Section A-Research Paper
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Effect of *Withania Coagulans* Dunal Leaves Extract on Reactive Oxygen Species (ROS) in Diabetes-Induced Rats by different methods of Molecular Docking

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1. INTRODUCTION

Aging and diseases associated with it develop because of free radicals and other reactive oxygen species causing oxidative stress and damage to the tissue, which has been implicated in cancer, atherosclerosis, other neurodegenerative diseases and diabetes [1, 2]. It is therefore required to measure it accurately. Oxidative stress is caused due to the over- production of the reactive oxygen species (ROS) and the disturbance developed in the antioxidant potential of bio-chemical processes [3, 4]. The ROS are hydrogen peroxide, superoxide anion, and hydroxyl radical [5, 6]. ROS mostly form in the brain due to high consumption of oxygen and insufficiency of endogenous antioxidant resistance mechanisms [7]. ROS is commonly generated by the mitochondrial electron transport chain (mETC), NADPH oxidases, xanthine oxidase, cyclooxygenase-I & II, and enzymatic stimulation of cytochrome P450 2E1 (CYP2E1) [8, 9]. CYP2E1 has an excessive percentage of NADPH oxidase activity, which causes the production of ROS and increases oxidative stress [10-14]. It can cause damage to the biomolecules [15], (lipids, proteins, DNA) [16], which ultimately develop numerous chronic diseases like atherosclerosis [17], cancer [18], diabetes [19, 20], rheumatoid arthritis [21], post- ischemic

perfusion injury [22, 23], myocardial infarction, cardiovascular diseases [24, 25], chronic inflammation, stroke and septic shock, aging and other degenerative diseases [26-31]. These ROS exposures demand a carefully balanced control of their production and disposition, which is largely achieved through the body's elaborate antioxidant system. The human antioxidant system consists of small antioxidants, antioxidant proteins, ROS-metabolizing enzymes, as well as many regulator proteins that mediate adaptive responses to oxidant stress. How such a complex system reacts with oxidants and achieves the required specificity and sensitivity for proper anti-oxidation is incompletely understood [32-34].

Diabetes-facilitated ROS could be produced both enzymatically and non-enzymatically [35]. In diabetic conditions, chronic hyperglycemia and consequent generation of ROS depreciate beta-cell activity, which intensifies the insulin resistance [36]. We have studied the effect of the ethyl acetate extract of *Withania Coagulans* Dunal. (EAWC) on ROS in the brain of diabetes-induced rats. We have also investigated the possible molecular mechanism of reduction in ROS through molecular docking. We performed molecular docking of flavonoids on CYP2E1 to study the inhibitory potential. As from the above literature, it is clear that CYP2E1 is mostly responsible for the production of ROS. The link between CYP2E1, ROS, diabetes, and oxidative stress is represented in Fig. (1).

