Evaluating *in-vitro* antioxidant and *in-silico* anti-inflammatory potential of Diosgenin

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Abstract

Chronic diseases are inextricably tied up with oxidative stress and inflammatory responses. Oxidative stress triggers an antioxidant imbalance that elicits a multitude of inflammatory reactions, inevitably culminating in death-causing diseases by integrating free radicals. Rising research approaches intensely focus on identifying natural antioxidants with efficient anti-inflammatory potency. Diosgenin is a potent bioactive therapeutic candidate spotted in Fenugreek and Yams and has varied pharmacological properties. Concerning the therapeutic importance of this compound, the current study intends to explore its antioxidant and anti-inflammatory potential. The antioxidant ability of diosgenin was tested by using free radical scavenging assays such as 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), 2, 2'-Azinobis-(3-ethyl benzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$), Hydroxyl radical and Superoxide anion. Furthermore, the screening of Diosgenin's anti-inflammatory potential was done by ligand interactions via Autodock software to explore its binding affinity with inflammatory receptors (NF-κB and TNF-α). On comparing this with standard ascorbic acid, Diosgenin has nearly equivalent IC_{50} values.

The IC_{50} values of Diosgenin and Ascorbic acid were found to be 48.24 µg/mL and 47.93 µg/mL (DPPH), 41.18 µg/mL and 40.19 µg/mL ($ABTS^{\bullet+}$), 50.14 µg/mL and 48.99 µg/mL (Hydroxyl radical), 44.33 µg/mL and 50.02 µg/mL (Superoxide anion). Additionally, Diosgenin has good binding scores (-7.7, -9.8 kcal/mol) with target inflammatory receptors (NF-κB and TNF-α). In-vitro and in-silico modeling proves that diosgenin has effectively high antioxidant and anti-inflammatory potency. Hereby, diosgenin seems to be a promising antioxidant against inflammatory diseases.

Keywords: Antioxidant, Anti-inflammatory, Diosgenin, Molecular docking, Ligand interactions, Auto Dock.

Introduction

Oxidative stress is described as a disruption of the equilibrium between the development of Reactive Oxygen Species (ROS) and antioxidant defenses. Free radicals are oxygen-containing molecules with an unequal quantity of electrons. Owing to the unequal number, they might readily interact with other molecules. As a consequence, multiple primary chain chemical events termed oxidation transpire in our bodies[11]. A diverse spectrum of transcriptional regulators was principally triggered via oxidative stress. This contributes to the altered expression of several genes implicated in inflammatory pathways[10]. Numerous inflammatory stimuli have been observed to ignite an inflammatory process via the production and ejection of pro-inflammatory cytokines like excessive ROS released in the metabolic oxidation response and predefined natural or artificial compounds.

For example, the activation of the Nuclear Factor Kappa-B (NF-κB) and the generation of the Tumor Necrosis Factor-alpha (TNF-α) were evidenced to play a crucial role in the inflammatory process. Inflammation sparked by oxidative stress is the root cause of several chronic diseases[5]. Antioxidants are substances that can offer an electron to a free radical without becoming unstable. It aids in the stabilization of free radicals and their reduction in reactivity. They play a key role in preventing free radical development and help to reduce various diseases such as cancer, aging, cardiovascular disease, cataracts, weakening of the immune system, brain disorders and several inflammatory diseases[15].

Although a variety of synthetic antioxidants are already accessible, the majority of them have harmful hazardous negative impacts. Many plant species that have high antioxidant ability without toxic side effects have been used as alternate preventive medicine. Nowadays, naturally-derived antioxidants are in high demand for use as nutraceuticals, bio-pharmaceutical and food additive[8].

Naturally derived steroids have garnered considerable attention in recent times owing to their outstanding antioxidant ability. These sterols antioxidant function is mainly due to their redox properties, allowing them to serve as reducing agents or donors of hydrogen atoms[2]. Among those, Diosgenin was considered a good target for scavenging free radicals due to the lack of toxic side effects and its therapeutic importance. Moreover, diosgenin is a key active ingredient in several conventional and proprietary Chinese drugs. It is a (3β,25R)-spirost-5-en-3-ol (Figure 1), is a spirostanol saponin with hydrophilic sugar moiety associated with hydrophobic steroid aglycone.

