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In silico bimolecular characterization of anticancer phytochemicals from *Fagonia indica*

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Abstract: The in *silico* molecular dynamics and structure-based site-specific drug design of indigenous plant biomolecules and selected proteins have remarkable potential for cancer therapy. A set of five proteins included for this research were epidermal growth factor protein (PDB ID; 1M17), crystal structure of mutated EGFR kinase (PDB ID; 2EB3), crystal structure of Bcl-xl (PDB ID; 2YXJ), apoptosis regulator protein MCL-1 BH3 (PDB ID; 3MK8) and apoptosis proteins (PDB ID; 5C3H). The present study on *in silico* investigation of fifteen indigenous medicinal plants were selected there one hundred thirty four ligands available literature were docked against five proteins involved in carcinogenesis. The highest scoring *in silico* plant, *Fagonia indica* was subjected to *in vitro* cytotoxic effects on HCT116, HepG-2 and HeLa human carcinoma cell lines. Molecular dynamics showed best ligand-protein inhibition interaction between Coumarin-2xyj and Kaempferol-2eb3 with promising binding affinities. Whereas, on HeLa human cervical cancer cell line IC_{50} was 28.3±0.102/ml. *Fagonia indica* could be potential source from natural products that have cytotoxic properties against cervical cancer cells by blocking mutant epidermal growth factor tyrosine or peroxisome proliferators activated receptor proteins.

Keywords: *Fagonia indica*, molecular modeling, HeLa, coumarin, anticancer.

INTRODUCTION

Cancer is unrestricted proliferation of abnormal malignant cells regardless of all significant clinical interventions World Health organization has estimated 18.1 million cancer by 2018. Present therapeutic prospects are not sufficient this devastating disease. Most common cancers include; skin, non-melanoma and non-melanoma, lungs, prostate and breast carcinoma. The rate of incidence for cancer is higher in developed countries than developing countries (Segura-Carretero and Curiel, 2018). The human Epidermal Growth Factor Receptor (EGFR) has an indispensable role in signalling pathways which includes cellular proliferation and activation of intracellular tyrosine kinase (Pfeffer and Singh) domain oncogenic mutant leads to uncontrolled oncogenesis 2eb3 over expression in growth factor ErB2 cause aberrant signaling in case of breast cancer 1m17 (Mahdavifar *et al.*, 2016). PPARs gamma (peroxisome proliferators-activated receptor proteins (2xyj) is responsible for the apoptosis defects which leads to uncontrolled proliferation and tumor generation. The defects in apoptosis machinery cause uncontrolled proliferation the BCL-2 and MCL-1 over expression leads to pathogenesis of cancers, including multiple myeloma and breast cancer 3mk8 (David and Manley, 2010). Multifunctional proteins maintain signaling path way, metastasis regulation and caspases their deregulation is associated to tumor formation 5c3h (Bull, 2013). In recent decades, phytochemicals have revolutionize the drug development being target specific, low cost, and less toxic and have a rapid effect. Nevertheless, phytochemicals are remarkable in targeting specifically on tumour cells and remain ineffective to normal cells. The potential of medicinal plants as therapeutic agents depends upon the quality and quantity of active phytochemicals in them, which vary with latitude, longitude, altitude, age, and climate and season from species to species (Zhang *et al.*, 2011). The Pharmacological functions and their level vary with plant parts. Plants contain phytochemicals triterpenoids, flavonoids, alkaloids and glycosides which are reported to effective in inflammation, pain, infection and cancers (Borokini and Omotayo, 2012). These compounds (ligands) are conveniently available for researchers to perform structure-based virtual screening by inhibiting or suppressing onco-proteins available in the literature obtained from different software free scientific databases. The structure-based drug designing was performed on selected proteins having pivotal role cancer development, molecular docking analysis elaborated the virtual

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inhibition of uncontrolled proliferation (Liu *et al.*, 2017). Over the years, molecular docking has developed as a major field in drug designing and development (Mortier *et al.*, 2015) the molecular docking interactions between ligand and active site of protein conducted the minimization of energy on a set of van der waals electrostatic grids and reduced the high energy produced as a result of interatomic interactions (Shiekh *et al.*, 2019). In this study, computer aided molecular simulation studies and *in vitro* cancer cell line experiments are compared for the anti-cancer cellular growth inhibition of *Fagonia indica* extracts. Aim of this research was, identification of bioactive or chemo preventive potential of *Fagonia indica* against human cancer cells.