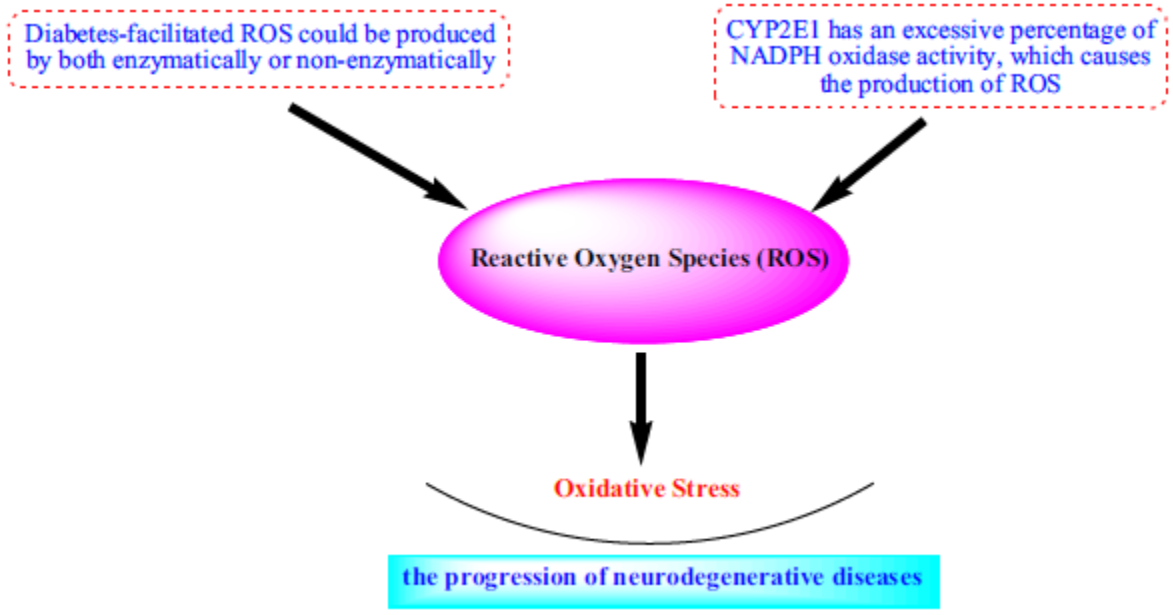

Figure 1: The link between CYP2E1, ROS, diabetes, and oxidative stress.

Withania (W.) coagulans Dunal belongs to family *Solanaceae*. It is distributed in the East of the Mediterranean region and extends to South Asia. Different parts of this plant have been reported to possess a variety of biological activities. The fruit, berries are used for commercial purposes for milk coagulation. Literature surveys on this plant revealed the presence of esterases, lignan, alkaloids, free amino acids, fatty oils, essential oils and withanolides. The fig. 3.1 represents the images of *W. Coagulans* Dunal.

2. MATERIALS AND METHODS

2.1 Ethical Considerations

The experimental protocol was approved by the Institutional Animal Ethics Committee of the Preclinical Research and Development Organization (PRADO), Pune (Approval No.-1723/PO/RcBiBt/13/CPCSEA).

2.2 Plant Authentication and Extraction

Collection and authentication of plant *Withania Coagulans* Dunal Linn. were carried out from Sri Venkateswara University Tirupati, India (Voucher number-2120). Leaves were dried and were macerated with petroleum ether for 24 hrs. The residue was extracted with methanol using the Soxhlet apparatus. The residue obtained was extracted with ethyl acetate and water (1:8:2 i.e. I ml residue+8 ml ethyl acetate 2 ml water) in a separating funnel. The filtrate was concentrated to get a dry mass of EAWC [54].

2.3 Chemicals and Reagents

CM-H,DCFDA (General Oxidative Stress Indicator, catalog number: C6827), Dulbecco's phosphate-buffered saline (DPBS), calcium, magnesium (catalog number. 14040224), Hank's balanced salt solution (HBSS) with calcium and magnesium and without red phenol (catalog number: 14025) and propidium iodide - 1.0 mg/ml (catalog number: P3566) were procured and purchased from Life Technologies, Invitrogen Ix Trypsin-EDTA (0.05% 0.02% in PBS, catalog number: L11-004) was purchased from PAA Laboratories GmbH.

2.4 Animals

Sprague Dawley male rats (180-250 g) were used for the current study. They were maintained with temperature (25+2°C), relative humidity (45-55%) and under standard environmental conditions (12 h light & dark cycles). The rats had free access to standard animal food and water ad-libitum [55].

2.5 Experimental Designs

The five groups of six rats in each group were made to fast overnight to use them for the experiment. Group I served as control. Group II served as diabetic control, group III and IV treated with an oral dose of EAWC 50 mg/kg and 100. mg/kg, respectively. Group V and VI served as a standard group that received metformin 120 mg/kg and gabapentin 100 mg/kg, respectively. (Oral route) [56, 57].

2.6 Induction of Diabetes

Hyperglycemia was induced by a single. intraperitoneal injection of freshly prepared streptozotocin (STZ) (55mg/kg body weight, in 0.1M citrate buffer (pH 4.5) to a group of overnight fasted rats. To control drug-induced hypoglycemia, a solution of 5% glucose overnight was given to rats. Hyperglycemia was confirmed on the third day after STZ injection by estimating glucose level by glucometer. The rats having a glucose level of 300 mg/dl were used for the study [39, 58-60].