In addition, it is a natural sterol found mostly in fenugreek (*Trigonella sp.*), legumes and yams (*Dioscorea sp.*). It has also proven potential in the therapy of a multitude of disorder including cancer, hypercholesterolemia, inflammation and infections[19].

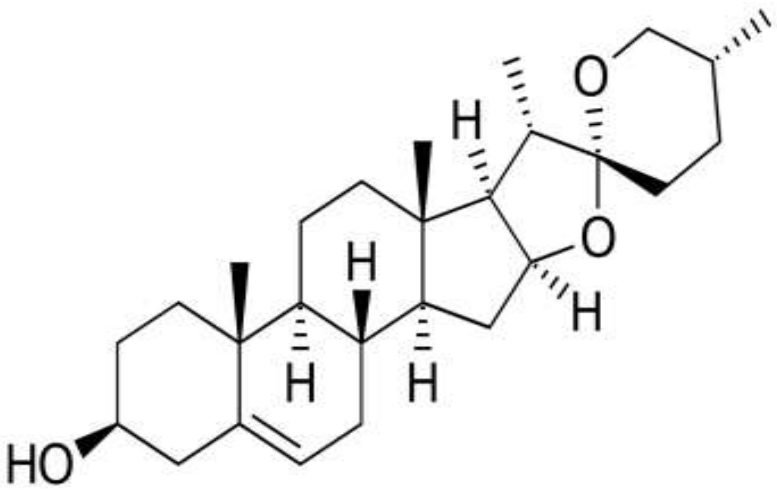

Figure 1: Structure of Diosgenin

Despite the rising therapeutic impact of diosgenin, the vital need to optimize its medicinal importance has sparked us to design the present study on the assessment of antioxidant and possible anti-inflammatory capacity. Thus, the main intent of the current study was to explore the antioxidant potential of Diosgenin by testing *in vitro* free radical scavenging assays such as 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), 2, 2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS $^{\bullet+}$), Hydroxyl radical, Superoxide anion and screening anti-inflammatory potential through *in silico* analysis using Auto-dock software.

Material and Methods

Antioxidant activity of Diosgenin:

Chemicals used: 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), 2,2-azino bis-(3-ethylbenzothiazoline-6sulfonic acid) (ABTS ●+), Nitro blue tetrazolium (NBT), trichloroacetic acid (TCA), potassium persulphate, ferric chloride, nicotinamide adenine dinucleotide (NADH), phenazine methosulphate (PMS), Ethylene Diamine Tetra Acetic acid (EDTA), thiobarbituric acid (TBA), hydrogen peroxide (H_2O_2), 2-deoxyribose, ethanol, phosphate-buffered saline (PBS), Ascorbic acid and Diosgenin were purchased from Sigma-Aldrich Pvt. Ltd., India.

DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay: DPPH is a strong free radical, widely accepted as a method for assessing an antioxidant's free radical scavenging activity[20]. The free radical scavenging behavior of diosgenin against DPPH was calculated spectrophotometrically in a dark room. DPPH accepts a radical electron or hydrogen to become a stable molecule of diamagnetic.

It reacts with an antioxidant compound capable of giving hydrogen and gets reduced. The color change (from deep violet to light yellow) has been measured. The yellow color frequency depends on the amount and type of the present radical scavenger[14]. This method was the most common framework for testing any new drug's free radical scavenging ability[12].

Different concentrations of diosgenin (10–50 µg/ml) were added in separate tubes and add 1ml of 0.5 mM DPPH solution in ethanol and then add 2 ml of 0.1 M acetate buffer (pH= 5.5). The solutions were held for 30 minutes at room temperature and then the absorption was measured at 517 nm using a UV-Visible Spectrophotometer (UV-1800 SHIMADZU). The decreased absorbance of the reaction mixture indicated higher free radical scavenging activity. Ascorbic acid was used as a comparison standard. The DPPH radical scavenging activity of Diosgenin was calculated using the equation:

