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Original article

Rutin inhibits proliferation, attenuates superoxide production and decreases adhesion and migration of human cancerous cells

Mohamed ben Sghaier[a,*], Alessandra Pagano[b,c], Mohamed Mousslim[b,c], Youssef Ammari[a], Hervé Kovacic[b,c], José Luis[b,c]

[a] Laboratory for Forest Ecology, National Institute for Research in Rural Engineering, Water and Forests, University of carthage, BP 10, 2080 Ariana, Tunisia
[b] Centre de Recherche en Oncologie Biologique et Oncopharmacologie (CRO2), INSERM UMR 911, Faculté de Pharmacie, Marseille, France
[c] Aix-Marseille Université, France

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ABSTRACT

Lung and colorectal cancer are the principal causes of death in the world. Rutin, an active flavonoid compound, is known for possessing a wide range of biological activities.

In this study, we examined the effect of rutin on the viability, superoxide anion production, adhesion and migration of human lung (A549) and colon (HT29 and Caco-2) cancer cell lines. In order to control the harmlessness of the tested concentrations of rutin, the viability of cancer cell lines was assessed using a 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. ROS generation was measured by lucigenin chemiluminescence detecting superoxide ions. To investigate the effect of rutin on the behavior of human lung and colon cancer cell lines, we performed adhesion assays, using various purified extracellular matrix (ECM) proteins. Finally, *in vitro* cell migration assays were explored using modified Boyden chambers. The viability of cancerous cells was inhibited by rutin. It also significantly attenuated the superoxide production in HT29 cells. In addition, rutin affected adhesion and migration of A549 and HT29 cell.

These findings indicate that rutin, a natural molecule, might have potential as anticancer agent against lung and colorectal carcinogenesis.

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1. Introduction

Lung cancer is one of the principal causes of death in the world, and non-small cell lung carcinoma accounts for approximately 75%–85% of all lung cancers [1]. Furthermore, colorectal cancer (CRC) is the second most common cancer diagnosed in women [2] and the third in men [3], with an overall survival rate of only 5 years due to resistance to cytostatic drugs [4]. Consequently, novel therapeutic agents are needed to deal with the increasing incidence of human lung and colorectal cancer. Several reports including animal studies have shown that increased consumption of plant-based diet can reduce the risk of cancer such as lung and colon cancer [5,6].

The production of reactive oxygen species (ROS) by eukaryotic cells is believed to be related to the formation of ROS as toxic by-products of various cellular metabolic processes. Recently, the importance of controlled ROS production by nonphagocytic cells, including lung epithelial cells, in the regulation of physiological functions has been suggested in many studies [7]. However, the bioactive cancer preventives remain to be identified. Herbal-based dietary supplements contain a large array of phytochemicals including the flavonoids which might mediate physiological functions related to cancer suppression.

Cancer preventive properties of natural compounds have been studied extensively; however, very little in relation to lung carcinogen [8]. In this context, there is no preceding work dealing with the action of rutin on lung cancer cells. Nevertheless, it was previously described the dietary intervention of polyphenols in cancer prevention, including the chemopreventive ability of dietary flavonoids, act against lung carcinogens. *In vitro* studies were performed over a wide range of polyphenols which showed significant effect in lung Cancer, such as epigallocatechin-3-gallate (EGCG), suppresses in a dose dependent manner, the hexavalent chromium (Cr (VI))-induced apoptosis, reduces activation of caspase-3 and nuclear poly (ADP-ribose) polymerase (PARP), and intracellular ROS [9]. It has also been demonstrated, that Caffeic acid can scavenge intracellular ROS and hence prevent lipid peroxidation in WI-38 cells [10]. Lim et al. [11] revealed that Catechol, a naturally-occurring compound found in fruits and vegetables such as onions,

* Corresponding author.
E-mail address: medbensghaier@gmail.com (M. ben Sghaier).

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apples and olive oil, suppressed anchorage-independent growth of murine KP2 and human H460 lung cancer cell lines in a dose-dependent manner and inhibited ERK2 kinase activity. *In Vivo* polyphenols studies of lung cancer prevention were also conducted in experimental mice models [12–17].

