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MOLECULAR MECHANISM UNDERLYING QUERCETIN INDUCED APOPTOSIS BY *insilico* ANALYSIS and in PROSTATE CANCER PC-3 CELLS

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ABSTRACT

The study was aimed to investigate the molecular mechanisms underlying triggering of apoptosis by quercetin using *in silico* analysis. The mechanism of binding of quercetin with NF-kB and other apoptotic proteins like BCL_2 and BCL_{XL} was analyzed *in silico* using Schrodinger suite 2009.Quercetin was found to exhibit high-affinity binding resulting from greater intermolecular forces between the ligand and its receptor NF-kB (-0.281 Glide score and -6.172 Docking Score). Quercetin binds to NFκB very strongly with metallic coordination.It can act as a inhibitor of apoptosis inhibitor when compared to BCL_{XL} and BCL_2.Quercetin down regulates the cell survival proliferative anti-apoptotic proteins thereby can prevent prostate cancer. To confirm this, in vitro study can be carried out with techniques like MTT Assay, Western blot, and polymerase chain reaction.

KEY WORDS: **Quercetin,NF-kB,TNF-α, Molecular interaction**

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INTRODUCTION

The majority of the case control studies focused on the use of fruits and vegetables because these items, which include soybean, ginger, onion, cabbage, cauliflower, turmeric, are the basis of most diets throughout the world, and represent important sources of potentially non-toxic molecules (dietary phytochemicals).These molecules can exert a cancer-preventive effect and therefore are termed as "chemopreventers"[1]. Among them, the most studied are curcumin, quercetin (Qu), resveratrol, luteolin, genistein, epigallocatechin-3-gallate (EGCG), lycopene and, in general, flavonoids and polyphenols[2]. Chemopreventers often have preferentially an antioxidant activity; however, they are also able to exert anti-proliferation and anti-inflammation actions. Indeed, they can directly modulate several proteins that are involved in cell cycle and cellular homeostasis and whose deregulation can play a role in carcinogenesis, such as p53, p73, p21, Bax, Bcl-2, COX-2, NF-kB, catalase, glutathione (GSH)-peroxidase [3,4]. Quercetin is a potent enhancer of TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis, through the induction of the expression of death receptor (DR)-5, a phenomenon that specifically occurs in prostate cancer cells[5]. The study of quercetin as potential chemopreventer is assuming increasing importance considering its involvement in the suppression of many tumor-related processes including oxidative stress, apoptosis, proliferation and metastasis. Quercetin has also received greater attention as pro-apoptotic flavonoid with a specific and almost exclusive activity on tumor cell lines rather than normal, non-transformed cells [6]. Nuclear factor-κB (NF-κB) regulates the transcription of genes involved in immune and inflammatory responses, cell proliferation or differentiation or cell transformation and acts as a survival factor by protecting tumor cells from apoptosis[7-10].A majority of cancer cells become resistant to TNF-α as a result of the activation of NF-κB and consequent induction of anti-apoptotic molecules(eg. BCL-XL) Identification of molecular and cellular targets, which are associated with the suppression of cell malignancy, is important in the prevention of cancer and will provide a better understanding of anticancer mechanisms. The conventional methods to identify the molecular mechanism of action of drugs are very tedious and time consuming. On the other hand, computational based methodologies have proved to be efficient and inexpensive tools. The computational strategies for determining protein targets of flavonoids including Quercetin have not yet received a great deal of attention. The present study aimed to employ computational molecular docking and invitro assays using PC-3 human prostate cancer cell line to determine the effective target for quercetin.

MATERIALS AND METHODS

Sample collection

The leaves of *Anredera cordifolia* were collected from Gudalur and have authenticated (Certificate No. BSI/SRC/5/23/2015/ Tech /406 dated: 24/2/2015) by Botanical survey of India (BSI), Coimbatore division, Tamil Nadu State, India. The leaves were washed with distilled water to remove the soil and other dust particles. After washing the leaves were shade dried and powdered. The powdered leaves were used for the assay.

Chemicals and reagents

The standard quercetin was procured from Sigma Aldrich.The solvents used in the chromatography such as toluene (Merck), methanol (Merck), ethyl acetate (Merck) and formic acid (Merck) were of AR grade.

