CHAPTER VII
IN SILICO STUDIES IN UNDERSTANDING THE MECHANISM OF ANTI-CANCER ACTION

7.1 INTRODUCTION

*J. tanjorensis* (Family, Euphorbiaceae) also known as the “Catholic vegetable” or “Reverend Father’s vegetable” is an exotic plant species found in India, Africa, and America. Ethnomedicinally *tanjorensis* is being used as a vegetable to make palatable soup. Besides it possesses antiseptic and anti-hypertensive properties (O’Hara et al., 1998). There are also reports existing on the use of this plant drug in the treatment of diabetes (Olayiwola et al., 2004). Studies carried out on the nutraceutical values of this plant drug, showed the abundant presence of bioactive phytoconstituents and minerals like flavonoids (3.69%), alkaloids (1.89%), calcium (5.69%), magnesium (4.22%) and potassium (2.15%). This provides scientific evidences for its use as herbal dietary supplement (Arun et al, 2012). Among the main constituents of medicinal plants, flavonoids and its glycoside derivatives are of the largest groups of molecules that are enriched with numerous biological activities and extensive studies are available on these bioactive molecules (Hertog and Katan, 1998). These phytomolecules besides possessing potent antioxidant property (Zhang et al., 2006 and Wang et al., 1996) were also found to be useful in combating various disorders like cancer. It was also reported that these flavonoids posses antiproliferative (Kawai et al., 1999) and anti-inflammatory activity (Garcia-Lafuente et al., 2009). Compounds with antioxidant and cytotoxic potentials
could be useful in preventing the secondary damage to the biological system caused by the release of free radicals. This property makes flavonoids and its derivatives to be more useful towards the development of safe and efficacious plant drugs. Present study showcases remarkable potential of methanolic leaf extract of J. tanjorensis in protecting cells from free-radical damages and uncontrolled proliferations through various in vitro assays. The extract is mainly composed of several glycosides and aglycones and structures of each phytochemical belonging to these classes were confirmed using their MS/MS data. Principle compound(s) accounting medicinal properties of the extract were evaluated for their anticancer efficacy through their binding interactions and affinities with 8 different proteins of Bcl-2 family by conducting molecular docking studies and the studies suggested that Vitexin and 6-C-Pentosyl-8-C-hexosyl apigenin are proved to be the lead active compounds. Present work also brought into limelight unique structural features of the compounds that could be developed as potent anticancer molecules.

7.2 MATERIALS AND METHODS

7.2.1 Targets selections and preparations for molecular docking studies

Three-dimensional (3D) structures of proteins such as Bcl-2 (1GJH), Bcl-B (4B4S), Bcl-W (1ZY3), Bcl-XL (2LPC), Bfl-1 (3MQP), Mcl-1 (2KBW), Bax (1F16) and Bak (2IMS) were retrieved from Protein Data Bank (PDB). Moreover, missing hydrogen atoms were added and bond orders were assigned to all proteins considered, herein, by using Schrodinger suite 9.2. The proteins were then energy minimized using ‘Steepest descent’ algorithm with a tolerance of 1000 KJ/mol/nm and step size of 0.01 with required number of minimization steps. The energy minimized structures were subjected to molecular dynamics (MD) simulations in near physiological conditions (pH 7.0, 1
atmospheric pressure and ionic strength of 0.1 M NaCl) for 5 ns at 310 K by Gromacs 4.5.1 (prior to productive runs, each protein was equilibrated for 200 ps and 500 ps under NVT and NPT conditions, respectively). In each case, trajectory structures were stored at every 25 ps and the data were analyzed in terms of change in potential energy and RMSD of backbone atoms of proteins. The data analyses revealed that all the proteins reached equilibrium under defined conditions well within the time span of 5 ns MD and a representative structure from the equilibrium phase of each protein was selected. The representative structures were used as 3D structures of the corresponding proteins for further analyses carried out in the present work.