Rutin is found in many typical plants such as buckwheat, apples, black tea, and vegetables. It also exists in many traditional chinese medicines [18]. Many beneficial effects of rutin have been identified, including inhibition of platelet aggregation, being anti-inflammatory, antioxidant, and reducing blood fat and cholesterol [19,20]. Furthermore, it was previously described that rutin induced *in vitro* cytotoxic effects on cancer cell lines [21,22] including human colon cancer cells [23,24]. Rutin and their analogues, such as EGCG and quercetin, act as efficient radical inhibitors and have been shown to have chemopreventive activity in both a variety of colonic cancer cell lines and in murine models [25–27]. Moreover, rutin has shown antitumor effects in some *in vivo* models such as NK/Ly ascites and B16F10 cells [21,22,28]. Therefore, it was also illustrated that rutin exerted significant beneficial effects on decreasing the amount of precancerous lesions and inducing apoptosis in the large intestine cancer and human neuroblastoma LAN-5 cells [29,30].

Despite the evidence for its several biological activities, the anticancer effects of rutin on human colon cancer cells remain to be explored. We also examined, for the first time, the effect of rutin on lung cancer cells adhesion and migration using several purified extracellular matrix (ECM) proteins. In addition, we investigated the potential antiproliferative and antioxidant activities of rutin in human lung (A549) and colon (HT29 and Caco-2) cancer cell lines.

2. Materials and methods

2.1. Chemicals and reagents

Rutin was purchased from Extrasynthèse (Genay,France) and it was of the highest available purity.

Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 medium were purchased from Lonza (Levallois-Perret, France). Penicillin, streptomycin, fetal bovine serum (FBS), trypsin-EDTA, L-glutamine, and sodium pyruvate were purchased from GIBCO (Cergy-Pontoise, France). Methylthiazolyldiphenyl-tetrazolium bromide (MTT), lucigenin, poly-L-lysine, human fibrinogen and mouse laminin-1 was from Sigma (St Quentin Fallavier, France). Rat type I collagen was from Upstate (Lake Placid, NY, USA) and human fibronectin from Millipore (St Quentin en Yvelines, France).

2.2. Tumors cell lines and culture conditions

Human lung (A549) cells were cultured in RPMI 1640 medium, Colon (HT29 and Caco-2) cells were maintained in Dulbecco's modified Eagle's medium (DMEM), all the mediums were supplemented with 10% foetal bovine serum (FBS), supplemented with 2 mM L-glutamine and 1% sodium pyruvate and were maintained at 37 °C in a humidified atmosphere with 5% CO2.

2.3. Cell viability assay

Cell viability was assessed by MTT assay after 24 h incubation. For each cell line, 70% confluent cell culture flask was trypsinized and the cells were seeded in 96 well plates at a density of 5000 cells by well in the appropriate complete media; 24 h after seeding, the cells were treated with increasing concentrations of rutin or control vehicle solution (DMSO 0.1% in phosphate saline buffer); 72 h after treatment, the cells were exposed to 0.5 mg/ml of MTT for 3 h at 37 °C in the appropriate complete medium. Medium and MTT were removed and after solubilization in dimethylsulfoxide (DMSO), the amount of insoluble formazan crystals was evaluated by measuring the optical density at 550 nm. Each condition was performed in triplicate.

2.4. Measurement of ROS

ROS generation was measured by lucigenin chemiluminescence detecting superoxide ions [31]. After incubation of the cells for the desired time with drugs in 96-well plates (50×10^3 cells/well), luminescence was detected by a Fluoroscan Ascent FL fluorimeter (Labsystems, France). The detected signal was assessed at each minute over the course of 45 min. The results represent the integration of the signal for 45 min which is associated with the inter-mediate time of measurement. All measurements were performed at 37 °C and the results were expressed as total reactive oxygen species measurements which represent the difference of reactive oxygen species production measured in untreated control.

2.5. Cell adhesion assay

Adhesion assays were performed as previously described [32]. Briefly, flat bottom 96-well microtiter plates were coated with one of the following purified extracellular matrix (ECM) proteins: fibronectin (10 μg/ml), vitronectin (10 μg/ml), laminin 1 (10 μg/ml), fibrinogen (50 μg/ml), collagen type I and IV (10 μg/ml), poly-L-lysine (10 and 50 μg/ml) and were blocked with BSA. The cells were harvested in single cell suspension and resuspended in DMEM and RPMI containing 0.2% BSA (adhesion buffer) in the presence or absence of rutin. After incubation for 30 min at room temperature, the cells were added to coated wells in a volume of 50 μl (10^6 cells/ml) and allowed to adhere to the substrate for 1 h (A549 cells) and 2 h (HT29 cells) at 37 °C. Unattached cells were removed by gently washing three times with adhesion buffer. Residual attached cells were fixed by 1% glutaraldehyde, stained by 0.1% crystal violet and lysed with 1% SDS. Absorbance was then measured at 600 nm by a microplate reader.

