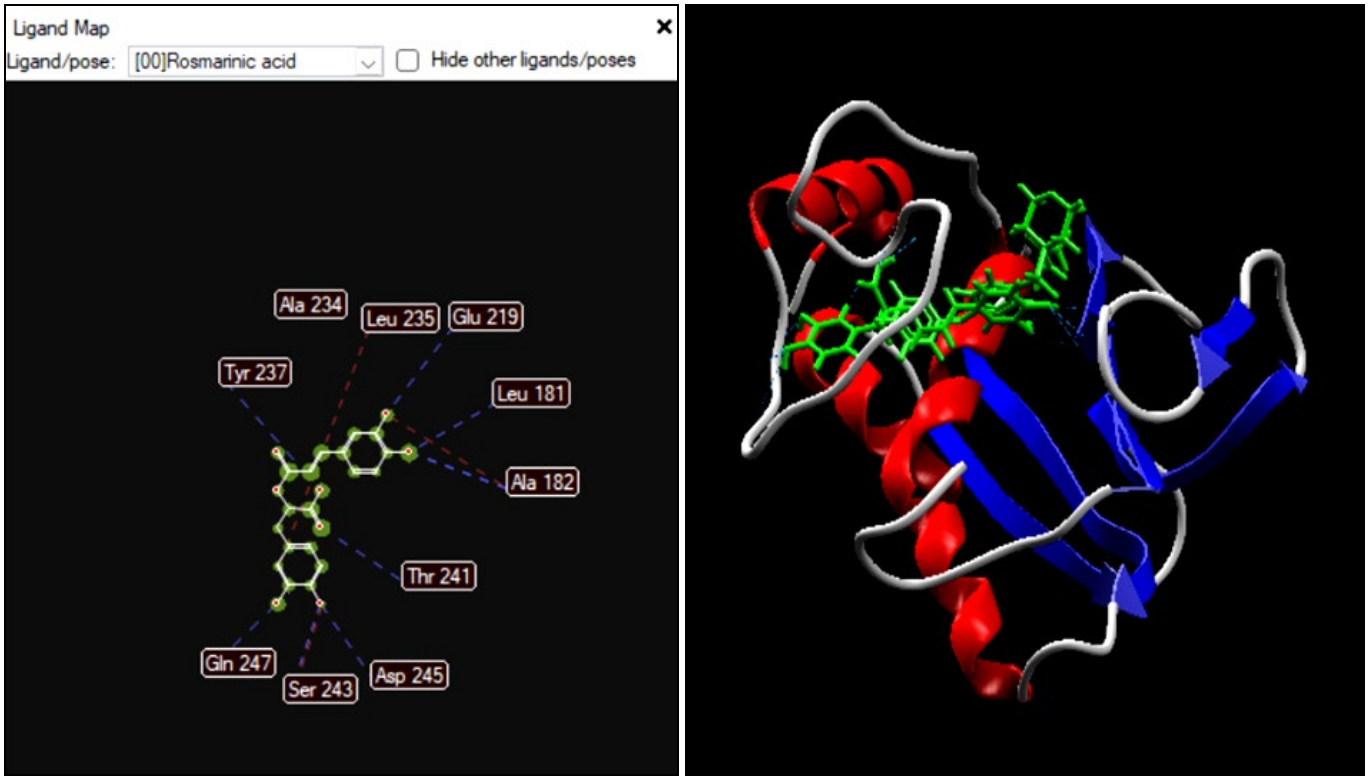