2.7 Tissue Preparation for Biochemical Analysis

Tissues were minced into 3 to 4 mm pieces with a sterile scalpel or scissors and washed several times with Hanks' Balanced Salt Solution (HBSS). The collagenase (50 to 200 U/ml in HBSS) was then added to it. It was incubated at 37°C for 4 to 18 hrs. and 3M CaCl, was added to increase the efficiency of dissociation. The cell suspension was filtered through a sterile nylon mesh to separate the dispersed cells and the tissue fragments from the larger pieces. The fresh collagenase was added to the fragments for further dis- aggregation of the suspension. The suspension was washed several times by centrifugation in HBSS. The pellets were suspended in the culture medium, followed by counting and seeding the cells for culture and further treatment [61]. The images of isolation of the rats' brains for measurement of ROS are represented in Fig. (2).

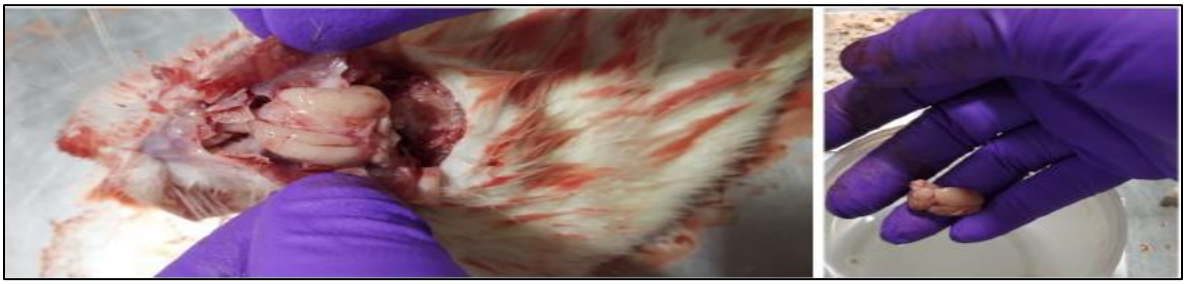

Figure 2: The isolation of rat for the measurement of reactive oxygen species (ROS) by Flow cytometry.

2.8 Measurement of Reactive Oxygen Species (ROS) Generation

The single-cell preparation was done by using collagenase treatment and seeded in culture plates. Sixteen hours later, the cells (90% confluence) were washed by warm DPBS, harvested by trypsinization (250 µl of trypsin/well). Then 1 ml of complete culture medium (10% FCS) was added to stop trypsinization and the cells were-suspended by gently pipetting up and down. The cells were collected into a 5 ml polystyrene round-bottom tube by centrifugation (5 min at 200X g) at room temperature and the supernatant was re- a moved. The cell pellet was washed with 4 ml DPBS and centrifuged for 5 min at 200 X g at room temperature. The cell pellet was re-suspended in HBSS with 10 µmol/L of CM- H_2 DCFDA and different concentrations of extracts. The cells were incubated (re-suspended in tubes) in a cell incubator [(37 °C) with high relative humidity (95%), and controlled CO_2 level (5%)] in the dark for 45 min. Propidium iodide 0.5 ul/0.5 ml/tube (final concentration 1 ug/ml) was added and the tubes were placed on ice (still in the dark) and immediately proceeded for flow cytometry analysis. A total of 5,000 events were analyzed in flow cytometry. The cellular viability was assessed by propidium iodide staining. The Mean Fluorescent Intensity (MFI) was obtained from the FL-3 channel (620 nm) of the Attune NxT flow cytometer from Thermofisher. Living cells, which were Pl negative, were selected by FACS gating. In these living cells, we recorded the fluorescence of DCF on the FL-1 channel (525 nm) of the Attune NxT flow cytometer from Thermofisher [55, 61- 64].

2.9 Statistical Analysis

Results were articulated as mean ± standard deviation (SD). Data were analyzed using analysis of variance (ANO- VA) followed by Bonferroni's a post hoc test. Values of $p \leq 0.05$ were measured as substantial. All the statistical analyses were done by Graph pad prism 7software [55, 56].

2.10 Safety Studies

Rats were injected with different doses of EAWC up to 2000 mg/kg and the observations found were free of any toxicity as per the range provided by the guid No. 425, and no mortality was found.