7.2.2 High-throughput virtual screening (HTVS) and lead optimizations

3D structures of all 10 small molecular compounds identified in methanolic extract of *J. tanjorensis* leaves were generated and energy minimized (till structural conformations are converged) using ArgusLab 4.0.1. Then, the resultant structures were processed at pH 7.0 using LigPrep module of Schrodinger suite and docked on the BH3-binding grooves (which were covered by grid boxes having about 80 x 80 x 80 Å dimensions) of Bcl-2 family proteins by Glide-XP with default parameters. However, total numbers of poses per run, total numbers of generated and selected poses per ligand were set as 10,000, 5,000 and 400 respectively. The docked ligands on each protein were ranked on the basis of their GlideScores and GlideEnergies. Hydrogen-bond (H-bond) and other non-covalent interactions between ligand and protein of the docked complexes were scrutinized using PyMol 1.5.0.1. Pharmacokinetic properties of the chemical compounds were assessed by subjecting each compound to QikProp module of Schrödinger software suite 9.2 and ADME-Tox prediction tool.
7.3 RESULTS AND DISCUSSION

Apart from various mechanisms, apoptosis induction in cancerous cell is considered to be the best way of tackling cancer (Rozalski et al. 2006). Vast number of natural products have been identified which possess the activity of inducing apoptosis in cancerous cells (Amit et al. 2001). Identifying the best active compounds from plant extract has gained huge importance in cancer treatment. Anticancer efficacy of flavonoids is reported earlier by Iwashita et al. (2000). Hence in silico docking studies were performed on flavonoids and its derivatives identified in the selected plant against various apoptosis related proteins, especially Bcl-2 family proteins which determine the cell fate by overexpression of pro- or anti-apoptotic proteins.

One of the major causes for the occurrence of cancer is inadequate activation of apoptosis (Johnstone et al., 2002). Bcl-2-family proteins are important regulators of apoptosis. Anti-apoptotic proteins such as Bcl-2 and Bcl-xL, contains a surface hydrophobic groove in which the BH3 domain of the pro-apoptotic counterparts binds (Reed 1995). This binding is important for regulating apoptosis. Bax and Bak which belongs to pro-apoptotic proteins mediate the permeabilization of mitochondrial membrane upon oligomerization, resulting in the release of intermembrane space proteins such as cytochrome c, OMI/HTRA2, SMAC/DIABLO and endonuclease G leading to the formation of apoptotic bodies, condensation and fragmentation of nuclei which brings cell death (Kuwana and Newmeyer 2003). Bcl-XL plays its anti-apoptotic role by forming complex with Bax protein thus bringing conformational changes to Bax and hence prevents its activity of releasing apoptosis execution signaling factors. Inhibition of anti-apoptotic proteins by various natural products are the recent advancement in exploring the possible mode of action of these molecules in treating cancer.
7.3.1 Lead anti-cancer compounds and their specificities towards the Bcl-2 family proteins

GlideScores of 10 small molecular compounds on the surface grooves of 8 Bcl-2 family proteins playing essential roles in cell homeostasis processes of *Homosapiens* have been enumerated in Table 23. Surprisingly, the ligand molecules showed differential binding preferences towards anti-apoptotic and pro-apoptotic proteins.

Table 23: GlideScore of various phytochemical identified from the methanolic leaf extract of *J. tanjorensis* on interacting with the BH3-binding grooves of 8 different proteins of Bcl-2 family as calculated by means of Glide-XP molecular docking tool.

<table>
<thead>
<tr>
<th>Name</th>
<th>Bcl-2</th>
<th>Bcl-XL</th>
<th>Bcl-B</th>
<th>Bcl-W</th>
<th>Bfl-1</th>
<th>Mcl-1</th>
<th>BAK</th>
<th>BAX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baicalein</td>
<td>-3.1</td>
<td>-2.5</td>
<td>-4.9</td>
<td>-6.8</td>
<td>-3.7</td>
<td>-4.4</td>
<td>-3.2</td>
<td>-6.6</td>
</tr>
<tr>
<td>Diosmetin</td>
<td>-4.2</td>
<td>-1.7</td>
<td>-4.6</td>
<td>-6.1</td>
<td>-3.8</td>
<td>-4.4</td>
<td>-1.5</td>
<td>-3.6</td>
</tr>
<tr>
<td>Homoorientin</td>
<td>-4.2</td>
<td>-5.3</td>
<td>-4.7</td>
<td>-10.0</td>
<td>-7.1</td>
<td>-5.3</td>
<td>---</td>
<td>-7.4</td>
</tr>
<tr>
<td>Kaempferide</td>
<td>-2.6</td>
<td>-1.9</td>
<td>-3.9</td>
<td>-5.3</td>
<td>-3.4</td>
<td>-3.8</td>
<td>-1.3</td>
<td>-3.0</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>-3.9</td>
<td>-1.8</td>
<td>-4.6</td>
<td>-5.0</td>
<td>-3.7</td>
<td>-3.97</td>
<td>-2.2</td>
<td>-6.0</td>
</tr>
<tr>
<td>Kaempferol-3-o-rutinoside</td>
<td>-7.1</td>
<td>-6.5</td>
<td>-6.8</td>
<td>-10.7</td>
<td>-7.0</td>
<td>-7.9</td>
<td>---</td>
<td>-6.6</td>
</tr>
<tr>
<td>Luteolin-7-O-glucoside</td>
<td>-4.9</td>
<td>-6.2</td>
<td>-5.9</td>
<td>-8.4</td>
<td>-5.1</td>
<td>-5.7</td>
<td>---</td>
<td>-5.2</td>
</tr>
<tr>
<td>6-c-pentosyl 8-c-hexosyl apigenin</td>
<td>-4.0</td>
<td>-3.0</td>
<td>-4.6</td>
<td>-8.5</td>
<td>-4.9</td>
<td>-6.6</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Naringin</td>
<td>-3.8</td>
<td>-3.0</td>
<td>-4.9</td>
<td>-8.6</td>
<td>-5.9</td>
<td>-6.5</td>
<td>---</td>
<td>-4.7</td>
</tr>
<tr>
<td>Vitexin</td>
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<td>-5.8</td>
<td>-7.7</td>
<td>-6.1</td>
<td>-4.9</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