Instrumentation

Microsyringe (Linomat syringe, Hamilton-Bonaduz Schweiz), precoated silica gel 60 F_{256} glass plates (10 × 10 cm with 200 µm thickness HPTLC; Merck,KGaA), Camag Linomat 55 automatic sample applicator (Camag), Camag 100 µL sample syringe (Hamilton), Camag twin trough chamber 10 × 10 cm (Camag), UV chamber (Camag), TLC scanner III-170418 (Camag), and win CATS version 1.4.0 software (Camag) were used in this study

Preparation of plant extract

The plant sample was washed, shade dried, powdered. 50g finely powered samples were extracted with ethanol using soxhlet for 72 hours. The resultant extracts were placed in water bath at 60°C for 15 mins and then the extract was collected. This extract was used for HPTLC analysis.

Phytochemical analysis

High performance thin-layer chromatography (HPTLC)

HPTLC was performed on precoated silica aluminium TLC plate 60F_{256} (E-Merk, KGaA) for qualitative evaluation of flavanoid in ethanolic extract of *Anredera cordifolia.* In brief, concentrated ethanolic extraction of *Anredera cordifolia* (10µl) and standard quercetin (5µl) were loaded on TLC plate with CAMAG linomat 55 applicator with inert gas supply. The mobile phase used for the flavonoid was toluene: ethyl acetate: formic acid: methanol (3:6:1.6:0.4) respectively. The plate was developed by the distance of 13.3mm in a Camag twintrough chamber previously equilibrated with mobile phase for 5 minutes.After development of flavonoid plate, derivation was carried out with 5% sulphuric acid in methanol and heated at 60°c on Camag TLC plate platform heater for 5 minute. Camag TLC visualize-170503 was used for photo documentation of flavanoid at 254nm. The flavonoid HPTLC chromatogram was obtained using Camag scanner-170418 in conjugation with Win CATS software.

Molecular docking and insilico studies

Docking studies

The molecular docking involves four steps: *i*) Ligand preparation, the ligand quercetin was created and energy minimized using Lig Prep module of Schrodinger suite version 9 keeping one conformer per ligand, and rest of the parameters were kept as default; *ii*) Protein preparation, the crystal structure of pro- and anti-apoptotic proteins BCL2 (2O2F), BCLXL(1R2D) and NF-kB (1SVC) was retrieved from Protein Data Bank and for FADD the structure was predicted using Schrodinger

software suite (version 9). All the target proteins were prepared using Protein preparation wizard in Maestro; iii) Next glide preparation, interaction between the ligand molecule and a receptor molecule. The shape and properties of the receptor are represented on a grid by different set of fields to provide accurate scoring ligand pose. iv) Docking was performed by using the glide integrated with Maestro 9(Schrodinger,LLC,2009). The structure of human BCL2 (2O2F), BCLXL (1R2D), and NF-kB (1SVC) was modified with the "protein preparation wizard" by deleting the substrate cofactor and crystallographically observed water molecules. The prepared protein receptor grid files and the minimized ligand database were given as input in the virtual screening workflow protocol. Then single low energy 3D structure of ligands with correct chiralities was docked with the binding site using the 'extra precision' Glide algorithm in Schrodinger.

Accession of target protein

The three-dimensional structure of quercetin was downloaded from the RCSB protein Data Bank.

Ligand selection

The chemical structure of quercetin was obtained from PubChem compound database. It was prepared by ChemBioDraw and MOL SDF format of this ligand was converted to PDBQT file using PyRx tool to generate atomic coordinates.

Target and ligand optimization

For docking analysis, PDB coordinates of the target protein and quercetin molecule were optimized by Drug Discovery Studio version 3.0 software and UCSF Chimera tool, respectively (adding missing residues). These coordinates had minimum energy and stable conformation.

Analysis of target active binding sites

The active sites are the coordinates of the ligand in the original target protein grids, and these active binding sites of target protein were analyzed using the Drug Discovery Studio version 3.0 and 3D Ligand Site virtual tools.

