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In Silico Molecular Docking Analysis of Nigella Sativa Bioactive Compounds as Potential Modulators of WNT/B-Catenin Pathway Proteins in Chronic Venous Insufficiency

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تحليل الالتحام الجزيئي الحاسوبي لمركبات الحبة السوداء الفعالة كمعدلات محتملة لبروتينات مسار إشارات *WNT/β-catenin* في القصور الوريدي المزمن

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Abstract:
Chronic venous insufficiency (CVI) is a vascular disease in which there is obstruction in the flow of venous blood either in the superficial vein or in the deep veins, which causes venous hypertension due to the development of reflux and inflammation. Recent research has emphasized the role of the WNT/β-catenin signaling pathway in the physiopathology of CVI. The aim of this in silico study is to explore the binding potential of two important bioactive molecules (carvacrol & thymoquinone) from Nigella sativa with WNT-related proteins (FZD2, DVL2, and AXIN1) underlying the pathology of venous insufficiency. Further proof of the highly functional interplay between FZD2, DVL2, and AXIN1 was provided through protein-protein interaction analysis using STRING, thereby justifying their selection as the major molecular targets. The docking study revealed that both carvacrol and thymoquinone showed a promising binding affinity towards the target molecules, especially AXIN1. The carvacrol-AXIN1 complex exhibited a better geometric complementarity value of -13.86 AC, as well as the binding free energy of -6.13 kcal/mol. Although thymoquinone had similar thermodynamic stability, carvacrol possessed a better fit in terms of geometric complementarity within the AXIN1. These findings suggest that the minor discrepancies may not establish therapeutic superiority for chronic venous insufficiency. The present in-silico study serves as a basis for future hypothesis-driven research on the therapeutic effects of such molecules in vascular diseases.

Keywords: Chronic venous insufficiency, Vascular remodeling, Carvacrol, Thymoquinone.

الملخص:
يُعد القصور الوريدي المزمن (CVI) مرضاً وعائياً يحدث فيه انسداد في تدفق الدم الوريدي إما في الأوردة السطحية أو العميقة، مما يسبب ارتفاع ضغط الدم الوريدي نتيجة تطور الارتجاع والالتهاب. وقد أكدت الأبحاث الحديثة دور مسار إشارات WNT/β-catenin في الفيزيولوجيا المرضية للقصور الوريدي المزمن. تهدف هذه الدراسة الحاسوبية إلى استكشاف قدرة الارتباط لجزأين حيويين هامين (الكارفاكرول والثايموكينون) المستخلصين من الحبة السوداء (Nigella

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sativa) مع البروتينات المرتبطة بـ WNT (وهي FZD2، وDVL2، وAXIN1) الكامنة وراء أمراض القصور الوريدي. وقد تم تقديم دليل إضافي على التفاعل الوظيفي العالي بين FZD2 وDVL2 وAXIN1 من خلال تحليل تفاعل البروتين-بروتين باستخدام أداة STRING، مما يبرر اختيارها كأهداف جزيئية رئيسية. أظهرت دراسة الالتحام الجزيئي أن كلًا من الكارفاكرول والثايموكينون أبديا تقارب ارتباط واعداً نحو الجزيئات المستهدفة، وخاصة AXIN1. أظهر معقد الكارفاكرول-AXIN1 قيمة أفضل للتكامل الهندسي بلغت -AC 13.86، بالإضافة إلى طاقة حرة للارتباط بلغت -6.13 كيلو كالوري/مول. وعلى الرغم من أن الثايموكينون كان له ثبات ديناميكي حراري مماثل، إلا أن الكارفاكرول امتلك توافقاً أفضل من حيث التكامل الهندسي داخل بروتين AXIN1. تشير هذه النتائج إلى أن الفوارق الطفيفة قد لا تؤسس لتفوق علاجي في حالات القصور الوريدي المزمن. وتُعد هذه الدراسة الحاسوبية أساساً لأبحاث مستقبلية مدفوعة بالفرضيات حول التأثيرات العلاجية لمثل هذه الجزيئات في الأمراض الوعائية.

الكلمات المفتاحية: القصور الوريدي المزمن، إعادة الهيكلة الوعائية، الكارفاكرول، الثايموكينون.

