RESEARCH ARTICLE

Plant Phenolics Ferulic Acid and P-Coumaric Acid Inhibit Colorectal Cancer Cell Proliferation through EGFR Down-Regulation

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Abstract

<u>Background</u>: **Colorectal cancer (CRC) or bowel cancer is one of the most important cancer diseases, needing serious attention. The cell surface receptor gene human epidermal growth factor receptor (EGFR) may have an important role in provoking CRC. In this pharmaceutical era, it is always attempted to identify plant-based drugs for cancer, which will have less side effects for human body, unlike the chemically synthesized marketed drugs having serious side effects. So, in this study the authors tried to assess the activity of two important plant compounds, ferulic acid (FA) and p-coumaric acid (pCA), on CRC.** <u>Materials and Methods</u>: **FA and pCA were tested for their cytotoxic effects on the human CRC cell line HCT 15 and also checked for the level of gene expression of EGFR by real time PCR analysis. Positive results were confirmed by *in silico* molecular docking studies using Discovery Studio (DS) 4.0. The drug parallel features of the same compounds were also assessed *in silico*.** <u>Results</u>: **Cytotoxicity experiments revealed that both the compounds were efficient in killing CRC cells on a controlled concentration basis. In addition, EGFR expression was down-regulated in the presence of the compounds. Docking studies unveiled that both the compounds were able to inhibit EGFR at its active site. Pharmacokinetic analysis of these compounds opened up their drug like behaviour.** <u>Conclusions</u>: **The findings of this study emphasize the importance of plant compounds for targeting diseases like CRC.**

Keywords: Colorectal cancer - EGFR - ferulic acid - p-coumaric acid - molecular docking - cytotoxicity

Introduction

Colorectal cancer (CRC) holds third position among all types of cancer in human males (746,000 cases, 10.0% of total) and second position in females (614,000 cases, 9.2% of total) worldwide (Ferlay et al., 2015). It is one of the most dominant causes of death rate throughout the world (Favoriti et al., 2016). The global burden of CRC is expected to increase by 60% to more than 2.2 million new cases and 1.1 million deaths by 2030 (Arnold et al., 2016). Occurrence of CRC can be prevented by maintaining a healthy lifestyle, consuming a diet low in red meat and high in fibre, and minimizing alcohol and nicotine intake (Torre et al., 2016).

Plenty of research work is regularly conducted from long time ago till date to screen and detect patients with CRC and help in its management (Wrafter et al., 2016). More often now a days, biomarkers are used to identify CRC victims (Yiu and Yiu, 2016). The human epidermal growth factor receptor (EGFR) gene in its mutated, overexpressed or amplified form, is always implicated as one of the important biomarkers responsible for pathogenicity of almost 60%-80% of CRC (Hammond et al., 2016). EGFR provokes CRC by activating mitogenic signalling pathways such as RAS-RAF- MEK-MAPK and PI3K-PTEN-AKT cascades (Pabla et al., 2015).

Consumption of active phytoceuticals/ functional foods from various plant sources can provide protective support to the body against CRC (Park et al., 2015; Koosha et al., 2016). Ferulic acid (FA, $C_{10}H_{10}O_4$) and p-Coumaric acid (pCA, $C_9H_8O_3$) are important naturally occurring phenolic acids highly abundant in most of the fruits, vegetables and grains (El-Seedi et al., 2012; Narayanankutty et al., 2016). It is evidenced from many reviews that these phytocompounds have beneficial effects on cancer diseases, as they act as antioxidants and help in free radical scavenging (Mancuso and Santangelo, 2014; Pei et al., 2016). This study is attempted to reconfirm the effect of above mentioned plant compounds on CRC by both wet lab and dry lab studies.

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Materials and Methods

Chemicals and cell lines

FA and pCA were procured from Sigma Aldrich Pvt. Ltd. A stock of 10 mg/mL was prepared for both the compounds which were made by dissolving in 400 μl ethanol and made up to 1 ml by PBS.

Human CRC cancer cell line HCT-15 was procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained in an asceptic animal tissue culture lab kept in RPMI 1640 media in 25 cm² tissue culture flask at 5% CO_2. The media was enriched with 10% Fetal Bovine Serum (FBS), 1% each of sodium pyruvate and glutamic acid and 4.5 gm of glucose per lt. of media as an additional source of energy.

Cell viability test

Cell viability percentage was assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Hatok et al., 2009; Sumantran, 2011). Human CRC cell line HCT-15 (1×10^5 cells/mL) was plated in sterile 96 well plate and allowed to attach at 37°C. At subconfluency, the cells were treated with the compounds at various concentrations (100-250 μg/mL). After 48 hours, 10 μL of MTT was added to each of the wells and again kept for incubation at 37°C for 4 hours.

