

## Original Article

# Malvidin induced anticancer activity in human colorectal HCT-116 cancer cells involves apoptosis, G2/M cell cycle arrest and upregulation of p21WAF1

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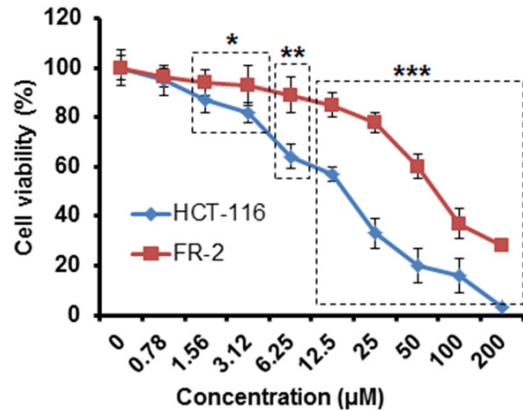
**Abstract:** Colorectal carcinoma is one of the most frequently detected cancers around the globe with an incidence of 1.4 million. Despite advances in the therapeutics and surgical interventions, there is a pressing need to explore safer and more effective therapeutic modalities. In the recent past natural products have gained attention for their diverse bioactivities. In the present study we evaluated the anticancer activity of malvidin, a natural product of plant origin, against human colorectal HCT-116 cancer and FR-2 normal human cell lines. The results of the present study revealed that malvidin exhibited an  $IC_{50}$  of 15  $\mu$ M against colorectal cancer HCT-116 cell line as compared to the  $IC_{50}$  of 65  $\mu$ M against the normal human FR-2 cell line. Malvidin caused significant inhibition of colony formation and triggered apoptosis in HCT-116 cells. Additionally, malvidin treatment induced G2/M cell cycle arrest which was also associated with concentration-dependent inhibition of cell cycle related proteins and upregulation of p21WAF1. Taken together, we propose that malvidin exhibited significant anticancer activity against colorectal cancer cells and may prove as an important lead molecule in cancer chemotherapy.

**Keywords:** Colorectal cancer, cell cycle arrest, apoptosis, malvidin

## Introduction

Colorectal carcinoma is one of the most frequently detected cancers around the globe with a incidence of 1.4 million [1, 2]. Despite advances in the therapeutics and surgical interventions there is a pressing need to explore safer and more effective therapeutic modalities to limit the spread of colorectal carcinoma and to ensure minimal adverse effects that affect the quality of life of the patient [1, 2]. Consistent with this, drugs from natural have gained considerable attention in the recent past. Among natural products, flavonoids represent an important part of the human diet and in United States the estimated regular dietary intake of mixed flavonoids ranges from 500 to 1000 mg. This figure may be even higher for people improving their diets with flavonoid rich herbal preparations [3]. With advancements in medical research, flavonoids are being evaluated for diversity of bioactivities. So far they have been reported to exhibit wide

range of activities which include, but are not limited to, anti-inflammatory, estrogenic, enzyme inhibition, antimicrobial, anti-antiallergic, antioxidant and antitumor [4, 5]. Owing to their fairly consistent structure, flavonoids impede the activity of a wide range of eukaryotic enzymes and therefore exhibit diversity of activities. The different parts of flavonoid molecules have been considered critical for their bioactivities [5, 6]. Moreover, flavonoids are ubiquitously present in edible plants and beverages; they are therefore expected to have minimal toxicity. Against backdrop, the present study evaluated the anticancer activity of a natural flavonoid, malvidin against colorectal cancer cells. It was observed that malvidin exerted strong antiproliferative effects against colorectal carcinoma cell line HCT-116 as compared to the normal human FR-2 cell line. Malvidin induced apoptosis in HCT-116 cells and triggered G2/M cell cycle arrest in HCT-116 cells. Malvidin-induced cell arrest was associated with the reduction of the expression of



**Figure 1.** Effect of indicated doses of malvidin on cell viability of colorectal HCT-116 and normal FR-2 human cells. Results are mean of three biological replicates and expressed as mean  $\pm$  SD. The values were considered significant at \* $p < 0.01$ , \*\* $p < 0.001$  and \*\*\* $p < 0.0001$ .

cell cycle associated proteins and upregulation of p21WAF1. Taken together, we propose that malvidin may prove beneficial in the treatment and management of colorectal carcinoma, however further research endeavors are still required urgently.

