doi.org/10.36721/PJPS.2021.34.6.SUP.2325-2331.1

In vitro antimutagenic, cytotoxic and anticancer potential of *Fagonia indica* phytochemicals

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Abstract: Cancer is one of the most diagnosed and life threatening disease throughout the world. Nevertheless present day clinical management for cancers are surgery, radiations which are insufficient to contain the disease burden. In the past two decades, more than half of chemotherapeutic drugs developed are either directly or indirectly dependent on medicinal base phytocompounds or their derivative. The present study aims to provide the base for chemotherapeutic phytochemicals. *Fagonia indica* showed significant antimutagenic potential with reference to control IC_{50} values were calculated as 146.33±5.2µg/ml, TA100 (AZS) 105.33±4.0µg/ml, TA98 (2AA) 113.6±5.2µg/ml followed and TA98 (AZS) 112.6±4.4 in Ames test. For this reason, the antiproliferation effect of extracts on cancer cell lines was studied through resazurin fluorescence. On HepG-2 cell lines 50% cytotoxic concentration (CC_{50}) of (FIWM) was recorded as 128.3±,2.43µg/ml. On the homo sapiens epithelial cell of lung tissue (A549), the high throughput instrumental analysis of *Fagonia indica* depicts maximum cytotoxic effect in 30hr. The electrical impedance displays the real-time evidence about qualitative apoptosis expressed. The impedance results were supported as palmitic acid from *Fagonia indica* virtually that inhibits Cyclin Dependent Kinase 2 (CDKs 2) in silico molecular docking studies. *Fagonia indica* extract possesses substantial antimutagenic, cytotoxic and anticancer activity which supports the potential of its phytochemicals for drug development.

Keywords: *Fagonia indica*, antimutagenicity, cytotoxicity, resazurin, Ames assay, *Salmonella typhimurium*, molecular docking.

INTRODUCTION

The abnormal cellular proliferation in any type of body tissue or cells that leads to the formation of anomalous masses causes cancers. Cancers are categorised into three major classes which include leukaemia, carcinoma and sarcoma. Out of these malignancies, carcinoma is most frequently more than 85% which is an abnormality of neoplastic epithelial cells (Hoadley *et al*., 2018). At the onset of cancer different factors leads to the initiation of DNA damage along with irreversible which exponentially transfers to next generation daughter cells during each cell division. The World Health organization estimated various types of cancer cases almost 18.1 million by 2018. At present day therapeutic interventions are insufficient to contain even the most common cancers like breast, melanoma, lungs and prostate (Dimple *et al*., 2018). The incidence rate is enhancing in developed countries as compared to underdeveloped countries. However, the mortality rate is increasing in underdeveloped countries due to lack of health care facilities, underprivileged diagnosis and exorbitant treatment prospects.

Most commonly uncontrolled abnormal tumour cells are lemmatized by chemotherapy (Khatak *et al*., 2018). Existing therapeutic preferences includes surgery, radiotherapy, cytostatic or cytotoxic DNA interactive mediators (Arya *et al*., 1992). Whereas, the cells with swift growth like bone marrow, hair follicle and cells of digestive tract cells are also distressed besides the malignant cells. These side effects are a huge challenge that ultimately causes inflammation, immunosuppression and condenses the production of blood cells. Moreover, more than half of the carcinoma is due to mutation in cell cycle regulator gene p53 (Delgado-Vargas *et al*., 2018).

Medicinal plants constitute precious phytochemicals and their derivatives contribute to the major part of most successful drugs against various diseases. From the traditional medicinal system of all continents, researchers

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have reported the number of potential anticancer phytochemicals after rigorous laboratory procedures on in vitro cell lines assays and animal model *in vivo* experiments. These cytotoxic evaluations comprehend the mechanistic, physiological and pharmacological anticancer potentials phytochemicals. The aqueous, *Fagonia creatica* extract has revealed remarkable p53 gene up-regulation potential on MCF-7 the breast cancer (Javed *et al*., 2016). Extract of *Swertia chirata* has presented promising inhibition against derma tumours during mice model experiments and structural elucidations (Fatima *et al*., 2019). In recent research, myelocytic HL-60 human cells have been contained by Berberis lycium Royle.