MATERIALS AND METHODS

In silico experimentation

MacBook with operating system, RAM 8 GB, with software for molecular modeling AutoDock Vina 4.2.6, PYMOL 2.0 and MGL tools

Ligand preparation

After, extensive scientific literature review, ligands and their molecular structures were downloading from various ethnobotanical and 3D chemical compound databases (table 2.2). These databases provide concise, validated information and chemical structure in 2D, 3D and different formats like XML, SDF and MOL etc. All 3D structures were downloaded in the SDF or MOL format and converted to the PDB format by using the online molecular format converter Open babel http://www.webqc.org/moleculerformatsconverter.php.

Protein preparation

The 3D structures of five different target proteins were retrieved from Protein Data Bank (PDB) (http://www.rcsb.org). To visualize protein structure PyMOL a user friendly software allows, graphical representation of protein structure as wire bonds, cylinders, dot surfaces, solid surfaces, ball and stick, wire mesh spheres, backbone ribbons and cartoon ribbons. It provides access to protein sequence, active site and chain, label or select amino acid residues and alteration in protein structures colours. H-atoms were added to the protein residues. The protonated states of residues, hydrogen's on hydroxyls and thiols optimize H-bond network.

Molecular docking analysis

In structure based drug design, binding conformations of ligands with targeted active site of protein can be determined. The Vina, uses the same PDBQT molecular structure file format for input and output files. Therefore, ligand and protein files were generated in PDBQT file format and viewed by MGLTools. The autodock tool 1.5.6. enables removal of water molecules, addition of hydrogen bonds and by torsion tools to make side chain of ligand movable. The structures of the molecules being docked were subjected to AutoGrid parameter files (GPF, DPF) and grid map files. The analyses of docked PDB files were uploaded in the PyMOL the results were analysed by binding affinities and residual interactions. Binding energies were sorted by highest to lowest the plants with most ligands with value greater than -7 kcal/mol were selected for further subjected to *in vitro* assessment.

Plant collection

The *Fagonia indica* (Family; Zygophyllaceae) the whole plant was collected from Fatima Mills, GPS coordinates (30°03'07.6"N 71°08'45.0"E) Muzaffargarh District, Punjab Province Pakistan (August 2018). The plant material was identified and cleaned dried at room temperature and milled with pastel and mortar, powder was further possessed for solvent extraction.

Extraction

Extraction was carried by taking 200 grams of *Fagonia indica* whole (FIW) powdered plant was dissolved in with 1000 ml of methanol sonnicated for 25 minutes and was kept at room temperature for 24 hours. The extracted supernatant was filtered through filter paper (Whatman Qualitative Filter Paper, 18.5cm). Methanol was evaporated under reduced pressure and recovered in round bottom flask by using rotary evaporator Bucchi (R200) extract was taken to dryness at 40˚C and extracts were kept at 4°C. The procedure was repeated thrice using same recovered methanol and weight in grams and % yield was calculated for each plant extract obtained. Further Liquid-Liquid extraction was performed in separating funnel using solvents methanol (M), dichloromethane (D) and hexane (H) and labeled extracts as (FIWM) (FIWD) (FIWH).

In vitro experiment

Human carcinoma cell lines culture

Human cell line culturing was performed on Homo sapiens colon colorectal carcinoma cells (HCT 116), Homo sapiens liver carcinoma cells (Hep G2), Homo sapiens cervix adenocarcinoma (HeLa) at The Department of Microbiology and Molecular Genetics, University of the Punjab Lahore. The cells were maintained in respective culture media HCT-116 in McCoy'S 5A (Gibco), HepG-2 in EMEM (Gibco) and HeLa in DMEM (Gibco) along with 10% Fetal bovine serum (Invitrogen) and 1% Penicillin/Streptomycin in humidified incubator, 5% CO_2 and 37°C.