Scavenging (%) = [(Control OD-Test OD)/ Control OD] ×100

2,2'-Azinobis-(3-ethyl benzothiazoline-6-sulfonic acid) (ABTS$^{\bullet+}$) radical cation decolorization assay: ABTS ● + radical cation production is the basis of one of the spectrophotometric techniques used to estimate the total antioxidant activity of pure substance solutions. The enhanced ABTS ● + generation technique involves direct blue/green ABTS ● +chromophore production through the ABTS ● +potassium persulfate reaction[18]. Diosgenin was added to compete with ABTS ● + and diminish color formation. By mixing with 2.5mM potassium persulfate, a 7mM ABTS radical cation solution was prepared. The reaction mixture contained Diosgenin at different concentrations (10–50 µg/ml) were added in separate tubes in a total volume of 3ml and then mixed with an ABTS solution (2.7 ml); then the reaction mixture was allowed to stand for 30 min and the absorption was recorded using a spectrophotometer at 734 nm. Ascorbic acid was used as a reference standard. The blank contained water in place of steroidal standards. The calculation of radical scavenging was determined by using the formula:

Scavenging (%) = [(Control OD-Test OD)/ Control OD] ×100

Hydroxyl radical scavenging assay: Hydroxyl radicals are generated in this assay by reducing H_2O_2 by the transition

metal ion in the presence of ascorbic acid. Hydroxyl radical generation was identified by its ability to degrade deoxyribose to form products that form a pink chromogenic color when heated with TBA[4]. Diosgenin addition competes for hydroxyl radicals with deoxyribose and decreases color formation. The incubation mixture contained 0.1 mL of buffer in a total volume of 1 mL, varying Diosgenin concentrations (10–50 µg/ml) in a separate tube, then 0.2 mL of ferric chloride, 0.1 mL of ascorbic acid, 0.1 mL of EDTA, 0.1 mL of H_2O_2 and 0.2 mL of 2-deoxyribose were added.

The substances were thoroughly mixed and incubated for 60 min at room temperature; then 1 mL TBA and 1 mL of TCA were added. All the tubes are held for 30 min in a boiling bath of steam. Ascorbic acid was used for contrast as a positive control. Using a spectrophotometer at 535 nm, the supernatant absorbance was read using a reagent blank containing water instead of steroidal standards. Decreased absorption of the reaction mixture showed increased activity of radical hydroxyl scavenging. The calculation of radical scavenging was determined by using the formula:

Scavenging (%) = [(Control OD-Test OD)/ Control OD] ×100

Superoxide anion radical scavenging assay: Superoxide anion radical scavenging assay was based on the production of superoxide anion dissolved oxygen by phenazine methosulfate-reduced coupling reaction and reduction of nitro blue tetrazolium (NBT) nicotinamide adenine dinucleotide (PMS–NADH). NADH oxidation by PMS releases PMS_{red}, which converts oxidized (NBT_{oxi}) to its reduced (NBT_{red}) form. This forms a complex of violet color suggesting superoxide anion productions which was measured at 560 nm spectrophotometrically[13]. With the introduction of the antioxidant, a decline in color formation was an indicator of its radical scavenging activity. Add and mix well 1 mL of NADH solution and varying Diosgenin concentrations in a separate tube (10–50 µg/ml) to 1 mL NBT. The reaction began with the introduction of PMS 100 RPM. For 15 minutes, the reaction mixture has been incubated at 30 ° C.

Incubation with water was used as blank instead of sterol standards. Ascorbic acid was used as a comparison guide and the absorption was measured on a spectrophotometer at 560 nm. Decreased reaction mixture absorbance suggested increased scavenging activity of superoxide anion. As shown below, the percentage of scavenging was calculated:

Scavenging (%) = [(Control OD-Test OD)/ Control OD] ×100

Statistical analysis: Each trial was carried out with three groups and the mean values were determined. The data were recorded as mean ± SD (n=3). The samples were detected in triplicates and one-way analysis of variance was carried out using SPSS software (version 11.5 for Windows 2000, SPSS Inc.) and the values of $P < 0.05$ were considered statistically significant. The graphical representation of the results was done by using Microsoft Excel 2007 (Roselle, IL, U.S.A).