The in silico molecular modelling analysis through virtual software is a more convenient method to observe the exact target protein and its behaviour with the ligand of choice. This has made it laborious work wet lab work to perform primarily in dry lab and get the drug ability information for the most important drug candidate to be performed *in vitro* or *in vivo* experiments. The present study was designed to evaluate the cytotoxic, anti-mutagenic and anticancer effect of *Fagonia indica* extract on the panel of human carcinoma cell lines and in silico effect. This research aims to identify the chemotherapeutic potential of bioactive agents from *Fagonia indica*.

MATERIALS AND METHODS

The study was conducted at the Lahore Pharmacy College (LMDC), University of Health Sciences Lahore, Department of Microbiology and Molecular Genetics, University of the Punjab Lahore, Pakistan Pakistan and Section Animal Physiology and Neurobiology, Department of Biology, KU Leuven, Belgium (2018-2020). *Fagonia indica* sample was submitted at herbarium to the University of the Punjab Lahore, voucher specimen deposited as the whole plant (FIW) LAH #28120.

Plant Collection Extraction and Preliminary Phytochemical Analysis

Fresh plants were collected from Fatima Mills, Muzafarghar, South Punjab Pakistan. The plant material was shade dried with active ventilation and powdered before extraction 100grams of plant material was dissolved in 500ml of methanol. After that, this mixture was sonicated for about 20minutes and placed for 24hours at room temperature. Methanol was recovered in a round bottom flask by using a Rotary evaporator Bucchi (R200) was used for the recovery of methanol and extract was dried at 35˚C. The extracts were weighed in grams (g) for % yield calculation from solvent systems. The extracts obtained in solvents hexane, dichloromethane and methanol on polarity order labelled as FIWH, FIWD and FIWM. Primary phytochemical analysis was performed for alkaloids, glycosides, cardiac glycosides, steroids, polysterol (Hussain *et al*., 2018), saponins, anthrhaquinones, phenol, flavonoids, catechins, tannins, triterpenes and diterpenoids (Dilshad *et al*., 2018).

AMES Test

Ames test evaluates the mutagenic proficiency of extract through the induction of mutation in his operon the reverse mutation. In the 100µl of nutrient broth 1×10^9 cell/ml of *Salmonella typhimurium*, TA98 and TA100 strain were cultured besides minimal glucose agar plates. The sodium azide AZS and 2-aminoanthracene 2AA were taken as mutagenic agents. Correspondingly, 100µl of *Salmonella typhimurium* were added to tubes incubated at 37^{o}C for 30 minutes accompanied with small histidine. Subsequently, the material was transferred on a minimal glucose agar plate at 37^{o}C for 48hrs, after this incubation counting of revenant colonies was employed

% Antimutagenic activity =A-B/Ax100

Where A=No. of revertants in control (Media + mutagen): B= No. of revertants sample (Media +mutagen + phytoconstitutes)

Cell culturing on human carcinoma cell lines

Cell culturing on human carcinoma cell lines was perform in resazurin colourimetric assay which provides information for cell proliferation and chemo sensitivity analysis. The purplish blue dye of resazurin has dye that has the ability to reduce it to pink in viable cells and the precipitates which become are insoluble in the aqueous environment (Mosmann, 1983; Van Meerloo *et al*., 2011) Resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide) blue, is a highly fluorescent redox dye it can be reduced by the metabolically active live cell to resorufin (7-hydroxy-3H-phenoxazin-3-one) pink. In 4×10^4 cells were seeded per in 96 well cell culture grade plates. The medicinal plant extracts were added to the plates with a conventional cell culture environment of 5% CO_2 at 37°C for 24 hours. After the incubation period, the fresh 100µl culture media and 20µl of resazurin solution were also dispensed and incubated for 4hours. The fluorescence of the plate was read at the wavelength of excitation 550 nm and emission 590 nm and measured by FLEX station II microplate reader (Molecular Devices).