Introduction

Chronic venous insufficiency (CVI) is described as a medical condition that involves dysfunction of the superficial or deep veins, causing venous hypertension and pathological changes [1, 2]. Worldwide, CVI stands for an important health problem with 25% of the world population being affected due to its outcomes and high frequency [3]. The factors that can contribute to CVI include age, gender, genetics, pregnancy, obesity, long-standing position, previous thromboembolism, injuries of the legs, and hormonal disorders [4-6]. Despite advances in medicine, the molecular basis of insufficiency remains poorly understood; thus, further studies in molecular processes controlling this disorder are needed. In recent years, there have been many investigations focusing on WNT signaling, which controls differentiation, proliferation, apoptosis, and migration of cells [7-9]. It has been shown before that WNT signaling is important for development, endothelial function, inflammation, and vascular remodeling [10, 11, 12]. Conversely, dysregulation in WNT signaling is associated with varicose veins, oxidative stress, inflammation, and fibrosis and is involved in remodeling through increasing β-catenin expression that results in dysfunction and severe consequences of CVI [12, 13-16]. Some of the crucial components in the WNT pathway, including FZD2, are receptors; DVL2 is a signal transducer; and AXIN1 is a negative regulator. But any malfunction in these proteins leads to disease [14, 17, 18]. Previous studies showed that FZD2 presents in vascular smooth muscle cells and upregulated by angiotensin II, connecting it to vascular remodeling in hypertension [19]. Also, DVL2, which is abundantly expressed in endothelial cells, facilitates both canonical and non-canonical WNT signaling pathways; inhibition of DVL2 leads to the abnormal regulation of the cytoskeleton organization and endothelial cell shape and vascular remodeling [20, 21]. Additionally, AXIN1 regulates the integrity of the endothelial cell barrier and angiogenesis, although its dysregulation affects vascular development. In general, disturbances in FZD2, DVL2, and AXIN1 signaling can affect endothelial functioning and smooth muscle proliferation. These disruptions are crucial components for venous wall remodeling and venous insufficiency [22, 23]. Among medical plants, Nigella sativa is a plant belonging to the family of Ranunculaceae, which is also referred to as black cumin or black seeds [24, 25]. According to the chemical analysis, the volatile oil fraction of Nigella sativa seeds consists of 2.85-3.65% carvacrol and 10.27-29.77% thymoquinone in relation to the method of isolation and seed origin [26-28]. Several studies have revealed that carvacrol and thymoquinone provide the traits of Nigella sativa [29] by showing anti-inflammatory properties, free radicals scavenging activity, and other antioxidant properties [30]. Moreover, studies of Nigella sativa have found that carvacrol and thymoquinone are the components responsible for the therapeutic effects in different diseases at various doses [31]. Carvacrol and thymoquinone have several health benefits, such as antioxidant and anticancer properties, diabetes prevention, cardiovascular benefits, antihistaminic properties, reproductive benefits, antibacterial properties, and immunity modulation [32-36]. Molecular docking is one of the bioinformatics approaches that determines the binding energies of molecules and protein and acts as the first step in structure-based drug design [37]. This in silico approach has several advantages in the case of natural product research, considering that it is inexpensive, can perform rapid screening, and involves the rational design of structures. [38, 39]. The current in silico docking study aims to determine the interaction between bioactive molecules (carvacrol and thymoquinone) and the WNT-associated proteins, namely FZD2, DVL2, and AXIN1, which may be involved in the condition of chronic venous insufficiency.

Material and methods

2.1. Protein–protein interaction analysis

The protein-protein interactions between FZD2, DVL2, and AXIN1 were examined using the STRING v12.0 database [40]. The default settings of the program and the medium confidence value ≥ 0.4 were utilized for drawing the interaction map and pathway relations in the WNT/β-catenin signaling pathway.

2.2. The docking study design

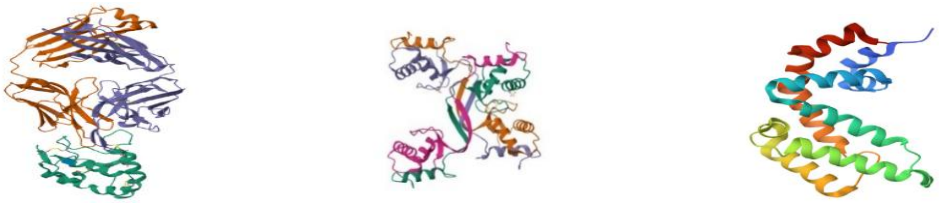
Molecular docking was used as a method to study the estimated binding free energies of carvacrol and thymoquinone with selected WNT-related proteins (FZD2, DVL2, and AXIN1) using SwissDock 2024

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software, with the AC 2.0 scoring function [41]. Further structural analysis of the docking procedure was carried out through the Protein-Ligand Interaction Profiler (PLIP) web service (2015) [42].