The insoluble formazan formed by MTT was dissolved by adding 100 μL DMSO (Dimethyl sulfoxide) to each well. The absorbance was measured at 570 nm at 37°C using a monochromatic ELISA Microplate reader (Molecular Devices, USA). From the readings of absorbance values, IC_{50} value of the compound was calculated using the following formula:

$$\text{Percentage of cell death} = \frac{\text{mean of control-mean of sample}}{\text{mean of control}} \times 100$$

Real time PCR analysis

Effect of test compounds on the expression of EGFR gene was studied through real time quantitative PCR assay. For this, HCT 15 cells were plated in 6 well plates at a concentration of 1×10^5 cells/mL and incubated for 24 hours. The cells were treated with both test compounds after 24 hours. The cells were again incubated for more 24 hours to proceed for cDNA synthesis using gene specific primers. Forward and reverse primers specific to human gene EGFR was procured from Sigma Aldrich Pvt. Ltd. The sequences were as follows:

Forward-5' GAGACGAGAACTGCCAGAA 3';
Reverse- 5 ' GTAGCATTTATGGAGAGTC 3'.

cDNA was synthesized using commercially available cDNA kits (Superscript cDNA synthesis kit, Invitrogen) as per the manufacturer's protocol.

Real time PCR reaction was then carried out by using readymade mastermix of SYBR Premix Ex Taq II in Applied Biosystems 7300 real time PCR system (Kubista et al., 2006). β- actin was used as a reference gene in the analysis. A total volume of 25 μL reaction mixture was made comprising of 12.5 μL of SYBR Premix Ex Taq II, 1 μL each of forward and reverse primers of EGFR

(100 nmoles), 0.5 μL of reference dye (ROX), 5 μL of nuclease free water and 5 μL of template cDNA. The cycle settings were as follows; (a) initial denaturation at 95°C for 2 minutes, then 40 cycles consisted of denaturation for 30 seconds at 95°C, annealing for 34 sec at 60°C and dissociation for 15 seconds at 95°C, for 1 minute at 60°C and for 15 seconds at 95°C. Ct values were calculated by the system software and used to calculate fold change compared to β- actin. The expression of target gene EGFR was analysed using the ΔΔCt method (Livak and Schmittgen, 2001). A positive ΔΔCT value indicates down-regulation of the target gene and a negative ΔΔCT value indicates upregulation of the target gene.

Procuring of test compounds and target protein and their preparation

Three dimensional (3D) structures of FA and pCA were downloaded from PubChem database (https://pubchem.ncbi.nlm.nih.gov/) (Kim et al., 2016). The Pubchem IDs for FA and pCA were 445858 and 637542 respectively. The compounds were prepared by 'Prepare ligand' protocol of DS 4.0, so as to find any formed isomers and tautomers, remove intra-magnetism, add hydrogen bonds and minimize energy using CHARMm force field (Vanommeslaeghe and MacKerell, 2015).

The 3D crystallographic structure of EGFR was downloaded from Protein Data Bank (PDB- http://www.rcsb.org/pdb/home/home.do) (Deller and Rupp, 2015; Rose et al., 2015). The PDB ID for the protein was 3UG2. All the non-standard residues such as water molecules, natural ligands and hetero atoms were removed by using 'Prepare protein' protocol of DS 4.0. Active sites and critical residues present in them were found out using poseview software molecular interactions as displayed in PDB (Stierand and Rarey, 2010). A grid receptor sphere was generated, including the selected binding active site and incorporating all the critical functional residues.

Docking study and confirmation of drug like property

A structure based docking work was performed by 'CDOCKER' protocol of Discovery studio 4.0 to find out if there is any binding affinity of the test compounds with EGFR at molecular level (Wu et al., 2003; Du et al., 2016). A maximum of ten poses were allowed to be analyzed. The pose which had the lowest binding energy calculation as the scoring function is considered as the best interaction. Number of hydrogen bonds formed between the target and compounds were recorded along with their lengths.

The test compounds after docking studies were forwarded for confirmation of drug like properties and oral bioavailability. These were measured by *in silico* filtration of the compounds through Lipinski's rule of five and Veber's protocol (Ro5&VP) and also by assessment of absorption, distribution, metabolism, excretion and toxicity (ADMET) parameters after human ingestion (Pollastri, 2010; Tian et al., 2015).