## Materials and methods

### Chemicals, reagents, cell line and culture conditions

All chemicals and reagents used in the present study were procured from Sigma-Aldrich Co. (St. Louis, MO, USA). Colorectal cancer cell line cell (HCT-116) were procured from Cancer Research Institute of Beijing, China, and it was maintained in RPMI-1640 medium and was supplemented with 10% FBS and antibiotics (100  $\mu$ g/ml streptomycin and 100 U/ml penicillin G) in an incubator at 37°C (5% CO<sub>2</sub> and 95% air).

### MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazoliumbromide] assay

MTT assay was used to determine the antiproliferative activity of malvidin against the colorectal cancer cell line cell (HCT-116) and normal human FR-2 cell line. The cells in 100  $\mu$ L culture medium were seed in a 96-well plate at a density of  $3 \times 10^3$  cells/mL and incubated at 37°C in 5% CO<sub>2</sub> for a time period of 24 h. After 24 h, an additional 100  $\mu$ L of complete medium with either: no additions or dif-

ferent concentrations (0-200  $\mu$ M) of malvidin were added. Thereafter the cells were incubated for 24 h. This was followed by the addition of 20  $\mu$ L of MTT solution (5 mg/mL) and an incubation of 4 h. afterwards, the medium was removed and 150  $\mu$ L DMSO added. The absorbance (OD) of each well was measured at 490 nm using a Tunable Mi-185 croplate Reader (EL-x 800, BioTek Instruments, USA).

### Colony formation assay

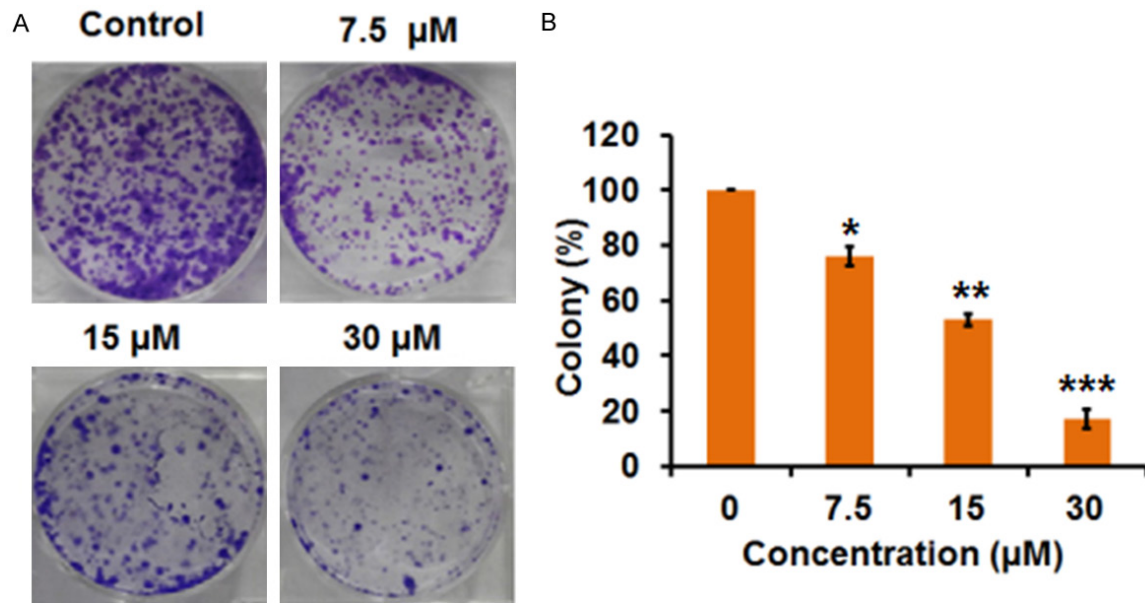
HCT-116 cells were plated in 12-well culture plate (500-600 cells per well) respectively and allowed to attach for 12 h, then treated with varied concentrations (0, 7.5, 15 and 30  $\mu$ M) of malvidin. After incubation for another 7 days, cells were washed with PBS, fixed with methanol for 30 min, and stained with 0.5% crystal violet solution. Colonies (>50 cells per colony) were counted under an inverted microscope.