2.6. Cell migration assays using modified Boyden chambers

In vitro cell migration assays were performed using modified Boyden chambers (NeuroProbe Inc, Bethesda, MD, USA) as previously described [33]. Briefly, membranes were coated with fibronectin or collagen type IV (10 μg/ml) for 2 h at 37 °C and blocked with a solution of 0.1% BSA/PBS. The cells harvested as a single cell suspension were added to precoated membranes and allowed to migrate for 5 h at 37 °C. Migrated cells were stained by 0.1% crystal violet, lysed with 1% SDS and cell migration was then quantified by measurement of the absorbance at 600 nm.

2.7. Wound healing assay

A549 and HT29 cells migration was assayed by wound/healing assay as described previously [34]. Confluent cells in 35-mm-diameter dishes were damaged by scraping with a sterile pipette tip (500 μm in diameter). The cultures were washed twice with phosphate-buffered saline to remove cellular debris. Vehicle control and various concentrations of rutin were added to the respective wells for the indicated times. After 18 h, the cells that had migrated into the denuded area were photographed using an Olympus inverted microscope. Migration was quantified by measuring covered surface.

2.8. Statistical analysis

The results were expressed as means ± S.D. from at least three independent experiments. Statistical analysis was performed using

unpaired Student's test. The value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cell viability in tumors cell lines

We first evaluated the cytotoxicity of rutin (31.25–1000 mM) after 24 h of incubation on the different cancer cell lines (A549, HT29 and Caco-2) using MTT assay. As illustrated in Fig. 1, rutin was able to inhibit the viability of A549, HT29 and Caco-2 cells in a dose-dependent manner. A significant difference in growth-inhibitory effects of this compound on A549, HT29 and Caco-2 cells was revealed. Rutin was most efficient in HT29 cell with an estimated IC_{50} value of 388.33 ± 4 μM after 72 h. However, A549 and Caco-2 cells seemed to be more resistant to flavonoid treatment with IC_{50} values of 559.83 ± 3.5 μM and 710.59 ± 3.5 respectively.

3.2. ROS scavenger activity of rutin

As high level of ROS production exists in tumor cells, ROS production has been linked either to the cell proliferation or cell death. We investigated the effect of rutin on superoxide anion production in the different cell lines. The superoxide reducing ability of rutin is shown in Fig. 2. It appears that the superoxide anion reducing effect was dose dependent in the presence of rutin. Therefore, the maximum reducing effect of rutin was observed at a concentration of 100 μM against HT29 cells with a reduction percentage of $64.41 \pm 2\%$. However, at this concentration, rutin was able to reduce superoxide anion production with a reduction percentage of 56.65 ± 3 and $31.12 \pm 1.5\%$ against A549 and Caco-2 cells respectively. This result indicates that rutin is more effective in HT29 cells.

3.3. Rutin affects adhesion of tumour cells

It has long been known that most cell types require attachment to grow. As rutin inhibits tumor cell proliferation, we investigated whether it can act through the blocking of cell attachment. In order to investigate the rutin effects on the behaviour of human non-small cell lung cancer A549 and HT29 colonic adenocarcinoma cells, we first performed cell adhesion assays using a large array of purified ECM proteins.

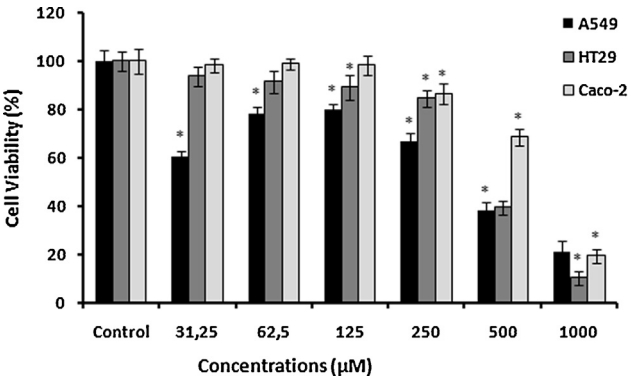


Fig. 1. The Inhibitory effect of rutin on the proliferation of Human lung (A549) and colon (HT29 and Caco-2) cancer cell lines. Cells were plated at 5×10^3 cells per dish in the absence and the presence of rutin (31.25–1000 μM). Time-dependent decrease in the number of Human lung (A549) and colon (HT29 and Caco-2) cancer cell at the indicated concentrations. As control, the cells were treated without or with 0.1% DMSO (vehicle). Data are means ± S.D., n = 3 per group.