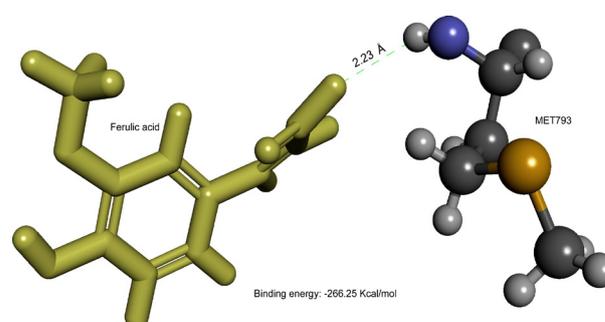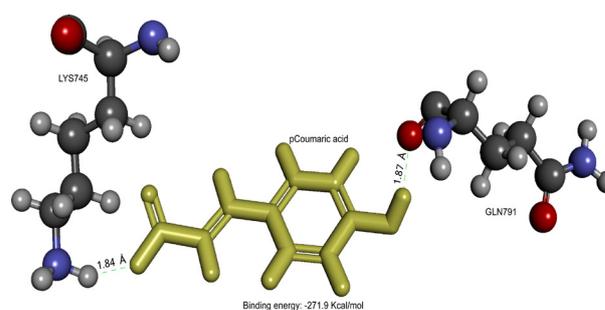## Results and Discussion

Cell viability test and qRTPCR assay

The cell viability test was assessed by MTT assay. This

Table 1. Pharmacokinetic Assessment of Test Compounds

Ro5 & VP filtration					
Compound name	MW (≤500 g/mol)	HBD (≤5)	HBA (≤10)	AlogP (≤5)	RB (≤10)	PSA (≤140 Å^2)
FA (Ferulic acid)	194.18	1	4	0.19	3	69.59
pCA (pCoumaric acid)	164.15	1	3	0.21	2	60.36
ADMET analysis					
Compound name	Solubility level (2-4)	BBB level (2-4)	CYP2D6 Prediction (False-non inhibitor)	Hepatotoxic Prediction (False-non toxic)	Absorption level (0-1)	
FA	4	3	FALSE	FALSE	0	
pCA	4	3	FALSE	FALSE	0	
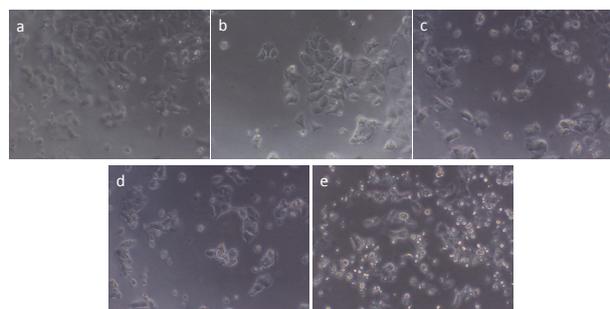
Figure 1. HCT 15 Cells Before Treatment with FA (a), Followed by Treatment with FA at 100 (b), 150 (c), 200 (d) and 250 μg/mL(e)
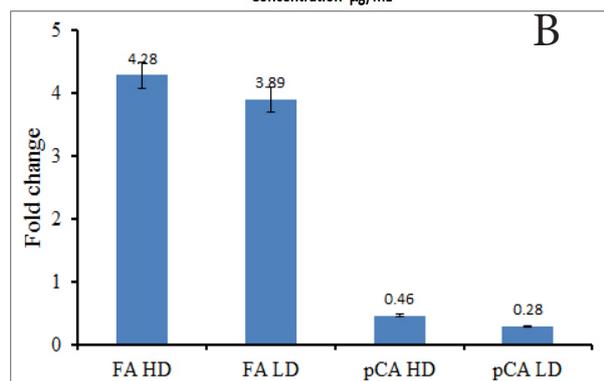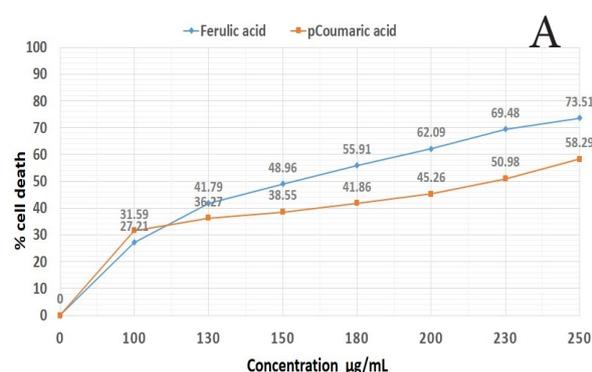
Figure 2. Effects of FA and pCA on the HCT-15 Cell Line. A) Cytotoxicity. B) Downregulation of EGFR gene expression by treatment with two different doses each of FA and pCA on HCT 15 cell line

