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Pharmacoinformatics and Antiangiogenic Activity of *Morinda tinctoria* Phytochemicals by Targeting Vascular Endothelial Growth Factor Receptor-2

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ABSTRACT

In cancer progression, angiogenesis plays a crucial role in the formation of newer blood vessels. Vascular endothelial growth factor (VEGF) is an important factor which stimulates angiogenesis. By blocking VEGF signaling, the anti-angiogenic potential can be accomplished. In this study, the potential can be achieved by the phytochemicals using *Morinda tinctoria* Roxb. belongs to the family Rubiaceae. The structures of the phytochemicals were downloaded from PubChem database and they were screened *in silico* for their physicochemical properties, pharmacokinetics parameters, drug likeness and toxicity profiles using various online tools and databases including Swiss ADME, pkCSM and ProTox II. All the phytochemicals were virtually screened against VEGFR-2 Tyrosine Kinase domain targets namely 3VHE, 4GA8 and 6GQQ) using iGEMDOCK. Molecular docking with VEGFR-2 target was performed by using AutoDock 4.2 software and the docking results were visualized using Biovia Discovery Studio version 16.1.0. Binding score and interaction profile were compared with the standard anti-angiogenic drugs Gefitinib and Pazopanib. The results revealed that Kaempferol-3-O-rutinoside, Ursolic acid, Acacetin-7-O-glucopyranoside and β-sitosterol showed greater binding energy and good interaction profile. In conclusion, these compounds may be taken as lead molecules to identify new chemical entities for cancer therapy.

***Keywords*:** *Morinda tinctoria*, Anti-angiogenic, Anticancer, Insilico analysis, Docking, VEGFR-2.r.

INTRODUCTION

Angiogenesis is the formation of newer blood vessels from existing blood capillaries, which plays an important role in ontogenetic development. It is an essential one in several physiological processes in the human body such as tissue growth and repairing mechanisms.[1] On the other hand, uncontrollable angiogenesis contributes to disorders like cancer, inflammation and ophthalmic disorders.[2] Vascular endothelial growth factor (VEGF) is an important growth factor that stimulates angiogenesis by signaling via VEGF receptors (VEGFRs). Among the VEGF receptors, VEGFR-2 is ten times stronger than other receptors. VEGFR-2 is an important receptor that transmits angiogenic signals and these receptor inhibitors can delay the angiogenesis by blocking the VEGR-2 mediated signaling pathway.[3,4] Recently a number of plant-derived natural compounds have been reported possessing potential antiangiogenic activity.[5,6,7,8] Hence, the blockade of VEFR-2 signaling is able to give a promising approach leading to develop anti-angiogenic agents.

Morinda tintctoria or Indian mulberry is well known medicinal plant in traditional medicine to have several medicinal benefits. This plant exhibited potent antidiabetic,[9] antimicrobial,[10] hepatoprotective,[11] anti-inflammatory,[12] antiradical,[13] free radical scavenging[14] and cytoprotective activity,[15] antidiabetic and antihyperlipidaemic.[16] *Morinda tinctoria* (Roxb.) roots were also found to have a therapeutically potent iridoid glycoside.[17] A phytochemical review of this plant revealed the presence of ursolic acid, quercetin, kaempferol-3-O-rutinoside, acacetin-7-O-β-D-glucopyranoside, apigenin-5,7-dimethyl ether 4'-galactoside, alizarin-1-methyl ether, rubiadin, anthragallol-2,3-dimethyl ether, soranjidiol, ibericin, morindone, morindine, tinctomorone, damnacanthan and nordamnacanthal.[18] Though it has more health

benefits but its efficacy on angiogenesis has not been established much. Keeping this in mind, the present investigation is aimed to evaluate the effect of various phytochemical constituents derived from an above-said plant on the VEGFR-2 using *in silico* approach. Further, the phytoconstituents were screened for pharmacokinetic and docked onto the kinase domain of VEGFR-2.

MATERIALS AND METHODS

Ligand preparation:

Structures of the phytochemicals from *Morinda tinctoria* namely Acacetin-7-O-glucopyranisode, damnacanthal, ibericin, kaempferol-3-O-rutinoside, morindone, nordamnacanthal, tubiadin, soranjidiol, ursolic acid and β-sitosterol was downloaded as 2D from PubChem database (https://pubchem.ncbi.nlm.nih.gov).[19] These structures were exported to ChemDraw Ultra 12.0 for the energy minimization and geometry correction. The outputs of the structures were saved in mol file (.mol), sdf (.sdf) and pdb (.pdb) formats which were used for further *in silico* studies.

Protein preparation:

Crystal three-dimensional structures of intracellular kinase domains of VEGFR-2 (PDB ID: 3VHE, 4GA8, 6GQQ) were downloaded from RCSB Protein Data Bank (http://rscb.org)in the pdb format.[20] The stereo chemical properties of each VEGFR-2 were assessed based on the information obtained from the PDB X-Ray structure Validation Report for each PDB structure. The resolution and R-values showed the goodness of the protein model being used. The X-ray crystal structure with resolution values of 2.0Å or less and R-values of 0.2 or less are considered acceptable. Moreover, the structures of the proteins were evaluated based on the Ramachandran plot. Proteins were energy minimized, water molecules were removed, and hydrogen along with charges was added. Finally, 3D structures of the target proteins were saved and used for virtual screening and docking studies.

Molecular properties and toxicity predictions:

The phytochemicals were screened for their molecular properties and drug-likeness using the SwissADME web server (http://www.swissadme.ch/).[21] Absorption, distribution, metabolism, elimination and toxicity (ADMET) using pkCSM web server (http://biosig.unimelb.edu.au/pkcsm/prediction)[22] and ProTox-II online tool (http://tox.charite.de/protox_II/).[23]

Virtual screening:

Virtual screening was performed using iGEMDOCK software version 2.1 (http://gemdock.life.nctu.edu.tw/dock/igemdock.php) with the following parameters: standard flexible docking option with the population size of 200, 70 generations and 2 solutions.[24] The binding site of the target was 8Å. The empirical screening function of iGEMDOCK was estimated as Fitness = vdw + Hbond+Elec.

Molecular docking and visualization:

Molecular docking was performed using AutoDock 4.2 (http://autodock.scripps.edu/) with the Lamarckian genetic algorithm. The docking parameters selected were 200 docking runs and a population size of 200. The binding energy of the protein coordinate was evaluated by a three-dimensional grid box of 60x60x60 (Number of grid points in xyz) created with a spacing of 0.375Å.[25] Biovia Discovery Studio version 16.1.0 (https://www.3dsbiovia.com/) was used to visualize the docking results.

Validation:

The docking energies of the phytochemicals were compared with the commercially available antiangiogenic drugs Gefitinib and Pazopanib for the validation of results. The docking energy for a given protein-ligand pair comprised of the intermolecular interaction energies including internal steric energy, hydrogen bond interaction energy, Van der Waals force and columbic electrostatic energy of the phytochemical were checked. The lowest binding energy of the protein-ligand complex has been considered to be the best.