### Apoptosis and cell cycle distribution

HCT-116 cells at a density of  $2 \times 10^5$  cells/well were seeded in 6 well plates and administered with 0, 7.5, 15 and 30  $\mu$ M malvidin for 24 h. The cells were then subjected to DAPI staining. Afterwards, the cell sample was studied and photographs taken under fluorescence microscopy. For cell cycle analysis, HCT-116 Cells were harvested, fixed in 70% ethanol, and stored at -20°C. Cells were then washed twice with ice-cold PBS and incubated with RNase and the DNA intercalating dye propidium iodide. Cell-cycle phase analysis was performed using a Becton Dickinson Facstar flow cytometer equipped with Becton Dickinson cell-fit software.

### Cell migration assay

Cell migration assay was carried out by Boyden chamber assay with some modifications. Cells at the density of  $5 \times 10^4$  cells/well were suspended in 2% FBS medium and placed in the upper chamber of 8  $\mu$ m pore size transwells. Afterwards, medium supplemented with 10% FBS was added to lower chamber. This was followed by an incubation of 24 h. On the upper surface of the membrane, unmigrated cells were removed while as on the lower surface of the membrane the migrated cells were fixed in methanol (100%) and giemsa stained. The cell migration was estimated by counting



**Figure 2.** Effect of indicated doses of malvidin on (A) Cell viability of colorectal HCT-116 cells (B) Quantification of cell viability. Results are mean of three biological replicates and expressed as mean  $\pm$  SD. The values were considered significant at  $*p<0.01$ ,  $**p<0.001$  and  $***p<0.0001$ .

the number of the migrated cells under a microscope.

#### Protein expression by western blotting

After administration with various concentrations of malvidin, cells were harvested and lysed in lysis buffer. Out of the total protein samples 20  $\mu$ g aliquot was separated on 10% SDS-PAGE gel. The gel was then transferred to nitrocellulose membranes, blocked with 5% BSA and probed with a primary antibody. This was followed by probing with the required secondary antibody. Finally, the signal was perceived with WEST-SAVE Up™ luminal-based ECL reagent.

#### Statistical analysis

Data are expressed as the mean  $\pm$  SD. One way ANOVA and Tukey's test was used to assess differences within treatment groups. Differences were considered significant when  $*p<0.01$ ,  $**p<0.001$  and  $***p<0.0001$ .

### Results

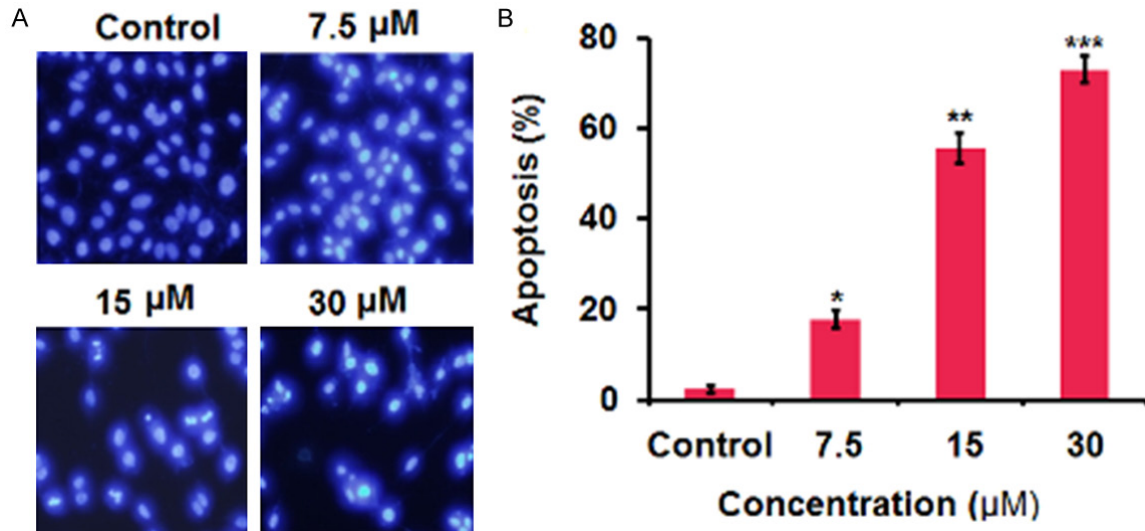
#### Anti-proliferative effects of malvidin on HCT-116 cell line

The anti-proliferation effect of malvidin against HCT-116 and normal human FR-2 cells was