2.11 Molecular Docking

Autodockvina 1.1.2 in PyRx-virtual screening tool 0.8 was used to perform the molecular docking studies [65]. The protein preparation and receptor-ligand interactions were studied by

using BIOVIA Discovery Studio Visualizer (version-19.1.0.18287) [66]. The structures of flavonoids (luteolin, quercetin, apigenin and hydroquinone) (SDF File) were downloaded from the official website of the U.S. National Library of Medicine PubChem (https://pubchem.ncbi.nlm. nih.gov/). Energy minimization (optimization) was per- formed by Universal Force Field (UFF) [67]. The elucidated crystal structure of human cytochrome P450 2E1 in complex with omega-imidazolyl-dodecanoic acid was obtained from the RCSB Protein Data Bank (PDB ID: 3LC4) (https://www.resb.org/structure/3LC4) [68]. There were two chains (Chain A& B) in the crystal structure of CYP2E1 Chain A was selected to perform the molecular docking. The complete molecular docking was performed as per the pro- cedure described by S. L. Khan et al. [69]. The structure of CYP2E1 (PDB ID: 3LC4); Chain-A with co-crystallized ligand in active cavity represented in Fig. (3A) was obtained from Discovery Studio, whereas Fig. (3B) represents the chemical structures of the flavonoids. The flavonoids used for the docking were checked whether they violate Lipinski's Rule of 5 or not. All the properties needed for it were noted from the U.S. National Library of Medicine PubChem from the chemical and physical properties tab. Table 1 represents the name of flavonoids, Pubchem CID and Lipinski's Rule of 5.

3. RESULTS & DISCUSSION

Extraction, Ethyl acetate fractionation, Pharmacognostical Evaluation, Phytochemical Screening by Qualitative Tests and Safety Studies

After getting methanolic extract, the ethyl acetate ethyl acetate fractions have been isolated. We have got 2.9% of EAWC and 1.1% of ethyl acetate fraction.

6.4.3 Measurement of reactive oxygen species (ROS) by Flow Cytometry

The ROS was estimated by flow cytometry in each group of animal models of both the ethyl acetate fractions. A determined ROS for animal models of both the ethyl acetate fractions are tabulated in table 6.12. The graphs of results obtained from determinations of ROS in the animal models of both the ethyl acetate fraction (EAWC) are illustrated in fig 6.10. ROS causes damage to the cellular tissues which results in diabetic neuropathic pain. It has been observed that both plant ethyl acetate fraction have reduces the ROS generation significantly compared to metformin treated group. This indicates the potential of these ethyl acetate fraction to treat neuropathic pain.

Table 2: A determined ROS for animal models of both the ethyl acetate fractions Median Florescence Intensity was measured by flow cytometry.

Normal Control	Diabetic Control	Metformin (120mg/kg)	Gabapentin (100mg/kg)	EAWC (50mg/kg)	EAWC (100mg/kg)
241.6 ± 9.83	450.3± 4.04***	449± 7.59	415.3333 ±8.81	382.333 ±3.75**	331± 9***

Results are expressed as means ± SEM;(n=6), One way ANOVA followed by Bonferroni test; Vs. respective controls; **p< 0.01, ***p< 0.001.

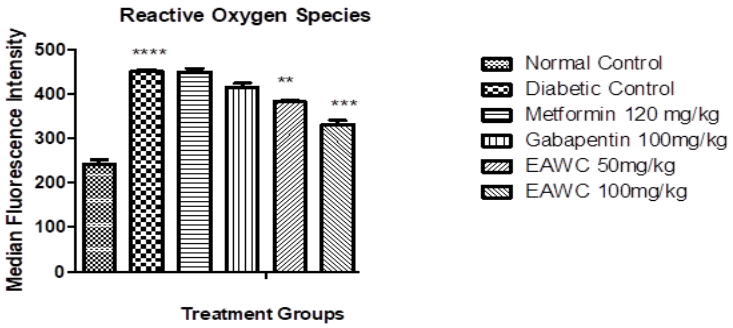

Figure 4. The graph of results obtained from determinations of ROS in the animal model of EAWC