RESULTS AND DISCUSSION

Angiogenesis is the formation of new blood vessels in order to aid wound healing, cell reproduction and development. It is strictly regulated; various inhibitors and stimulators play a vital role to maintain it in balance. Angiogenesis is required for tumor growth and metastasis, and stimulators are upregulated disturbing the angiogenesis equilibrium. Tumor blood vessels differ from normal blood vessels in that they contain irregular branches and are more heterogeneous.[6]Various pathways including Vascular Endothelial Growth Factor (VEGF), Epidermal Growth Factor (EGF), Fibroblast Growth Factor (FGF) and Hepatocyte Growth Factor (HGF) ensure the heterogeneity of tumor blood vessel structure and increased angiogenesis. VEGF signaling through the VEGF receptor 2 (VEGFR2) has been shown to play an important role in regulating tumor angiogenesis, where the binding of VEGF to VEGFR2 leads to receptor dimerization, autophosphorylation of tyrosine residues in the intracellular kinase domain

resulted in potent chemotactic and mitogenic effects on endothelial cells.[26] VEGFR2 is an attractive signaling receptor and it is regulated by tumor-induced endothelial cell proliferation, permeability and differentiation.[27] VEGF is a potent therapeutic target for the development of new anticancer agents and the number of approaches to inhibit VEGF activity is currently being assessed in preclinical and clinical trials.[28]

Secondary plant metabolites are great sources of small molecules identified from plants. A combined therapy approach using phytochemicals to target different components of angiogenesis potentially results in an effective anticancer treatment.[29] Phytochemicals have a variety of therapeutic uses, including cancer treatment due to their impact on cancer-related biological pathways including angiogenesis. Several phytochemicals exhibit anti-angiogenic effects that include flavonoids, triterpenoids, polyphenols, tannins and other aromatic substance has been reported. Many whole extracts have been investigated for anti-angiogenic activities to identify molecules targeting different pathways of angiogenesis. Among the pathways, VEGF is of special importance for its interaction with the plant secondary metabolites from several plant sources.[6] *Morinda tinctoria* is an Indian medicinal plant with several medicinal properties. Earlier studies in our laboratory revealed that the plant extract has potent anticancer activity. Phytochemical screening also revealed that the plant contains several phytochemicals including flavonoids, terpenoids, steroids and other aromatic compounds. Hence the present study focused on the identification of phytochemicals responsible for the observed anticancer activity by *in silico* methods against VEGFR2 targets.

The phytochemicals present in the plant *M. tinctoria* have been selected from the literature and the three-dimensional structure, canonical SMILE format of the compounds were downloaded from PubChem database and presented in Table 1 & Figure 1.

Potential drug substance should be readily absorbed ad distributed throughout the biological system for effective action and metabolism. All the selected phytochemical compounds, except kaempferol-3-O-rutinoside, passed the required measures of the Lipinski rule of five showing drug-likeness. Lipophilicity as indicated by ClogP was found to be <5 for all compounds, except β-sitosterol showing good penetrability and absorption across cell membranes.[30,31] The molecular weight of all the selected phytochemical compounds was<500 except kaempferol-3-O-rutinoside, indicating that these

Table 1: **List of phytochemicals from *Morinda tinctoria***

Name of the compound with PubChem CID	Molecular formula	Canonical SMILES
Acacetin-7-O-glucopyranoside (5321954)	$C_{22}H_{22}O_{10}$	$COC_1=CC=C(C=C_1)C_2=CC(=O)C_3=C(C=C(C=C_3O_2)OC_4C(C(C(C(O_4)CO)O)O)O)O)O$
Damnacanthal (2948)	$C_{16}H_{10}O_5$	$COC_1=C_2C(=CC(=C_1C=O)O)C(=O)C_3=CC=CC=C_3C_2=O$
Ibericin (28578)	$C_{17}H_{14}O_5$	$CCOCC_1=C(C=C_2C(=C_1O)C(=O)C_3=CC=CC=C_3C_2=O)O$
Kaempferol-3-O-rutinoside (5318767)	$C_{27}H_{30}O_{15}$	$CC_1C(C(C(C(O_1)OCC_2C(C(C(C(O_2)OC_3=C(OC_4=CC(=CC(=C_4C_3=O)O)O)C_5=CC=C(C=C_5)O)O)O)O)O)O)O)O$
Morindone (442756)	$C_{15}H_{10}O_5$	$CC_1=C(C_2=C(C=C_1)C(=O)C_3=C(C_2=O)C=CC(=C_3O)O)O$
Nordamnacanthal (160712)	$C_{15}H_8O_5$	$C_1=CC=C_2C(=C_1)C(=O)C_3=CC(=C(C(=C_3C_2=O)O)C=O)O$
Rubiadin (124062)	$C_{15}H_{10}O_4$	$CC_1=C(C=C_2C(=C_1O)C(=O)C_3=CC=CC=C_3C_2=O)O$
Soranjidiol (124063)	$C_{15}H_{10}O_4$	$CC_1=C(C_2=C(C=C_1)C(=O)C_3=C(C_2=O)C=CC(=C_3)O)O$
Ursolic acid (64945)	$C_{30}H_{48}O_3$	$CC_1CCC_2(CCC_3(C(=CCC_4C_3(CCC_5C_4(CCC(C_5(C)C)O)C)C)C_2C_1C)C)C(=O)O$
β-sitosterol (222284)	$C_{29}H_{50}O$	$CCC(CCC(C)C_1CCC_2C_1(CCC_3C_2CC=C_4C_3(CCC(C_4)O)C)C)C(C)C$
Gefitinib (123631)	$C_{22}H_{24}ClFN_4O_3$	$COC_1=C(C=C_2C(=C_1)N=CN=C_2NC_3=CC(=C(C=C_3)F)Cl)OCCCN_4CCOCC_4$
Pazopanib (10113978)	$C_{21}H_{23}N_7O_2S$	$CC_1=C(C=C(C=C_1)NC_2=NC=CC(=N_2)N(C)C_3=CC_4=NN(C(=C_4C=C_3)C)C)S(=O)(=O)N$