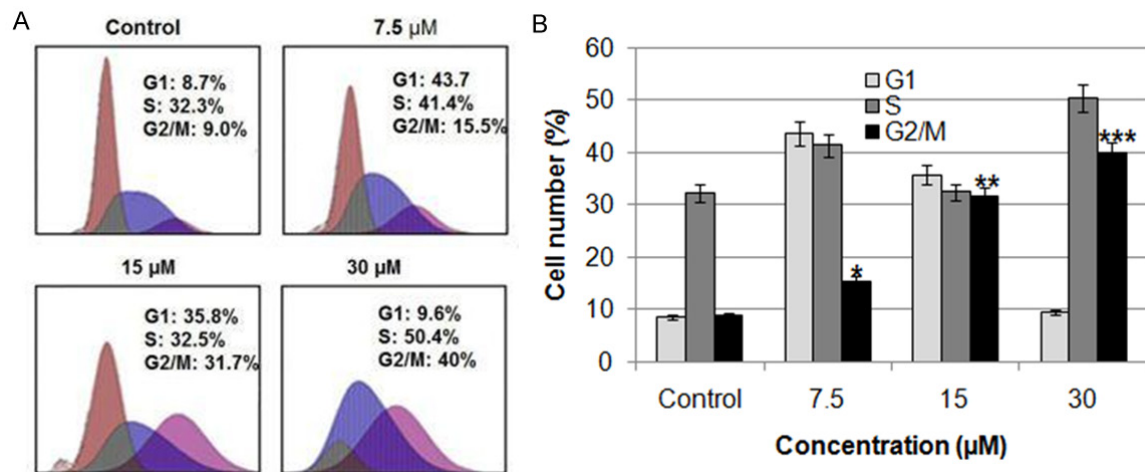
investigated by MTT assay. To examine the anti-proliferative effect of malvidin, the cells were treated different concentrations of malvidin and  $IC_{50}$  was determined. Malvidin displayed potent anti-proliferative effect against HCT-116 cells with an  $IC_{50}$  of 15  $\mu$ M against HCT-116 cells as compared to the  $IC_{50}$  of 65  $\mu$ M against the normal FR-2 cells. Additionally, malvidin exhibited dose dependent effect on the cell viability of HCT-116 cancer cells (Figure 1). The results of MTT assay were complemented by the colony formation assay wherein, we observed that malvidin treatment lead to significant reduction in the number of colonies in a dose-dependent manner (Figure 2A, 2B).

#### Malvidin induced apoptosis in HCT-116 cells

To investigate whether malvidin induced anti-proliferative effects were due to induction of apoptosis, the HCT-116 cells were treated with different concentrations of malvidin and apoptotic damage was detected by DAPI staining. Our results indicated that malvidin induced apoptosis in a dose-dependent manner as evident from the greater density of white color nuclei (Figure 3A, 3B). The percentage of apoptotic cells increased from 2% in control upto 77% at 30  $\mu$ M concentration of malvidin.



**Figure 3.** Effect of indicated doses of malvidin on (A) Induction of apoptosis in colorectal HCT-116 cells (B) Quantification of % apoptotic cells. Results are mean of three biological replicates and expressed as mean  $\pm$  SD. The values were considered significant at \* $p$ <0.01, \*\* $p$ <0.001 and \*\*\* $p$ <0.0001.



**Figure 4.** Cycle distribution of HCT-116 cells at indicated doses of malvidin determined by (A) Flow cytometry (B) Estimation of cell percentage in each phase. Results are mean of three biological replicates and expressed as mean  $\pm$  SD. The values were considered significant at \* $p$ <0.01, \*\* $p$ <0.001 and \*\*\* $p$ <0.0001.