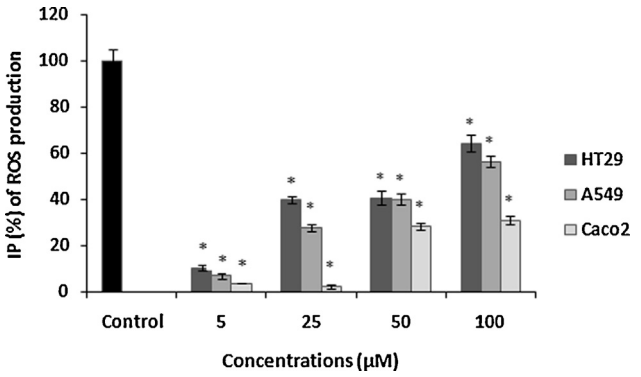


Fig. 2. Production of O_2^- in Human lung (A549) and colon (HT29 and Caco-2) cancer cell lines exposed to rutin (5–100 μM). Production of O_2^- was determined by lucigenin luminescence. The cells were plated at 50×10^3 cell/well. Data from at least three independent experiments have been pooled. Rutin significantly decreased O_2^- production in the A549 cell compared with untreated cells (*$P < 0.05$). Data are means ± S.D., n = 3 per group.

As illustrated in Fig. 3, rutin impaired attachment in a dose-dependent manner of both A549 and HT29 cells to fibronectin (inhibition of cell adhesion by $47 \pm 2.5\%$ for A549 cells and $38 \pm 1.9\%$ for HT29 cells in presence of rutin 200 μM). An