Quercetin binding to NF-kB

The active site of quercetin coordinates to the ligand in the protein grids of NF-$_K$B, and these active binding sites of target protein were docked using the ligand site virtual tools.

Quercetin binding to BCL_2

The active site of quercetin coordinates to the ligand in the protein grids of $BCL_{2,}$ and these active binding sites of target protein were docked using the ligand site virtual tools.

Quercetin binding to BCL_{XL}

The active site of quercetin coordinates to the ligand in the protein grids of $BCL_{XL,}$ and these active binding sites of target protein were docked using the ligand site virtual tools.

RESULTS

HPTLC fingerprinting profile

HPTLC profile of ethanol extract of *Anredera cordifolia* plant powder was recorded in mainly at 254nm. The extract shows the presence of flavanoids in chromatograph as well as in UV 254nm. The R_f value of the extract were found to be 0.03, 0.18, 0.23, 0.36, 0.42, 0.62 and 1.07(Table: 1). Among them, peak 6 were found as flavanoid with R_F value of 0.62.

Table 1
HPTLC for flavanoid

PEAK	R_F	HEIGHT	AREA	ASSIGNED SUBSTANCE
1	0.03	0.1	3676.4	Unknown
2	0.18	23.8	937.1	Unknown
3	0.23	45.1	1489.9	Unknown
4	0.36	69.3	6764.1	Unknown
5	0.42	43.5	2171.4	Unknown
6	0.62	70.5	18877.1	Flavanoid
7	1.07	0.7	27903.1	Unknown

HPTLC quercetin profile

HPTLC confirmed that *Anredera cordifolia* consists of flavanoid at 0.62 R_F value. The ethanol extract of *Anredera cordifolia* also showed the presence of quercetin in table 2 and Figure 1-3.

Table 2
HTPLC for quercetin

Peak	R_F	Height	Area	Assigned substance
1	-0.03	105.1	5.40	Unknown
2	0.04	47.3	2.92	Unknown
3	0.46	22.8	3.83	Unknown
4	0.60	734.3	87.84	Quercetin

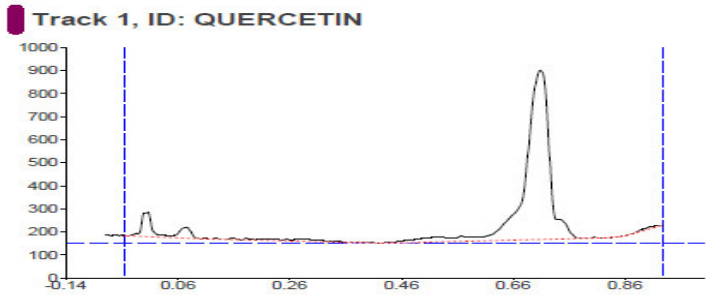

Figure 1
Graphical representation of quercetin

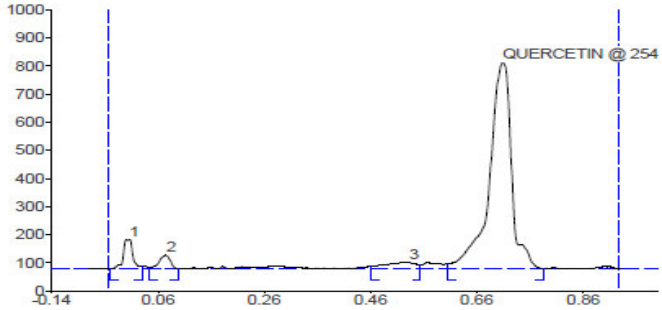

Figure 2
The ethanolic extract of Anredera cordifolia showing the presence of quercetin at 254nm