Electric impedance Spectroscopy

The electric impedance Spectroscopy was developed to measure the real-time effect on cellular viability on human carcinoma cells. This spectroscopic method is based on advanced biosensor and microelectronic based dynamic systems. The cellular analyser reports events in real-time providing information regarding viability, motility or adhesiveness of cells in terms of impedance (Z). The Cell Sine® digital system continuously monitors any change in morphology of cells and the device interpreters it in the form of impedance. In this 48hr

experiment, the effect of plant extract on cells was monitored by linking the Cell Sine device analyser 100Hz to 60Hz for measuring any change in quantitative impendence. Meanwhile, the electrical impedance was recorded by impedance signals the data obtained was intended graphically.

|Z| (normalized)=|Z|timeX/|Z| time

The impedance "Z" is measured by the relation between its two components Z and theta. The statistical analysis was performed using Graph pad prism 8.1.

In silico Ligand-Protein Molecular Docking and simulations

For, in silico studies software for molecular docking analysis were included Auto Dock Vina 4.2.6, Pymol 2.0 and VDM. From literature ligands of *Fagonia indica* were noted and searched precisely from Drug Bank in 3D PDB format. 3D protein structure of Cyclin Dependent Kinase 2CDKs was downloaded from http://www.rcsb.org structural amino acid residues and quality was evaluated through Ramachandran plot https://zlab.umassmed. edu/bu/rama/ salt bridge and protonation of residues was developed in Autodock vina. (PDB ID: 2FVD) Cyclin Dependent Kinase 2 (CDK2) 10.2210/pdb2FVD/PDB X-RAY diffraction method with resolution 1.85 Å, R-value free: 0.235 and R-value work: 0.207. In target ligand-protein drug associations the conformations for the ligand binding on protein active site with binding energies were observed for all conformations. Pymol software was used for accurately analysing and visualizing the overall ligand protein expressions.

Statistical Analysis

The results were calculated thrice as replicates (n=3) and expressed in mean ± SD by using statistical software, Graph pad Prism, version 8.2.1.

RESULTS

The ethnobotanical field surveys were arranged based on literature information after the identification of spots for the collection of medicinal plants. The site was identified in Southern, Punjab Pakistan, by global positioning system GPS, the geographic coordinates were noted regarding latitude as 30°03'07.6"N 71°08'45.0"E. After that solvent systems were selected for the extraction of plant extracts on a small scale and large scale. The small-scale extraction was done in methanol, hexane and dichloromethane whereas, large scale extraction was carried out in methanol to get the maximum possible phytochemical in the extract. After the percolation extraction, solvents were recovered by rotary evaporator and extracts were labelled with initials, part and solvent used as for Fagonia indica whole plant, FIWM; FIWC; FIWH. Phytochemical analysis showed moderate to abundant presence of cardiac glycosides, steroids, saponins, anthrhaquinones and tannins (table 1).

The substantial method to identify mutagenic effect ic was developed by Novick and Szilard in 1952 at first (Khan and Ahmad, 2020). The *Salmonella typhimurium* based AMES assay is widely used for the assessment of mutagenicity and antimutagenicity (Vijay *et al*., 2018). Researchers are provided evidence about the chemopreventive herbs and their ability to protect DNA the genetic material and contains the mutations which lead to carcinoma (Estrela *et al*., 2017).

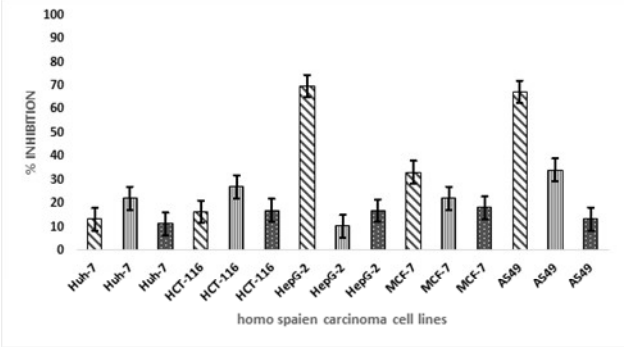

Fig. 1: Resazurin experiment for the analysis of % inhibition of *Fagonia indica* extracts in the order of FIWM, FIWD and FIWH each tested on Huh-7, HCT-116, liver HepG-2, MCF-7 and A549 carcinoma cells, expressed in ±SEM. Whereas the most prominent, 50% cytotoxic concentration CC_{50} was recorded for FIWM on HepG-2 as 128.3±2.43µg/m followed by on 146.1±1.32µg/ml A549µg/m.