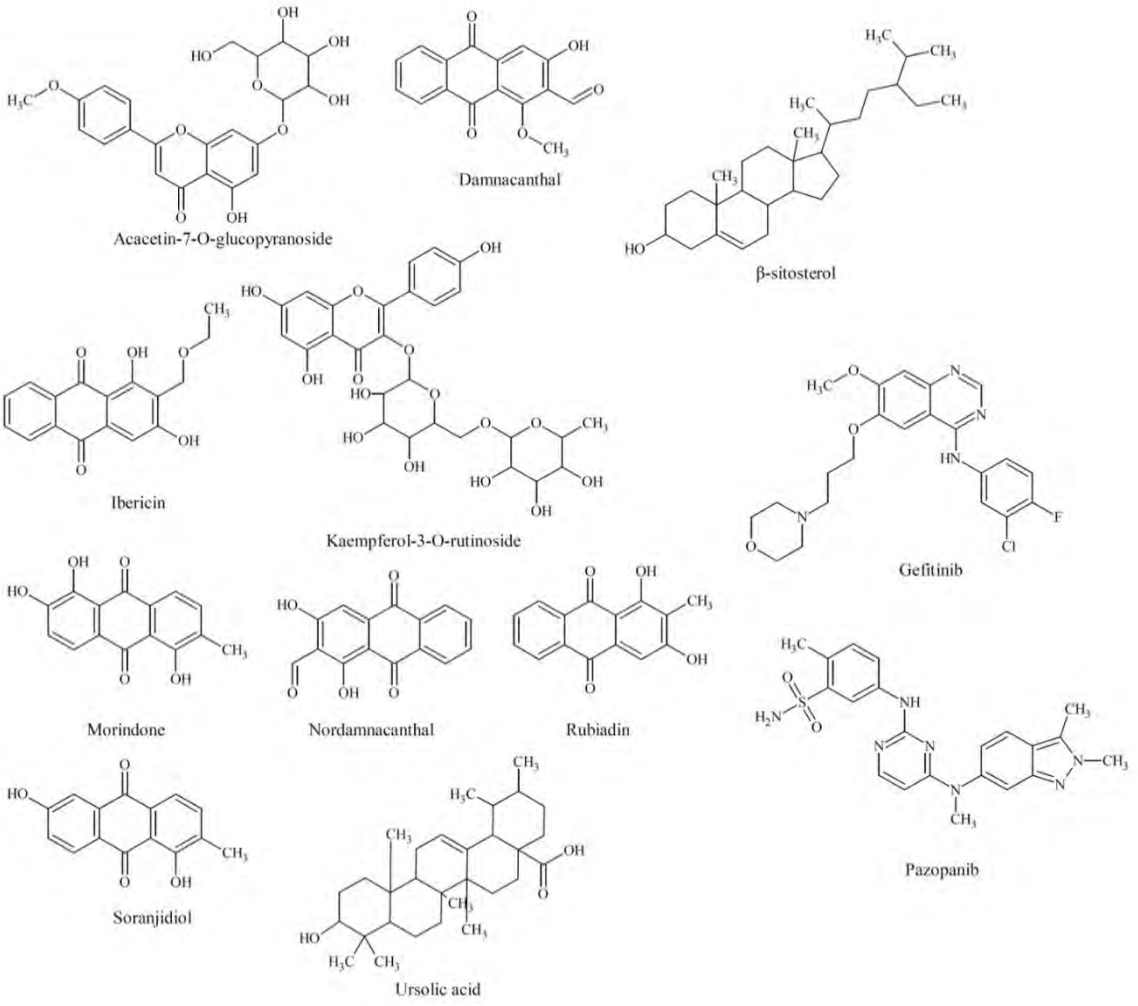

Figure 1: *Phytochemical compounds identified from M. tinctoria and Standard drugs used for the present study*

phytochemicals can be easily absorbed, diffused and transported. All the compounds showed less than 10 rotatable bonds representing less molecular flexibility. Total Polar Surface Area (TPSA) is correlated with the surface belonging to polar atoms in the molecule. It is an important property related to membrane permeability, hence lower TPSA is an important parameter for drug likeness.[32] Drug molecules exhibit poor absorption when their TPSA exceeds 140 $Å^2$, which is standard for marketed drugs. TPSA has a positive correlation with mass and the molecules with a mass greater than 500 are found to have TPSA beyond the range of 0–140.[33] In the present study, the TPSA of flavonoid compounds acacetin-7-O-glucopyranoside and kaempferol-3-O-rutinoside was found to be above 140 indicating poor absorption and for the remaining compounds it was in the range indicates good oral bioavailability. The results were compared with the standard antiangiogenic drugs Gefitinib and Pazopanib.

The Caco-2 cell line is made up of human epithelial colorectal adenocarcinoma cells. The Caco-2 monolayer of cells is widely used as an in vitro tool for the human intestinal mucosa to evaluate the absorption of orally administered medications.[34] If a compound is considered to have a high Caco-2 permeability if it has predictive values greater than 0.9. In the present study, all the compounds showed high Caco-2 permeability except acacetin-7-O-glucopyranoside, kaempferol-3-O-rutinoside, morindone, nordamnacanthal and the standard drug Pazopanib.

The intestine is normally the primary site for the absorption of a drug from an orally administered solution. A molecule with absorption is less than 30% is

considered to be poorly absorbed. Our results revealed that the majority of the compounds are highly absorbed (>90%). Acacetin-7-O-glucopyranoside and kaempferol-3-O-rutinoside showed poor intestinal absorption, whereas, morindone showed moderate absorption.

The volume of distribution (VDss) is the theoretical volume that the total dose of a drug must be distributed uniformly to achieve the same concentration as in blood plasma. If VD is higher, then the drug distributed to tissue is higher than plasma.[22] It can be affected by renal failure and dehydration. VDss is considered low if the value is below 0.71 L/kg (log VDss < -0.15) and high if above 2.81 L/kg (log VDss > 0.45). Our results revealed that most of the compounds have a moderate volume of distribution. Among the screened compounds, acacetin-7-O-glucopyranoside and damancanthal showed the low volume of distribution, whereas, kaempferol-3-O-rutinoside and standard drug gefitinib showed high value.

The brain is protected from exogenous compounds by the blood-brain barrier (BBB). The ability of a drug to cross into the brain is an important parameter to consider in helping reduce side effects and toxicities or improve the efficacy of drugs whose pharmacological activity is within the brain. Blood-brain barrier permeability is measured *in vivo* in animal models as log BB, the logarithmic ratio of brain to plasma drug concentrations. For a given compound, a logBB value greater than 0.3 is considered to readily cross the blood-brain barrier, while molecules with logBB value is less than -1 are poorly distributed to the brain. Our results revealed that all the compounds showed moderate permeability to cross the blood-brain barrier except, acacetin-7-O-glucopyranoside, kaempferol-3-O-rutinoside and standard drug pazopanib. Among the compounds tested, β-sitosterol showed the highest value which indicates that the compound can readily penetrate the blood-brain barrier and this property may be due to its high ClogP value.

Measuring blood-brain permeability can be difficult with confounding factors. The blood-brain permeability-surface area product (logPS) is a more direct measurement and it is obtained from in situ brain perfusions with the compound directly injected into the carotid artery. There are no systemic distribution effects, which could cause brain penetration to be distorted. Compounds with a logPS greater than -2 are believed to penetrate the central nervous system (CNS), while those with a logPS less than -3 are believed to be unable to penetrate the CNS. Among the screening compounds, ursolic acid and β-sitosterol showed good CNS penetration, whereas, acacetin-7-O-glucopyranoside, kaempferol-3-O-rutinoside and standard drug gefitinib were unable to penetrate. The remaining compounds showed moderate penetrability.