Figure 3. Interaction of FA with Active Site Residues of EGFR

Figure 4. Interaction of pCA with Active Site Residues of EGFR

is a colorimetric assay which detects living, but not dead cells. The proliferation activity of cell populations under different treatment conditions is determined based on the detection of mitochondrial dehydrogenase enzyme activity in living cells (Berridge et al., 2005). By this assay, it was observed that the viability of HCT-15 CRC cells decreased significantly by the action of FA and pCA in a controlled concentration basis. The activity of FA on HCT-15 can be seen in Figure 1. Half of the cells of HCT-15 were found dead at an IC_{50} value of 154 μg/ml of FA and 222 μg/ml of pCA (Figure 2A). pCA was noted to be little less potent in killing the cancer cells than FA.

For qRT-PCR assay, HCT-15 cells received 2 doses of the test compounds FA and pCA at two concentrations i.e. a low dose (LD) and a high dose (HD) than the IC_{50} value. For FA, the LD and HD were 135 and 175 μg/ml respectively, whereas for pCA, the LD and HD were 200 and 250 μg/ml respectively. cDNA was extracted from the treated cells. The ΔΔCT values obtained with HD and LD of FA were 4.28±0.15 and 3.89±0.15 respectively, whereas for HD and LD of pCA, the ΔΔCT values were 0.46±0.15 and 0.28±0.15 respectively. From these values, it was interpreted that both the test compounds were able to reduce the expression of the target gene EGFR dose dependently, with respect to β- actin. pCA was noticed to have less effect on EGFR, although it do possess the ability to decrease or down regulate the gene. FA with its high dose remarkably reduced the expression of the target gene EGFR up to 4.28 fold (Figure 2B).

Binding ability of test compounds

Molecular docking studies are generally done to check the placement of compounds in the binding site of any protein (Yuriev et al., 2015). Preparation of the ligand compounds did not yield any isomers and tautomers (Pospisil et al., 2003). After preparing the protein, 2 active sites were found present. The best active site (AC 1) was chosen looking at the PDB site records (poseview software interactions) for receptor grid preparation. In our studies, it was found that both the test compounds interacted very perfectly at the selected active site of the target protein EGFR (Doak et al., 2016). FA interacted with EGFR with a binding energy -266.25 kcal/mol by making a hydrogen bond of length 2.23 Å with the residue MET793 (Figure 3). pCA interacted with EGFR with a binding energy -211.91 kcal/mol by making 2 hydrogen bonds of length less than 1.9 Å with the residues LYS745 and GLN791 (Figure 4). The hydrogen bonds formed by both the test compounds with EGFR are of length less than 2.4 Å, which depicted strong interaction (Stierand and Rarey, 2010).

Pharmacokinetic assessment

To reduce cost and chance of clinical failures of new drugs, the compounds are effectively screened via *in silico* Ro5&VP filter and ADMET descriptors provided by DS 4.0 (Veber et al., 2002; Lipinski, 2004; Moroy et al., 2012). Filtration of the test compounds by Ro5 & VP, which screened the compounds on the basis of molecular weight (MW), no. of hydrogen bond donors (HBD) and hydrogen bond acceptors (HBA), no. of rotatable bonds (RB), logP value and polar surface area (PSA) allowed all of them to pass (Table 1). This implies that both the test compounds have drug like properties.

To check the oral bioavailability of the compounds, ADMET properties of the test compounds were examined (Table 1). It was noticed that both FA and pCA having no toxicity over human health and non-inhibitor of CYP450 enzyme i.e. there is no chance of unwanted drug drug interactions (Mohan et al., 2007; Branden et al., 2014). The aqueous solubility and intestinal absorption level of both the compounds was examined as very good (Bergstrom, 2005; Hou et al., 2009). The BBB level of both FA and pCA were examined as low and therefore, there is less possibility of casing of undesirable effects to the central nervous system (Katya et al., 2009).

In conclusion, CRC is one of the top 5 types of cancers most commonly detected in human beings. It is high time as a matter of worry to find out drugs for CRC, which will have less side effects on humans. Phenolic acids such as FA and pCA are long been in discussion for curing of many lifestyle diseases due to their radical scavenging properties. The present study as aimed reconfirmed the effects of FA and pCA on CRC. Both the 2 compounds were efficient in killing cancer cells as well as reduce the expression of target gene EGFR *in vitro*, and even showed good binding efficiency with the same target gene at molecular level *in silico*. The pharmacokinetic profile of both the compounds were found favorable for transforming them into long awaited herbal drugs in place of commercially available synthetic drugs.

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APJCP.2016.17.8.4019

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