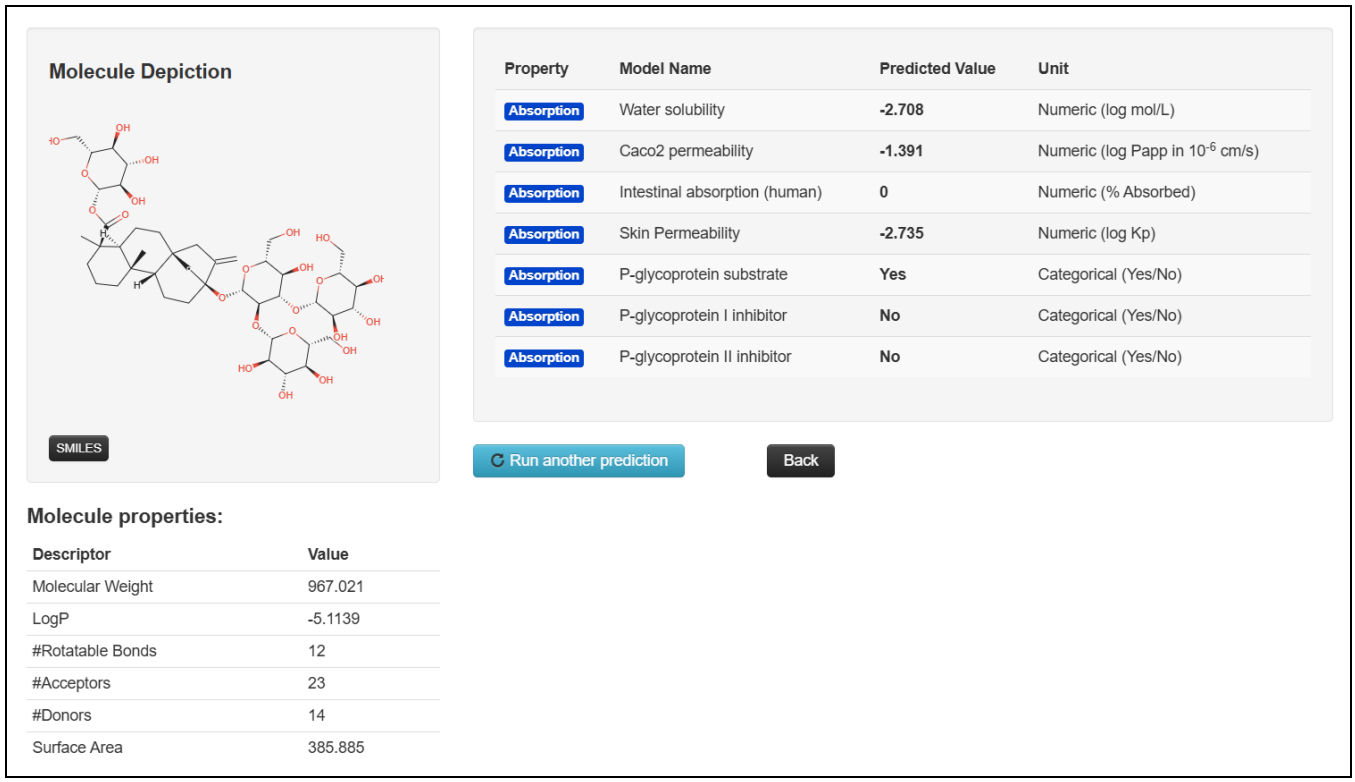