Cytochrome P450 is an important detoxification enzyme in the body, mainly found in the liver. It metabolizes the xenobiotics and facilitates their excretion. Many drugs are deactivated by the cytochrome P450s and some can be activated by it. Inhibitors or substrate of this enzyme can affect drug metabolism and are contraindicated. It is therefore important to assess a compound's ability to inhibit the various isoforms of this enzyme and also to assess whether the compound is likely to be a substrate. Our investigation revealed that the compounds such as ibericin, rubiadin and standards gefitinib and pazopanib acts as both inhibitor and substrate for the cytochrome P450 group of enzymes. Morindone, soranjidiol act as inhibitors whereas damnacanthal, ursolic acid and β-sitosterol act as substrates. Flavonoids like acacetin-7-O-glucopyranoside and kaempferol-3-O-rutinoside act as neither inhibitor nor substrate.

The proportionality constant CLtot is used to calculate drug clearance, which is essentially a combination of hepatic (liver metabolism and biliary clearance) and renal clearance. It is highly related to bioavailability and is essential to determine the dosing rates in achieving steady state concentrations.[22] The predicted total clearance of the phytochemicals is given in log (ml/min/kg). Renal Organic Cation Transporter 2 (OCT2) is a renal uptake transporter that plays an important role in the disposition and renal clearance of drugs and endogenous substances. OCT2 substrates have the potential for adverse interactions with co-administered OCT2 inhibitors. Assessing the potential of a molecule to be transported by OCT2 provides useful information regarding not only its clearance but potential contraindications. Our study reveals that none of the compounds except standard gefitinib acts as renal OCT2 substrate.

The prediction of chemical toxicity is a crucial step in the drug development process. Not only the computational toxicity estimates faster than determining harmful levels in animals, but they can also help to reduce the number of animal experiments. Drug-induced liver injury is a major safety concern for drug development and a significant cause of drug attrition. A compound was classed as hepatotoxic if it had at least one pathological or physiological liver event which is strongly associated with the disrupted normal function of the liver. Carcinogenicity is the tendency of a molecule to induce cancer, increase the

incidence of cancer or shorten the time of cancer onset. Immunotoxicity is an adverse event on the functioning of both local and systemic immune systems that result from exposure to chemical compounds. Mutagenicity is the ability of a compound to cause genetic changes. On continuous exposure, many chemical substances damage DNA causing cell death or mutation. Cytotoxicity is the property of being toxic to living cells. Hence it is essential to predict these toxic potentials of the selected phytochemicals. The results obtained revealed that all the compounds are found to be non-hepatotoxic except ursolic acid and standard drug gefitinib. Ursolic acid is carcinogenic and all other compounds are found to be non-carcinogenic. Interestingly all of the compounds showed immunotoxicity except acacetin-7-O-glucopyranoside and nordamnacanthal. Ibericin, morindone, rubiadin and sorajidiol were found to be mutagenic. All the compounds were found to be safe on living cells whereas the standard drug pazopanib was found to be cytotoxic.

It is important to predict the lethal dose of a potential compound. The lethal dosage values are a standard measurement of acute toxicity used to assess the relative toxicity of different molecules. The LD50 is the amount of a drug that, when administered all at once, causes 50% death in a group of test animals. Ursolic acid, β-sitosterol and the standard drug pazopanib fall on toxicity class 4, in which the predicted LD50 was between 300 to 2000 mg/kg. The remaining compounds belong to class 5 where the predicted lethal dose is between 2000 to 5000 mg/kg.

Inhibition of the potassium channels encoded by hERG (human ether-go-go gene) is the principal cause for the development of acquired long QT syndrome and leads to fatal ventricular arrhythmia.[35] Inhibition of hERG channels has resulted in the withdrawal of many drug substances from the pharmaceutical marker. In the present study, the result revealed that none of the compounds are found to be hERG inhibitors and hence they were found to be safe. The molecular properties, drug-likeness, pharmacokinetic properties and toxicity profile of the selected phytochemicals and standards are presented in Table 2-4.

In the present study, three different VEGFR2 targets (PDB ID: 3VHE, 4GA8, 6GQQ) were identified from the literature. The resolved crystal structures were downloaded from the Protein data bank. Further, the proteins were validated by the Ramachandran plot and the percentage residues present in the favorable region was calculated. The entire target proteins showed more than 98 % of favorable region, indicates all the target proteins are valid targets for drug screening. The experimental data for the selected targets is given on table 5 and the protein structures and their Ramachandran plot is given in figure 2.

Virtual screening of selected phytochemicals from *M. tinctoria* revealed that Acacetin-7-O-glucopyranoside and Kaempferol-3-O-rutinoside have good binding affinity against all three targets. The binding energy of

Table 2: **Molecular properties of selected phytochemicals and standards**

Phytochemicals	MW	HBD	HBA	NrotB	Clog P	TPSA	No. of violations	Drug likeness
Acacetin-7-O-glucopyranoside	446.4	5	10	5	0.81	159.05	0	Yes
Damnacanthal	282.25	1	5	2	1.84	80.67	0	Yes
Ibericin	298.29	2	5	3	2.23	83.83	0	Yes
Kaempferol-3-O-rutinoside	594.52	9	15	6	-0.73	249.2	3	No
Morindone	270.24	3	5	0	2.03	94.83	0	Yes
Nordamnacanthal	268.22	2	5	1	1.65	91.67	0	Yes
Rubiadin	254.24	2	4	0	2.23	74.6	0	Yes
Soranjidiol	254.24	2	4	0	2.22	74.6	0	Yes
Ursolic acid	456.7	2	3	1	5.94	57.53	1	Yes
β-sitosterol	414.71	1	1	6	7.19	20.23	1	Yes
Gefitinib	446.9	1	7	8	3.92	68.74	0	Yes
Pazopanib	437.52	2	6	5	2.56	127.41	0	Yes

MW: Molecular weight; HBD: Hydrogen bond donor; HBA: Hydrogen bond acceptor; NrotB: Number of rotatable bonds; ClogP: Consensus log P; TPSA: Total polar surface area.

