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Original Article

Anti-diabetic Properties of Thymoquinone is unassociated with Glycogen Phosphorylase Inhibition

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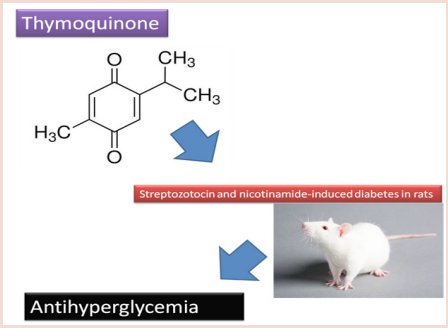
ABSTRACT

Background: *Nigella sativa* L. (Black seed), is commonly used by traditional healers as a remedy for more than four thousand years. The antidiabetic property of *N. sativa* seeds oil is attributable to the presence of Thymoquinone (TQ). On the other hand many studies have been designed to investigate the possible effects of the TQ in Streptozotocin (STZ) and nicotinamide (NA)-induced diabetes in rats. **Aim of the study:** The aim of this study was to elucidate the mechanisms underlying the glucose lowering effects of thymoquinone. **Methods**: *In vitro* and *in silico* using glycogen phosphorylase (GPa) enzyme assay and docking tools were used. **Results:** Oral administration of TQ for 60 days, dose dependently improved the glycemic status in STZ-NA induced diabetic rats. GPa activity was measured in the direction of glycogen synthesis by the release of phosphate from glucose-1-phosphate. TQ at a concentration of 0.05 Mm inhibits GPa activity by only 14.9%. **Conclusion:** These results show that TQ at 60 mg/kg b.w is associated with potential antihyperglycemic effects. Furthermore, anti-diabetic properties of TQ are unassociated with glycogen phosphorylase inhibition.

Key words: Diabetes, Docking, Enzyme, Glycogen phosphorylase inhibition, Streptozotocin, Thymoquinone.

SUMMARY

- Oral administration of Thymoquinone for 60 days, dose dependently improved the glycemic status in STZ-NA induced diabetic rats.
- Thymoquinone at a concentration of 0.05 Mm inhibits GPa activity by only 14.9%.
- Anti-diabetic properties of TQ are unassociated with glycogen phosphorylase inhibition.



PICTORIAL ABSTRACT

Abbreviations used: TQ: Thymoquinone, STZ: Streptozotocin, NA: Nicotinamide, GTT: Glucose tolerance test, ADT: Auto Dock Tools, GPa: Glycogen phosphorylase, GFP: Grid parameter file, PDB: Protein data bank.

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INTRODUCTION

Nigella sativa L. (Black seed), is commonly used by traditional healers as a remedy for more than four thousand years.[1,2] It has been used to treat many ailments including urine retention, hyperglycemia, cancer, pain, inflammation and diabetes.[3-6] Phytochemicals with versatile structures (contains alkaloids, tannins, steroids and flavonoids) were isolated from black seeds.[2,7,8] The antidiabetic property of *N. sativa* seeds oil is attributable to the presence of Thymoquinone (TQ). TQ is the main ingredients of black seeds oil and has been reported to have various pharmacological activities. On the other hand many studies have been designed to investigate the possible effects of the TQ in streptozotocine (STZ)-induced diabetes in rats. Previous findings demonstrated that the anti-diabetic action of TQ is mediated through a decrease in hepatic gluconeogenesis or by modifying the levels of glycoprotein components in plasma and tissues of diabetic rats. TQ was also found to exert curative protective properties in diabetes by diminishing oxidative stress and conserving pancreatic β-cell integrity. TQ was also found to successfully abrogate induction of diabetes mellitus with streptozotocin via nitric oxide inhibitory mechanism.[6]

Glycogen phosphorylase is one of the phosphorylase enzymes and it has been anticipated as one ways for curing diabetes-2. Since glucose production in the liver has been shown to increase in type 2 diabetes patients, inhibiting the release of glucose from the liver's glycogen's supplies appears to be a valid approach.[6] The aim of this study was to elucidate the mechanisms underlying the glucose lowering effects of thymoquinone *in vitro* and *in silico* using glycogen phosphorylase enzyme assay and docking tools, respectively.