Anti-inflammatory activity of Diosgenin:

Tools and materials used: The tools used in this study include the HP computer system (Intel Core i3, 4 GB RAM, Windows 10 operating system), PubChem database, protein data bank, Auto-dock tools in auto dock 4.2.6 program and Discovery Studio Visualizer.

Protein and Ligand preparation: The 3-D crystal structures of both inflammatory target proteins were retrieved from the Protein Data Bank (PDB) utilizing their PDB ID's such as NF-κB (1NFK) and TNF-α (6RMJ) separately. During the preparation of the protein, the non-essential water molecule was removed. Then charges and polar hydrogen were added and the packed protein target was finally saved in PDBQT format. Although during the ligand preparation, the Diosgenin formulation mentioned in this study was extracted from the PubChem compound database with its PubChem CID: 99474. The Diosgenin 3-D conformer has been exported as an SDF file. Then it was translated to a PDB format and opened in the ligand menu. Then pick the torsion menu to detect the root in the ligand and save the prepared ligand in the PDBQT format.

The graphical user interface was used to prepare PDBQT files from PDB files. Then the ligand and the protein were opened in the grid menu. After this, the grid box was built and the current file was saved in the grid parameter file (GPF) format. The Auto-Grid was run and generated the respective macromolecular file output with the molecular.glg extension.

Molecular docking and virtual screening: Auto-dock uses some of the multiple conformational search algorithms to explore the conformational status of a flexible ligand and the designed auto grid maps were used to determine the interaction between ligand and protein at each docking simulation stage. Similar binding sites and ligand conformations have been tested.

Docking results were analyzed using Biovia Discovery Studio Visualizer to find binding affinity, to visualize 2D and 3D interaction as well as to quantify these interactions between receptors and ligands and scrutinize all the unique amino acid residues that interfered in binding.

Results and Discussion

Diosgenin's antioxidant potency: *In-vitro* antioxidant activity was carried out with diosgenin by investigating DPPH, ABTS●+, hydroxyl, superoxide anion radical scavenging assays with ascorbic acid as a comparison guide. The IC_{50} value was determined for each compound and summarized as shown graphically in figures 2-5. In this, the free radical scavenging property of diosgenin increased with increasing concentrations.

This assay measures the ability of diosgenin to reduce radical DPPH by transforming unpaired electrons into paired electrons to the corresponding hydrazine. Figure 2 shows the diosgenin's radical capacity for DPPH scavenging compared to ascorbic acid. The ascorbic acid inhibitory concentration (IC_{50}) is 47.93 µg / ml which is close to 48.24 µg / mL of diosgenin (IC_{50}). In DPPH-free radical scavenging, both ascorbic acid and diosgenin have shown the same powerful activity.

ABTS ● + radical scavenging was measured based on blue/green ABTS ● +chromophore reduction generated by an antioxidant donating electron from the reaction between ABTS ● + and potassium persulphate. Figure 3 shows the diosgenin ABTS ● + radical scavenging activity compared to the Ascorbic acid standard. In this, the ascorbic acid IC_{50} value is 40.19 µg / ml while the diosgenin IC_{50} value is 41.18 µg / mL. Diosgenin scavenges ABTS ● + radicals with equal effect as ascorbic acid potential.

Hydroxyl radicals (OH), produced in the human body can play an important role in diseases caused by oxidative stress. Figure 4 shows diosgenin with ascorbic acid as a comparison guide for hydroxyl radical scavenging action. This indicates that the IC_{50} value for ascorbic acid in hydroxyl radical scavenging activity is 48.99 µg / mL and is equal to the diosgenin IC_{50} (50.14 µg / mL). This result shows that diosgenin was a strong hydroxyl radical scavenger.

