

In vitro and *in silico* Evaluation of Some Natural Molecules as Potent Glutathione Reductase Inhibitors

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Abstract: Glutathione reductase inhibitors are very popular antimalarial and anticancer agents. In this study, *in vitro* inhibition effects of β -sitosterol, stigmasterol, diosgenin and jervine which containing steroidal structure were determined against glutathione reductase enzyme. β -sitosterol, diosgenin and jervine were isolated from *Veratrum album* and stigmasterol was isolated from *Artemisia dracuncululus* L. by chromatographic methods. According to the results obtained, IC₅₀ values of β -sitosterol, stigmasterol, diosgenin and jervine were found as 1.2580, 5.2116, 0.1916 and 0.7701 μ M, respectively. Among test compounds, diosgenin showed the strongest inhibitory effect against glutathione reductase with Swissdock docking figure. In current study first time, β -sitosterol, stigmasterol, diosgenin and jervine were found to be much more glutathione reductase inhibitors.

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1. INTRODUCTION

Glutathione reductase (GR, EC 1.8.1.7), found in most organism is an important homodimeric flavoprotein and an antioxidant enzyme in protection a critical intracellular reducing environment against oxidative stress. It catalyzes the reduced glutathione (GSH) generation from oxidized glutathione using nicotinamide adenine dinucleotide phosphate (NADPH). Oxidized glutathione is generated via the oxidation of GSH by oxidants such as reactive oxygen species (ROS) that occur during oxidative stress. The cell is protected against oxidative stress through discontinuation of oxidants by GSH. GSH prevents the removal of oxygen radicals and lipid intracellular peroxidation. It is a reaction partner for the detoxification of endobiotics and xenobiotics, and is the mode of storage and transport of cysteine. It has important functions in the protection of the cell against oxidative stress, in the maintenance of thiol redox potential in the cell and in the production of deoxyribonucleotides. The lack of GR and GSH leads to oxidative damage in the cell, which can cause many diseases such as malaria and cancer [1-5].

Malaria is an essential worldwide most dangerous disease and seen especially in tropical regions. The most fatal malaria parasite is *Plasmodium falciparum*. The antimalarial drug developing is very difficult due to this parasite has high mutation rating [6]. Because, when the drugs destroy sensitive parasites, the resistant mutant parasites reproduce and infect other host.

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It has been reported in the literature, *P. falciparum* developed resistance to chloroquine, which is commonly used as an antimalarial drug [7]. Antimalarial drugs target GR enzyme inhibition and host cells in the malaria parasites. GSH plays a fundamental role in antioxidant defense in both malaria parasite and host cells [6].

The steroidal compounds β -sitosterol (1), stigmasterol (2), diosgenin (3) and jervine (4) are important bioactive natural products that are quite common in plants and animals (Figure 1) [8, 9]. Jervine is the major active steroidal alkaloid compound found in *Veratrum* species belonging to the *Liliaceae* family. It was reported that jervine is a teratogenic ingredient responsible from the malformation in birth after *Veratrum* species were consumed by pregnant animals [10]. It has been determined to inhibit the hedgehog signaling pathway, which is directly associated with cancer cell proliferation [11]. β -sitosterol, a steroid found in almost all plant species, has been reported to display a wide variety of biological activities, such as estrogenic, immunomodulatory, anti-arthritic, antioxidant, and anti-ulcer [9, 12-14].

