JBACHS
Journal of Basic and Clinical Health Sciences

J Basic Clin Health Sci 2022; 6: 569-578
https://doi.org/10.30621/jbachs.1056769
RESEARCH ARTICLE

THE EFFECT OF QUERCETIN AND QUERCETIN-3-D-XYLOSIDE ON BREAST CANCER PROLIFERATION AND MIGRATION

Tugba Nurcan Yuksel [1], Esra Bozgeyik [2], Muhammed Yayla[3]

[1] Tekirdag Namik Kemal University, Faculty of Medicine, Department of Pharmacology, Tekirdag, Turkey.
[2] Adiyaman University, Department of Vocational School of Health Services, Medical Laboratory Techniques, Adiyaman, Turkey.
[3] Kafkas University, Faculty of Medicine, Department of Pharmacology, Kars, Turkey

ORCID: T.N.Y. 0000-0001-5092-1674; E.B. 0000-0002-8726-3182; M.Y. 0000-0002-0659-3084

Address for Correspondence: Tugba Nurcan Yuksel, **E-mail:** tnyuksel@nku.edu.tr
Received: 12.01.2022; **Accepted:** 14.03.2022; **Available Online Date:** 30.05.2022

Cite this article as: Yuksel TN, Bozgeyik E, Yayla M. The Effect of Quercetin and Quercetin-3-D-Xylosıde on Breast Cancer Proliferation and Migration. J Basic Clin Health Sci 2022; 6: 235-244.

ABSTRACT

Background and Purpose: The aim of this study is to investigate the migration, wound healing, colony formation, and cytotoxic effects of Quercetin-3-D-xyloside (reynoutrin), a quercetin derivative, in breast cancer cells

Methods: In the present study, CRL-4010, MCF7 and MDA-MB-231 cells were used to evaluate the cytotoxic, antiproliferative and migration effects of reynoutrin on breast cancer. The IC50 concentration (400 µg/ml) of reynoutrin, quercetin and cisplatin in the cells was determined. For cytotoxicity assessments, varying concentrations of quercetin, reynoutrin and cisplatin were applied and incubated 24h and 48h. In addition, to examine effects of reynoutrin on migration, cells were seeded in 6-well plates and incubated for 24 hours. For the colony formation assay cells were seeded to 12-well plates at a concentration of 1000 cells/well and incubated overnight.

Results: These results indicated that reynoutrin markedly inhibit the cell viability in breast cancer.

Conclusion: We have demonstrated for the first time with the present study that reynoutrin suppressed the progression of breast cancer cell proliferation induction and may provide a potential therapeutic target for breast cancer treatment. However, these results should be further confirmed by future more comprehensive studies.

Keywords: Reynoutrin, quercetin, cytotoxicity, breast cancer, migration

INTRODUCTION

Breast cancer is a continues a very widespread disease among women, with an yearly global rate of estimated 2.3 million new cases (1) and one of the highest counts of cancer-related deaths among women (2). In recent years, the incidence of breast cancer in young women has continued to increase (3). In addition, breast cancer has the potential to metastasize to secondary tissues such as liver, lung and bone (4) , which is the primary reason of cancer-related deaths (5).

The prognosis of breast cancer patients has improved, thanks to advances in early diagnosis and comprehensive treatment strategies. (6). Nevertheless it is not easy to find an efficacious therapy for breast cancer due to the complex heterogeneity of different molecular subtypes (7) and

course of breast cancer disease varies from patient to patient (8).

Breast cancer therapy comprises a multiple complex strategy which involves neoadjuvant chemotherapy, endocrine therapy, radiotherapy and adjuvant chemotherapy or surgery of operable tumors (9). Despite the treatment methods used, the recovery rate of breast cancer is still highly poor, and therapy induces significant adverse effects, such as hematopoiesis and immune compromise (10). In addition, cost and resistance to drug therapy limit current treatment strategies (2). The restrictions and high incidence of therapeutic strategies emphasize the importance of exploring prevention strategies that lack important side effects.