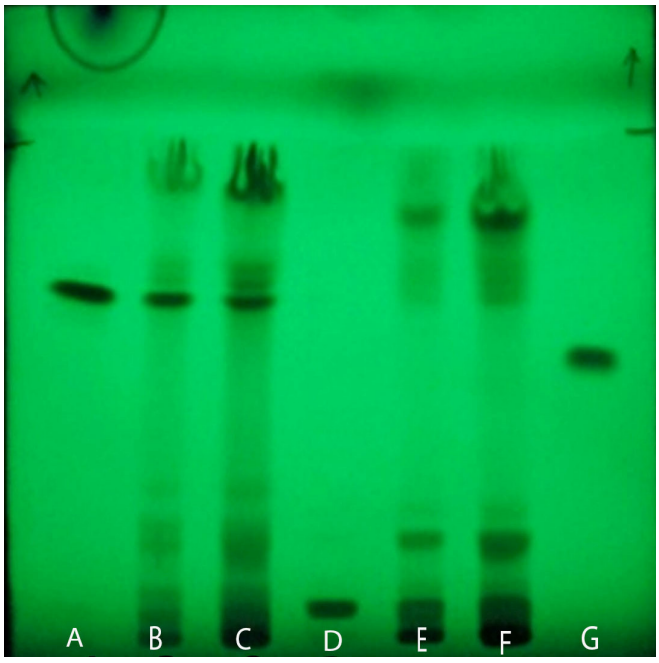

Figure 3

A-Standard Quercetin(Rf Value-0.62)
B-Ethanolic extract of Anredera cordifolia (Rf value-0.62)
C-Aqueous extract of Adredera cordifolia(Rf value-0.62)
D-Standard Rutin (Rf value-0.13)
E-Ethanolic extract of Basella rubra(Rf value-0.13)
F-Aqueous extract of Basella rubra(Rf value-0.13)
G-Standard Gallic acid (Rf valu-0.52)

Quercetin in chromatograph

The extract shows the presence of quercetin after deviation. A good separation of flavanoid has been observed.

Quercetin

HPTLC flavanoidal fingerprinting confirmed the presence of quercetin present in *Anredera cordifolia*. Quercetin may help protect against heart disease and cancer. Quercetin can also help stabilize the cells that release histamine in the body and thereby have an anti-inflammatory and antihistamine effect.

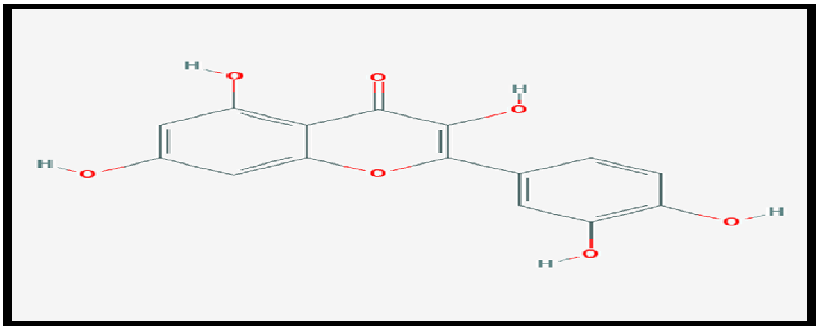

Figure 4
2D confirmation of quercetin
The structure of quercetin was drawn from PDB entry

Docking studies

To investigate the detailed intermolecular interactions between quercetin and its target protein, an automated docking program Glide (version 5.5) was used. Three-dimensional structure data for the other target proteins was obtained from the PDB entry 1R2D, 2O2F, 1SVC. Processing of the proteins included the deletion of the co-crystallized ligand and the solvent molecules as well as the addition of hydrogen atoms. Molecular docking revealed the interaction of quercetin with the active site of target proteins BCLXL, BCL2 and NF-kB. All the protein–ligand complexes possessed multiple hydrogen bonds. On the basis of G-score parameter the binding affinity of ligand towards receptors was determined.The higher negative value of glide score indicates a greater binding affinity of the ligand with receptor. When compared to the other target proteins, quercetin showed high affinity towards NF-kB with glide score of -0.281KJ/mol and docking score (-6.172). The Lipophilic EvdW is the lipophilic term derived from hydrophobic grid potential and fraction of the total protein-ligand vdW energy was very low for NF-kB (-0.295) when compared with other proteins. The HBond which is the Chem score H-bond pair was also low in NF-kB with score of -0.295.