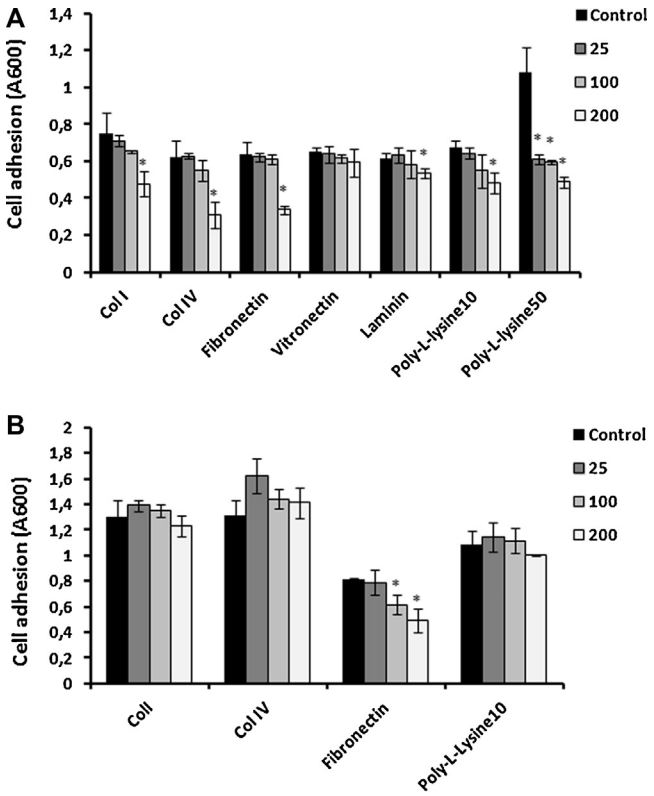


Fig. 3. Ruitn inhibits cell adhesion. (A) A549 cells were seeded at 10^6 cells/ml and were preincubated with various concentrations of rutin (25, 100 and 200 μM) for 30 min at room temperature. The cells were then added to 96-well microtiter plates coated with fibronectin (10 μg/ml), vitronectin (10 μg/ml), laminin 1 (10 μg/ml), fibrinogen (50 μg/ml), collagen type I and IV (10 μg/ml), poly-L-lysine (10 and 50 μg/ml) and allowed to adhere for 1 h at 37 °C. (B) HT29 cells were seeded at 10^6 cells/ml and were preincubated with various concentrations of rutin (25, 100 and 200 μM) for 30 min at room temperature. The cells were then added to 96-well microtiter plates coated with fibronectin (10 μg/ml), collagen type I and IV (10 μg/ml) and poly-L-lysine (10 μg/ml) and allowed to adhere for 2 h at 37 °C. After washing, adherent cells were stained with crystal violet, solubilized by SDS and absorbance was measured at 600 nm. Data are means (±S.D) of at least three independent experiments and expressed as a percentage of adhesion. * indicates statistically significant results compared to control ($p < 0.05$).

impairment of attachment to collagen type I and IV was observed in A549 (inhibition of cell adhesion by 36.6 ± 2 and 50.2 ± 3% respectively). In contrast, no effect of rutin in attachment of HT29 cells to collagen type I and IV was observed (Fig. 3).

3.4. Rutin inhibits tumor cell migration

Cell migration can be considered as a finely regulated process including successive steps of cell adhesion and de-adhesion. Because rutin inhibits A549 and HT29 cell adhesion, we also examined its effect on cell migration using a haptotaxis assay in modified Boyden chambers. As shown in Fig. 4, rutin at 100 and 200 μM blocked A549 and HT29 cell migration. This inhibition was dose-dependent with migration percentage of 47.9 ± 1.5 and 38.61 ± 1% in the presence of type IV collagen and 52.75 ± 1 and 9.48 ± 0.5% in the presence of fibronectin towards A549 cells with rutin at 100 and 200 μM, respectively. For HT29 cells, the migration percentage values were 55.19 ± 2.5 and 37.63 ± 1.5% towards type IV collagen and 54.49 ± 3 and 37.82 ± 2% towards fibronectin with rutin at 100 and 200 μM, respectively. The effect of rutin was also confirmed by using wound/healing assay. Scrape damaged A549 and HT29 monolayers were incubated in the presence of rutin (25–200 μM) for 18 h. Control cells completely covered the wounded area after 18 h of incubation at 37 °C. In contrary, treatment with rutin strongly reduced wound repair and significantly inhibited the migration of both A549 (Fig. 5A and B) and HT29 (Fig. 5C and D) cells in a concentration-dependent manner.

4. Discussion

Natural plant extracts and their flavonoids have been shown to display anti-cancer properties both *in vitro* and *in vivo*. The discovery of bioactive compounds as well as the clarification of their mechanism in the anticancer activity is crucial. Rutin, one of the major representatives of flavonoids, is present in many natural plants [35]. It has been used in a wide range of pharmacological applications. However, current research has shown its multispectral pharmacological benefits for the treatment of various chronic diseases such as cancer, diabetes, hypertension, and hypercholesterolemia [24,36–38]. In this study, we investigated the anti-tumour potential of rutin in terms of inhibition of cell viability, superoxide production, adhesion and migration of human cancerous cells.

The effect of rutin on the proliferation of human lung (A549) and colon (HT29 and Caco-2) cancer cell lines was studied for 72 h period. The proliferation of the various tested cell lines was significantly inhibited by rutin over a concentration range of 31.25–1000 μM in a dose-dependent manner. Rutin has shown cytotoxic effects on several human cancer cells [21,22,39] including human colorectal cancer cells [23,24]. Our results corroborate the cytotoxic effects of rutin previously reported. Araujo et al. [40] showed that rutin is able to inhibit cancer cell growth by cell cycle arrest and/or apoptosis, along with inhibition of proliferation, angiogenesis, and/or metastasis in colorectal cell lines. Moreover, other studies demonstrated the same effect of rutin on human U138MG and GL-15 glioma cell lines and murine leukemia WEHI-3