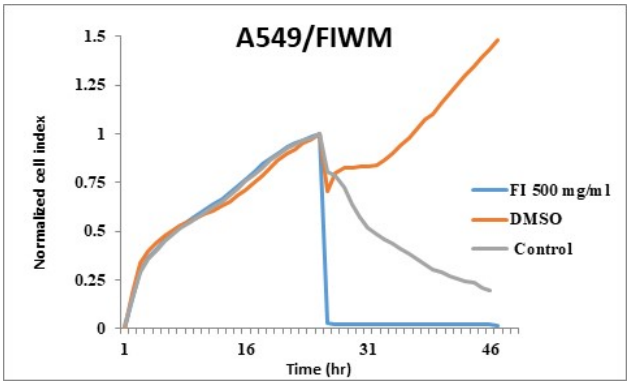

Fig. 2: *Fagonia indica* effect on epithelial lung A549 carcinoma cells monitored by real-time quantitative electrical impedance_analyser

These chemopreventive drug discovery and development efforts can be aided by phytochemical extracts which are responsible for containing the factors present in the environment which helps DNA damage and oxidative stress. (Klaunig and Wang, 2018). In genetically modified strains of *Salmonella typhimurium* strains, TA98; frame-shift mutation and TA100 base-pair substitutions are used as genetically modified DNA strains of bacteria (Janosch

et al., 2019). Here, *Fagonia indica* whole plant methanol (FIWM) were verified for their antimutagenic effect with 0.2% DMSO as negative control and bacterial inoculum tested substance as sterility control. FIWM exhibited a decent activity against AZS mutagen TA98 51.62% and TA100 63.62% inhibition independently at the highest concentration. The antimutagenic potential of *Fagonia indica* with reference to IC_{50} was recorded for control 146.33±5.2µg/ml, TA100; AZS 105.33±4.0µg/ml, TA98; 2AA 113.6±5.2µg/ml and TA98; AZS 112.6±4.4µg/ml (table 2).

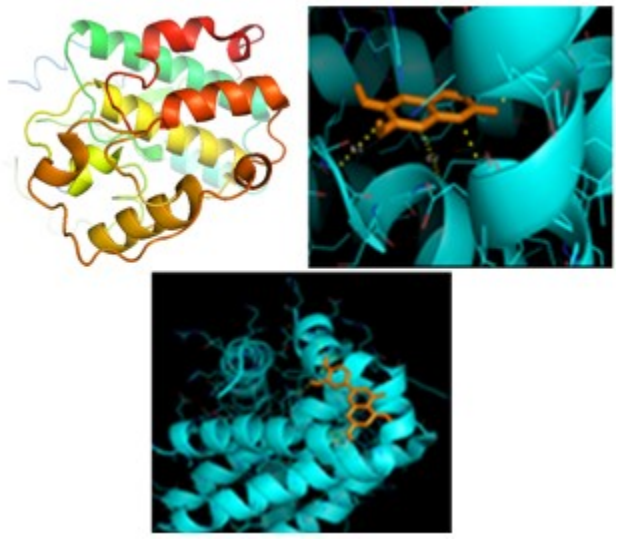

Fig. 3: (A) cyclin-dependent kinases (CDKs) (PDB ID: 2FVD) (B) inhibition conformations with palmitic acid.

Furthermore, a resazurin in vitro cytotoxicity assay was performed for the screening of cytotoxic potential on the panel of cell lines. The cell lines included were liver Huh-7; ATCC: 01042712, colon colorectal HCT-116; ATCC: CCL-247, liver Hep-G2; ATCC: HB8065, breast MCF-7; ATCC: HTB-22, and A549 lung; ATCC: CCL 185. The promising result was observed in the case of Fagonia indica whole plant (FIWM) (FIWC) (FIWH) and *Fagonia indica* FIWM remained effective on liver Hep-G2 and A549 carcinoma cells (Shumaila Saba1). During the resazurin assay colourimetric fluorescence-based experiment the resofrin production resorufin is proportional to viable cells and inversely proportional to nonviable cells in this assay monitored through Flexstation II fig. 1.