MTT (3-[4, 5-dimethylthiazol-2-y1]-2, 5-diphenyltetrazolium bromide) Cytotoxicity assay

MTT assay is a homogeneous colorimetric assay used for analysing the cell proliferation and chemosensitivity. This, assay was used for preliminary evaluation of

anticancer potential of selected plant extracts which have been nontoxic on normal fibroblast cell lines. MTT (3-[4, 5-dimethylthiazol-2-y1]-2, 5- diphenyltetrazolium bromide) is a quick, precise and sensitive *in vitro* method to evaluate the toxicity of plant extracts in cell cultures. The MTT is a yellow colour dye which is reduced to succinic dehydrogenases in living cells to purple colour formazan crystals precipitated and become insoluble in aqueous medium. The spectrophotometric absorption wavelength of dissolved purple colour formazan in the visible range shows a direct relationship with the quantity of cells attached to the bottom (viable cells) (Van Meerloo *et al.*, 2011; Dou *et al.*, 2016). This experiment was performed with, 4×10^4 cells/well cultured in 96 well culture plates. The test plant extracts were added in culture plates after 24 hours, and kept in an atmosphere of 5% CO_2 at 37°C for 24 hours. After that the media having test plant extracts was removed and 100 µl fresh media and 30 µl of MTT mixture was dispensed and the plate was wrapped with aluminium foil and placed in 37°C incubator for 4 hours. After that the media was removed and the formazan crystals were dissolved in 200 µl of Dimethyl sulfo-oxide (DMSO) in all wells and spectrophotometric absorbance was taken at 570nm, test wavelength and 620 nm reference wavelength. Each experiment included negative control, untreated cells and one positive control Gossypol. All experiments were performed in triplicate and repeated at least twice. The Cell viability was calculated by equation given:

Cell Viability % = (Test 570 nm-620 nm)/(Control 570 nm-620 nm) × 100

STATISTICAL ANALYSIS

The results were calculated on three (n=3) replicates and expressed in (±) standard mean error. One-way ANOVA analysis of variance and IC_{50} were calculated by recreational curve where, $P<0.05$ was considered as statistically significant. For statistical analysis we used Graphpad-Prism 8.2.1.

RESULTS

In silico docking

On the basis of hierarchical filters, the ligand-protein interaction function was evaluated by the gride program. The necessary and favorable interactions were made in this filter to activate the ligand for protein's spatial fit. The automatization ensured the assessment of locations for the ligand to be positioned in the active binding site of the protein (Paul *et al.*, 2019). A set of five proteins involved in cancer enhancement or cause due to mutation were selected for *in silico* these proteins were epidermal growth factor protein PDB ID; 1M17, crystal structure of mutated EGFR kinase PDB ID; 2EB3, crystal structure of Bcl-xl PDB ID; 2YXJ, MCL-1 BH3 PDB ID; 3MK8 and apoptosis proteins PDB ID; 5C3H fig. 1.

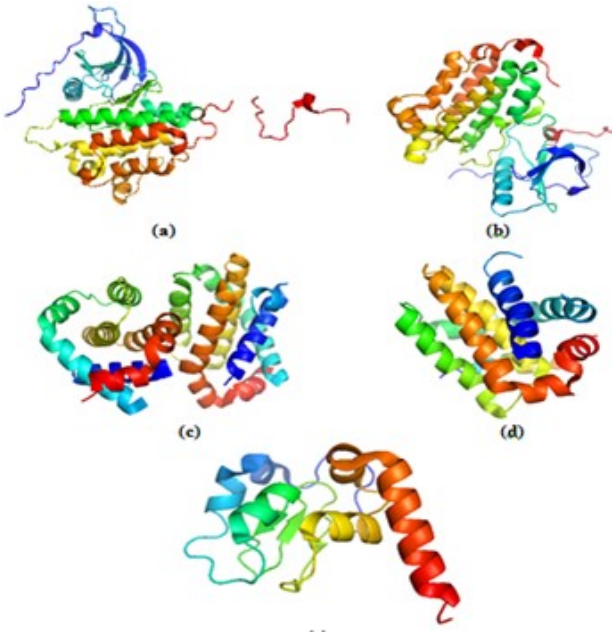

Fig. 1: Crystal structures of Docking Proteins (a) Epidermal growth factor Protein (PDB ID; 1M17) (b) Crystal structure of mutated EGFR kinase (PDB ID; 2EB3) (c) Crystal structure of Bcl-xL (PDB ID; 2YXJ) (d) The MCL-1 BH3 (PDB ID; 3MK8) (e) Apoptosis Proteins (PDB ID; 5C3H)