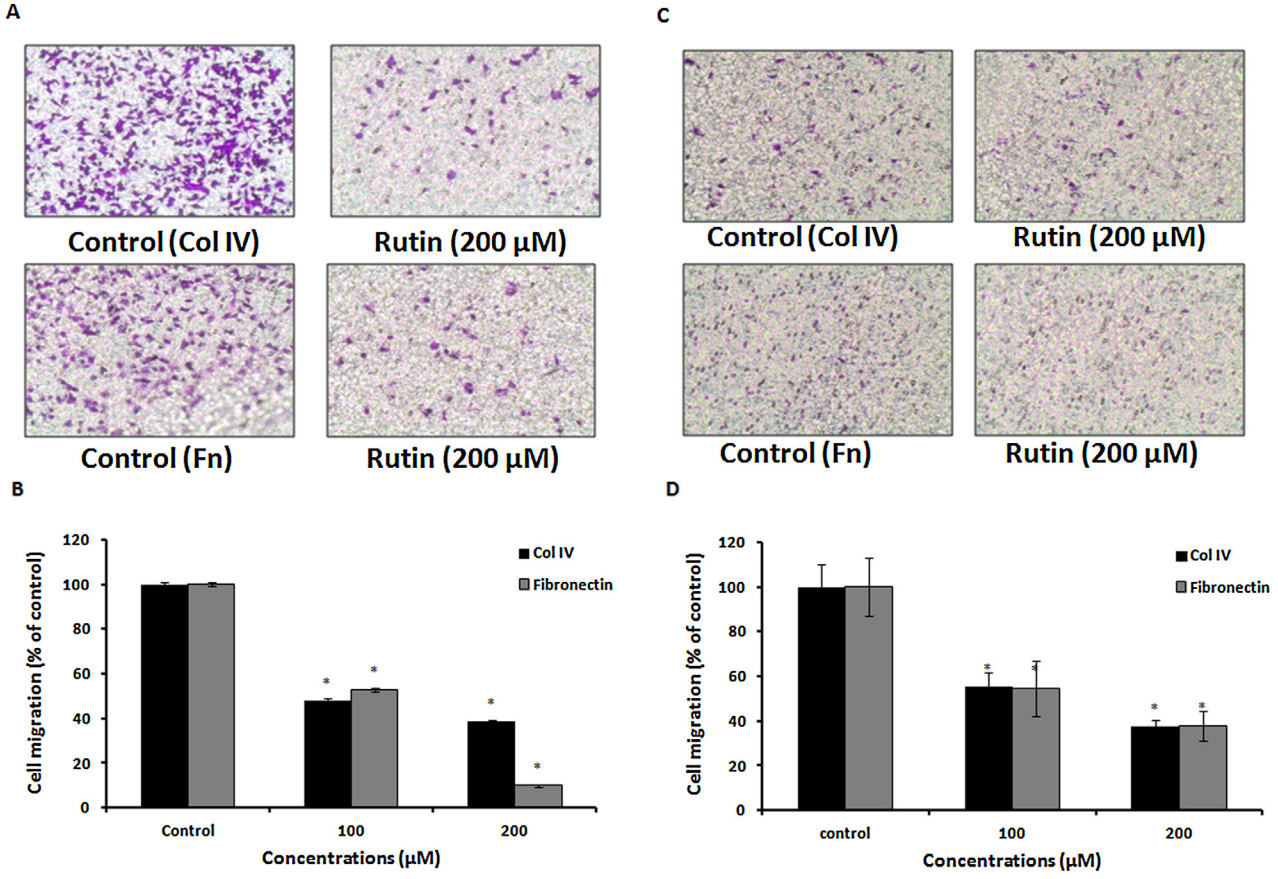


Fig. 4. Rutin inhibited Human lung A549 cell migration and colon (HT29) cancer cell lines. (A) and (C) Cell motility was measured in a modified Boyden chamber using porous membrane (8 μm) coated with 10 μg/ml of fibronectin and collagen type IV. After pre-treatment with 200 μM of samples for 30 min at room temperature, cancer cells were seeded into the upper reservoir and allowed to migrate through the filter towards the lower reservoir for 5 h at 37 °C. The cells that migrated to the underside of the filter were stained with 0.1% crystal violet. Scale bar = 100 μm (B) and (D) to quantify cell migration towards fibronectin and collagen type IV, crystal violet was solubilized with 1% SDS and absorbance was measured at 600 nm. Data are means ± S.D; n = 3 per group. * indicates statistically significant results compared to control ($p < 0.05$).