2.3. Target protein retrieval and choice

The 3D structures of the chosen proteins (FZD2: 7X8P, DVL2: 5LNP, AXIN1: 7Y1P) were retrieved from the Protein Data Bank [43] (Figure 1). Functional regions represented in the chosen structures include: FZD2 (7X8P)—Cysteine-rich domain (CRD) that is important for the interaction with WNT ligands [44]; DVL2 (5LNP)—Disheveled, Egl-10, and Pleckstrin domain (DEP) dimerization region that plays an important role in signalosome assembly [45]; and AXIN1 (7Y1P)—Regulator of G-protein Signaling domain (RGS) that forms a small-molecule regulatory pocket in the β-catenin destruction complex [46]. Additional pre-processing in the PyMOL 2.0 program included the removal of the water molecules, heteroatoms, and ligands bound to the protein [47].

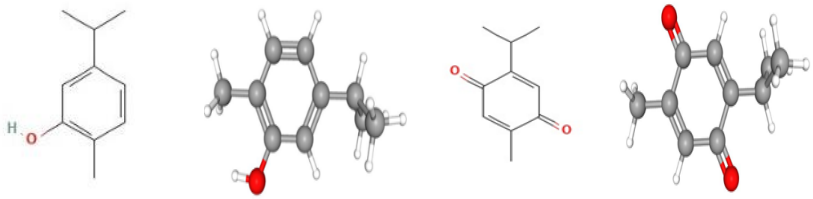


(A) (B) (C)

Figure 1. Crystal structures of human WNT proteins (A) FZD2 (B) DVL2 (C) AXIN1.

2.4. Preparation of ligands

The chemicals used for analysis were retrieved from the PubChem database in SMILES format [48]. They include carvacrol (CID10364) and thymoquinone (CID10281) **(Figure 2).**



(A) (B) (C) (D)

Figure 2. Chemical structures of (A) 2D representation of binding mode of carvacrol (B) 3D representation of binding mode of carvacrol (C) 2D representation of binding mode of thymoquinone as ligands (D) 3D representation of binding mode of thymoquinone as ligands.

2.5. Protein-ligand molecular docking

Protein and ligand molecules were uploaded to the SwissDock web server, and the possible binding cavities of the target proteins were automatically detected using attracting cavities (AC). Multiple binding poses of the ligand-protein complex were generated through the docking process. The ranking of these docking poses was based on the scoring function of the SwissDock and the binding free energies (ΔG) calculated by the docking tool. Binding poses were classified and ranked based on their AC. The best energy conformations and binding poses that have been classified and ranked by their AC were selected for further structural analysis using the PLIP web server.

Results and discussion

This research paper deals with the computational molecular docking of carvacrol and thymoquinone in relation to the FZD2, DVL2, and AXIN1 proteins associated with the WNT/β-catenin signaling pathway.

3.1. STRING network analysis

STRING network analysis showed 3 nodes and 3 edges, with the average node degree and clustering coefficient being 2 and 1.0, respectively. In addition, since the PPI enrichment p-value is 0.00022, these proteins are interacting much more than we would expect, proving that there is indeed a functional connection between FZD2, DVL2, and AXIN1. The network contained high-confidence edges depicting experimentally confirmed and database-supported significant interactions, adding further value to the selection of these proteins as the molecular target for studying protein-ligand interactions **(Figure 3).**

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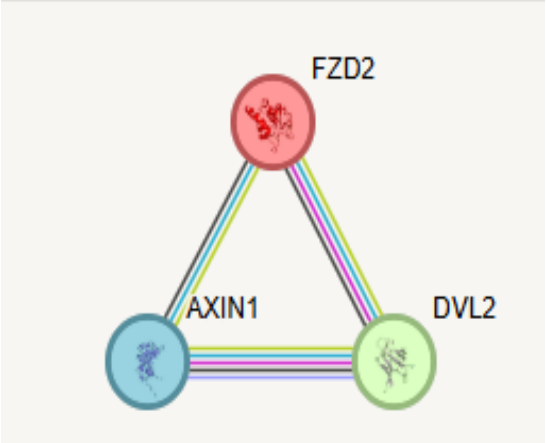


Figure 3. STRING interaction network depicting high-confidence functional connections among FZD2, DVL2, and AXIN1 within the WNT/β-catenin pathway.