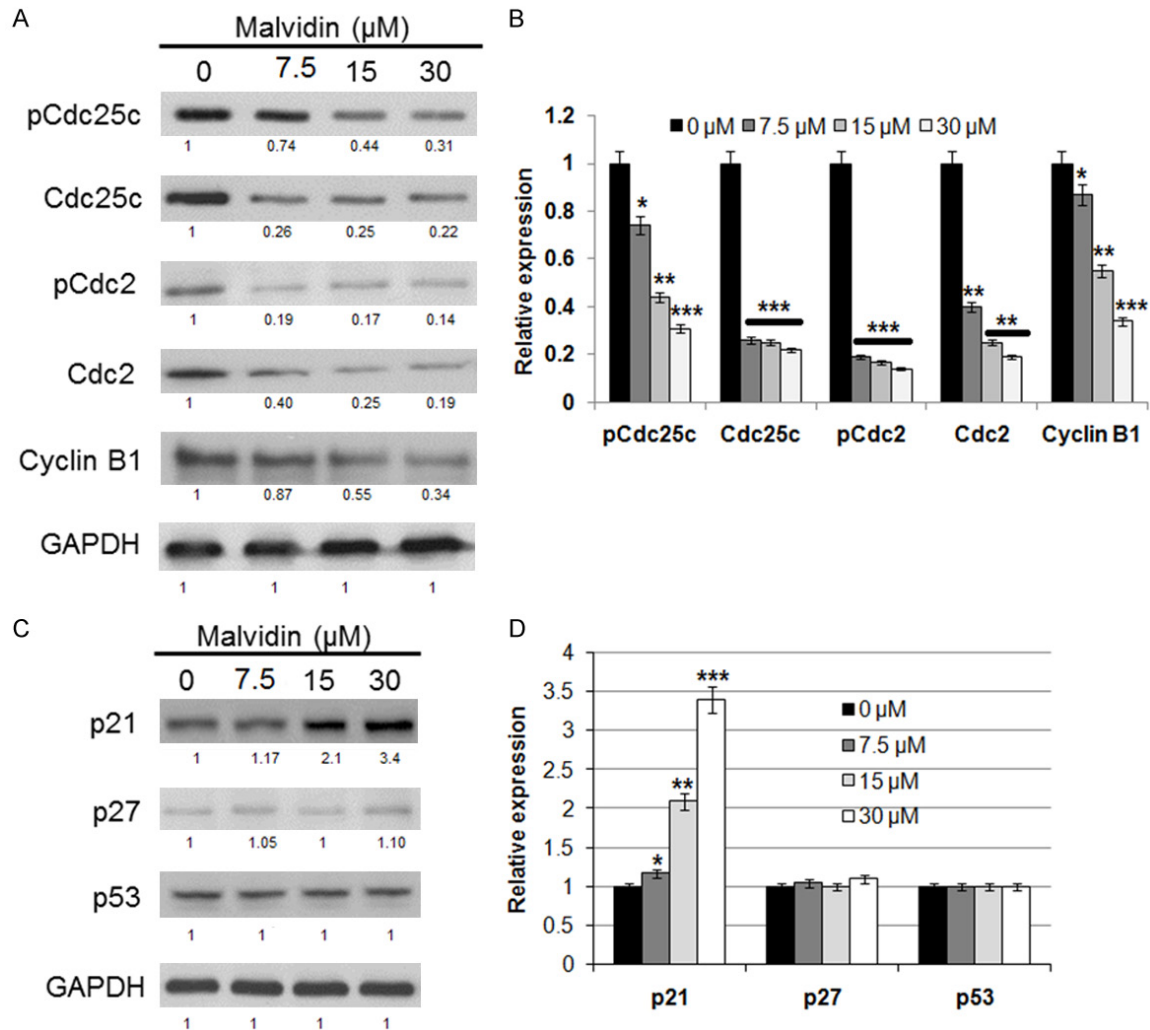
#### Malvidin caused G2/M cell cycle arrest of HCT-116 cancer

To further investigate the mechanism responsible for malvidin-mediated inhibition of cell growth, we examined cell-cycle distribution using flow cytometry. The results showed that malvidin triggered an increase in G2/M-phase arrest in cells after 24 h of treatment (Figure 4A, 4B). We next used immunoblotting to examine the effects of malvidin on the expression of G2/M cell-cycle regulatory proteins including cyclin B1, Cdc25c and Cdc2 in HCT-116 cells. As depicted in Figure 5A, 5B, malvi-

din treatment of the HCT-116 cells lead to a concentration-dependent reduction in the protein expression of pCdc25c (Ser216), Cdc25c, pCdc2 (Tyr15) and Cdc2 in HCT-116 cells. In addition, administration of cells with malvidin for 24 h resulted in a significant decrease in the protein levels of cyclin B1.