Flavonoids are a huge group of polyphenolic compounds that are ubiquitously found in plants and appear as glycosides, aglycones and methylated derivatives. The activities of flavonoids are significantly affected by their features include the degree of conjugation, polymerization and hydroxylation. A lot of proofs showed that flavonoids protected the body from diverse health problems such as, ulcers, cardiovascular illness, inflammation and diabetes (11, 12). Further, flavonoids are well known for potential for use as nutraceuticals (13), including antioxidant (14), antimicrobial (15), antithrombotic (16) and anticancer (17) effects (18).

The most of flavonoid structures are based upon the quercetin skeleton (19). Among diverse flavonoids, quercetin is of great interest because of its strong bioactivities (20). Quercetin is a natural compound ubiquitously found in various food sources such as fruits, vegetables, seeds, nuts, and wine (21). One of these forms, the quercetin-3-*O*-flavonoid glycoside has been accepted as a pattern compound for the research of flavonoids (22).

Quercetin-3-D-xyloside (reynoutrin), one of the quercetin glycosides, is a natural flavonoid that exists in the fruits and leaves of an assorted of natural plants and has been demonstrated to have potential antiviral properties (23) and antioxidant properties (24). Results showed that reynoutrin substantially remedied cardiac function, inhibited cardiomyocytes apoptosis, depressed the release of inflammatory factors, decreased oxidative stress, and attenuated myocardial fibrosis in rats with ischemic heart failure (25).

There are many studies on breast cancer in the literature. Studies on reynoutrin, which is found in different plant extracts, are scarcely no more important, there is no study in the literature examining the effects of non-extract reynoutrin on breast cancer. In the light of all this information, in the present study, we investigated the effects of reynoutrin on breast cancer.

METHODS

Cell culture

CRL-4010, MCF7 and MDA-MB-231 were commercially obtained and maintained in carbon dioxide (5 %) incubator at 37 °C and 95 % humidity. For the maintenance of cells Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Germany) complete growth medium containing 10 % fetal bovine serum (FBS) was used. Trypsin-EDTA solution was used to detach cells reaching 90 % confluency and pelleted at 1500 rpm for 4 minutes and used for further application.

Cytotoxicity Experiments

Confluent cells were trypsinized, counted using Thoma cell counting chamber and $1x10^5$/ml cells plated to 96-well plates for the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (Sigma-Aldrich, Germany) cell viability assay. After overnight incubation, varying concentrations of quercetin, reynoutrin and as a reference potent anti-cancer drug cisplatin (26, 27) were applied and incubated for 24h and 48h. Subsequently, supernatants were discarded, and cells were rinsed with 1X PBS (pH: 7.2) buffer several times. Following that cells were subjected to MTT incubation until the formation of insoluble formazan particles. Formed formazan particles were dissolved with Dimethylsulfoxide (DMSO) and viability of cells were measured under 570 nm in a Thermo Scientific Multiskan GO (Thermo Fisher Scientific, USA). GraphPad prism 8 software were used for the calculation of IC_{50} values.

Wound healing assay

For the wound healing assay $1x10^6$ cells were seeded into 6-well plates and incubated for 24 hours to obtain a confluent cell monolayer. Using a 100 µl pipette tip, a gap was formed at the center of the culture plates from top to bottom and wells were carefully washed twice with 1X PBS solutions and 0. hour pictures of gaps were visualized and recorded. Effective doses of reynoutrin and quercetin were applied to the cells that reached the appropriate confluency and

Table 1. IC50 concentration (400 µg/ml) of reynoutrin, quercetin and cisplatin in cells

Chemicals	CRL-4010		MCF7		MDA-MB-231	
	24h	48h	24h	48h	24h	48h
Reynoutrin	487.3	641.8	337.4	302.0	469.2	552.8
Quercetin	75.9	47.8	36.2	32.9	46.8	25.1
Cisplatin	13.1	11.3	6.25	6.15	21.4	18.4

incubated for 24 hours. In order to observe the effects of reynoutrin and quercetin on the migration capacities of cells, the first photographs of the wounded area was taken at every 24-hour time periods using an inverted microscope. Gaps were measured and analyzed using ImageJ software.