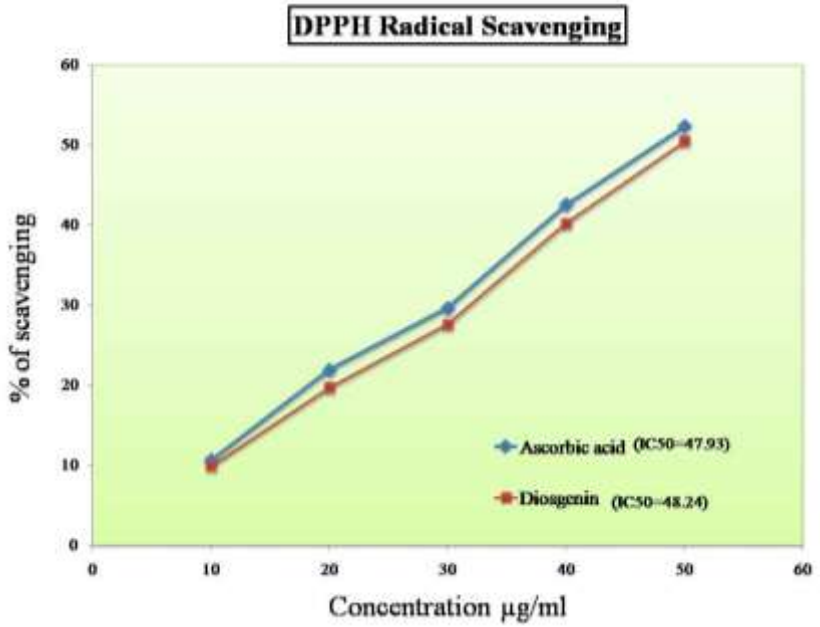

Figure 2: DPPH radical scavenging effect of diosgenin and ascorbic acid at different concentrations

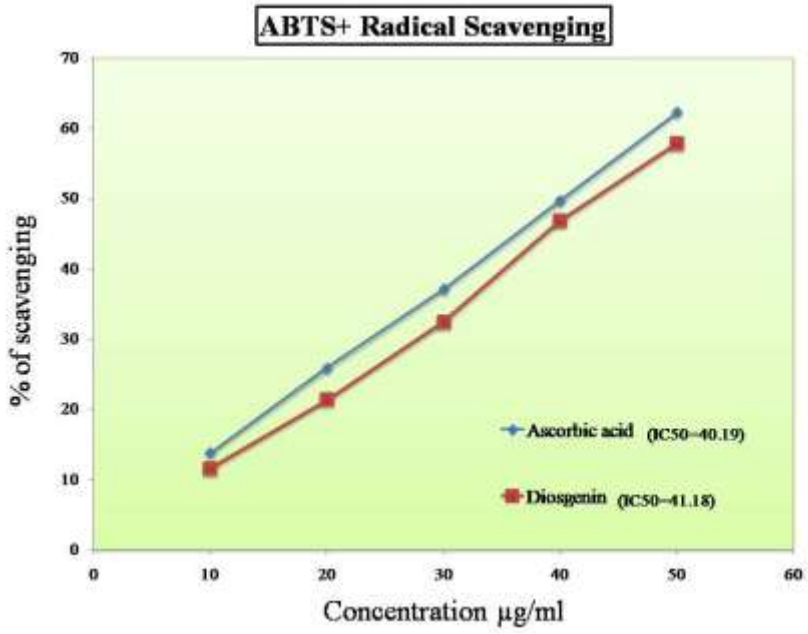

Figure 3: ABTS●+ radical scavenging effect of diosgenin and ascorbic acid at different concentrations

Superoxide anion radicals are the most significant ubiquitous ROS produced by the enzymatic cycle, autoxidation reaction and non-enzymatic electron transfer processes in which an electron is converted to molecular oxygen. Figure 5 shows the activity of Diosgenin to scavenge Superoxide anion.

The result obtained shows that the ascorbic acid IC_{50} value is 50.02 µg / mL equivalent to the diosgenin IC_{50} value (44.33 µg / mL). Thus, diosgenin's inhibitory effect shows that it was the powerful target for superoxide anion scavenging. Therefore, raising the concentration of diosgenin results in an increased inhibitory effect.

Diosgenin's anti-inflammatory potency: In a quest to reconcile the anti-inflammatory potential of diosgenin, the ligand was docked with the crystal structure of the target proteins obtained from the PDB. Thus, diosgenin shows the best binding poses with suitable binding energy for both targets.

The 2D and 3D interactions of diosgenin with inflammatory targets were well pictured in fig.6 and fig.7 by showing all of its binding residues using Biovia Discovery Studio Visualizer. Table.1 shows the binding energy and the interactions residues of ligand-protein interactions.