#### p21WAF1 is involved in malvidin-triggered G2/M cell-cycle arrest

Next, we investigated the effects of malvidin on induction p21WAF1, which is associated with suppression of the G2/M phase-transition



**Figure 5.** Effect of indicated doses of malvidin on (A) Expression of pCdc25c, Cdc25c, pCdc2, Cdc2, cyclin B1 by western blotting (B) The quantification of the western blots of pCdc25c, Cdc25c, pCdc2, Cdc2, cyclin B1 was carried out by densitometric analysis. (C) Expression of p21, p27 and p53 by western blotting (D) The quantification of the western blots of p21, p27 and p53 was carried out by densitometric analysis. The values were considered significant at \* $p < 0.01$ , \*\* $p < 0.001$  and \*\*\* $p < 0.0001$ .

checkpoint via formation of a cyclin B1/Cdc2 complex [7, 8]. Results from concentration-dependent studies revealed that malvidin increased the expression of p21WAF1 in HCT116 cells in (Figure 5C). There was about 3.4 fold increase in the expression of p21WAF1 at 30 μM concentration of malvidin as compared to control (Figure 5D). However, malvidin failed to affect the expression of either p27 or p53 tumor suppressor proteins (Figure 5B).

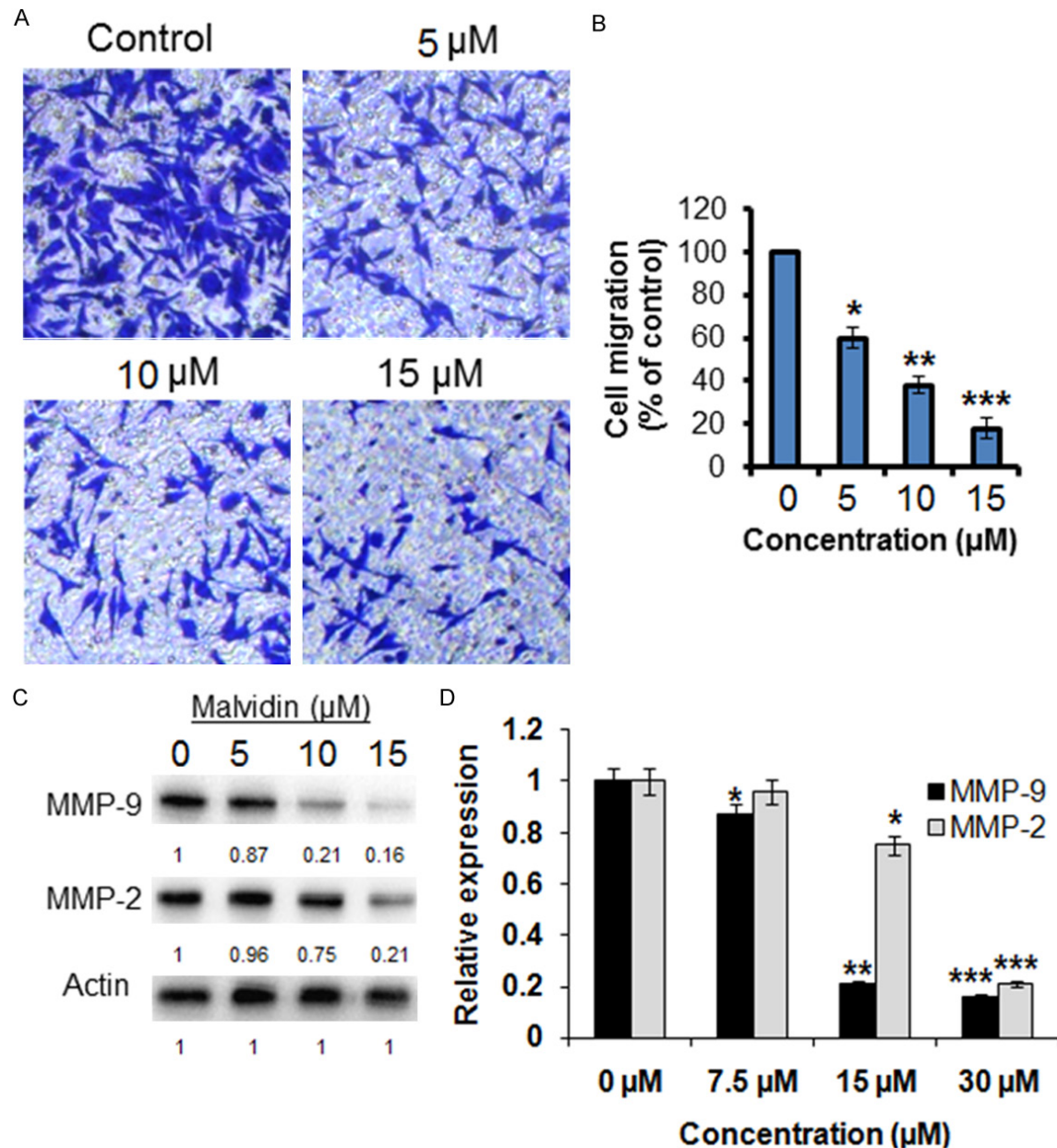
#### Effect of malvidin HCT-116 cancer cell migration