MATERIALS AND METHODS

In vivo hypoglycemic properties of Thymoquinone

Sprague Dawley rats (average body weight 150-200 g) were used in this study. Rats were housed in special clear sided cages at controlled temperature (37°C) with a 12:12-h light: dark cycle and had free access to water and laboratory standard commercial food over a 2-week adaptation period. Rats were fasted for 12 h prior to induction of diabetes. Diabetes was induced in twenty five rats by a single intra-peritoneal injection of STZ (65 mg/kg b. w freshly dissolved in 5 mmol/l citrate buffer, pH 4.5) 15 minutes after intra-peritoneal injection of Nicotinamide (NA) (120 mg/kg b. w dissolved in normal saline). Rats were given 5% dextrose instead of water for the first 24 hours to avoid hypoglycemia. Diabetes

was confirmed by blood glucose level of ≥ 200 mg/dl. After confirmation of diabetes, diabetic rats were randomly divided into five groups. The 1st, 2nd and 3rd groups were treated with TQ at doses of 60, 30 and 10 mg/kg, respectively, for sixty days. The 4th group was treated with metformin at a dose of 140 mg/kg whilbetic control group. The sixth group served as non-diabetic normal control. At day zero fasting blood glucose was done and the animals were weighed. 4 weeks after commencement of the treatment, GTT before sacrificing animals. Any reduction in blood sugar level in comparison to that of untreated controls was taken as anti-diabetic activity. Ethical approval for this study was obtained from the Internal Board Review, Medical Research Centre, Jazan University.

Glycogen phosphorylase enzyme assays

Materials

Glycogen phosphorylase a (from rabbit muscle), glycogen, glucose-1-phosphate, malachite green, and ammonium molybdate were purchased from the Sigma–Aldrich Corporation. Reagents and solvents were obtained from commercial suppliers and used without further purification. Solvents used were AR grade.

Enzymatic activity assay

The enzymatic inhibition of phosphorylase activity was monitored using multiskan spectrum (Thermo-Scientific) based on the published methods. In brief, GPa activity was measured in the direction of glycogen synthesis by the release of phosphate from glucose-1-phosphate. 0.5 mM. Thymoquinone was dissolved in DMSO. The enzyme was added into the 100 µL buffer with compounds dissolved in containing 50 mM Hepes (pH 7.2), 100 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM glucose-1-phosphate, and 1 mg/mL glycogen in 96-well microplates. After the addition of 150 µL of 1 M HCl containing 10 mg/mL ammonium molybdate and 0.38 mg/mL malachite green, reactions were run at 25°C for 20 min. And then the phosphate absorbance was measured at 620 nm.[9]

Docking with Auto-Dock 4.0 software

Auto Dock Tools (ADT) is a program package of automated docking tools. It is designed to predict how small molecules, such as substrates or drug candidates, bind to a receptor of known 3D-structure. Besides generating binding energies in these docking studies, the position of the ligand in the receptor's binding site can be visualized. It can be useful for designing better ligands and understanding the nature of the binding. In this experiment you will model the binding of an inhibitor ligand to its target enzyme. Auto-Dock 4.0 search for the best ways to fit a ligand molecule into a receptor results in a docking log file that contains a detailed record of the docking. By convention, these results files have the extension (.dlg). Reading a docking log or a set of docking logs into Auto-Dock 4.0 is the first step in analyzing the results of docking experiments.

Docking experiment

Each docking requires at least four input files PDBQT file for the ligand that encodes a torsion tree, PDBQT file for the receptor, Grid parameter file (GPF) for the Auto Grid calculation and Docking parameter file (DPF) for the Auto Dock calculation. If some residues in the receptor are to be modeled as flexible, a fifth PDBQT file containing the flexible receptor residues will be necessary. Protein Data Bank (PDB) files may have a variety of problems that need to be corrected before they can be used in ADT. These potential problems include missing atoms, added waters, more than one molecule, chain breaks, alternate locations etc.

Preparation of the ligand file

The ligand originally is drawn in different visualization programs such as Chem3D Ultra to obtain standard 3D structures (.mol2) format. All hydrogen's was added to the ligands and gastiger partial atomic charges were computed and saved in the required format. Auto-Dock 4.0 automatically assigned type to each atom and detected root (this is the rigid part of the ligand) also choose torsions option was used as an interactive browser to choose the number of rotatable bonds that move the fewest atoms, finally the ligands was saved as pdbqt format.