Colony Formation Assay

For the colony formation assay cells were seeded to 12-well plates at a concentration of 1000 cells/well and incubated overnight. Subsequently, effective doses of the substance were administered and the medium was changed after 24 hours. The growth medium was refreshed every three days. After the formation of visible colonies, the medium was discarded and wells were rinsed with the PBS several times. Cells were later fixed using 70 % ethanol solution and incubated at room temperature for 30 minutes. Following fixation, ethanol was discarded, and colonies were stained with 0.5 % crystal violet dye for 20 minutes. Excess dye was subsequently

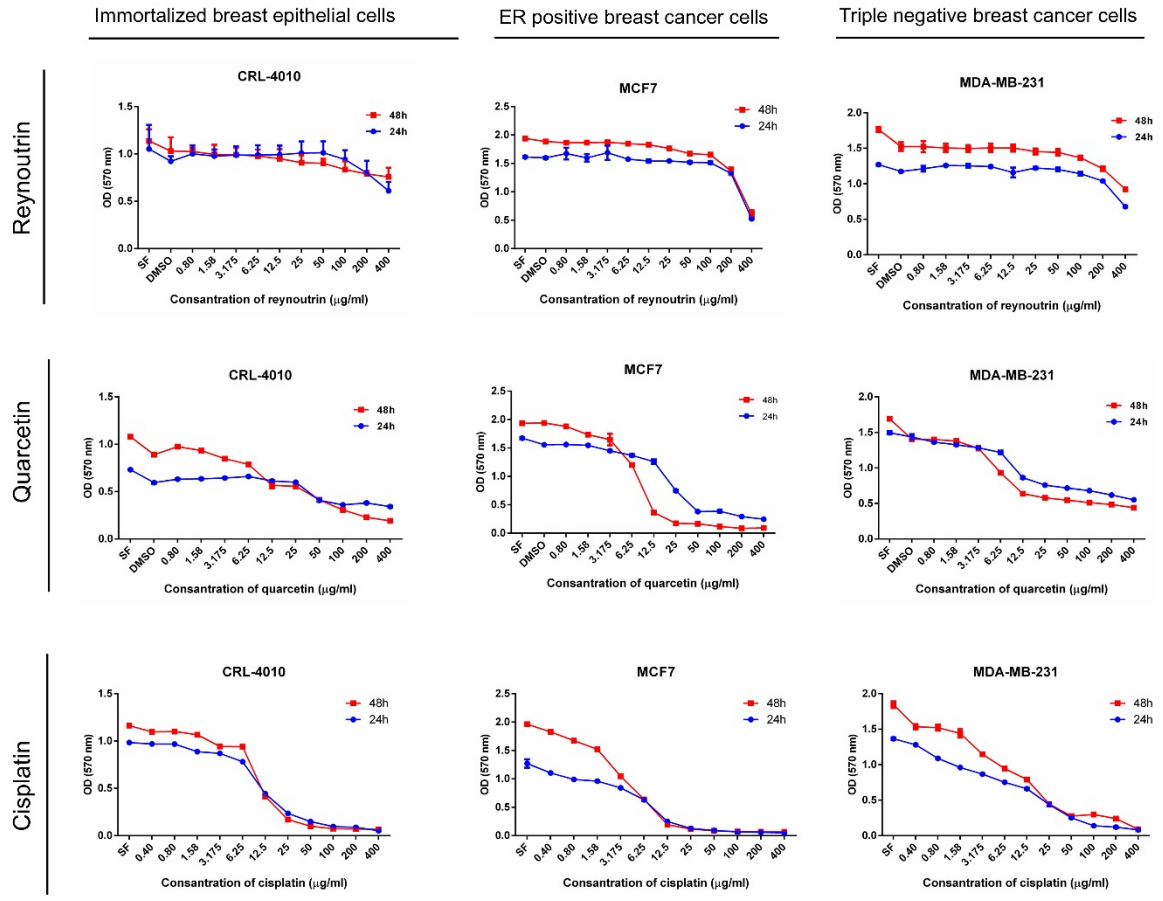

Figure 1. Demonstration of the cytotoxic effects of different concentrations (0 to 400 µg/ml) of reynoutrin, quercetin and cisplatin on breast epithelial cells and breast cancer cells for 24- and 48-hour time periods.