Table 3: **In silico pharmacokinetic properties of selected phytochemicals and standards**

Phytochemicals	Absorption		Distribution				Metabolism		Excretion	
	$CaCO_2$ Permeability (log Papp in 10^{-6} cm/s)	HIA (%)	VDss (log L/kg)	Fraction unbound	BBB Permeation (log BB)	CNS Permeation (log PS)	Cyp450 Inhibitor	CYP2D6/ CYP3A4 Substrate	Total Clearance (log ml/ min/kg)	Renal OCT2 substrate
Acacetin-7-O-glucopyranoside	0.407	46.53	-0.312	0.138	-1.52	-3.875	No	No	0.644	No
Damnacanthal	1.183	98.34	-0.303	0.103	-0.297	-2.438	No	Yes	0.071	No
Ibericin	1.048	95.34	0.14	0.054	-0.104	-2.405	Yes	Yes	0.24	No
Kaempferol-3-O-rutinoside	0.189	30.74	1.71	0.157	-1.669	-5.015	No	No	-0.16	No
Morindone	-0.011	69.47	0.331	0.184	-0.906	-2.391	Yes	No	-0.073	No
Nordamnacanthal	0.016	92.84	0.014	0.137	-0.157	-2.348	No	No	-0.024	No
Rubiadin	1.159	95.18	0.086	0.085	0.166	-2.067	Yes	Yes	0.151	No
Soranjidiol	1.222	96.74	0.106	0.156	0.197	-2.177	Yes	No	0.066	No
Ursolic acid	1.171	100	-1.088	0	-0.141	-1.187	No	Yes	0.083	No
β-sitosterol	1.201	94.46	0.193	0	0.781	-1.705	No	Yes	0.628	No
Gefitinib	1.246	90.99	1.08	0.111	-0.738	-3.198	Yes	Yes	0.937	Yes
Pazopanib	0.383	92.48	0.268	0.043	-1.161	-2.541	Yes	Yes	0.322	No

Table 4: ***In silico*** **toxicity studies of selected phytochemicals and standards**

Phytochemicals	Hepato-toxicity	Carcino-genicity	Immuno-toxicity	Muta-genicity	Cyto-toxicity	hERG Inhibitor	Predicted LD_{50} (mg/kg)	Toxicity Class
Acacetin-7-O-glucopyranoside	No	No	No	No	No	No	5000	5
Damnacanthal	No	No	Yes	No	No	No	5000	5
Ibericin	No	No	Yes	Active	No	No	5000	5
Kaempferol-3-O-rutinoside	No	No	Yes	No	No	No	5000	5
Morindone	No	No	Yes	Yes	No	No	7000	5
Nordamnacanthal	No	No	No	No	No	No	5000	5
Rubiadin	No	No	Yes	Yes	No	No	7000	5
Soranjidiol	No	No	Yes	Yes	No	No	7000	5
Ursolic acid	Yes	Yes	Yes	No	No	No	2000	4
β-sitosterol	No	No	Yes	No	No	No	890	4
Gefitinib	Yes	No	Yes	No	No	No	2935	5
Pazopanib	No	No	Yes	No	Yes	No	2000	4

hERG: Human ether-a-go-go-gene; LD_{50}: Lethal Dose; Toxicity class 4: 300<LD_{50}<2000; Toxicity class 5: 2000<LD_{50}<5000

Table 5: **Selected Protein Targets (VEGFR-2) and their Experimental Data**

PDB ID	Resolution (Å)	Wilson plot B factor ($Å^2$)	R-value	Favoured Regions (%)	Ramachandran outliers (%)
3VHE	1.55	16.1	0.209	98.0	0
4AG8	1.95	20.8	0.241	98.6	0
6GQQ	1.52	21.3	0.204	98.1	0

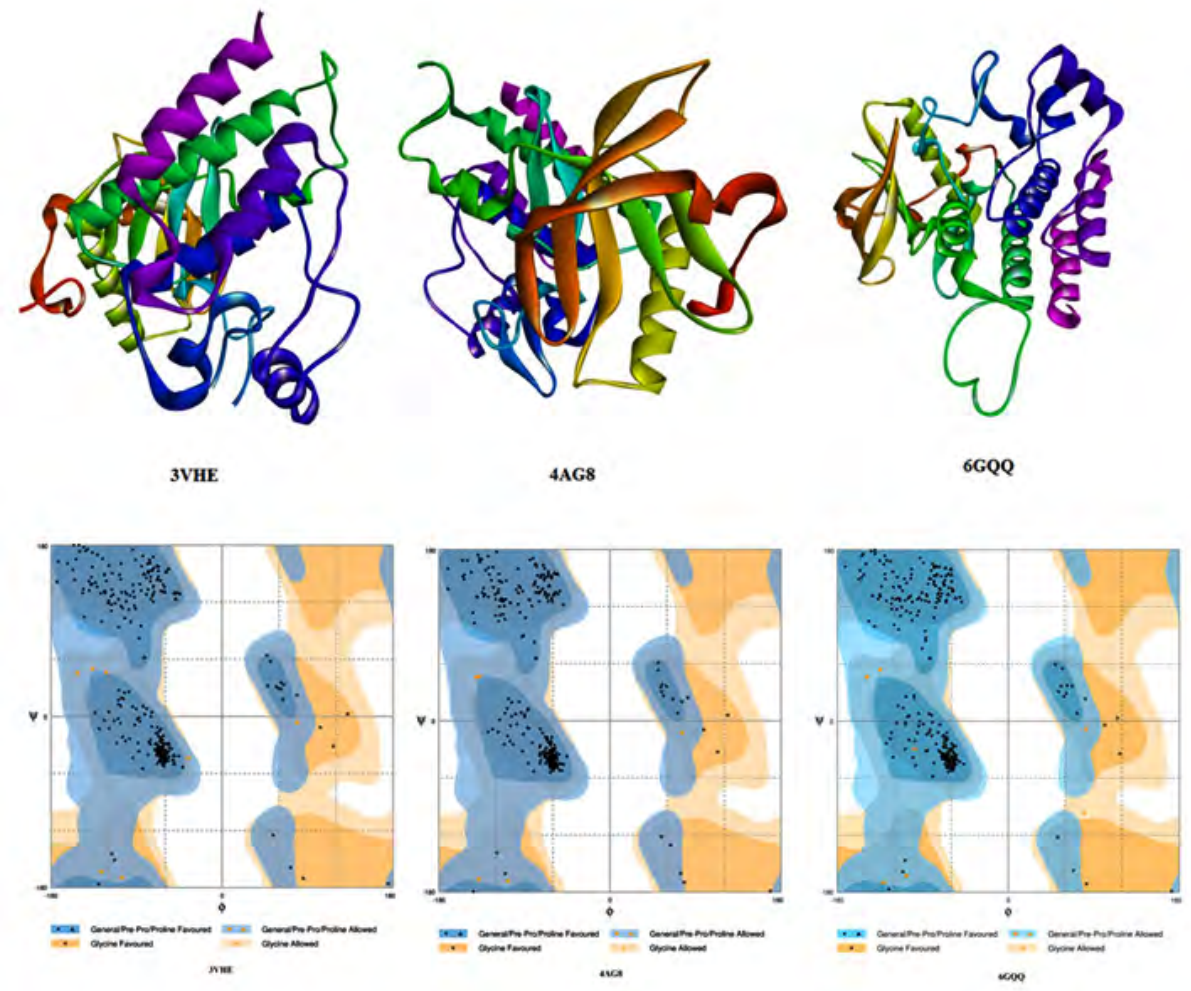

Figure 2: *Protein structures and Ramachandran plot of selected VEGFR-2 targets used for the present study*

these compounds is equal to the standard Pazopanib. The total binding energy, hydrogen bond interactions and interacting residues are presented in Table 6-8.

Further to confirm the results obtained by virtual screening, all the phytochemicals and standards were docked using AutoDock. The results of the final molecular docking study are presented in table 9. The docking pose and 2D interactions are shown in Fig.3 to Fig.5.