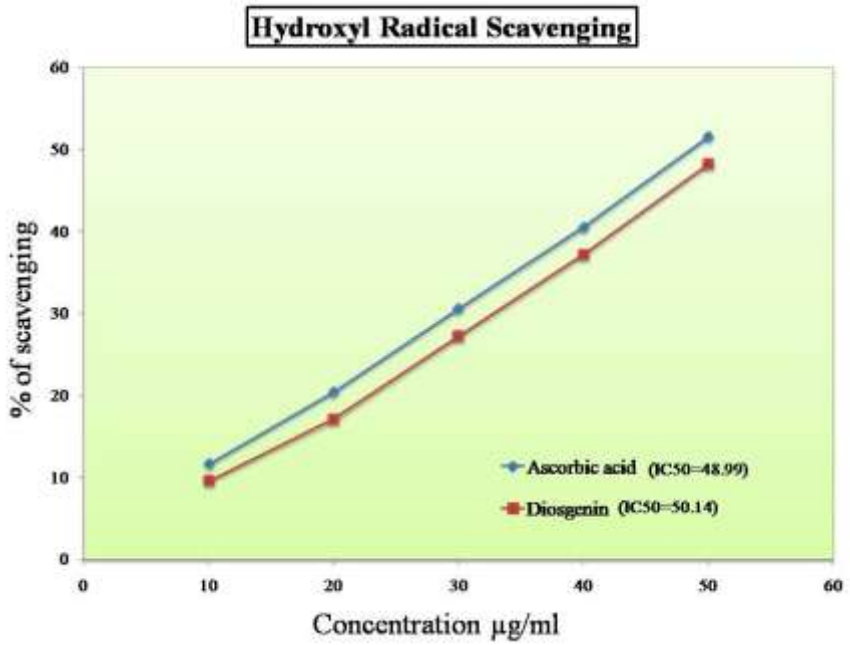

Figure 4: Hydroxyl radical scavenging effect of diosgenin and ascorbic acid at different concentrations

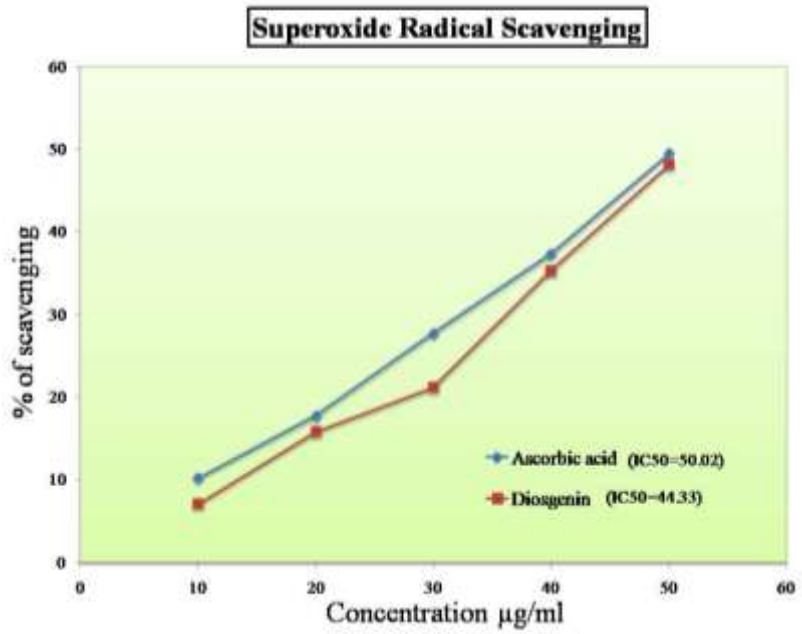

Figure 5: Superoxide anion radical scavenging effect of diosgenin and ascorbic acid at different concentrations