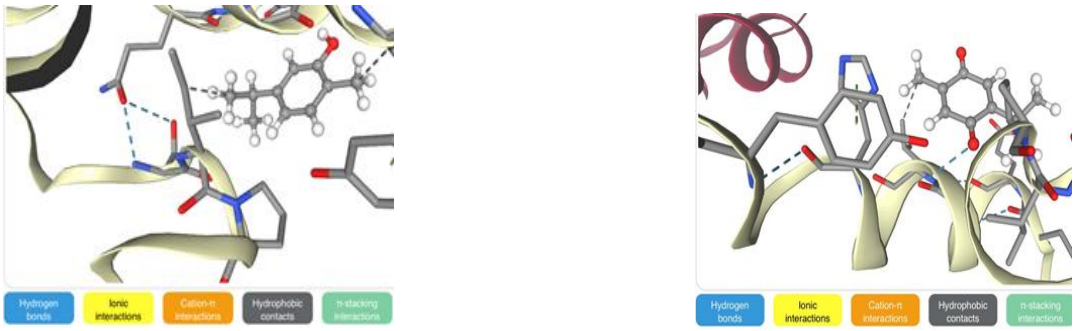
The molecular docking of the aforementioned compounds was performed using the SwissDock 2024 software tool that involves the Attracting Cavities (AC) 2.0 approach [38, 41]. Attracting cavities technology has the ability to identify the possible sites for ligand binding within molecules and is able to operate successfully both in blind and targeted ligand docking studies, allowing this technology to operate on both polar and non-polar sites as well as charged and uncharged ligands.

Protein-ligand complex binding conformations were studied by SwissDock and ranked by the score known as AC score (FullFitness), depending on the geometric complementarity of the ligand and the binding pocket. On the other hand, the values of binding free energy (ΔG) obtained from SwissDock predictions were used for the evaluation of the binding conformations, depending on the higher negativity of the values ΔG.

In light of the fact that the ΔG values represent rough values of CHARMM energy that include the impact of solvation by both polar and non-polar parts of the solvent. The type and strength of any stabilizing forces like hydrogen bonds were examined to test the structural validity of the docked complex [39]. Identification of docking poses was accomplished by ranking based on the AC scoring function as well as the ΔG calculation that was later verified at the interaction level through the Protein-Ligand Interaction Profiler tool of PLIP web.

3.2. Interaction of FZD2 with carvacrol and thymoquinone

Docking analysis revealed that carvacrol successfully docked into the CRD of the FZD2 protein with an AC value of (−12.62) and a ΔG value of (−5.86 kcal/mol), with minimal hydrophobic bonds. PLIP identified no hydrogen bonds and two hydrophobic bonds (Glu67 and Pro45) with residues (Gln66, Leu71, His74, Tyr77, and Gly70). Such interactions were formed in the hydrophobically dominated deep binding pocket, which indicated the nature of the hydrophobic binding site mechanism. In contrast, the complex of FZD2-thymoquinone interacted via the formation of only one hydrogen bond (Gln66) and one hydrophobic bond (Leu71), while the residues included Ser43, Ile44, Pro45, Glu67, and Gly70. The ligand bound to the buried pocket of the protein. This interaction showed the binding energy and stability, as represented by ΔG (−5.96 kcal/mol) and AC (−7.73) **(Figure 4; Table 1)**.



(A) (B)

Figure 4. Binding interaction of (A) Carvacrol-FZD2 and (B) Thymoquinone-FZD2.

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Table 1: Docking analysis of FZD2 with carvacrol and thymoquinone.

Ligand	AC Score (Fullfitness)	ΔG (kcal/mol)	Total Hydrogen Bonds	Total Hydrophobic Bonds
Carvacrol	−12.62	−5.86	0	2 (Glu67, Pro45)
Thymoquinone	−7.73	−5.96	1 (Gln66)	1 (Leu71)

3.3. Interaction of DVL2 with carvacrol and thymoquinone

Carvacrol demonstrated binding to the DEP domain of DVL2. It maintained an interaction with AC (−11.48) and ΔG (−5.94 kcal/mol). These showed favorable geometry and lay in a deep pocket, consistent with stronger complex stabilization despite the one hydrogen bond (Gln500) and two hydrophobic bonds (Leu445 and Lys446) with residues (Trp444, His490, Thr491, Val492, Lys494, Cys501, and Tyr502).