Further, we examined if malvidin can inhibit the migration of HCT-116 cancer cells at the

varied concentration at different time intervals by transwell assay. The results of transwell assay showed that malvidin reduced the migratory capability of HCT-116. While as in control the cells show fairly good capacity to migrate, in treatment, the cell showed less potential to migration as depicted in Figure 6A, 6B. The inhibition of cell migration was also associated with the concomitant decrease in the expression of metalloproteinases (MMPs), MMP-9 and MMP-2 (Figure 6C, 6D).

#### Discussion

Colorectal cancer is the third most lethal cancer worldwide. Both genders, male and female



**Figure 6.** Inhibition of cell migration HCT-116 cells at indicated concentrations of malvidin. A. Malvidin inhibited HCT-116 cell migration as tested by transwell assay carried out for 24 h. B. Quantification of % cell migration. C. HCT-116 cells were treated with indicated concentrations of malvidin for 24 h, and the protein levels of MMP-2 and MMP-9 were determined by western blot analysis. D. Quantification of western blots of MMP-2 and MMP-9 by densitometric analysis. Results are mean of three biological replicates and expressed as mean  $\pm$  SD. The values were considered significant at \* $p$ <0.01, \*\* $p$ <0.001 and \*\*\* $p$ <0.0001.

are equally affected by this deadly disease. In the past year about 140,000 people were diagnosed with colon cancer in the United States and the estimated survival is 50% or less [1, 9]. Hence, there is an urgent need to find out safer and more effective therapeutic modalities to limit the spread of colorectal carcinoma and

to ensure minimal adverse effects that affect the quality of life of the patients. In this malvidin a natural product of plant origin was evaluated for its anticancer activity against colorectal carcinoma. It was observed that malvidin exhibited potent antiproliferative effects on HCT-116 cells and reduced colony forming

potential of HCT-116 cells as evident from the proliferation assay (**Figure 1**). Additionally, the IC<sub>50</sub> of malvidin against HCT-116 was found to be 15 µM which was significantly lower than the IC<sub>50</sub> of 65 µM observed against the normal FR-2 cells indicating lower toxicity of malvidin. The antiproliferative effects of malvidin were further confirmed by the colony formation assay (**Figure 2**). As reported in literature, many drugs show anticancer activity by triggering apoptosis. For instance, several chemotherapeutic drugs, such as cisplatin have been shown to induce apoptosis in cancer cells [10, 11]. Additionally, the resistance of the cancer cells to drugs is in part due to their capacity to escape apoptosis [12]. To examine whether malvidin induces apoptosis in HCT-116, we carried out the DAPI staining of the malvidin-treated cells. The results of our study indicated that malvidin induces DNA apoptosis concentration dependent manner (**Figure 3**). Moreover, the apoptotic cell population increased significantly in a concentration dependent manner (**Figure 3B**).

To further explore the mechanisms involved in the anticancer activity of malvidin on HCT116 cells, we examined the events that cause inhibition of colon cancer cell proliferation. Our results revealed that malvidin triggered G2/M phase arrest of HCT-116 cells (**Figure 4**). The accumulation of the G2/M phase prevents cells from entering into mitosis [13, 14]. Treatment HCT-116 cells with malvidin lead to incomplete cell division, indicating that cells at the G2/M phase failed to progress through mitosis of the cell cycle due to the inhibition of the G2/M regulatory proteins such as cyclin B1, Cdc25C, and Cdc2 (**Figures 4, 5A**). p21-WAF1 is an important inhibitor in regulating cell-cycle progression [15]. There are concrete evidences that indicate that p21WAF1 is associated with inhibiting the expression of the Cdc2/cyclin B1 complex [7, 8]. The results obtained from the present study revealed that malvidin suppresses the expression of cyclin B1, Cdc25C, and Cdc2 and at the same time increases the protein expression levels of p21/WAF1 (**Figure 5B**). However, malvidin had no significant effect on the expression of either p27 or p53. These results indicate that malvidin stimulated 21WAF1 expression which in turn triggered G2/M-phase arrest of the p53-independent pathway. Malvidin also inhibited

the cell migration of HCT-116 cells as evident from the transwell assay (**Figure 6A, 6B**). Cell migration is the key feature of cancer progression and metastasis [16] and suppression of cell migration may prove essential in inhibition of metastasis *in vivo*. This may ensure comparatively longer survival period of patients. Moreover, the inhibition of cell migration was also associated with the decreased expression of MMP 9 and MMP-2 (**Figure 6C**). Therefore, the potential of malvidin to suppress migration of HCT-116 cancer cells indicates that it may prove as efficient molecule in inhibiting the metastasis of cancer cells.