Preparation of the macromolecule file

The receptor is usually taken from the Protein Data Bank (PDB) in (.pdb) format. This is a special format for protein structures that are obtained by X-ray crystallography studies. All polar hydrogens were added, and the partial kollman charge was computed for arbitrary molecule. The protein molecule was then saved as pdbq file. Protein was defined as two parts which are flexible residue (amino acids in the binding site region of the protein that are able to alter their position via conformation charge upon ligand binding) was selected to prepare flexible residue file, the second file was the rigid residue part.

Preparation of the Grid parameter file

Autogrid builds as many files as the number of probe atoms used. Each one shows the interaction energies for a particular atom type, such as aliphatic carbons, aromatic carbons, hydrogen bonding oxygens, and so on. The grid itself is a box with determined dimensions that is located in a place on the surface of the receptor where we expect the ligand to interact with the receptor. The center of such a grid was precalculated earlier and will be used in the following operations. An additional feature of Auto-Dock 4.0 is the ability to model not only how the ligand docks to the receptor but also the position of flexible residues. Flexible residues are amino acids that change their positions while the ligand interacts with the receptor. They are found by comparison of different crystallized structures or by molecular dynamic simulations. The grid volume should be large enough to at least allow the ligand to rotate freely, even when the ligand is in its most fully-extended conformation. To set up the grid size you can inspect it visually and adjust the dimension by using the three thumbwheel widgets. So for instance the suitable grid size was determined according to flexible residues, using the default grid spacing of 0.375. In the case you do not have any ligand position information the grid should be centered in the putative binding site and sized to embrace all residues making the binding pocket. Adjust all the values and save the information, save the gpf file and check the written file.

Preparation of the docking parameter file

When the preparation of the input files (ligand and protein) and the calculation of the affinity maps are properly performed Auto-Dock will carry out the docking automatically using the newest docking algorithm (Lamarckian Genetic Algorithm). Prepared docking parameter file was the final step. The resultant structure files of Auto-Dock 4.0 software was analyzed using Discovery studio visualization (DSV) and ligplot programs.

Statistical analysis

Values are expressed as mean ± SEM. Unpaired Student t-test was used for statistical comparison. In case of *in vivo* studies comparison were made between normal and diabetic, diabetic versus diabetic treated animals. Changes were considered significant if the P-value was less than 0.05.

RESULTS

The current study employed a well established animal model of diabetes to investigate the hypoglycemic properties of TQ. Diabetes was induced using STZ and NA. As shown Table 1., the effect of this natural compound on the body weights and blood glucose levels. Diabetic rats treat-

Table 1: Effects of Thymoquinone on body weight and fasting blood glucose of STZ induced type-2 rats

Groups	Initial weight (gm)	Final weight (gm)	Blood Glucose Levels (mg/dl)		
			Fasting (0 hr)	1 hr	2 hr
STZ-induced + thymoquinone 10 mg/kg	156.33 ± 3.6	135.83 ± 2.9	180.33 ± 10.5	346.5 ± 5.1	260.5 ± 2.7
STZ-induced + thymoquinone 30 mg/kg	164.44 ± 1.5	140.97 ± 6.4	137.38 ± 1.9	285.58 ± 2.9	172.75 ± 10.8
STZ-induced + thymoquinone 60 mg/kg	173.5 ± 5.8	307.83 ± 8.4	177 ± 2.4	306 ± 5.7	222 ± 8.1
STZ-induced + metformin	179.6 ± 6.4	175.33 ± 5.9	102.6 ± 65.1	255 ± 1.8	87 ± 5.8
STZ-induced (Diabetic)	155.25 ± 2.9	291.5 ± 2.5	257.5 ± 5.7	440 ± 6.4	326 ± 6.4
Normal group	171.83 ± 1.5	184.16 ± 3.5	84.33 ± 5.8	122.16 ± 3.8	93.16 ± 4.9
ANOVA					
F-statistic	1.249	2.374	2.178	5.980	3.981
P-value	0.322	0.068	0.04	0.002	0.011