Kaempferol-3-O-rutinoside shows conventional hydrogen bond interactions with PHE921 and CYS919 amino acid residues of VEGR2 (3VHE). Apart from the conventional hydrogen bond interactions, the compound shows van der Waals interaction, π-σ interactions, π-loan pair and π-alkyl interactions with the aminoacid residues present in the target. Ursolic acid showed conventional hydrogen bond interaction between ASP814, VAL899, ILE1044 and ARG1027. It also shows van der Waals interaction alkyl and π-alkyl interactions with the amino acid residues present. β-sitosterol did not show any hydrogen bond interaction but it interacted to the target through van der Waals interaction, alkyl and π-alkyl interactions. Acacetin-7-O-glucopyranoside shows conventional hydrogen bond interaction between the benzopyran ring and amino acid residues present in 3VHE target protein.

Kaempferol-3-O-rutinoside and 4AG8 complex was stabilized with five hydrogen bond interactions with GLU885, ALA881, CYS817, ILE1025 and LYS1023. Van der Wall's interactions were observed with LYS868, SER884, HIS816, CYS1024, LEU889, VAL898, VAL899, ILE1019 and ILE1044. A C-H bond between the phenyl ring and ARG1027 was also observed. Apart from these interactions, a π-alkyl interaction with ILE888 and CYS817 residues of VEGFR2 receptor kinase (4AG8) was also observed. In addition, an amide-π stacked interaction between the benzopyran moiety

Table 6: **Binding energy scores and molecular interactions of phytochemicals against 3VHE using iGEMDOCK**

Phytochemicals	Total Binding Energy (kcal/mol)	Interacting residues
Acacetin-7-O-glucopyranoside	-119.042	LYS 868, CYS 919, GLY 922, CYS 1045, ASP 1046, PHE 1047
Damnacanthal	-94.1905	LEU 840, VAL 848, CYS 919, LYS 920, GLY 922
Ibericin	-95.7088	GLU 885, CYS 1045, ASP 1046, PHE 1047
Kaempferol-3-O-rutinoside	-105.713	ALA 881, SER 884, GLU 885, ASP 1046
Morindone	-93.3851	LYS 868, GLU 885, GLU 917, CYS 919, ASP 1046, PHE 1047
Nordamnacanthal	-96.7028	LEU 840, CYS 919, LYS 920, GLY 922
Rubiadin	-93.9608	LEU 840, CYS 919, LYS 920, GLY 922
Soranjidiol	-89.961	CYS 191, LYS 920, GLY 922
Ursolic acid	-90.1	GLU 885, ASP 814, LYS 868, ILE 888, LEU 889, ILE 892
β-sitosterol	-82.9473	ASP 814, LEU 840, VAL 848, LYS 868, VAL 899, ILE 1044
Gefitinib	-98.11	LEU 840, VAL 848, PHE 918, CYS 919, LYS 920, ARG 1051
Pazopanib	-117.429	LEU 840, VAL 848, LYS 868, GLU 885, GLU 917, LYS 920

Table 7: **Binding energy scores and molecular interactions of Phytochemicals against 4AG8 using iGEMDOCK**

Phytochemicals	Total Binding Energy (kcal/mol)	Interacting residues
Acacetin-7-O-glucopyranoside	-109.307	CYS 919, THR 926, ASP 1046, ARG 1051
Damnacanthal	-88.6119	CYS 919, LYS 920, GLY 922
Ibericin	-93.4038	LEU 840, CYS 919, LYS 920, GLY 922
Kaempferol-3-O-rutinoside	-108.487	PRO 839, LEU 840, CYS 919, ARG 1051
Morindone	-92.2047	LYS 868, GLU 885, GLU 917, CYS 919, ASP 1046
Nordamnacanthal	-90.6618	LEU 840, CYS 919, LYS 920, GLY 922
Rubiadin	-88.2599	LEU 840, CYS 919, LYS 920, GLY 922
Soranjidiol	-85.8711	CYS 919, LYS 920, GLY 922
Ursolic acid	-74.2663	GLU 888, LEU 889, ILE 892, VAL 899, VAL 914, PHE 918
β-sitosterol	-87.2967	GLU 885, LEU 889, ILE 892, VAL 899, PHE 918
Gefitinib	-103.563	GLU 885, LEU 889, ILE 892, PHE 918, HIS 1026, ASP 1046
Pazopanib	-102.928	ALA 866, LYS 868, GLY 885, VAL 914, ASP 1046

of the ligand and the CYS1045 amino acid residue was present. Ursolic acid showed three conventional hydrogen bond interactions with VAL899, ILE1025 and ILE1044 residues. Apart from this, van der Waals interaction, carbon-hydrogen bond interaction and alkyl interactions with the target were also observed. Acacetin-7-O-glucopyranoside and VEGR2 receptor kinase (4AG8) complex were stabilized by four hydrogen bond interactions. The interactions were observed between ARG1027, VAL899 and GLU885 amino acid residues. A number of Van der Walls interactions were observed viz. VAL898, HIS1026, LEU1019, ILE1025, CYS1045, ASP1046, LEU1035, PHE1047, VAL848, ALA866, VAL867, VAL914 and ILE1044. In addition, a π-σ interaction with LEU889 and π-alkyl interaction with ILE892, CYS1024 and ILE888 was observed. In the case of β-sitosterol, one hydrogen bond interaction with VAL899 residue was observed. It also binds with the target protein through van der Waals interactions, alkyl and π-alkyl interactions with several amino acid residues.