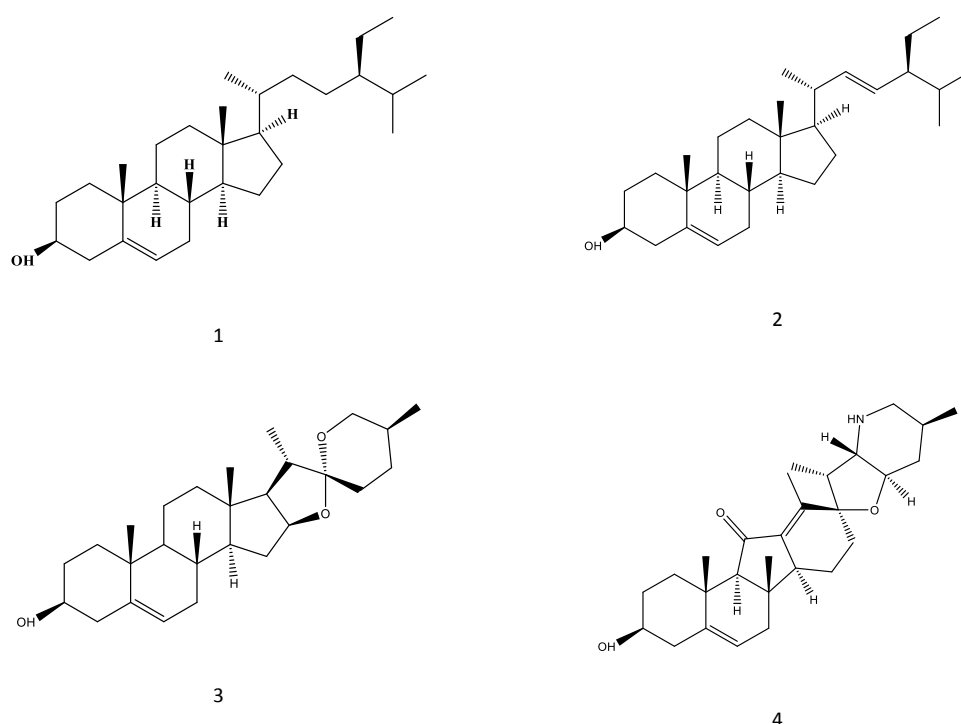


Figure 1. The chemical structures of test compounds 1-4. β -sitosterol (1); stigmasterol (2); diosgenin (3); jervine (4).

Diosgenin, which is used as a starting material for the synthesis of hormones cortisone and progesterone, is a very important natural steroidal sapogenin using in synthesize of new synthetic drugs. It has proapoptotic and anticancer properties [15]. In the previous study by our team, the stigmasterol was isolated from tarragon (*Artemisia dracuncululus* L.) [16]. Stigmasterol is a common plant steroid and has anti-inflammatory and anti-angiogenic activities [17].

In this study, we have tried to find out that β -sitosterol, stigmasterol, diosgenin and jervine can enter the enzyme active site more easily compared to bulky molecules such as NADPH and GSH, which are the substrates of GR. Thus, it was considered to evaluate these substances as GR inhibitors and to obtain important pharmacological data. Compounds 1-4 are important steroidal bioactive natural compounds, and no studies have been conducted related to the inhibitory effect of these metabolites on GR enzyme. In study first time, the inhibitory effects of β -sitosterol, stigmasterol, diosgenin and jervine were investigated on the GR enzyme.

2. MATERIAL and METHODS

2.1. Plant Materials and Chemicals

As in our previous study, β -sitosterol, diosgenin and jervine compounds were isolated from rhizomes of *Veratrum album* and stigmasterol was isolated from *Artemisia dracunculus* L. [9, 16]. GR enzyme was purchased from Sigma-Aldrich. All other chemicals used in the study were purchased commercially from Tekkim and Sigma-Aldrich.

2.2. Extraction and Isolation

β -sitosterol, diosgenin and jervine compounds were purified from the acetone extract of *Veratrum album* according to the procedure in our 2014 study [9]. The stigmasterol was purified from the dichloromethane extract of *Artemisia dracunculus* L. according to the procedure in our previous study [16]. The spectroscopic findings of the compounds were showed as in the our previous study [9, 16].

2.3. Inhibition of GR

GR enzyme activity was measured by Beutler's method [18]. An enzyme unit was defined as 1 μ M NADPH oxidation per minute under test conditions (25 °C, pH: 8.0). Using a spectrophotometer, a time of 3 minutes was determined at 340 nm absorbance. Different inhibitor concentrations were used and compounds were tested in triplicate at same process. Control cuvette activity was accepted as 100% in the absence of inhibitor. An Activity %-[Inhibitor] graph was drawn for each inhibitor [19, 20].

2.4. In silico Docking Studies

In silico docking studies were performed in order to investigate the interactions between structure containing molecules and amino acid residues within the GR active site. *In silico* docking figures were taken from Swissdock.

3. RESULTS and DISCUSSION

GR is a primary enzyme which maintains the reduced state of cell. Therefore lack of GR is be directly associated with various disease related to oxidative damage [21]. In this study, we report *in vitro* inhibitory effects of the steroidal natural compounds β -sitosterol, stigmasterol, diosgenin and jervine on GR enzyme. These compounds were found to be highly potent inhibitors of GR enzyme at micromolar level as shown in the Table 1. The IC₅₀ (μ M) values of the compounds was determined as 1.2580, 5.2116, 0.1916, 0.7701 for β -sitosterol, stigmasterol, diosgenin and jervine respectively. The IC₅₀ value of the *N,N*-bis(2-chloroethyl)-*N*-nitro used as reference substance was obtained from the literature as 647 μ M [22].

Table 1. IC₅₀ values, FullFitness and Estimated Δ G scores of test compounds on GR enzyme.