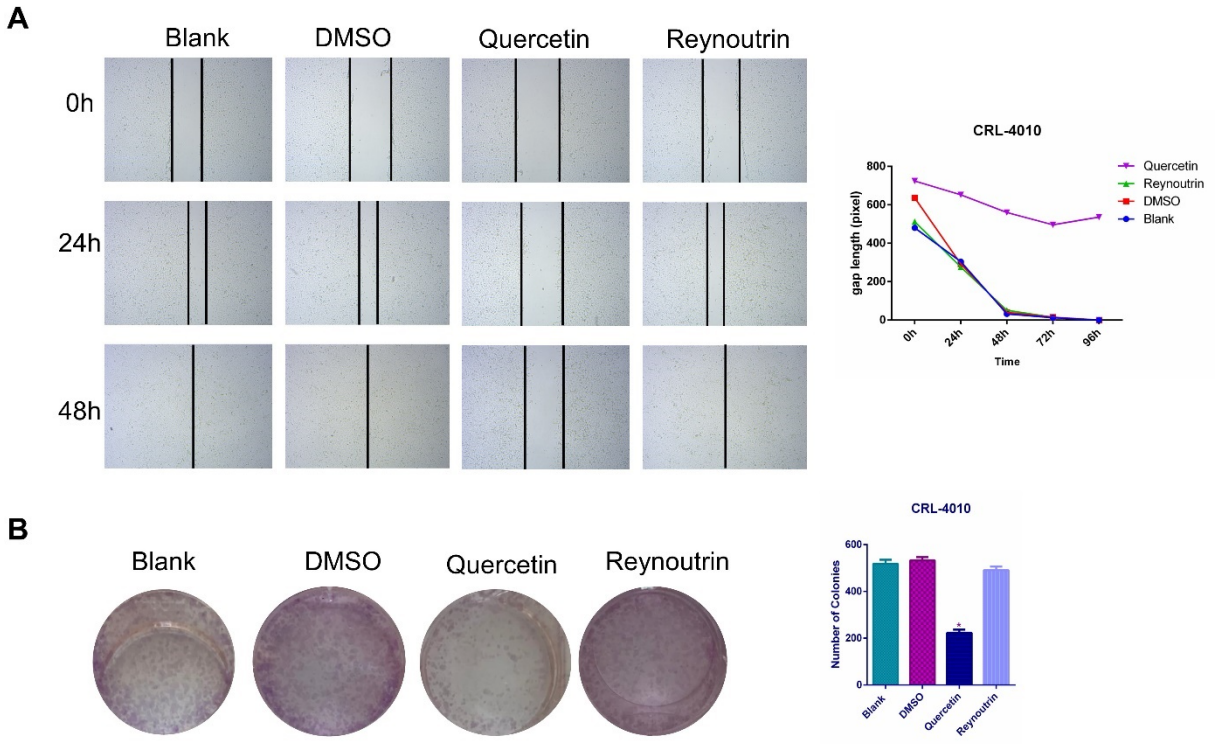

Figure 2. Demonstration of the effects of reynoutrin and quercetin on cell migration and colony formation in CRL-4010 cells. A. Cell migration, B. Colony formation. "*" indicates p=0.039.

rinsed with ddH_2O. Plates were air-dried, and colonies were counted.

RESULTS

Cytotoxic effects of reynoutrin

To reveal the cytotoxic effects of reynoutrin we used CRL-4010 (normal breast epithelial cells), MDA-MB-231 (triple negative breast cancer cells) and MCF7 (ER positive breast cancer cells). Given that reynoutrin is a quercetin derivative, we explored effects of both reynoutrin and quercetin in our study. Also, as shown in Figure 1, the potent anti-cancer drug cisplatin was used as reference. As a result, reynoutrin did not show any cytotoxic effect on CRL-4010 cells. When we evaluated anti-proliferative effects of reynoutrin; it was found to be not effective in MDA-MB-231 cells after 24 and 48 hours of incubation, even at the maximum concentration of 400 µg/ml. In contrast, reynoutrin was found to inhibit cell viability of MCF7 cells at the highest concentration of 400 µg/ml. At this concentration, reynoutrin was not cytotoxic in CRL-4010. Moreover, quercetin was found to significantly inhibit the cell viability of CRL-4010, MDA-MB-231 and MCF7 cells in a dose dependent manner. Also, cisplatin as an anticancer agent significantly inhibited the cell viability of CRL-4010, MCF7 and MDA-MB-231 cells in a dose dependent manner. It is very interesting that reynoutrin has an antiproliferative effect only in MCF-7 cells at a dose of 400 mg and that it has no toxic effect especially in normal breast cells. In this context, while quercetin and cisplatin show cytotoxic effects in healthy and cancer cells, the fact that reynoutrin only shows activity in MCF-7 cells in a certain dose and it suggests that it may act with a different mechanism rather than a cytotoxic effect. This situation might be an advantage in anticancer treatment because one of the most significant problems in cancer therapy is the cytotoxicity of anticancer drugs on healthy cells.