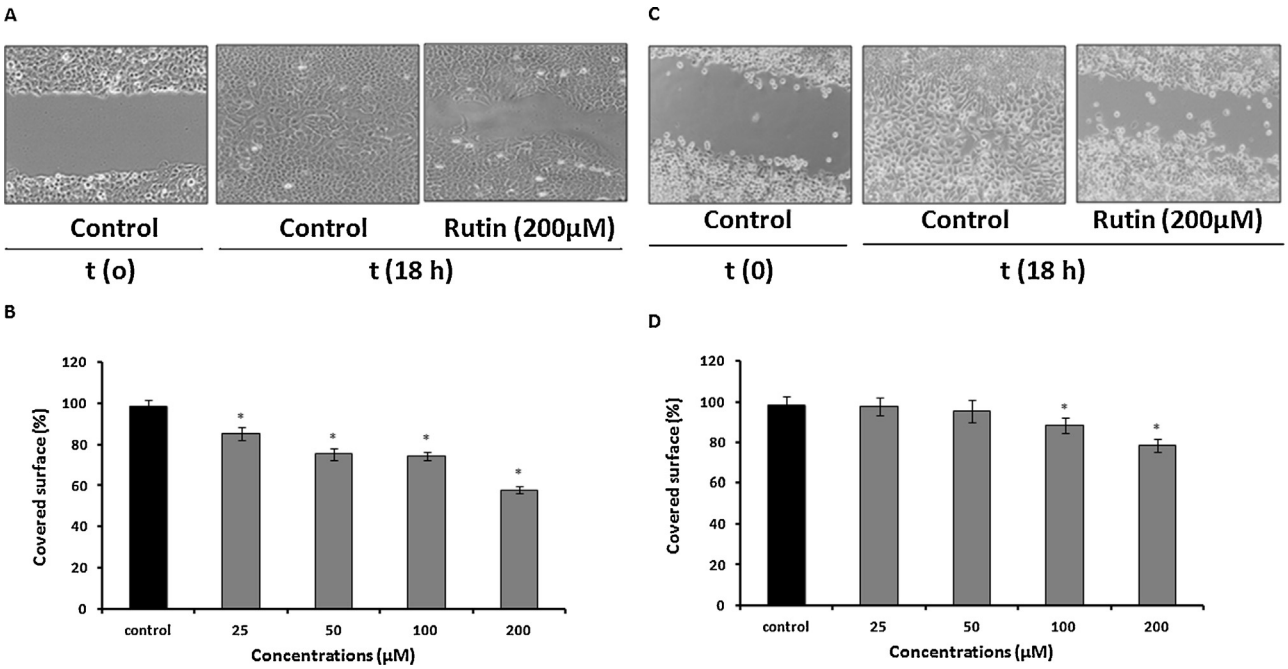


Fig. 5. The Inhibitroy effect of rutin on the migration of Human lung (A549) and colon (HT29) cancer cell lines. The cells were stimulated with rutin (25–200 µM) for 18 h as a negative control; the cells were incubated with 0.1% DMSO (vehicle). (A) and (C) Photomicrographs showing A549 and HT29 migration into the denuded area in the absence and presence of rutin (25–200 µM). The zone between the two lines indicates the area occupied by the initial wound. Scale bar = 100 µm the data are representative of three independent experiments. (B) and (D) Covered surface of migrated A549 and HT29 cells in the absence and the presence of rutin (25–200 µM). Data are means ± S.D; n = 3 per group. * indicates statistically significant results compared to control ($p < 0.05$).

cells [41]. Compared to quercetin, rutin demonstrated a range of different effects, either inhibiting [42–45] or not altering colorectal cancer cell proliferation [46]. Furthermore, rutin showed a less antiproliferative potential against HT-29, HCT 116, MCF-7, HepG2, and A549 cells, but the transformation of rutin to quercetin-3-β-D-glucoside showed the most potent growth inhibition [47].

ROS are highly reactive molecules that can impair cell structures such as carbohydrates, nucleic acids, lipids and proteins and alter their function [48]. The oxidative stress is considered a fundamental factor in increasing persistent degenerative disorders, such as coronary heart diseases, cancer and aging [49,50].

Previous studies have suggested that the anticancer effect of many antioxidants may be due to their capability to scavenge free radicals [51,52]. Some tumor cells, such as human colon adenocarcinoma HT29 and melanoma HCT15 cells can produce high levels of ROS [53]. In the present study, we confirmed that the ROS level decreased in all the tested tumor cell lines after rutin treatment particularly in HT29 cells.