The possible role of identified compounds from *J. tanjorensis* in inducing apoptosis in cancer cells are discussed in detail through *in silico* modeling studies.

Identifying the best active compounds from plant extract has gained huge importance in cancer treatment. Based on the previous reported studies on anticancer efficacy of flavonoids (Iwashita et al., 2000) and through *in vitro* experiments findings it was clear that *J. tanjorensis* posse’s potent biological activity against cancer. Apart from various mechanisms, apoptosis induction in cancerous cell is considered to be the best way of managing cancer. Hence *in silico* docking studies were performed on molecules
identified in selected plant against various apoptosis related proteins, especially Bcl-2 family proteins.

7.3.2 Specificities of small molecular compounds towards Bcl-2 family proteins

GlideScores of 10 small molecular compounds on the surface grooves of 8 Bcl-2 family proteins, VEGF and VEGFR-2 playing essential roles in cell homeostasis processes of *Homosapiens* have been enumerated in Table 23. All the chemical molecules showed interactions with VEGF and VEGFR-2. In contrary to the observations, the ligands showed differential binding preferences towards anti- and pro-apoptotic proteins. The chemical molecules could be classified into three groups on the basis of their binding preferences with those proteins. Group 1 consists of baicalein, diosmetin, kaempferide and kaempferol and these ligands are interacting with all the Bcl-2 family proteins. Chemical molecules such as naringin, homoorientin, kaempferol-3-rutinoside and luteolin-7-O-glucoside are belonging to Group II and they are also interacting with all protein targets and also with Bak, a pro-apoptotic protein. Interestingly, vitexin and 6-C-pentosyl-8-C-hexosyl apigenin (PHA) belonging to Group III interact with anti-apoptotic proteins but showed bereft of interaction with pro-apoptotic proteins implying that these compounds may act as lead anti-cancer compounds of the plant extract.

The Group II compounds may play significant roles in a unique Bak-mediated apoptosis on retarding the process of cell proliferation. Of the 4 compounds, kaempferol-3-rutinoside was ranked as top lead compound to 8 of 10 proteins considered in the present study (Table 14), on the basis of GlideScores. The compound was ranked to second position on interacting with Bfl-1 and Bax, which depicted subtle stronger interaction with homoorientin than their affinities with kaempferol-3-rutinoside (KF3R).
Strikingly, the binding affinities of KF3R are stronger with the Bcl-2, Bcl-B, Bcl-W, Bfl-1, Mcl-1 and VEGFR-2 than that of Bax pro-apoptotic protein and its interaction with Bcl-XL and VEGF are comparable, only differing in the first decimal, with Bax-KF3R interaction. By tie together, vitexin, PHA and KF3R may be active principles of the plant extract showing *in vitro* cytotoxic activity against A431, EAC and Caco-2 cells.