The inhibitory concentrations of reynoutrin and quercetin were calculated according to the DMSO and SF group. The inhibitory concentrations of reynoutrin, quercetin and cisplatin were presented in Table 1. Collectively, these finding suggest that reynoutrin has no significant anti-cancer activity in breast cancer. The activity of reynoutrin was found to be much lower than that of quercetin.

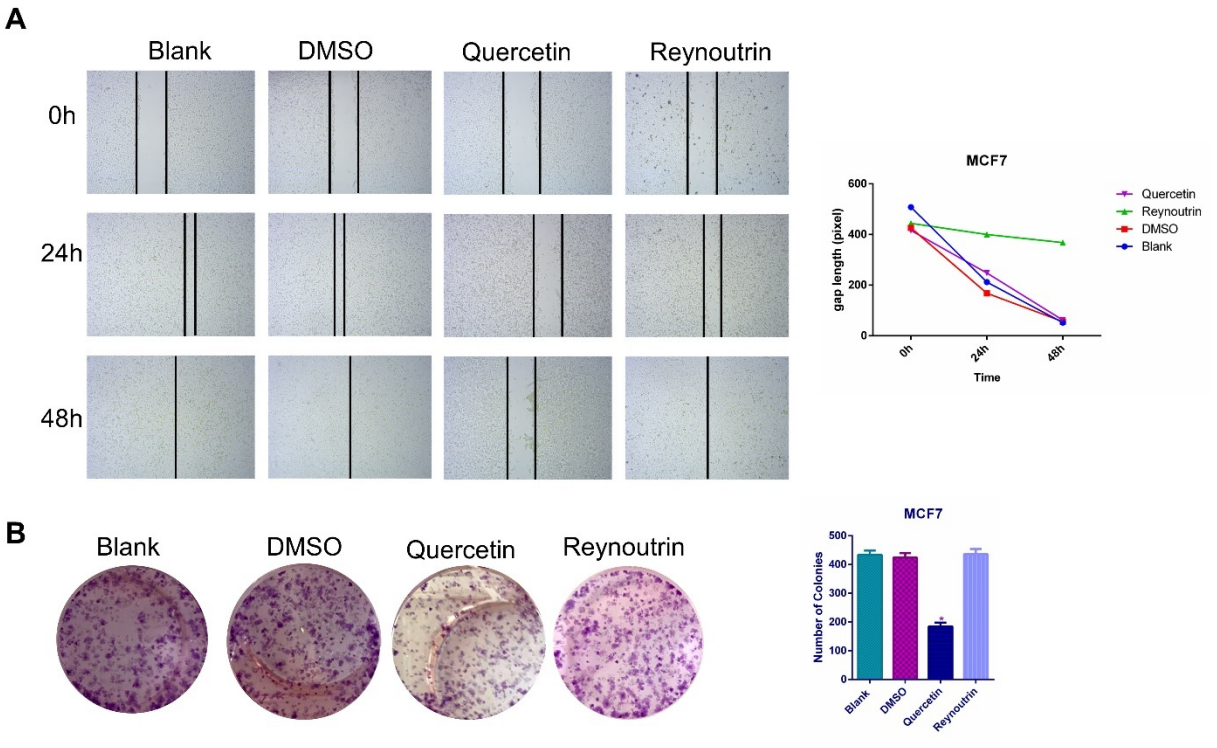

Figure 3. Demonstration of the effects of reynoutrin and quercetin on cell migration and colony formation in MCF7 cells. A. Cell migration, B. Colony formation. "*" indicates p=0.023.