6.5 Molecular Docking Studies of Flavonoids on Target Enzyme to Propose the Possible Mechanism behind the suppression of Diabetic Neuropathy

6.5.1 Molecular Docking of Flavonoids Present in EAWC

The docking score, type of interactions, and active amino acid residues with bond length (A^0) of all the docked flavonoids are represented in table 6.13. For molecular docking simulation, the three-dimensional grid box (size_x = 45.7138A^o; size_y = 37.6096A^o; size_z = 32.2988A^o) was designed (to define the active cavity) using Autodock tool 1.5.6 with exhaustiveness value of 8. All the dock scores were selected in which the RMSD values were zero. The 2D & -3D docking poses of quercetin and myrecitin are represented in fig. 6.11 and 6.12 respectively.

Table 3: The dock score, type of interactions and active amino acid residues with bond length (A^0) of all the docked flavonoids present in EAWC

Name of Compound	**Dock Score (*kcal/mol*)**	**Type of Interaction**	**Active Amino Acid Residues with Bond Length (A^0)**
Quercetin	-8.9	Hydrogen Bond	Leu-A:363 (2.23), Gln-A:358 (2.12)
		Pi-Alkyl	Val-A:363 (5.18), Pro-A:429 (5.18), Leu-A:363 (5.27)
		Pi-Sigma	Leu-A:363 (3.79)
		Pi-Sulfur	Cys-A:437 (3.96)

		van der Waals	Arg-A:435, Arg-A:100, Leu-A:368, Asn-A:367, Ser-A:431, Thr-A:362, Leu-A;397, Ile-A:361, Thr-A:307, Leu-A:447, Thr-A:304, Ala-A:443, Thr-A:303, Phe-A:430
Myrecitin	-8.8	Hydrogen Bond	Gln-A:358 (2.66), Phe-A:430 (2.33)
		Pi-Sigma	Leu-A:363 (3.79)
		Pi-Sulfur	Cys-A:437 (3.90)
		Pi-Alkyl	Val-A:364 (5.25), Pro-A:429 (5.19), Leu-A:363 (5.27)
		Unfavorable Donor-Donor	Thr-A:307 (2.79)
		Unfavorable Acceptor-Acceptor	Leu-A:363 (1.29), Gln-A:358 (3.00)

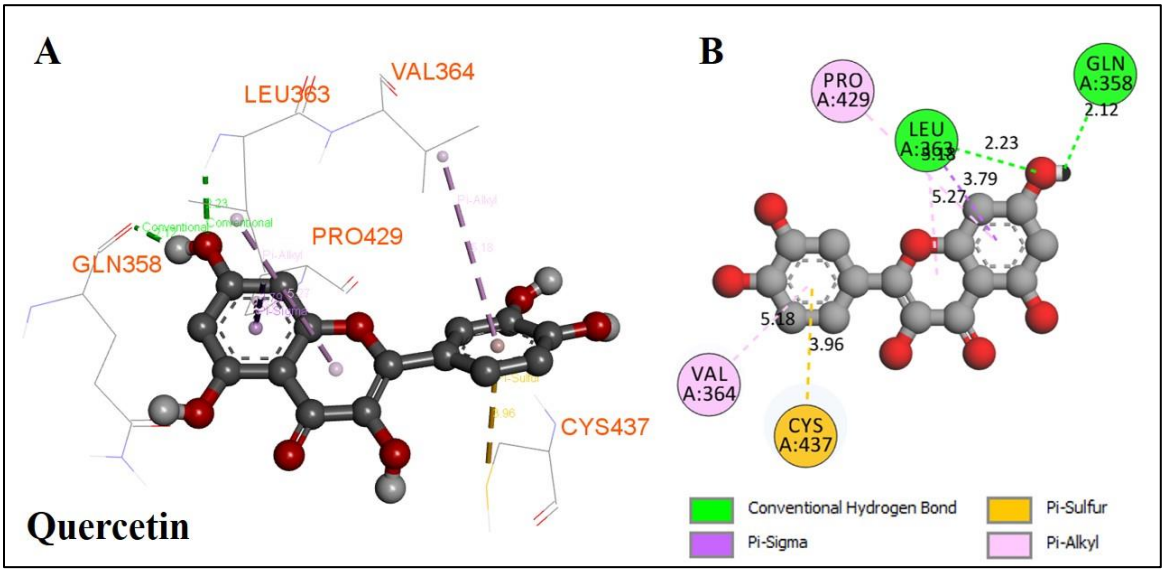

Figure 5: A) The 3D docking pose; B) The 2D docking pose of Quercetin with CYP2E1