## Conclusion

Based on our results we conclude that malvidin exhibits significant anticancer activity against colorectal cancer line by inducing apoptosis, cell cycle arrest, and inhibition of cell migration therefore may prove as an important lead molecule. Therefore we strongly believe that this molecule deserves to be carried forward for *in vivo* studies.

## Disclosure of conflict of interest

None.

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## References

- [1] WHO; IARC (international agency for research on cancer); latest world cancer statistics, global cancer burden rises to 14.1 million new cases in 2012, 12th December 2013 Press Release No. 233.
- [2] GLOBOCAN 2012 estimated cancer incidence, mortality and prevalence about colorectal carcinoma, Worldwide in 2012.
- [3] Aitken JF, Elwood M, Baade PD. Clinical whole-body skin examination reduces the incidence of thick melanomas. *Int J Cancer* 2010; 126: 450-458.
- [4] Nagaoka T, Banskota AH, Tezuka Y, Saiiki I, Kadota S. Selective antiproliferative activity of caffeic acid phenethyl ester analogues on highly livermetastatic murine colon 26-L5 car-

- cinoma cell line. *Bioorg Med Chem* 2002; 10: 3351-3359.
- [5] Takagaki N, Sowa Y, Oki T, Nakanishi R, Yogo-sawa S, Sakai T. Apigenin induces cell cycle arrest and p21/WAF1 expression in a p53-independent pathway. *Inter J Oncol* 2005; 26: 185-190.
- [6] Alonso DF, Farías EF, Urtreger A, Ladedá V, Vidal MC, Bal De Kier Joffé E. Characterization of F3II, a sarcomatoid mammary carcinoma cell line originated from a clonal subpopulation of a mouse adenocarcinoma. *J Sr Oncol* 1996; 62: 288-297.
- [7] Baus F, Gire V, Fisher D, Piette J, Dulić V. Permanent cell cycle exit in G2 phase after DNA damage in normal human fibroblasts. *EMBO J* 2003; 22: 3992-4002.
- [8] Hsu YL, Kuo PL, Lin LT, Lin CC. Asiatic acid, a triterpene, induces apoptosis and cell cycle arrest through activation of extracellular signal regulated kinase and p38 mitogen-activated protein kinase pathways in human breast cancer cells. *J Pharmacol Exp Ther* 2005; 313: 333-344.
- [9] ACS. Colorectal cancer facts and figures. American Cancer Society 2014: 1-25.
- [10] Azuma M, Tamatani T, Ashida Y, Takashima R, Harada K, Sato M. Cisplatin induces apoptosis in oral squamous carcinoma cells by the mitochondria-mediated but not the NF-kappaB-suppressed pathway. *Oral Oncol* 2003; 39: 282-289.
- [11] Yoneda K, Yamamoto T, Osaki T. p53- and p21-independent apoptosis of squamous cell carcinoma cells induced by 5-fluorouracil and radiation. *Oral Oncol* 1998; 34: 529-537.
- [12] Ferreira CG, Epping M, Krut FA, Giaccone G. Apoptosis. *Clin Cancer Res* 2002; 8: 2024-34.
- [13] Gautier J, Solomon M, Boher R, Bazan J, Kirschner M. Cdc25 is a specific tyrosine phosphatase that directly activates Cdc2. *Cell* 1991; 65: 197-211.
- [14] Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, Linn S. Molecular mechanisms of mammalian DNA repair and DNA damage checkpoints. *Annu Rev Biochem* 2004; 73: 39-85.
- [15] Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. The p21 Cdk interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* 1993; 75: 805-816.
- [16] Zhang M, Liu Y, Gao Y, Li S. Silibinin-induced glioma cell apoptosis by PI3K-mediated but Akt-independent downregulation of FoxM1 expression. *European J Pharmacol* 2015; 765: 346-54.