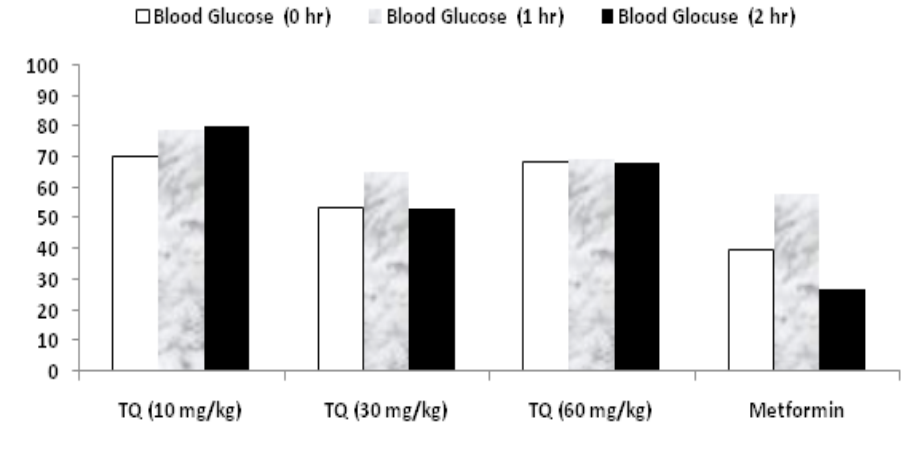

Figure 1: Effect of Thymoquinone on blood glucose levels (%) of STZ induced diabetic rats. Vertical axis represents the percentage changes on blood glucose levels as compared to diabetic control group.

ed with TQ oral dose of 10, 20 and 30 mg/kg showed 70.03%, 53.35%, 68.74%, respectively, reduction in blood glucose (at zero time) in comparison to untreated diabetic rats. Figure 1 showed the effect of TQ on blood glucose levels (%) of STZ-NA induced diabetic rats. Vertical axis represents the percentage changes on blood glucose levels as compared to diabetic control group. Blood glucose levels of control diabetic and treated animals were 102.6 ± 65.1 and 257.5 ± 5.7 mg/dl respectively ($P<0.05$). Changes in body weight are depicted in Table 1.

As shown in Table 2, TQ at a concentration of 0.05 Mm inhibits GPa activity by only 14.9%. To investigate the interaction of TQ, molecular docking simulations of the binding of this molecule with glycogen phosphorylase active site were carried out using Autodock 4.2. Biochemical interactions of TQ with GPa is shown in Figure 2. Figure 2a is schematic diagram obtained by DSV software for docking results of Auto-Dock while Figure 2b is a schematic diagram obtained by Ligplot software for docking results of Auto-Dock. Caffeine was used as standard in glycogen phosphorylase assay.

DISCUSSION

The antidiabetic property of *N. sativa* seeds oil is attributable to the presence of thymoquinone (TQ)[10,11] On the other hand many studies have been designed to investigate the possible effects of the TQ in STZ-NA induced diabetes in rats. The aim of this study was to elucidate the mechanisms underlying the glucose lowering effects of Thymoquinone *in vitro* and *in silico* using glycogen phosphorylase (GPa) enzyme assay and docking tools, respectively.

Table 2: Effects of Thymoquinone on Glycogen phosphorylase enzyme (Protein code: 1GFZ from protein data bank)

Compound	Inhibition % of G Pase (0.5 Mm)	Docked Energy
Thymoquinone	14.9	-5.58

For the study of antidiabetic agents, STZ induced hyperglycemia in rodents is considered to be a good preliminary screening model and is widely used.[12,13] STZ, N-[methylnitrocarbamoyl]-D-glucosamine is a potent methylating agent for DNA and acts as nitric oxide donor in pancreatic cells. Beta cells are particularly sensitive to damage by nitric oxide and free radicals because of their low levels of free radical scavenging enzymes.[14-17] From the results obtained, it is evident diabetic rats had much higher glucose levels than of control rats. Oral administration of TQ decreased the blood glucose level in diabetic rats. Anti-hyperglycemic effect of medicinal plant extracts is generally dependent upon the degree of β-cell destruction.[18,19]