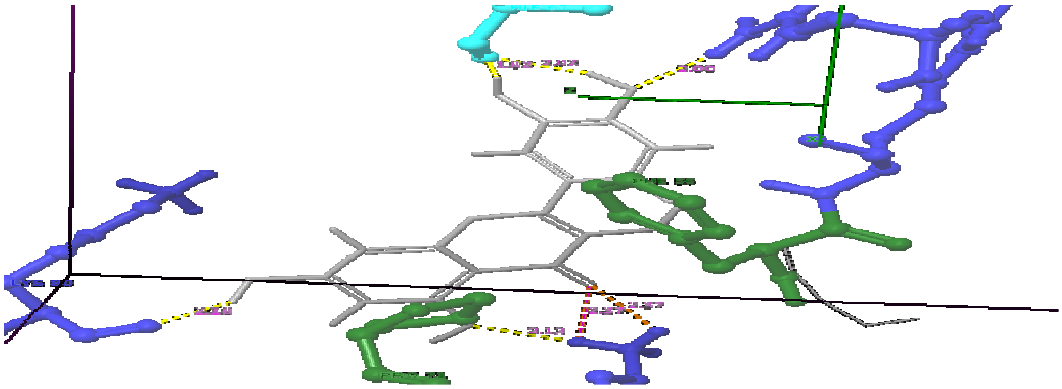

Figure 5
Binding site of quercetin vs NF-κB (1svc)

NF-κB (1SVC) interacts with Quercetin mainly through LYS 80 at a distance of 2.18, ARG 59 at a distance of 2.37 and GLY 68 at a distance of 2.00. The interaction between quercetin and 1SVC is positively charged and hydrophobic in nature. There are certain unspecified residues present during interaction. These interactions undergo metal coordination along with a salt bridge.

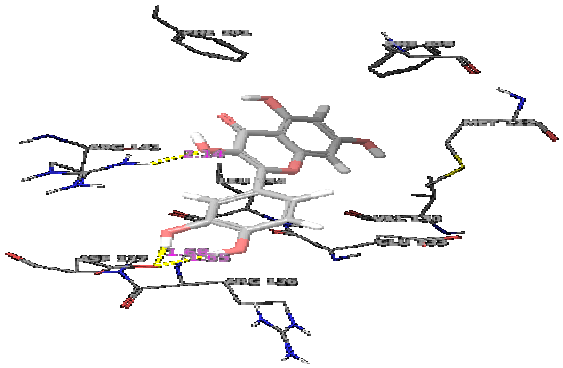

Figure 6
Binding site of quercetin Vs BCL_2 (202F)

BCL_2(202F) interacts with Quercetin mainly through ARG 137 at a distance of 2.14, ASP 137 at a distance of 1.68 and PHE101 at a distance of 1.85. The interaction between Quercetin and 202F is negatively charged. There are certain unspecified residues present which undergo metal coordination.

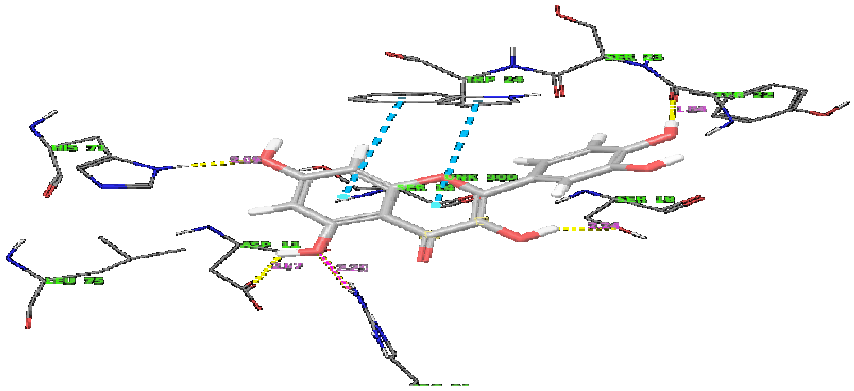

Figure 7
Binding site of quercetin Vs BCL_{XL} (1R2D)

BCL_{XL} (1R2D) interacts with Quercetin mainly through ASP11 at a distance of 2.67, HIS 71 at a distance of 2.00, SER 18 at a distance of 2.24 and TYR 22 at a distance of 1.84. Quercetin interacts with 1R2D very strongly only with specified residues and a salt bridge.

Table 3
Target proteins (1SVC, 202F, 1R2D) Vs quercetin interaction profile for NF-κB, BCL_2 and BCL_{XL}.