Conversely, thymoquinone bound effectively to DVL2, showing AC (−7.07) and ΔG (−6.05 kcal/mol). The thymoquinone–DVL2 complex established two hydrogen bonds (Tyr502 and Thr491) and two hydrophobic bonds (Trp444 and Leu445) with residues (Lys446, His490, Val492, Lys494, and Cys501). These results suggested that interactions reinforced the thymoquinone–DVL2 complex, supporting favorable geometric complementarity within the predicted binding pocket (**Figure 5**; **Table 2**).



(A) (B)

Figure 5: Binding interaction of (A) Carvacrol-DVL2 and (B) Thymoquinone-DVL2.

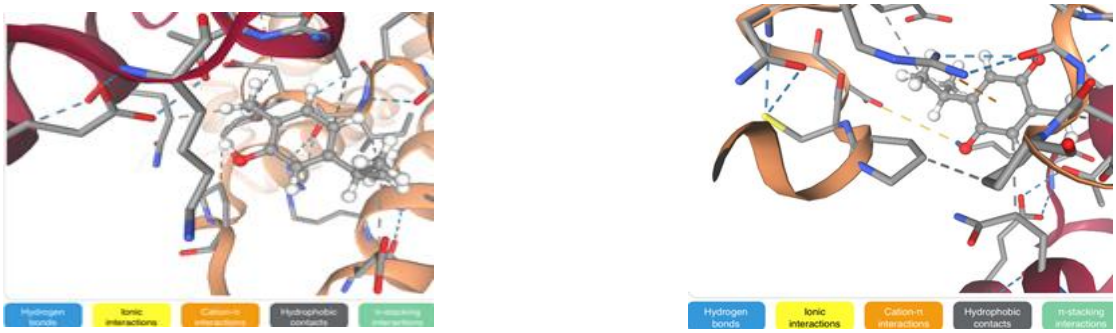
Table 2: Docking analysis of DVL2 with carvacrol and thymoquinone.

Ligand	AC Score (Fullfitness)	ΔG (kcal/mol)	Total Hydrogen Bonds	Total Hydrophobic Bonds
Carvacrol	−11.48	−5.94	1 (Gln500)	2 (Leu445, Lys446)
Thymoquinone	−7.07	−6.05	2 (Tyr502, Thr491)	2 (Trp444, Leu445)

3.4. Interaction of AXIN1 with carvacrol and thymoquinone

The binding energy of carvacrol to the RGS domain pocket of AXIN1 was high for all targets, ΔG (−6.13 kcal/mol), with good geometric complementation, as well as a negative AC (−13.86). This compound was involved in interaction with a deep hydrophobic pocket, forming the following interactions with no hydrogen bonds and five hydrophobic bonds (Pro129, Gln184, Lys161, Glu188, and Asp178) with residues (Cys130, Thr164, and Ile177). Thus, favorable geometrical interactions increased the binding energy of this compound. On the other hand, thymoquinone had a negative binding energy toward AXIN1 with the values of ΔG (−6.37 kcal/mol) and AC (−8.94). PLIP identified one hydrogen bond (Ile177), and one hydrophobic bond (Glu134) of thymoquinone with AXIN. Residues included Pro129, Cys130, Lys161, Ala163, Thr164, Gln175, Leu176, Gln184, Asp178, and Glu188. The ligand was located inside a semi-buried pocket; therefore, the ligand binding was not stable. These results showed that AXIN1 is the most appropriate molecular target for both ligands **(Figure 6; Table 3)**.

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(A) (B)

Figure 6: Binding interaction of (A) Carvacrol- AXIN1 and (B) Thymoquinone-AXIN1.