The effect of reynoutrin on wound healing and colony formation

We further showed the effect of reynoutrin on migration and colony forming capabilities of CRL-4010, MCF7 and MDA-MB-231 cells. In consistent with the cytotoxic effects of reynoutrin, our results showed that reynoutrin has no marked inhibitory activity on the migration and colony forming capabilities of CRL-4010 cells as compared to blank wells and DMSO treated wells as presented Figure 2. In contrast, quercetin was significantly interfered with the migration and colony forming capabilities (p=0.039) of CRL-4010 cells as shown in Figure 2. Similarly, reynoutrin was not significantly altered the colony forming and migration capabilities of MCF7 and MDA-MB-231 cells whereas significant inhibition of migration and colony formation was observed in MDA-MB-231 and MCF7 cells treated with quercetin as shown in Figures 3 & 4.

DISCUSSION

Cancer is one of the most important causes of death in both underdeveloped and economically developed countries. It has been determined that cancer is increasing in economically developed countries due to low birth rate, smoking and adopting a sedentary lifestyle. (28).

Cancer, which has a high mortality rate worldwide, is a major threat to people's mental and physical health (29). Previous studies have indicated that cancer markers include maintenance of proliferation, inhibition of cell death, avoidance of growth inhibitory factors, angiogenesis, replicative immortality, and enhanced metastasis and invasion. The maintenance of these biological functions is due to genetic variation caused by genomic instability (30).

Breast cancer is one of the most widespread malignant cancer and the leading cause of cancer-related deaths among women in the world. Lately, breast cancer patients are categorized by the AJCC staging system and histologic classification (31). Moreover, because of the high level of heterogeneity in breast cancer, patients with analogous clinical status may have dissimilar prognoses. Therefore, it becomes essential to include other significant factors to better guide the clinical treatment and develop the prognosis of breast cancer patients (32).

Combinations of chemotherapeutic agents are preferred because surgery alone increases the likelihood of recurrence. However, depending on the