Fig 7: 2D and 3D Images of *Rozmarinic Acid* Compound Bonding to Collagenase Enzyme.

The results of PKCSm *Stevia Rebaudiana* obtained data according to the figure below.



Absorption of stevia compounds through the skin -2.725 logKp (<-2.5) indicates this compound has low permeability in the skin. So if it is used for skin-applied preparations, preparations that improve permeation must be selected, thus requiring the development of Transdermal Drug Delivery using stevia raw materials.

Steady State Volume of Distribution (VDss) describes the volume of dose required for the drug to be distributed in the same concentration as the blood plasma. The VDss of a drug is considered low if <0.15 log L/Kg and is considered high if >0.14 log L/Kg. Stevia has a VDss of 0.113 log L/Kg. Its metabolism, excretion and toxicity are still being investigated. From the results of toxicity analysis using the Protox application, the data obtained is as shown below:

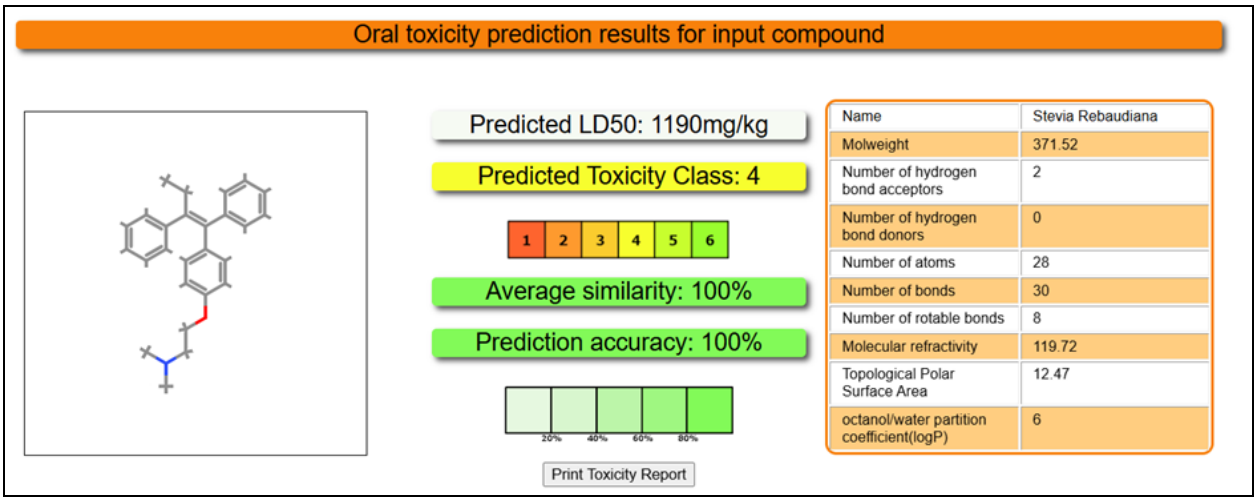


Fig 8: Figure Stevia Toxicity Analysis Results in Protox II

From the figure above, it can be seen that the LD50 of stevia is 1190mg/kg, which is less than 5000mg/kg. This means high acute toxicity. Class 4 LD 50 values are generally within a certain range that indicates the dose required to cause death in 50% of the test animal population. This substance causes adverse health effects if ingested, causes gastrointestinal irritation, or even organ damage in high enough doses so that the dose must be observed and labelled.

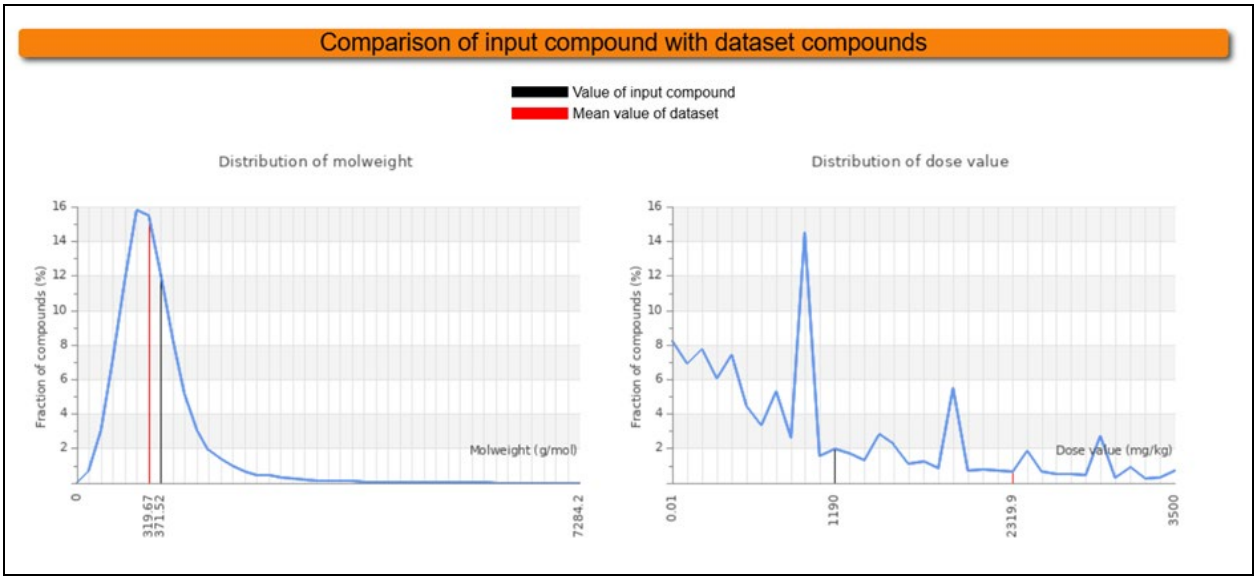


Fig 9: Image. Comparison of Input Compound with Dataset Compounds

From the data it can be seen to get a safe dose if the fraction is less than 2%.

4. Conclusion and Suggestion

The results of insilico study about interaction between Polyphenol compounds in Stevia 96% ethanol leave extract against Four Antiaging Enzymes showed the strongest binding of *Rozmarinic Acid* (one of the stevia Polyphenols) compounds with elastase and collagenase enzymes with RMSD ≤ 2Å and the best rerank score. Absorption of stevia compounds through the skin -2.725 logKp (<-2.5) indicates this compound has low permeability in the skin. So if used for skin-applied preparations, preparations that improve permeation must be selected, thus requiring the development of transdermal drug delivery using stevia raw materials. It has a low *Steady State Volume of Distribution* (VDss) value of 0.113 log L/Kg so that in small doses it can be distributed into blood plasma. The Lethal dose 50 (LD50) of stevia is 1190mg/kg meaning that acute toxicity is high. So that the dose, metabolism, excretion and toxicity are still being researched.

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