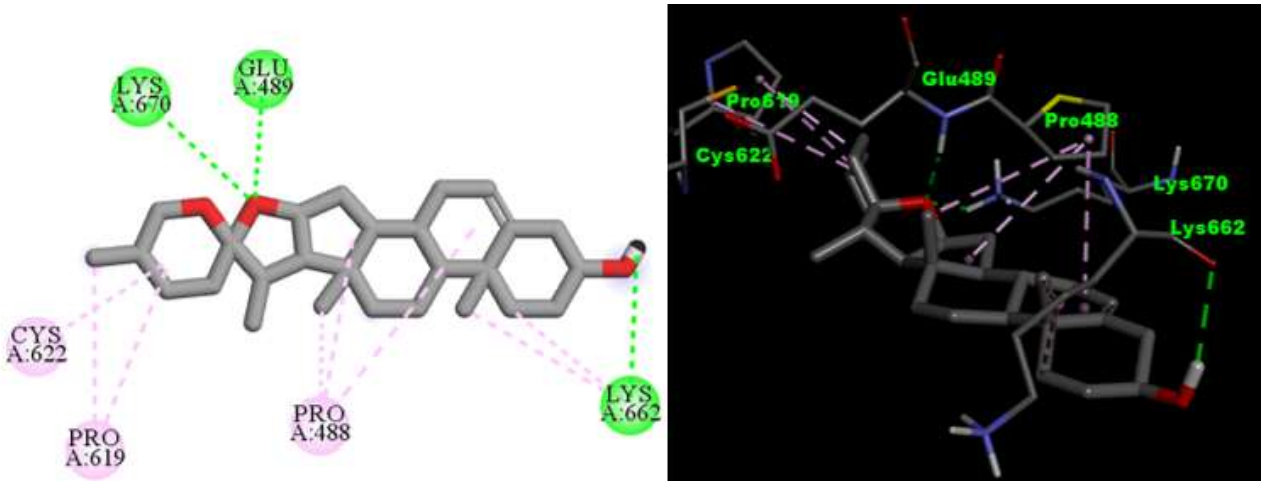

Figure 6: 2D & 3D interactions of diosgenin with NF-κB

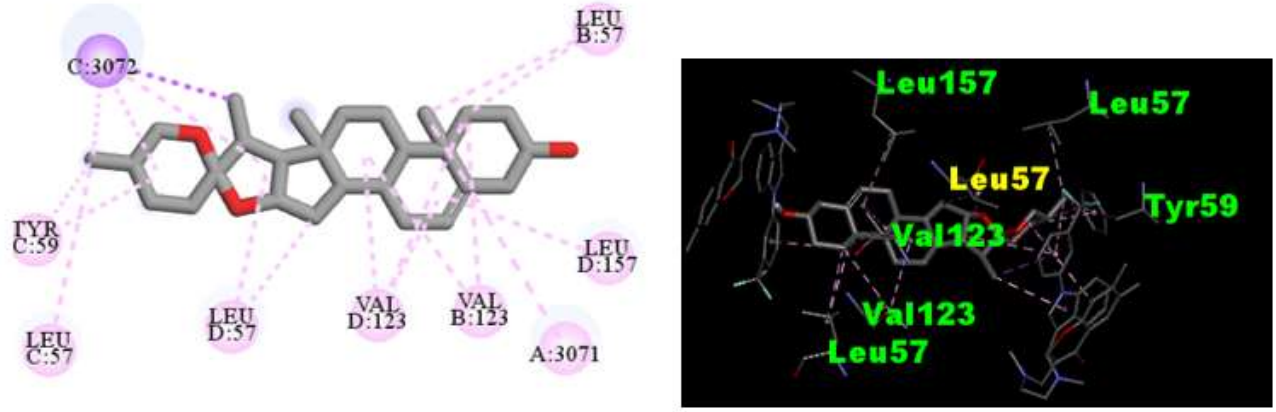

Figure 7: 2D & 3D interaction of diosgenin with TNF-α

Table 1
Diosgenin docking results with inflammatory targets

S.N.	Target	PDB ID (Target)	Ligand	Binding energy	Interactive residues
1.	NF-κB	1NFK	Diosgenin	-7.7	LYS A: 670, GLU A: 489, LYS A: 662, PRO A: 488, PRO A: 619, CYS A: 622.
2.	TNF-α	6RMJ	Diosgenin	-9.8	TYR C: 59, LEU C: 57, LEU D: 57, VAL D: 123, VAL B: 123, LEU D: 157, LEU B: 57.