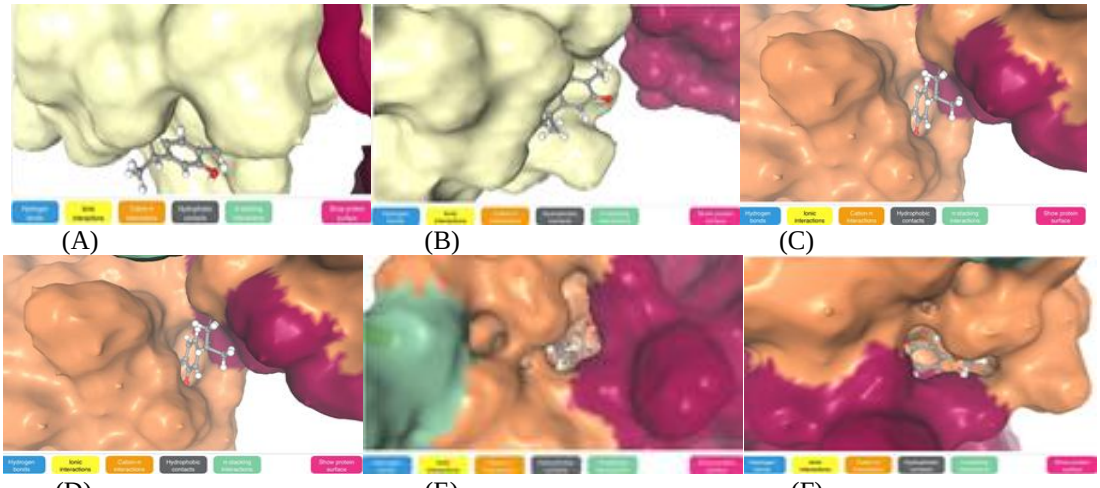
Table 3: Docking analysis of AXIN1 with carvacrol and thymoquinone.

Ligand	AC Score (Fullfitness)	ΔG (kcal/mol)	Total Hydrogen Bonds	Total Hydrophobic Bonds
Carvacrol	−13.86	−6.13	0	5 bonds (Pro129, Gln184, Lys161, Glu188, Asp178)
Thymoquinone	−8.94	−6.37	1 (Ile177)	1(Glu134)

It is essential to gain insight into the molecular WNT pathways to understand their roles in the context of CVI. Mechanically, the canonical and non-canonical WNT signaling pathways control physiological functions and tissue remodeling and play a critical role in vascular biology and in diseases. According to interaction analysis by the STRING database, the functional association between FZD2, DVL2, and AXIN1 in the WNT/β-catenin signaling pathway was verified with a PPI enrichment p-value of 0.00022, and their choice as the potential targets in the current research was validated. Controlled experimental studies have revealed that *Nigella sativa* seeds improve vasoreactivity, vascular functionality, and vascular inflammation [26]. One other research has found that aqueous extract of *Nigella sativa* stimulated proliferation of human gingival fibroblasts when used as a monolayer with rapid wound closure properties without affecting collagen synthesis [27]. The safety and good tolerability properties of carvacrol and thymoquinone make them safe and protective agents with low toxicities in all studies [29, 31, 32]. In alignment with another study, the research findings revealed that the carvacrol complex had a better geometric fit with a low AC score, hence deep pocket insertion in all targets (FZD2, DVL2, AXIN1) [50].

3.5. In Structural analysis

The docking site structures were analyzed to prove that hydrogen bond and hydrophobic interactions were the main driving forces stabilizing the ligands in the predicted binding sites. The polar and non-polar amino acids were found to be key players at the molecular level, where the proper positioning of the ligand and pocket complementation took place. Analysis showed that there was a difference in the interaction behavior of thymoquinone and carvacrol. As a result, the ligand-binding site of carvacrol was deeper and hydrophobicity was higher, especially in the case of the AXIN1 protein **(Figure 7)**.



(A) (B) (C)

(D) (E) (F)

Figure 7: Ligand binding cavities in target proteins: (A) FZD2-carvacrol (B) FZD2-thymoquinone (C) DVL2-carvacrol; (D) DVL2-thymoquinone (E) AXIN1-carvacrol (F) AXIN1- thymoquinone.

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The above-mentioned findings prove that carvacrol acquired markedly larger negative AC values, ranging between -11.48 to -13.86, relative to thymoquinone. This implies that carvacrol has a better geometry and spatial compatibility. On the contrary, the thermodynamics of thymoquinone complexes were better than those of carvacrol complexes for all WNT-selected proteins, since the former had marginally better binding free energy. The ΔG values of thymoquinone obtained from the SwissDock simulation were ranging between -5.96 to -6.37 kcal/mol. This suggests that thymoquinone is relatively more stable in aqueous medium. In general, the range of binding free energy between -5 to -7 kcal/mol is considered favorable in research utilizing SwissDock simulation [49, 51].