Roche applies science has developed a digital device system electrical impedance spectrometer along with ACEA Bioscience. This system can digitally monitor the quantitative high throughput measurement of changes in cellular condition in real-time (Ferrer *et al*., 2017). The xCELLigence® electrical impedance instruments microelectronic and biosensor technology in 96 well plates of cell culture system as impedance Z produced. Predominantly this analysis provides information regarding cellular events continuously taking place including adhesiveness of cells, morphological changes, variability and nonviability at the same time. The cytotoxic potential of *Fagonia indica* was monitored for the impedance frequency on lung A549 carcinoma cells with proapoptotic compound gossypol (fig. 2) and extreme influence was noted at |Z| at 20 kHz. It is a sensitive screening method for recoding impedance frequency with the addition of extracts on cells as their ability of apoptosis induction is one of the major objectives to design and develop a drug with chemotherapeutic potential.

The revolution in drug designing and development is through high put virtual screening methods. The protein-ligand interactions with the minimized energy results in interatomic interactions. (Rout *et al*., 2019). The cyclin-dependent kinases (CDKs) along with other cyclin associates are vital to cell cycle regulators. Promising inhibition was observed in conformations with palmitic acid with Cyclin Dependent Kinase 2 (CDKs 2) as given in fig. 3.

As per observation the in many homo sapiens carcinomas cells the CDKs deregulations with high frequency. Ramachandra plot: for 2FVD (PDB) highly preferred observations shown as 270 (96.429%). Herein, the most prominent ligands of *Fagonia indica* with exceptional binding affinities with residual interactions are given in table 3.

DISCUSSION

Cancer is the disease with the highest rate of mortality and complications worldwide (Kligerman *et al*., 2019). The formation of malignant cellular mass during cancer are the result of abnormal cellular accumulation, morphological and genetic material deformations and alterations (Lim and Leprivier, 2019). Today all available clinical manifestations and managements have severe adverse effects which include toxicity to other normal cells, generalization and limited bioavailability. In recent decades some of the phytochemicals with drastic have been discovered from traditional medicinal systems against antiviral, antibacterial, antioxidant and even anti-cancer effects. Presently, 80% of the approved drugs are either d derivatives or analogues of compounds derived from natural product systems, however, in Pakistan, most of these traditional drugs are actinically still unexplored (Rathore *et al*., 2017; Riaz *et al*., 2020; Javed *et al*., 2021).

In this research, the *Fagonia indica* whole plant was extracted with the different solvent systems to obtain the best solvent with maximum phytochemicals that can be tested for its potential. Mutagens are agents present in the environments and their exposure may cause mutations in

Table 1: Preliminary Phytochemical analysis of *Fagonia indica*

Phytochemical Tests	*Fagonia indica*		
	FIWH	FIWD	FIWM
Alkaloids	-	+	+
Glycosides	-	+	+
Cardiac Glycosides	-	++	+++
Steroids	++	+++	+++
Polysterol		-	+
Saponins	+	++	+++
Anthrhaquinones	-	+	+
Phenol	-	+	-
Flavonoids	-	+	+
Catechins	-	-	-
Tannins	++	++	+++
Triterpenes	+	-	+
Diterpenoids	+	-	-

Where active constituent are represented by (-) absent (+) weak (++) moderate (+++) abundant

Table 2: Anti-mutagenicity by AMES assay

Plant Extracts	No. of Revertant Colonies				Concentration
	TA98	TA98	TA100	TA100	(µg/plate)
	(AZS)	(2AA)	(AZS)	(2AA)	
Control	216.31±4.5	187.4±9.3	263.3±14.1	305.4±8.1	
FIWM	158.2±1.6	142.4±5.4	130.7±6.3	202.6±6.3	125
	142.2±6.4	134.3±0.8	128.66±3.4	168.3±7.2	250
	112.6±4.4	117.0±0.7	119.00±0.7	153.5±4.4	500
	118.2±1.3	113.6±5.2	105.33±4.0	146.5±5.2	1000

Antimutagenic potential of *Fagonia indica* whole plant methanol (FIWM) as mean ± SD; µg/p means microgram per plate; *Salmonella typhimurium strains* TA98; frame-shift mutations, TA100; base-pair substitutions, Mutatgens AZS Sodium azide direct-acting mutagen, 2AA; 2-aminoanthracene indirect-acting mutagen.