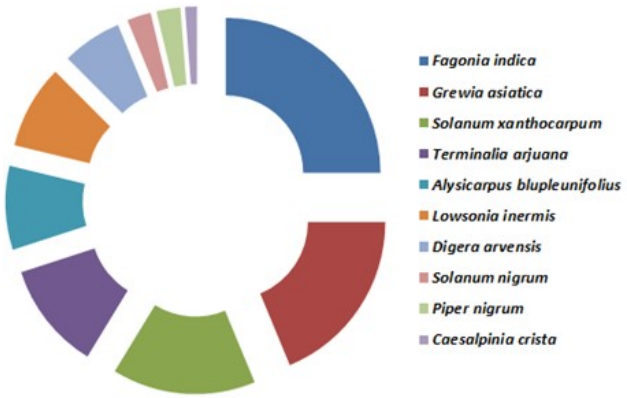

Fig. 2: *In silico* interaction between one hundered thirty three ligands with five set of carcinogensis protein showed medicinal plants with highest inhibitor ligands *Fagonia indica* (20%), *Grewia asiatica* (15%) and *Solanum xanthocarpum* (12%).

The sets of proteins after molecular modeling, each set contained one hundred thirty three ligands 3D structure which resulted in altogether generating 1200 molecular docking results. However, the criteria for further *in vitro* biological activities of medicinal plants were proteins-ligand interactions in all set of proteins with score -7 kcal/mol or above. The high throughput virtual screening describes the order medicinal plants with potential ligands *Fagonia indica, Grewia asiatica* and *Solanum xanthocarpum* respectively remained to be the plants with maximum number of compounds with crystal structure of

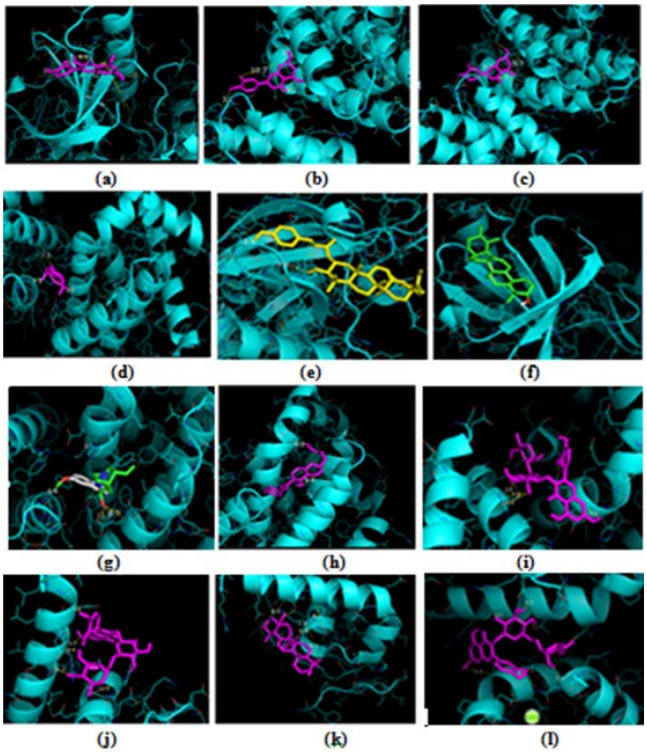

Fig. 3: *Fagonia indica* 3D ligand structures and Protein docking complex (a) Coumarin- 5c3h (b) Chinova acid- 5c3h (c) Benzyl salicylate-5c3h (d) Ursolic acid-5c3h (e) Coumarin-2xyj (f) Benzyl salicylate-3mk8 (g) Kaempferol-2eb3(h) Daidzein- 2eb3 (i) Benzyl salicylate- 2xyj (i) Betulinic acid- 2xyj (k) cis-4-Decenoic acid- 2xyj (l) p-Coumaric acid-3mk8

Table 1: Binding affinities and residual interactions of activated proteins with lead