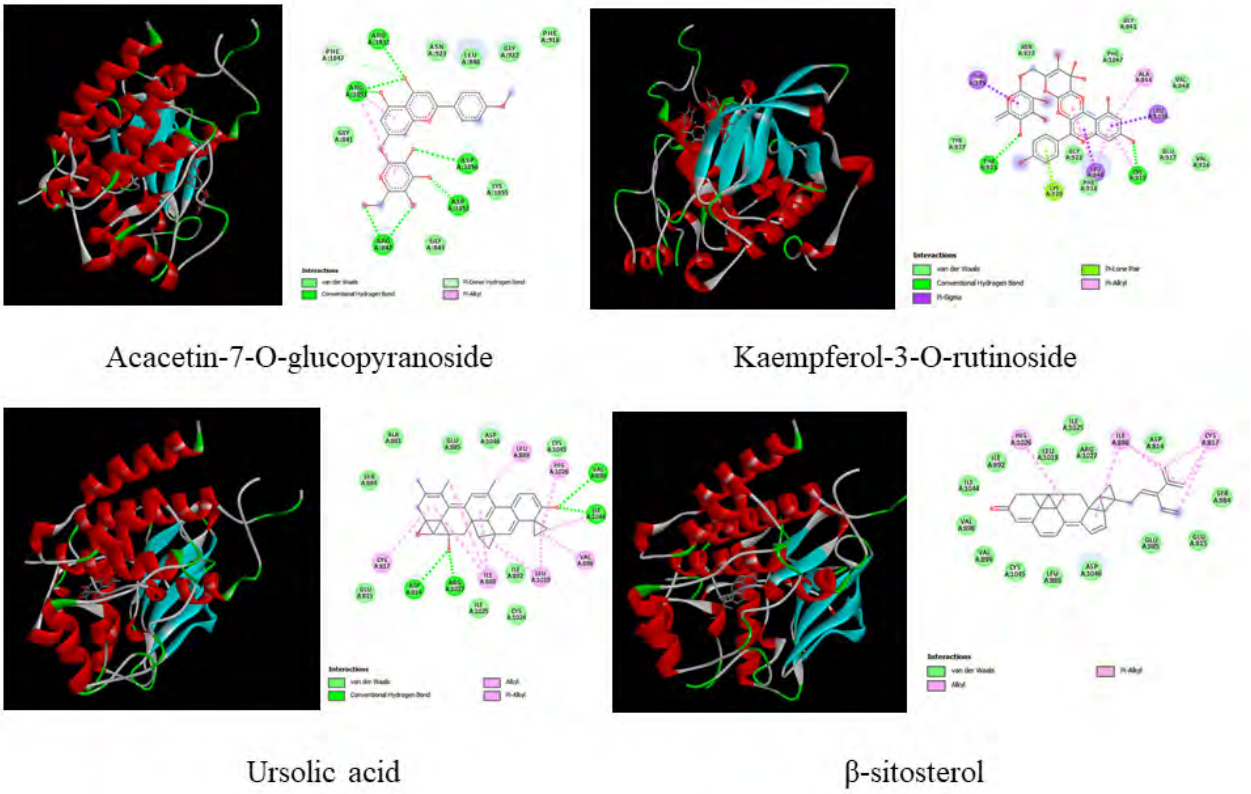

Figure 3: *Docking pose and 2D interaction of top four phytochemicals from M. tinctoria with VEGFR-2 (3VHE)*

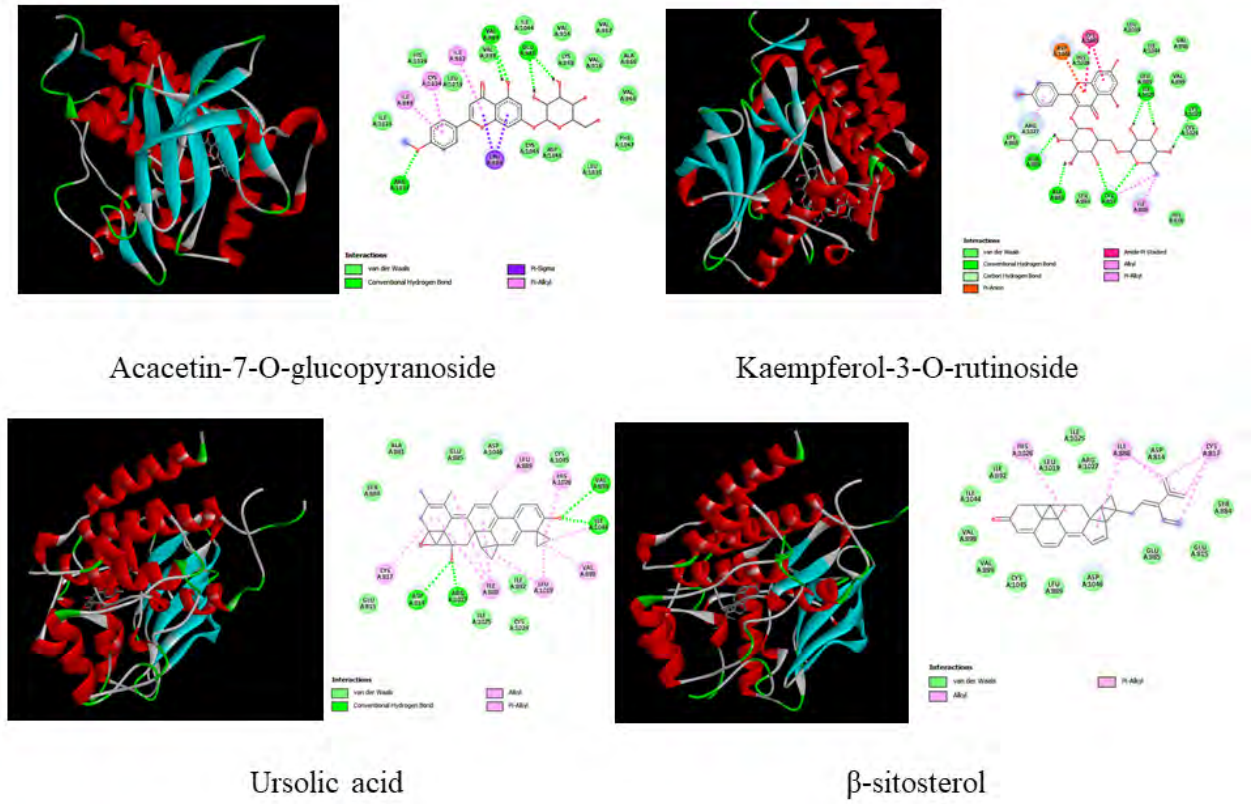

Figure 4: *Docking pose and 2D interaction of top four phytochemicals from M. tinctoria with VEGFR-2 (4AG8)*

Table 8: **Binding energy scores and molecular interactions of Phytochemicals against 6GQQ using iGEMDOCK**

Phytochemicals	Total Binding Energy (kcal/mol)	Interacting residues
Acacetin-7-O-glucopyranoside	-113.992	LYS 868, CYS 919, CYS 1045, ASP 1046, PHE 1047
Damnacanthal	-85.7383	VAL 899, ASP 1046, LYS 868, GLU 885, ILE 888, LEU 889
Ibericin	-98.7214	LYS 868, GLU 885, VAL 899, ASP 1046
Kaempferol-3-O-rutinoside	-109.948	GLU 885, VAL 899, CYS 1024, HIS 1026, ASP 1046
Morindone	-89.9707	VAL 899, ILE 1025, HIS 1026, ILE 1044, ASP 1046
Nordamnacanthal	-92.0818	LYS 868, GLU 885, VAL 899, ILE 1044, ASP 1046
Rubiadin	-88.2219	LYS 868, GLU 885, VAL 899, ILE 1044, ASP 1046
Soranjidiol	-86.8147	VAL 899, ILE 1025, HIS 1026, ILE 1044, ASP 1046
Ursolic acid	-80.1047	LYS 868, GLU 885, ILE 888, HIS 1026
β-sitosterol	-79.3071	VAL 848, LYS 868, ILE 1025, HIS 1026
Gefitinib	-99.3773	VAL 848, LYS 868, LEU 889, ILE 892, ASP 1046
Pazopanib	-109.785	LEU 840, VAL 848, LYS 868, GLU 885, ASP 1046