Table 3: Residual interactions of CDKs (2FVD) activated protein with lead

Chem ID	IUPAC names	Mol. Formula	Energy Affinity	Residues in contact To ligand
323	p-Coumarin	$C_9H_6O_2$	-8.3	VAL 154,PRO 155,VAL156,HIS161
361	Dihydrophloroglucinol	$C_6H_8O_3$	-4.1	GLU162,TYR168,ARG169
4444395	Kaempferol	$C_{15}H_{10}O_6$	-6.2	VAL 154,PRO 155,VAL156,HIS161
8363	Benzyl salicylate	$C_{14}H_{12}O_3$	-5.4	THR165,LEU166,TRP167
58496	Betulinic acid	$C_{30}H_{48}O_3$	-4.2	VAL 154,PRO 155,VAL156,HIS161
4444100	Palmitic Acid	$C_{16}H_{32}O_2$	-8.7	VAL 154, PRO 155
960	P- Apigenin	$C_{15}H_{10}O_5$	-5.2	TYR168,ARG169,ALA179,PRO171

DNA the genetic material which may lead to cancers. Phytochemical analysis showed abundant presence of steroids, saponins, anthrhaquinones and tannins which concords with previous study (Anil *et al*., 2012; Patel and Kumar, 2020).

Here, *Fagonia indica* showed drastic antimutagenicity at the concentration of 500µg/ plate for TA98 AZS) and at 1000µg/plate for TA98 (2AA), TA100 (AZS) and TA100 (2AA) respectively. Several secondary metabolites obtained from plant extracts have been identified experimentally with the ability to contain the mutation in genetic material which is triggered by various mutagens (Ramadan *et al*., 2019; Sarwari *et al*., 2019).

The medicinal plants which are known for their potential anticancer ability by cellular pathway their phytoconstituents are recognized as chemo preventative or chemotherapeutic or carcinogenesis properties (George *et al*., 2017). The resazurin assay is a colourimetric method for screening cellular viability (Zheng *et al*., 2019). In this study, it was performed on liver Huh-7, liver Hep-G2, breast MCF-7 and lungs A549 human carcinoma cell lines to observe the cytotoxicity effect and

concentration of extract with best %inhibition potential. The results were recorded for *Fagonia indica* (FIWM) CC_{50}128.3±2.43µg/ml on HepG-2 followed by A-549 CC_{50}146.1±1.32µg/ml indicating decent potential anticancer ability. *Fagonia indica* FIWM was then evaluated its apoptosis induction potential for electrical impedance in real-time in high throughput digital instrumental experiment following previous study protocol (Cerignoli *et al*., 2018). Moreover, there is great potential for small molecules like palmitic acid can virtually inhibit CDKs and develop an effective cancer treatment strategy. Furthermore, protein-ligand direct targeted interactions showed promising binding site affinity along with energy. Future pharmacological, animal model studies will enhance the understanding of the mechanistic approaches for the development of indigenous anticancer drug candidates.

CONCLUSION

Fagonia indica whole methanol (FIWM) extract was found to have potential antimutagenic, chemotherapeutic effect in vitro homo special liver and lung carcinoma cell culturing based approaches moreover, in silico approaches supported the results. This provides the basis for the potential anticancer from phytochemical from *Fagonia indica* can be a drug candidate for designing and developing the chemotherapeutic drug.

ACKNOWLEDGEMENTS

IRSIP fellowship at KU Leuven Belgium, The Higher Education Commission of Pakistan, this research is part of PhD thesis

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