Pub Chem ID	IUPAC names	Mol. Formula	PDB ID	Binding Affinity Kcal/mol	Residues Interact via H-bonding	Residues in contact To ligand
	Fagonia indica					
323	Coumarin	$C_9H_6O_2$	5c3h	-9.3	Val312, Thr253	Pro251, Leu256, Thr271,Lys 281
107733	Chinova acid	$C_{14}H_{12}N_2O_2$	5c3h	-8.1	Phe291, His413, Ile328	Ser261, Ile276, Asp296, Phe301
8363	Benzyl salicylate	$C_{14}H_{12}O_3$	5c3h	-10.4	Val305, Phe324	Thr271, Phe301,Lys311, pro316
58472	Ursolic acid	$C_{30}H_{48}O_3$	5c3h	-9.2	Ile328, Thr253	Pro251, Ilys281, Lys311
323	Coumarin	$C_9H_6O_2$	2xyj	-14	Phe143, Phe144, Met170	Val86, Lys187, Agr91, Gln89
8363	Benzyl salicylate	$C_{14}H_{12}O_3$	3mk8	-7.3	Tyr247, Leu275	Val,141, Phe146, Cys151, asp156
4444395	Kaempferol	$C_{15}H_{10}O_6$	2eb3	-12.1	Gln182, Ala185	Val161, Ser164, Agr165, Ala171
5281708	Daidzein	$C_{15}H_{24}$	2eb3	-7.0	Pro236, Tyr247	Pro116, Gln121, Val126, Phe131
8363	Benzyl salicylate	$C_{14}H_{12}O_3$	2xyj	-7.4	Leu275, Ser428, Phe 274	Asn175, Trp181, Gly186, Phe191
58496	Betulinic acid	$C_{30}H_{48}O_3$	2xyj	-7.3	Lys745, Asp855, Leu844	Agr836, Ala840, Lys846
637542	Cis-4-Decenoic acid	$C_{10}H_{18}O_2$	2xyj	-8.2	Gln791, Lys745	Val876, Ile878, Met881, Leu883, Gln886
637542	P-Coumaric acid	$C_{14}H_{12}N_2O_2$	3mk8	-9.4	Glu738, Asp831	Asp172, Ile176, Leu186,Thr191

Table 2: MTT Cytotoxic cell proliferation assay

	HCT-116	HepG-2	HeLa
	IC_{50} µg/ml		
FIWM	352±0.110	216±0.130	28.3±0.102
FIWC	256±0.074	371±0.102	97±0.035***
FIWH	581±0.165	611±0.093	728±0.139
Gossypol	34.7±0.031***	12.67±0.051***	18.3±101

*** Statistical significance P<0.05

selected proteins. Out of seventeen plants and there one thirty three non-repeated ligands *Fagonia indica* possess most compounds with *in silico* chemotherapeutic potential belong to these plants fig. 2.

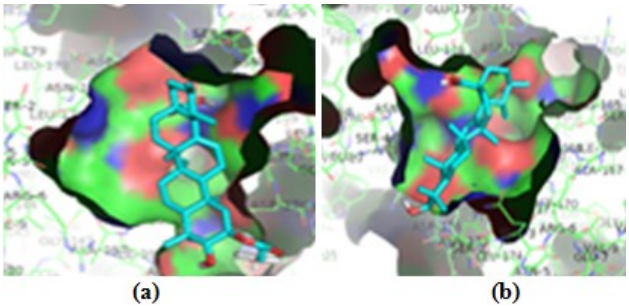

Fig. 4: 3D binding interactions of (a) Coumarin and (b) kaempferol cavity pockets docked with peroxisome proliferators activated receptor proteins (PPARs) and Crystal structure of mutated EGFR kinase respectively, images were generated with PyMOL 1.7.4.

The structure-based drug designing was performed selected proteins having pivotal role cancer development molecular docking analysis elaborated the inhibition of uncontrolled proliferation virtual (Yadav *et al.*, 2018). The molecular docking has developed as a major field in drug designing and development the molecular docking interactions between ligand and active site of protein conducted the minimization of energy on a set of van der waals electrostatic grids and reduced the high energy produced as a result of interatomic interactions (Rout *et al.*, 2019). The binding affinities for most promising ligands of *Figonia indica* along with residual interactions with proteins are given in table 1.