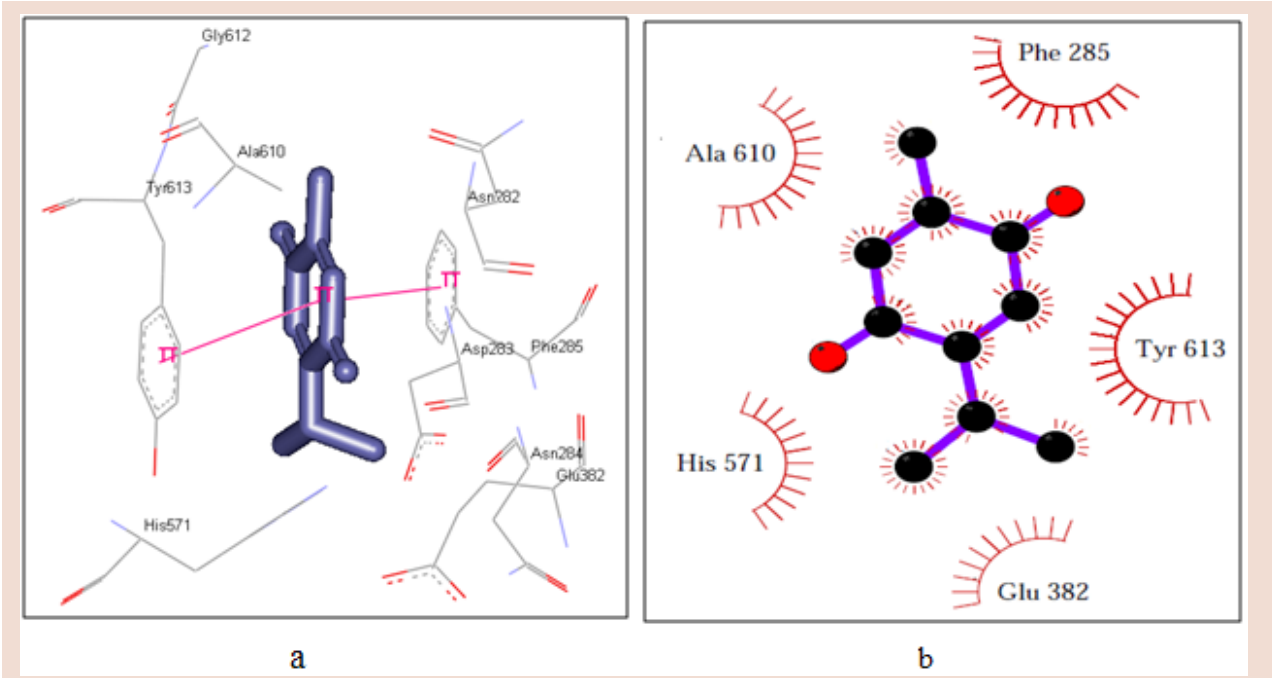

Figure 2: Biochemical interactions of Thymoquinone with Glycogen phosphorylase: (A) is schematic diagram obtained by DSV software for docking results of Auto-Dock. (B) is schematic diagram obtained by Ligplot software for docking results of Auto-Dock. Protein code: 1GFZ from protein data bank.

In spite of the availability of different classes of hypoglycaemic drugs, treatment regimens are often unable to achieve an intensive degree of glucose control known to most effectively reduce the incidence and severity of diabetic complications.[20,21] Hepatic glucose output is elevated in type 2 diabetic patients and current evidence indicates that glycogenolysis (release of monomeric glucose from the glycogen polymer storage form) is an important contributor to the abnormally high production of glucose by the liver.[22,23] Glycogen phosphorylase is the enzyme that catalyses this release and recent advances in new inhibitors of this structurally and kinetically well studied enzyme have enabled work which further delineate the pharmacological and physiological consequences of inhibiting glucose production by this pathway.[24,25] Most notably, these agents lower glucose in diabetic animal models, both acutely and chronically, appear to affect both gluconeogenic and glycogenolytic pathways and demonstrate potential for a beneficial effect on cardiovascular risk factors. Results of the current study did not show any significant inhibition of glycogen phosphorylase by TQ. As shown in Table 2, TQ at a concentration of 0.05 Mm inhibits GPa activity by only 14.9%. To investigate the interaction of TQ, molecular docking simulations of the binding of this molecule with glycogen phosphorylase active site were carried out using Autodock 4.2.

More studies are needed to demonstrate the exact mechanism of action of TQ on diabetes. Consequently, TQ may be clinically useful for the treatment of diabetes.

CONCLUSION

The current results show that TQ is associated with potential antihyperglycemic effects. Furthermore, anti-diabetic properties of TQ are unassociated with glycogen phosphorylase inhibition.

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CONFLICT OF INTEREST

The authors report no conflicts of interest in this work.

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