Table 9: **Binding energy of selected phytochemicals using AutoDock**

Phytochemicals	Binding Energy (kcal/mol)		
	3VH3	4AG8	6GQQ
Acacetin-7-O-glucopyranoside	-10.54	-10.29	-10.03
Damnacanthal	-9.09	-8.15	-7.15
Ibericin	-9.79	-8.45	-7.81
Kaempferol-3-O-rutinoside	-12.67	-12.34	-11.06
Morindone	-9.96	-8.72	-7.87
Nordamnacanthal	-9.39	-8.78	-7.95
Rubiadin	-9.2	-8.49	-7.71
Soranjidiol	-9.54	-8.45	-7.86
Ursolic acid	-12.29	-11.5	-10.76
β-sitosterol	-12.38	-9.33	-8.93
Gefitinib	-10.13	-8.56	-8.33
Pazopanib	-11.08	-11.13	-10.81

Kaempferol-3-O-rutinoside and VEGFR-2 (6GQQ) complex were stabilized by six conventional hydrogen bond interactions with LEU840, ARG842, ASN923, ARG1032, ARG1051 and ASP1052. In addition to the hydrogen bond interactions, the compound showed van der Waals interactions, carbon-hydrogen bond interaction and π-alkyl interactions. Ursolic acid showed conventional hydrogen bond interactions with VAL899 and ILE1044. Additionally van der Waals interactions and alkyl interactions were also observed. Acacetin-7-O-glucopyranoside and VEGR2 receptor kinase complex showed one hydrogen bond interaction with CYS919 residue. Apart from this, van der Waals interactions, π-cation, π-sulphur and π-alkyl interactions were also observed. β-sitosterol also showed similar kinds of interactions.

Among the ten identified phytochemicals from *M. tinctoria*, four compounds show good binding energy with all three tested targets. The binding energy of these four compounds is more or equal to the binding energy of the standards used. Since angiogenesis plays a vital role in tumor growth and metastasis, several studies have focused on the antiangiogenic evaluation of promising cytotoxic agents. Many flavonoids such as apigenin, quercetin, kaempferol and acacetin significantly down-regulate the VEGF levels. The antiangiogenic activity of the flavone wogonoside was evaluated on the estrogen receptor-positive breast cancer cells which show excellent effect.[36] Similarly Ursolic acid in nontoxic doses reduced vessel development in the rat aortic ring. Several studies clearly showed the inhibitory effect of ursolic acid on the proliferation of HUVECs, endothelial cell migration and invasion. The role of metalloproteinases has been shown to be important in angiogenesis. Ursolic acid was found to have an inhibiting effect on matrix metalloproteinase protein expression. All these findings clearly state that ursolic acid has antiangiogenic activity.[37] Because

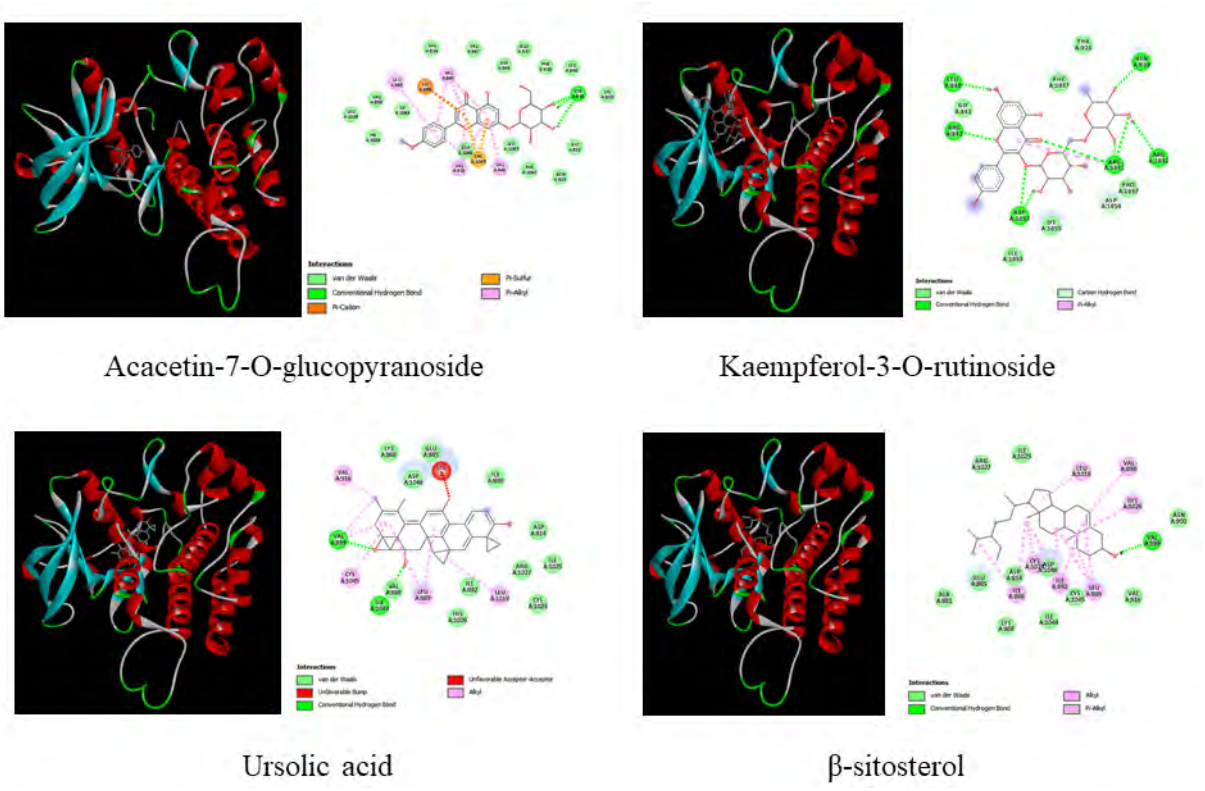

Figure 5: *Docking pose and 2D interaction of top four phytochemicals from M. tinctoria with VEGFR-2 (6GQQ)*

of the strongest affinity and good interactions with vascular endothelial growth factor receptors, as well as appropriate physicochemical, pharmacokinetic and toxicity properties of the identified phytochemicals from *M. tinctoria*, it can be concluded that these compounds are good candidates for cancer chemotherapy.

CONCLUSION

Angiogenesis is required for the formation of new blood vessels and hence nourishment of tumor cells is one of the many processes in the growth of cancer. Various signaling pathways play a vital role in angiogenesis. Targeting the VEFG (vascular endothelial growth factor) signaling pathway with phytochemicals might be a promising option. Four phytochemicals namely, Kaempferol-3-O-rutinoside, Ursolic acid, Acacetin-7-O-glucopyranoside and β-sitosterol have been identified to be antiangiogenic and observed to bind with VEGFR-Tyrosine Kinase domain at high affinity as validated by molecular docking. Furthermore, research is needed studies to understand the angiogenesis mechanism and synergism to identify new therapeutic strategies with phytochemicals.

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CONFLICTS OF INTEREST

The authors declared that they have no conflict of interest.

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