Top hits scoring for *Fagonia indica* phytochemicals were selected and the scoring function provides information about in silico efficiency, highest binding affinities, polar bonds and interactions with active residues. The result showed coumarin had highest binding or inhibiting affinity with 2xyj protein followed by kaempferol 2eb3 fig. 3.

The kaempferol showed second highest scoring function offers evidence about the *in silico* proficiency with 2eb3 residual binding affinities and tendencies, covalent bond and interactions with activated residues fig. 4.

In vitro cytotoxicity via MTT cell proliferation assay

MTT assay was performed on HCT-116, HepG2 and HeLa cell lines, the MTT is reduced in viable cells by succinic dehydrogenases inside cell mitochondria which produces insoluble formazan crystals in purple coloration the absorption of dissolved formazan predicts the number of alive cells. (Rai *et al.*, 2018). *Fagonia indica* extract was investigated on all the three carcinoma cell lines with concentration ranging from 10 µg/ml to 500 µg/ml. IC_{50} was calculated by regression analysis as the lower value shows more cytotoxic effect the *Fagonia indica* extract (FIWM) was found to possess most cytotoxic effect on HeLa cells with IC_{50} value 28.3±0.102µg/ml in comparison to standard drug gossypol 18.3±101µg/ml used as positive control. In case of HCT-116 and HepG-2 less cytotoxicity was observed which showed that carcinoma cell proliferation was unaffected on these cells as in table 2.

DISCUSSION

The cytotoxic activity *Fagonia indica* has been evaluated against some cancer HepG2, HT29, HCT-116, and normal cell lines (Tariq *et al.*, 2017). In another *in silico* study on cyclin-dependent kinase 2 CDK2 receptor the best dock score were observed by plant compounds from *Angola vetiver* essential oil compound, tetrahydroxy flavanone showed anticancer potential on human breast cancer cells (Jaikumar and Jasmine, 2016; Khan *et al.*, 2020). The set of proteins selected proteins epidermal growth factor protein, crystal structure of mutated EGFR kinase, MCL apoptosis regulator protein and apoptosis protein showed moderated residual binding affinity with ligands. Moreover, crystal structure of Bcl-xl (PPARδ) showed most significant binding affinity and covalent interaction with most of the ligands (Xu *et al.*, 2016). In previous study *Fagonia* aqueous extract showed remarkable potential in breast MCF-7 cell line (Kumbi, 2019) and cervical cancer. The coumarin is a prominent natural compounds which has several pharmacological activities which includes chemo preventive effect. A research on panel of human carcinoma cell lines showed effect of coumarin on NIH3T3 cells (Emami and Dadashpour, 2015). A previous study concords with these results on HeLa cells coumarin induced inhibition was studied by cell cycle arrest by mitochondrial and caspase-3 mechanism (Dini *et al.*, 2019). Studies showed, *Syzygium cumini* kaempferol has cytotasic potential and can possess anticancer potential as natural dietary bioactive phytochemical (Smruthi *et al.*, 2016; Bijauliya *et al.*, 2017). As per recommendation of American National Cancer Institute crude phytochemical extract need to be purify when IC_{50} value is less than 30µg/ml (Suffness and Pezzuto, 1990). On this criteria *Fagonia indica* on HeLa has potential for further mechanistic studies for its anticancer potential.

The present study shows that *Fagonia indica* contains phytochemical with antiproliferative potential *in silico* and *in vitro* experiments. Further, high through put screening via bioassay guided procedures is needed for identification of bioactive cytotoxic phytocompounds from *Fagonia indica* on MCF-7 cell line. Additionally, cytotoxic effect monitoring on normal cell lines may also provide basis for the development of natural anti- breast cancer herbal supplement from *Fagonia indica.*

CONCLUSION

The *in silico* molecular docking simulations of coumarin and kaempferol from *Fagonia indica* showed remarkable inhibition potential. The cancer cell growth was inhibited by blockage of protein pathway for EGFR and PPARδ. The promising *in vitro* antiproliferation effect of methanol extract of *Fagonia indica* on HeLa cervical carcinoma cells provides the basis for potential anticancer drug.

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