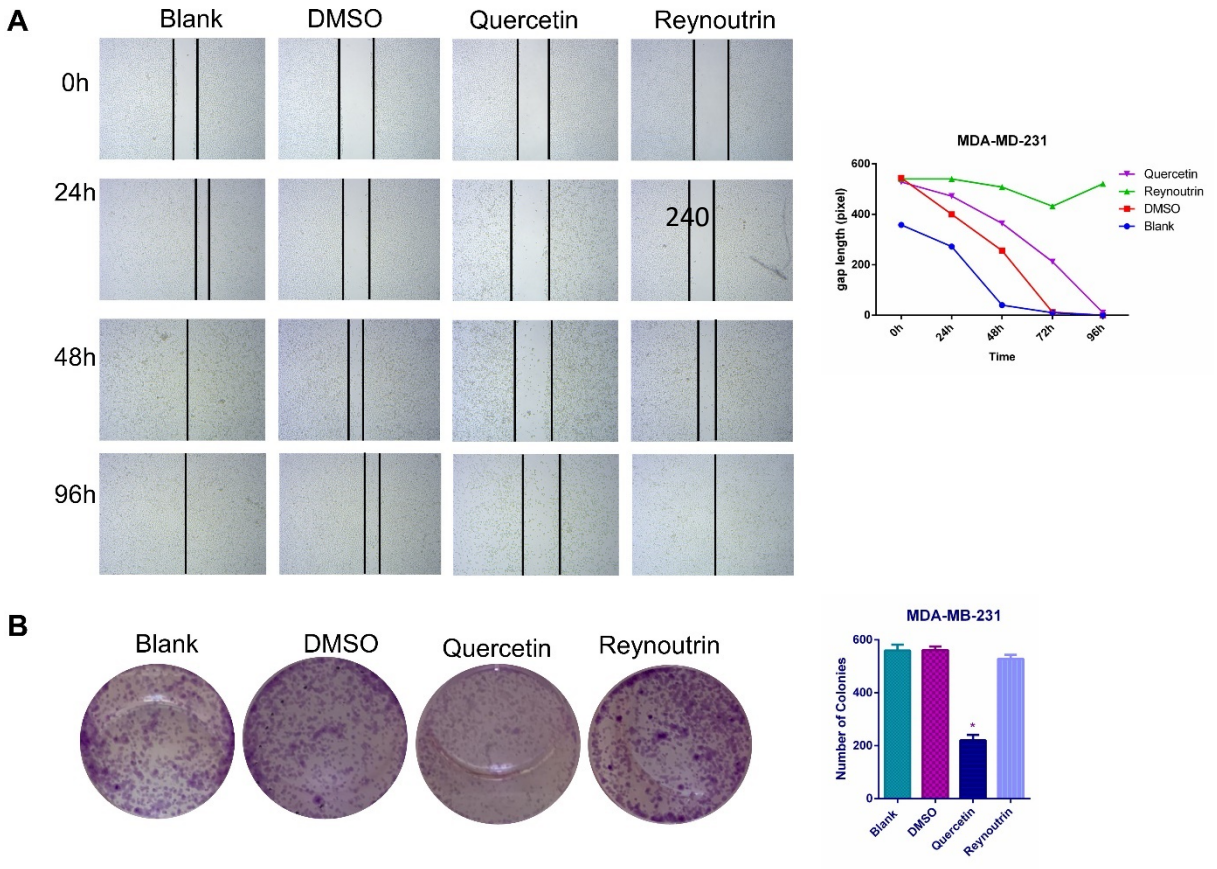

Figure 4. Demonstration of the effects of reynoutrin and quercetin on cell migration and colony formation in MDA-MB-231 cells. A. Cell migration, B. Colony formation. "*" indicates p=0.0408

person, the drug and the dose, nausea, vomiting, mouth sores, loss of appetite, diarrhea, fatigue, hair loss, and bone marrow suppression may occur.

Today, it is well established that women with breast cancer are treated with combined methods, including radiation therapy, chemotherapy, and surgery. Ongoing research efforts are making treatment modalities more individualized in hopes of both minimizing side effects and improving overall survival (33). Despite recent advances in cancer treatment, current proof-based demonstrates against breast cancer has been slow over the past decade. This translates to several months of survival in the metastatic area. Unfortunately, this is not surprising given the significant restriction of the targeted therapies existing. Reasons for the high rates of acquired and intrinsic resistance to current targeted drugs contain transient antitumor activities and failure to take into account intra-tumor and inter-patient heterogeneity. Understanding this highly complex heterogeneity is of great importance in the fight against breast tumorigenesis and metastasis (34).

In most of the breast cancer cases, the expression level of estrogen receptor alpha is directly proportional to tumor cell growth (35). For this reason, the MCF-7 cell model has been investigated commonly to define the mechanism of estrogen-stimulated growth in tumor (36). A luminal cancer cell type and providing many data for practical information matched to other cell types, MCF-7 becomes a suitable cell line model for discovering new breast cancer entities (37). Further, an estrogen receptor-positive adenocarcinoma, MCF-7 is a breast cancer cell line displaying an intermediate step of neoplastic transformation, with low metastatic potential and a less aggressive profile (38). In addition, MDA-MB-231 cells are known to be resistant to several anti-cancer agents (39).

Unluckily, with quantum leap in compelling treatment and enhanced diagnostic skills, breast cancer remains the primary cancer among women all over the world. To tackle this complex and multifactorial illness, interceptive approach can be thought as a levelheaded and superior choice. Literature disclosed

that most patients except for cancer treatment are self-medicating with older plant-derived drugs (40). The herbs obstruct damage by progressing detoxification, decrease deadly reactions, change the action of endogenous hormones and chemicals and ailments of chemotherapy and radiotherapy. Phytoconstituents acquired from the herbs such as Allium sativum, Taxus wallichiana, Vinca rosea, Tinospora cordifolia, Panax pseudoginseng, Zingiber officinale, Viscum album have been used as a part of diverse regulations to help the body to fight malignancy all the more influentially and reduce the stinging symptoms of radiotherapy and chemotherapy (41).

Based on the status of cancer, which is a very malignant diseases, it is exigent to search a kind of drug with lower toxicity, lower side effects, and effective drug for adjuvant therapy or cancer therapy. The tumor formation and progress include multiple pathways, links and targets. The confusion of the interplay among the various links may lead to clinical reactions such as large side effects and limited therapeutic effect. Quercetin is a flavonoid compound and a natural product, and treating with a suitable dose of quercetin is non-toxic and has diverse inhibitory impacts on diverse ways of tumor creation (42).

Reynoutrin is a natural flavonoid from quercetin glycosides. At present, there are limited studies on reynoutrin. In this respect, in our study, we examined the cytotoxic, antiproliferative and migration effects of reynoutrin to evaluate whether it has a protective effect on tumor breast cells in vitro.