Pdb complex	Glide score	Docking score	Lipophilic	H bond	Site map	Energy
Quercetin Vs NF-Kb (1SVC)	-0.281	-6.172	-1.892	-0.295	0	-35.75
Quercetin Vs BCL_2 (2O2F)	-0.249	-5.469	-1.21	0	0	-35.62
Quecetin Vs BCL_{XL} (1R2D)	-0.244	-5.36	-0.462	-0.163	0	-33.69

Table 4
Quercetin interaction with NF-κB, BCL_2, AND BCL_{XL}

PDB complex	Residues	Distance
Quercetin Vs NF-KB (1SVC)	LYS 80	2.18
	ARG 59	2.37
	GLY 68	2.00
Quercetin Vs BCL_2 (2O2F)	ARG 137	2.14
	ASP 137	1.68
	PHE 101	1.85
Quercetin Vs BCL_{XL} (1R2D)	ASP 11	2.67
	HIS 71	2.00
	SER 18	2.24
	TYR22	1.84

The more negative value on the scoring function indicates better docking, and the docking of ligand and protein were ranked according to the corresponding Glide scoring function. The scoring function of Glide docking program was exhibited in the form of G-score. G-score indicates the binding ability of a ligand to the specific conformation of the protein receptor. The accuracy of a docking procedure is determined from

the object scoring function which was predicted even in lowest energy pose. It was observed that successful docking has been correlated with good intermolecular hydrogen bonding and liphophilic interactions between the ligand and the receptor [11].

DISCUSSION

NF-κB is normally located in the cytoplasm as an inactive dimer. The activity of NF-κB is regulated by interaction with inhibitory IκB proteins, which repress the potential of NF-κB to translocate to the nucleus and bind with DNA . Upon activation, IκB is phosphorylated, which marks the inhibitor for ubiquitination and degradation via a proteasome-dependent pathway [12].It has been well established that inhibition of NF-kB signaling pathway sensitizes TNF-α induced cell death and TNF-α is often used to provoke cell apoptosis. NF-kB signaling antagonizes TNF-α and chemotherapeutic agents induced apoptosis by promoting the transcription of anti-apoptotic genes, such as c-FLIP, cIAP-1, and cIAP-2, XIAP, survivin, Bcl-xL and Bcl-2. In this way, blocking of NF-kB signaling can potentiate TNF-α induced apoptosis [13, 14]. NF-kB proteins are transcription factors induced in response to inflammatory and other stress stimuli[15]. A majority of cancer cells become resistant to TNF-α as a result of the activation of NF-kB and consequent induction of anti-apoptotic molecules (e.g. IAPs/Bcl-XL), as the pro-survival effects of TNF-α out-perform the pro-apoptotic effects. Literature shows that blocking NF-kB activation can overcome TNF-α resistance, although a constitutive NF-kB activation, rather than the inducible one, has been suggested to be more important[15]. *In silico* docking studies confirmed the binding of hesperetin a flavanoid to DNA binding domain of NF-kB in preventing the expression of NF-kB mediated cell survival genes, in which hespertin has greater binding affinity within the DNA binding domain of NF-kB in comparison with the other pro- and anti-apototic proteins[16].

CONCLUSION

On the basis of Interaction, the binding affinity of quercetin towards receptors was determined. Abnormal proliferation of cells lead to mutation. Activated quercetin along with pro and anti-apototic proteins like BCL_2 , BCL_{XL} and NFκB may lead to inhibition of growth of PC-3 cancer cells.All the target proteins had a good score of interaction 1SVC=-6.172, 202F= - 5.469 and 1R2D=-5.36.When compared to all the three interactions by target protein, quercetin binds to NF-κB very strongly with metallic coordination.It can act as a inhibitor of apoptosis inhibitor when compared to BCL_{XL} and BCL_2. Quercetin down regulates the cell survival proliferative anti-apoptotic proteins thereby can prevent prostate cancer. To confirm this, in vitro study can be carried out with techniques like MTT Assay, Western blot, and polymerase chain reaction.

CONFLICT OF INTEREST

Conflict of interest declared none.

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