Inhibitor	IC ₅₀ , μ M	FullFitness, kcal/mol	Estimated Δ G, kcal/mol
β -sitosterol	1.2580	-2327.41	-7.30
Stigmasterol	5.2116	-2334.39	-6.76
Diosgenin	0.1916	-2347.70	-8.24
Jervine	0.7701	-2347.71	-7.75
<i>N,N</i> -Bis(2-chloroethyl)- <i>N</i> -nitrosourea	647	-	-

As we were unable for the moment to crystallize a GR in complex with one of the natural steroidal structure containing molecules, we performed *in silico* docking studies in order to investigate the interactions between natural steroidal structure containing molecules and amino acid residues within the GR active site. To this end, fully flexible docking methodology for

both receptor residues (1GRA pdb file was used for the target structure) and docked ligands was used by FullFitness and Estimated ΔG which was implemented with the Swissdock. β -sitosterol, stigmasterol, diosgenin and jervine were docked at the binding site of the target (hGR). FullFitness and Estimated ΔG scores of docked inhibitors at hGR targets and corresponding binding interactions were shown in Table 1. Also diosgenin docking figure of GR enzyme was shown in Figure 2.

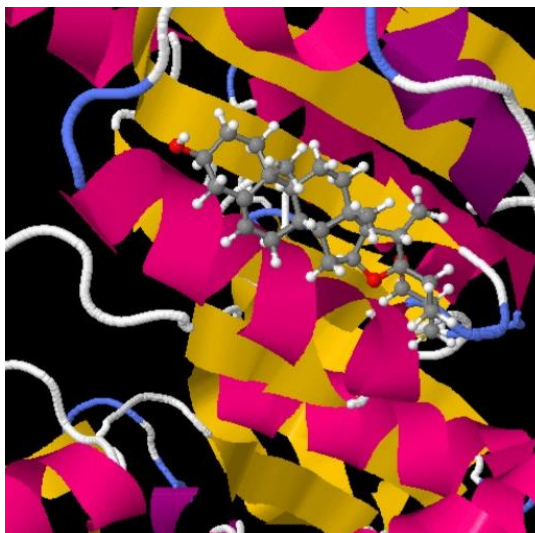


Figure 2. GR enzyme with diosgenin docking figure.

Among the test compounds, the diosgenin showed the strongest effect and stigmasterol showed the weakest effect. When we compare these results with the docking results, docking supports the results that diosgenin is the most potent GR inhibitor. Besides, it appears that the diosgenin is a stronger GR inhibitor than the reference compound *N,N*-bis(2-chloroethyl)-*N*-nitrosourea.

In literature, IC_{50} values of 1,4-naphthoquinone, 4-nitrobenzothiadiazole and methylene blue which are inhibitors of *P. falciparum* GR, have been reported as 2.71, 8.38 and 19.23 μM respectively. These inhibitors were greatly reduced GSH formation [4]. In a study of some *N*-methyl pyrrole derivatives, 0.104 to 4.942 μM results were obtained for GR enzyme [23]. Similar results were obtained in other studies on GR enzyme of natural substances such as thiamine, tyrosine, dopamine, lysine and glutamic acid [24, 25]. These values are similar to our results.

In terms of structure and activation relationship, steroidal molecules with little electron density have shown better GR inhibitor effect. The only difference in the chemical structure of 1 and 2 compounds is the double bond in the stigmasterol. According to results, β -sitosterol with IC_{50} value 1.2580 μM is a more potent GR inhibitor than stigmasterol. This result can be interpreted as stigmasterol has higher IC_{50} value because its double bonds is surrounded by hydrophobic amino acids. GR has a better inhibitory effect on structures with less electron density. Jervine has more electronegative atoms. Therefore, it has less interaction with the active site of GR enzyme. In the current study, IC_{50} results support this.

The results of our study showed that diosgenin was a very potent GR inhibitor with an IC_{50} value, 0.1916 μM (3376 times more effective than the reference molecule). So we may suggest to diosgenin as potent GR inhibitor. Diosgenin is a raw material in the drug industrial that using in the synthesis of steroidal agents such as progesterone, testosterone, norethisterone and glucocorticoids. It has cardioprotective, anticancer, antiaging and contraceptive properties and high economic value. Progesterone which produced by many pharmaceutical companies is synthesized from diosgenin [26].

4. CONCLUSION

In the current study, it has been presented β -sitosterol, stigmasterol, diosgenin and jervine as novel GR inhibitors that have IC₅₀ values at micromolar level. The most widely used anticancer drug *N,N*-bis(2-chloroethyl)-*N*-nitrosourea in the literature has an 647 μ M IC₅₀ value against the GR enzyme. However, this drug which is used as a GR inhibitor, leads to toxicity and inhibition of DNA synthesis [22]. We report natural steroidal compounds as novel natural GR inhibitors. The IC₅₀ values of the substances used in the study were found to be significantly lower than the positive control so these compounds were evaluated as strong inhibitors. Strong inhibitors may exhibit inhibitory effects at low concentrations, therefore it is thought that their side effects will be less. In this study, the natural steroidal molecules were exhibited much potent inhibitory activities against GR with IC₅₀ values ranging between 0.1916 and 5.2116 μ M. Inhibition of GR leads to death of malaria parasite and cancer cell due to an abnormal increase in ROS levels. Therefore strong GR inhibitors are very important for antimalarial and anticancer drug research and development.

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