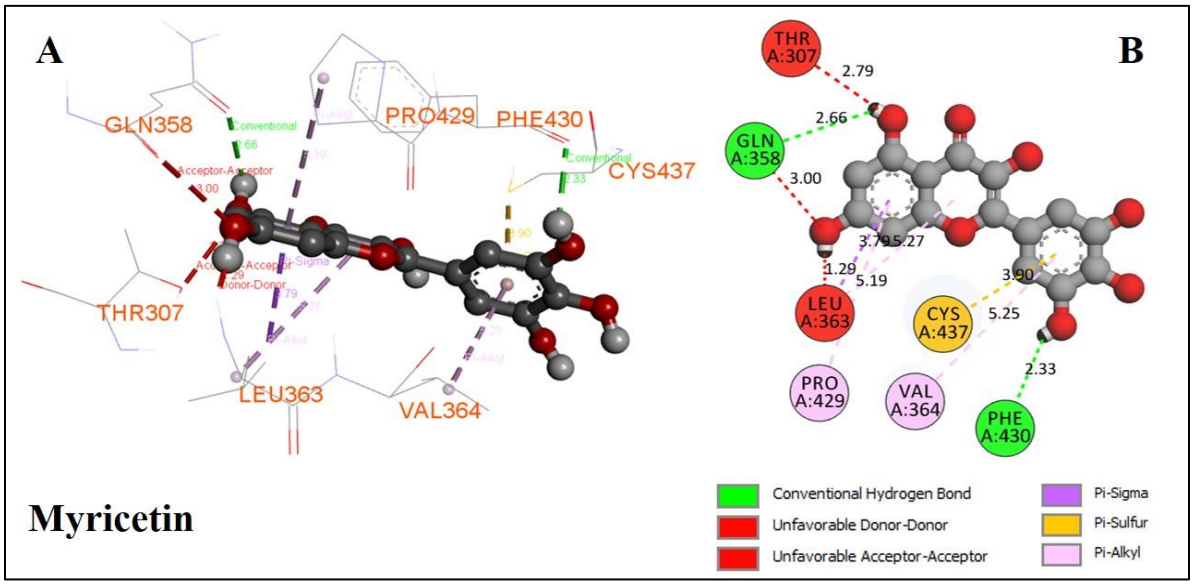

Figure Error! No text of specified style in document.. A) The 3D docking pose; B) The 2D docking pose of Myrecitin with CYP2E1

CONCLUSION

This study indicates that the glucose-induced cell toxicity in diabetes could be mediated through ROS production. The presence of EAWC lowers the glucose-induced production of ROS. ROS are mostly formed in the brain due to the high consumption of oxygen and the insufficiency of endogenous antioxidant resistance mechanisms. We have studied the effect of the Ethyl Acetate Extract of *Withania Coagulans* Dunal. on ROS in the brain of diabetes- induced rats. We have investigated the possible molecular mechanism of reduction in ROS through molecular docking. Luteolin, quercetin, and apigenin have inhibited the CYP2E1 very effectively. Luteolin formed 4 hydrogen bonds with CYP2E1, which indicates its potential inhibition. Although, luteolin and apigenin have shown a very good binding affinity with the enzyme. The ROS measurement in the rats' brain by flow cytometry also supports these results. The results have shown that EAWC reduces the generation of ROS in the diabetes-induced rat in a dose-dependent manner. The oral dose of EAWC 50 mg/kg and 100 mg/kg was given to the rat and the ROS generation got affected accordingly. From the present work, we concluded that the ethyl acetate extract of *Withania Coagulans* Dunal can effectively inhibit the ROS generation in the diabetes-induced rat by inhibiting the activity of CYP2E1.

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