Based on these perspectives, it can be seen that both carvacrol and thymoquinone are structurally suitable for chosen proteins involved in the WNT pathway. Even though carvacrol was a more geometrically favorable molecule, thymoquinone had a marginally better ΔG; docking scores alone cannot establish that either compound inhibits the WNT/β-catenin pathway. Remarkably, among all the targets, the AXIN1 complex was found to interact very effectively with both carvacrol and thymoquinone.

However, AXIN1 had better geometry and pocket depth in case of carvacrol compared to thymoquinone. This might be due to the presence of the deep groove within AXIN1 scaffold proteins that seem to be flexible and adaptive in nature and can modify themselves to bind with various partners, as has been seen in earlier studies [52, 53]. Binding of these molecules in the WNT/β-catenin components might result in enhancing intracellular β-catenin degradation.

Consequently, degradation of cytosolic β-catenin through the APC/AXIN1 destruction complex is necessary for activation of the WNT pathway and is, therefore, a negative regulator of the canonical WNT pathway [15, 23, 54]. Besides, carvacrol and thymoquinone interaction with AXIN1 implies the possibility of the modulation of the β-catenin degradation process, which results in the inhibition of canonical WNT signaling. It has been experimentally shown that AXIN1 affects vascular barrier function and explains its participation in the vascular tissues. In other studies, the RGS domain of the AXIN1 protein was found to be one of those that can be targeted by drugs; specifically, the small molecule KYA1797K binds to the RGS domain to promote β-catenin degradation by destroying complex formation [55]. Another relevant background for these results is our prior research on the transcriptional coordination of the WNT pathway, including FZD2, DVL2, and AXIN1 in human great saphenous veins obtained from CVI patients and controls. It identified significant positive correlations of these genes in each group, particular the correlation between DVL2 and AXIN1, which was $r = 0.722$ ($p < 0.0001$) in CVI samples compared to control samples [56]. This descriptive difference in coordination suggests a CVI-related reason for studying these two proteins together; however, in order to establish the statistically significant change between them, we should carry out specific correlation tests. The current docking analysis goes beyond it by raising a new structural question about the possibility of the binding of small natural molecules with the target WNT-related proteins. In the present docking, carvacrol and thymoquinone showed their most favorable ΔG values with the AXIN1 RGS domain (−6.13 and −6.37 kcal/mol, respectively) among the three targets. Thus, AXIN1 can be considered a candidate target for modulating the WNT signaling pathway in CVI, pending experimental validation.

In terms of the receptors, the geometric fit of carvacrol with FZD2 (AC score −12.62; ΔG −5.86 kcal/mol) only tentatively indicated its possible inhibition of the interaction between the WNT ligand and receptor, thereby interfering with the WNT signaling at the cell membrane level. The carvacrol–DVL2 interaction (AC score −11.48; ΔG −5.94 kcal/mol) suggested possible interference with signal transduction, potentially disrupting DVL2-dependent WNT signaling in both canonical and non-canonical WNT pathways. More specifically, in silico strategies, such as molecular docking and virtual screening, have proven to be highly efficient in the process of drug discovery at an early stage. As they allow for quick screening of compound libraries, prediction of binding modes, and selection of candidates for testing using costly and time-consuming methods [37, 51, 57]. However, despite the significant amount of useful data that the current research has yielded regarding the potential modulation of the WNT pathway, there are certain limitations of the molecular docking method itself that need to be considered. The fact that docking is a static method means that there are no factors like protein flexibility, solvation dynamics, or entropic effects. Further studies should be conducted using molecular dynamics simulations that assess the stability of binding over time.

Conclusion

This computational study explored the interaction between bioactive molecules (thymoquinone and carvacrol) derived from *Nigella sativa* and proteins involved in the WNT pathway (FZD2, DVL2, and AXIN1) in chronic venous insufficiency. The binding results revealed that both of the molecules exhibit a geometrically favorable binding towards the target proteins, especially towards AXIN1, exhibiting the most favorable geometric complementarity (AC score). Although the results suggest promising binding affinities, they do not confirm biological activity or therapeutic efficacy. Therefore, further in vitro and in vivo studies are required to validate these computational findings and determine their potential therapeutic relevance in chronic venous insufficiency.

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Compliance with ethical standards

Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

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