Rutin, is also known as Vitamin P and has antiplatelet, antiviral, and antihypertensive properties, as well as strength in the capillaries, which are the result of its high radical scavenging activity and antioxidant capacity [54]. Gong et al. [55] found that rutin protected human vein endothelium cells (HUVEC) against H_2O_2-induced apoptotic cell death. It that study, the protective effects of rutin was attributed to regulation of glutathione reactive oxygen species.

Rutin analog quercetin (quercetin rutinoside) has been reported to reduce both the risk and progression of cancer through their decrease in ROS production [56,57]. It protects cells from oxidative stress, inflammation, and DNA damage due to its antioxidant properties and modulates growth of many cancer cell lines by blocking cell cycle progression in the G0/G1 phase, the G2/M-phase, and the S-phase, reducing tumor cell proliferation, inducing apoptosis and caspase-3 activity and inhibition of VEGF production [58–63].

Furthermore, quercetin also known to enhance the GSH/GSSG ratio and the catalase activity in melanoma cells. In the study of Kim [64], it was described that quercetin decrease and modulate the ROS production induced melanogenesis not only by reducing the total level of all reactive species but also by developing the antioxidative $ONOO^-$ scavenging performance, the GSH/GSSG ratio and catalase activity in melanoma cells.

A relevant interest is present on the mechanisms of action of chemopreventers, especially concerning the identification of molecular and cellular targets of natural compounds, and the molecular basis of their cancer-preventive action. Indeed, the possible role in cancer chemoprevention of flavonoids can be attributed to their capacity of quenching ROS, reactive nitrogen species (RNS) and other radicals [65]. Additionally, flavonoids exert their potential antioxidant effect not only by quenching ROS, but also by modulating the activity of several detoxifying enzymes, including lipoxygenase, cycloxygenase, inducible nitric oxide synthase, monoxygenase, xanthine oxidase and NADH oxidase [66–68]. Among enzymes that are inhibited by flavonoids, thioredoxin reductases have to be quoted, as they are involved in cellular redox control, and are overexpressed in different aggressive tumors [69]. Moreover, Flavonoids induce apoptosis or caspase activity, inhibit cell proliferation and/or cyclin-dependent kinases cancer (CDK) activities and also block different cell cycles from cell lines [50].

Considering the presence of polyphenols in vegetables and other foods [70], they can therefore be used in the remedy or as complementary agents in the treatment of cancer [50,71]. Currently, the development of herbal medicines for the inhibition of angiogenesis, in combination with conventional anticancer therapies, is amongst the most intensively studied approaches for cancer prevention [72,73].

Cell adhesion is a critical process in many biological phenomena such as development, tissue structure maintenance, angiogenesis, and tumour metastasis. In the absence of appropriate ECM contacts, mainly mediated by integrins, the cells undergo

programmed cell death [74,75]. Therefore, the characterization of new anti-integrin agents is of considerable utility for the development of therapies [76].

In the present study, we demonstrated that rutin affected adhesion of A549 mainly on all the tested matrix and HT29 cells, particularly on fibronectin. This effect could lead to the suppression of the constitutive integrin activation, disrupt cell–ECM interactions and block the tumor adhesion process via influencing the CAMs expressions. In relation study, Nasr Bouzaiene et al. [77] demonstrated that the phenolic acids affect adhesion of A549 and HT29-D4 cells.

Cancer cell metastasis involves multistep cellular processes, commencing with cell migration and invasion. Cell migration is tightly regulated by coordinated remodeling of membrane and actin cytoskeleton. Although the detailed mechanisms are not yet understood, it is clear that dynamic and reciprocal interactions between cell adhesion molecules, ECM and soluble factors are essential.

The inhibitory effect of rutin on cell migration is likely due to the reduced attachment to ECM proteins observed in the presence of this flavonoid. The same effect was observed with others flavonoids against lung cancer A549 cells and human colorectal adenocarcinoma DLD1 and SW480 cells [78,79].

5. Conclusion

In summary, the findings of the current study show, for the first time, that rutin was used as a multifunctional agent to decrease cell adhesion and migration of human lung and colon cancer cells, to inhibit proliferation and to reduce the production of ROS. All these results showed that rutin can be one of the major effective ingredients in the therapeutic effect of cancer diseases. Further studies are necessary to improve our understanding of thetherapeutic effects of rutin.

Conflict of interest

The authors declare that they have no conflict of interest.

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