To reveal the cytotoxic effects of reynoutrin we used CRL-4010 cells, MCF7 cells and MDA-MB-231 cells. A study by Alraouji et.al showed that cisplatin exhibited cytotoxic activity in MDA-MB-231 cell (43). Also, Nadal-Serrano et.al. in a study showed that cisplatin inhibited the cell viability of MCF7 (44) significantly, and inhibited the cell viability of CRL-4010, MCF7 and MDA-MB-231 cells in a dose dependent manner. Similar to these results, the results of our study showed that cisplatin significantly inhibited the cell viability of CRL-4010, MCF7 and MDA-MB-231 cells in a dose dependent manner. Choi et. al. showed that quercetin induces growth in the human breast carcinoma cell line MCF-7 in their study (45). In a study by Chien et al. showed that quercetin causes cell death in human breast cancer MDA-MB-231. Also, Kabała-Dzik et. al. in a comparative study on flavonoids proved that quercetin exhibited cytotoxic activity in both MDA-MB-231 and MCF7 cells (46). In line with these results, the results of our study showed that quercetin significantly inhibited the cell viability of CRL-4010, MCF7 and MDA-MB-231 cells and produced a cytotoxic effect depending on the dose. In contrast, even at the highest concentration of 400 µg/ml, reynoutrin did not show cytotoxic effect in CRL-4010 cells and MDA-MB-231 cells. The fact that reynoutrin does not show a cytotoxic effect on normal breast cells even at high concentration indicates that it may be a safe substance. In this way, reynoutrin provides an advantage over existing anticancer drugs since it does not show cytotoxic effects on healthy cells. Thus, it can improve the patients' life qualities. In addition, it inhibited cell viability of MCF7 cells at the highest concentration of 400 µg/ml, while it had no effect on MDA-MB-231 cells. This suggests that reynoutrin may act on an estrogen-related or a different mechanism against breast cancer. More importantly, although there are studies on different subjects related to reynoutrin found in some plant extracts in the literature, there is no specific study examining the effects of reynoutrin as a raw material on breast cancer. Although these results reveal the originality of our study, more detailed studies are needed to show this effect of reynoutrin.

Further, we demonstrated the migration and antiproliferative effect of reynoutrin in our study. Umar et. al. showed that quercetin impairs migration of triple-negative breast cancer cells (47). In addition, Jia et. al. revealed that quercetin effectively suppresses cell invasion and migration in breast cancer (48). Alike, in our study, quercetin significantly inhibited the migration and colony forming capabilities of CRL-4010, MCF7 and MDA-MB-231 cells. Reynoutrin is on the other hand in parallel with its cytotoxic effects, has no marked inhibitory activity on the migration and colony forming capabilities of CRL-4010 cells as compared to blank wells and DMSO. Additionally, reynoutrin did not significantly alter the migration and colony forming capabilities of MCF7 and MDA-MB-231 cells. Additionally, the lack of a specific study in the literature examining the migration and antiproliferative effects of reynoutrin as a raw material on breast cancer reveals the originality of our study. Furthermore, to demonstrate these effects of reynoutrin are needed more and detailed studies.

CONCLUSION

In the present study, the cytotoxic, antiproliferative and migration effects of reynoutrin on breast cancer were evaluated using CRL-4010, MCF7 and MDA-MB-231 cells. We have demonstrated for the first time with the present study that reynoutrin suppressed the progression of breast cancer induction and may provide a potential therapeutic target for breast cancer treatment. These results provided new insights on the anticancer activity of reynoutrin. Also, the results of the present study showed that reynoutrin could be a promising natural product for the treatment of breast cancer in the future. More preclinical and clinical studies are needed to use reynoutrin as a natural anticancer drug in the future.

Author contributions: TNY: Conception, design and finansman for research, supervision, literature review, writing and critical review for manuscript. EB: Materials, data collection and processing analysis-interpretation for research, writing, critical review for manuscript. MY: Conception and design for research, supervision, literature review, writing and critical review for manuscript.

Conflict of interest: No conflicting relationship exists for any author in this study.

Ethical statement: Ethics committee approval was not required as it was a cell culture study.

Funding: This study was carried out without any financial support.

Peer-review: Externally peer-reviewed.

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