By tie together, all flavones/flavonols studied in the present study from the *J. tanjorensis* are interacting with all the 8 proteins of Bcl-2 family and only flavone glucosides present in the source are depicting differential binding preferences with those protein targets. Interestingly, flavone glucosides of Group II and Group III are especially differing from each other in their glycosidic linkages. Flavone glucosides of Group III have glycosidic bond between 8\textsuperscript{th} carbon atom of flavonoid moiety and their corresponding sugar groups, whereas different hexose groups are attached to various positions (exclusively other than 8\textsuperscript{th} carbon atom) of flavonoid structures of flavone glucosides classified into Group II. While the flavone glucosides of Group II are interacting with all the anti-apoptotic proteins and Bak, the flavone glucosides (VN & AN) of Group III are interacting only with anti-apoptotic proteins (they neither interact with Bak nor with Bax). It implies that the unique structural architectures of the VN and AN are crucial in accounting their specific interactions with anti-apoptotic proteins over pro-apoptotic proteins. BH3-binding grooves of all the 8 proteins of the Bcl-2 family and binding modes of the VN and AN on the surface grooves of the 6 anti-apoptotic proteins are shown in Figure 20.
Figure 20. BH3-grooves (shown in red surface models) of 2 pro-apoptotic proteins and binding locations of the leads (VN in red sticks models and AN in blue sticks models) on the BH3-binding grooves of the 6 anti-apoptotic proteins are illustrated.

A quick inspection to the figures clearly suggests that the surface grooves of Bax and Bak are with incompatible geometries to accommodate structures of the VN and AN (Fig. 21).

In the case of the anti-apoptotic proteins-AN/VN interactions, it could be noted that the sugar moieties of the leads were tightly surrounded by a few hydrophobic residues (such as alanine, leucine, methionine, phenylalanine and valine) and at least one
polar residue (such as arginine, asparagine, lysine, glutamic acid, histidine, serine, threonine and tyrosine) from the proteins to which they showed bindings.

Figure 21. Molecular structure of A. Vitexin (VN) and B. 6-C-pentosyl-8-C-hexosyl apigenin (AN).

It could also be observed that at least anyone of 4 hydroxyl groups (at 3″, 4″, 5″ and 7″ of VN and 3″, 4″, 5″, 7″, 2‴, 3‴ and 6‴ of AN) of sugar moieties of the leads invariably established hydrogen-bonding (H-bond) interactions with anyone type of polar residues that are within 4 Å proximity to the corresponding groups. However, these chemical complementarities were not apparent features in the grooves of pro-apoptotic proteins and consequently they were unable to accommodate the leads in their BH3-
grooves. Thus, the unique structural and chemical features of the lead compounds (AN & VN) to interact with anti-apoptotic proteins as figured-out herein may pave a way to develop highly efficient and specific inhibitors to the anti-apoptotic proteins. Moreover, toxicities and bioavailability of the two lead compounds were predicted by using ADME-Tox and QikProp computational tools, respectively and inferred that both leads are non-toxic in nature and qualitative human oral absorption of both compounds are medium suggesting that they are also promising lead compounds for developing them as therapeutic drugs against cancer.

*In silico* studies carried out using the molecules identified in the selected plant extract docked effectively against Bcl-2 family proteins suggesting the possible induction of apoptosis in studied cancer cells by the extract. Supporting anticancer and epifluorescence data obtained in *in vitro* studies were presented and discussed in sequel. As discussed, the development of tumors arises as a consequence of dysregulated proliferation and a suppression of apoptosis, and each of these primary defects provides an obvious opportunity for clinical intervention. Many of the current chemotherapeutics designed to perturb proliferation in such a crude manner that the resulting damage to normal cells limits their clinical efficacy. Small molecule-based strategies for disrupting the activities of antiapoptotic Bcl-2 proteins have arisen from our extensive understanding of the structures of these proteins. These include the design of small molecules that are able to bind to and inactivate the hydrophobic pocket in Bcl-2 and Bcl-xL, which is essential for its antiapoptotic function and which houses the docking site for the death-promoting members of the family (Bax, Bak, and Bad) via their BH3 domains.
7.4 CONCLUSION

In silico modeling experiments performed on various apoptosis and cell proliferation proteins as targets have clearly proved that vitexin and 6-c-pentosyl 8-c-hexosyl apigenin possess much better anticancer efficacy as compared to positive anticancer standard drugs such as doxorubicin, vincristine, vinblastine, 5-FU and cyclophosphamide. This suggests that the methanolic extract of *J. tanjorensis* induced anticancer action through an intrinsic apoptosis pathway in EAC and Caco-2 cells. Present investigation constitutes the first comprehensive report on the constituents and anticancer activity of the methanolic extract of *J. tanjorensis* and contributes in providing scientific evidences for the traditional uses of selected plant drug *J. tanjorensis*. We can predict that extract containing all these 10 compounds can act as a better oral potent drug for promoting apoptosis. Based on the extensive in silico experiments it was understood that methanolic extract of *J. tanjorensis* brings its anticancer activity through inhibiting anti-apoptotic proteins and inducing the activity of pro-apoptotic proteins. In vitro and in vivo studies carried out further supported this hypothesis.