The present study used four different antioxidant test system such as DPPH, ABTS+, hydroxyl radical and superoxide anion scavenging activity to confirm diosgenin's antioxidant potential. DPPH (1,1-diphenyl-2-picrylhydrazyl) is a stable, deep purple free radical with strong absorption at 515-520 nm. Diosgenin can adopt an electron or hydrogen atom from antioxidant scavenger molecule culminating in a more stable DPPH molecule with a pale yellowish tinge[1]. The degree of discoloration reveals diosgenin's ability to scavenge free radicals because of its capacity to provide hydrogen proton[6]. The concentration-dependent curve of DPPH radical scavenging activity of Diosgenin fits well with ascorbic acid as standard drug.

Therefore, the result obtained confirm Diosgenin's DPPH radical scavenging activity. The 2,2, - azino bis (3-ethyl benzoline-6-sulfonic acid) (ABTS+) assay is based on the inhibition of the absorbance of the radical cation ABTS+, which has an attribute in the long-wavelength absorption spectrum[17]. The concentration-dependent curve of ABTS+ radical scavenging activity of Diosgenin compared well with ascorbic acid as a standard drug. The results obtained in our

study revealed that the activity of the sterol was either by inhibiting or scavenging the ABTS+ radical.

These results are in agreement with the antioxidant effect of diosgenin. Radical hydroxyl in living cells is known to be the most reactive radicals that can attack and damage nearly every bio-macromolecule. The hydroxyl radical is mainly known for its ability to induce lipid peroxidation, which occurs when a hydroxyl radical is formed near the membranes and attacks the fatty acid side chains of the membrane's phospholipids[16]. In our present study, diosgenin showed the percentage of inhibition in a dose-dependent manner. Superoxide anion is one of single oxygen's precursors, thereby potentially inhibiting lipid peroxidation. Besides, the presence of a superoxide anion can increase cell damage because it produces other free radicals and oxidizing agents[21].

In biological systems, radical superoxide can be converted to hydrogen peroxide by superoxide dismutase enzyme and the H_2O_2 can then create extremely reactive hydroxyl radicals in the presence of specific transition metal ions. The scavenging or preventative activity of diosgenin against superoxide anion-free radicals was studied in this work. As a result, the superoxide anion radical scavenging properties of diosgenin increased as the concentration was raised. In comparison to the benchmark for ascorbic acid, our data shows that diosgenin has strong free radical scavenging capabilities.

Molecular docking was further employed to explore diosgenin's anti-inflammatory properties. Before *in-vitro* analysis, molecular docking is a promising approach for evaluating ligand-protein interactions[3]. We utilized an auto-dock to detect bound conformations in this investigation. Nuclear factor-κB (NF-κB) is the term for a novel genomic family of transcription factors that regulates a large number of genes involved in immunological and inflammatory responses[9]. Diosgenin shows the binding energy (-7.7) with NF-κB. LYS A: 670, GLU A: 489, LYS A: 662, PRO A: 488, PRO A: 619, CYS A: 622 are the major residues involved in NF-κB - DG interaction.

Tumor Necrosis Factor - α (TNF-α) is an inflammatory activator secreted by macrophages following acute inflammation that regulates a variety of cell signaling cascade leads to apoptosis[7]. Diosgenin displays the assurance of binding energy (-9.8) to TNF-α. TYR C: 59, LEU C: 57, LEU D: 57, VAL D: 123, VAL B: 123, LEU D: 157, LEU B: 57 are the primary residues of TNF-α – DG interactions respectively. As a result, the aforesaid findings support diosgenin's anti-inflammatory properties.

Conclusion

Natural nutrients have indeed been exploited in herbal medicine for eons and are now garnering recognition primarily due to their potent antioxidant properties. Diosgenin can inhibit radical assaults and eventual oxidation of biological components by stabilizing the radicals emitted by reactive oxygen species.

Furthermore, diosgenin has a considerable influence on radical stabilization and has high antioxidant activity as a consequence. We conclude that diosgenin has the equivalent antioxidant capacity as the well-known standard ascorbic acid. The anti-inflammatory potential of diosgenin was also extensively explored in this study owing to its high binding affinity. As a consequence, our present research expects that diosgenin will pave the way for novel strategies to address inflammatory disorders.

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