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The Role of Morus Nigra Extract and Its Active Compounds as Drug Candidate on Human Colorectal Adenocarcinoma Cell Line HT-29

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Abstract: According to laboratory-based *in vitro* researches, there are numerous medicinal plants and natural compounds that indicate potential as an anticancer agent. Morus nigra (M.nigra) (black mulberry) and its active components are strong candidates to be anticancer agents. The purpose of the present study was to investigate antiproliferative and antimigratory effects of M.nigra extract, its leptin Morniga G (MorG) and one of its component Chalcone 4 hydrate; and their synergistic effect in combination with cetuximab application on colorectal adenocarcinoma cell line HT-29. The antiproliferative effect was determined by impedance based proliferation assay following exposure to M.nigra extract (10%, 1%, 0.1%), MorG (0.5, 5, 50 µM) and Chalcone 4 hydrate (0.5, 5, 50 µM) for 48 hours. The antimigratory effect of M.nigra extract and its coponents, was investigated by a wound-healing assay for 48 h. According to results, Morus nigra extract and its leptin MorG reduced cell viability. After 48 hours of 200 µg/ml Cetuximab exposure with M.nigra extract and MorG at different concentrations, a significant decrease on the cell viability was detected when compared to Cetuximab application. MorG can be suggested as a potantial conjugate for targeted drug. However, futher studies are required to fully understand its mechanisms of action.

Keywords: Morus Nigra, Morniga G, Chalcone 4 Hydrate, Colorectal Adenocarcinoma, HT-29

1. Introduction

Colorectal cancer is one of the most common malignancies and a major cause of mortality throughout the world [1]. Despite abundance of different chemotherapeutic agents and studies on colorectal cancer, still a great number of patients suffer from poor prognosis. Therefore, studies on new efficient anticancer agents with lesser side effects have come into prominence. Plant phytochemicals from natural products (fruits, vegetables and herbs), are promising candidates to be anticancer agents due to the fact that they have been shown to decrease cell proliferation, induce apoptosis, inhibit angiogenesis [2] and enhance drug binding to cancerous cells [3]. Morus nigra (M. nigra) is a commonly used fruit in traditional Chinese medicine for cancer and atherosclerosis because of its antioxidant effect. Antioxidant activity of M.nigra is generally attributed to its phytochemicals such as anthocyanins and lectins. Plant lectins are a very diverse group of proteins that can bind to specific sugars or glycans found in glycoproteins and glycolipids selectively and reversibly [4]. Morniga G (MorG), a heterotetrameric lectin derived from M. nigra, was found to show specifity to T/Tn antigens [Thomsen-Friedenreich antigens (Gal β (1-3) GalNac α (1-O)-Ser/Thr)/ (GalNAcαO1-Ser/Thr)] expression

of which increase in certain cancer cells [5]. Therefore, this lectin can be used in drug targeting against tumor cells expressing T and/or Tn antigens potently.

Chalcone derivatives are one of the major classes of natural compounds and they widely present in vegetables, herbs and fruits especially in M. nigra [6].

They were shown to have anti-inflammatory, antimicrobial, antioxidant, and anticancer properties [7]. Chalcones are the secondary metabolite precursors of flavonoids and isoflovonoids [8] and were shown to have anti-inflammatory, antimicrobial, antioxidant, and anticancer properties [7]. Chalcone 4 hydrate (Figure 1) is a potent and selective inhibitor of chemokine SDF-1α [9] which has been shown to induce tumor growth, increase angiogenesis, play role in metastasis and involved in immunosupressive network of tumor microenvironment [10]. Chalcone 4 hydrate is also a small molecule anti-ligand of CXCR4 and CXCR7, receptors of SDF-1α [9]. Because they are safe to use, can be used orally and synthesized easily studies on the discovery of their pharmacological effect is increasing.

The purpose of the present study was to investigate its antiproliferative and antimigratory effects of Morus nigra extract, its lectin Morniga G (MorG) and one of its component Chalcone 4 hydrate; and their synergistic effect in combination with cetuximab application on colorectal adenocarcinoma cell line HT-29.

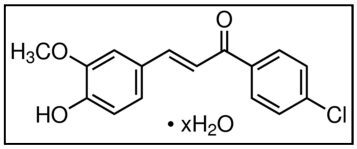

Figure 1. *Chemical structure of Chalcone 4 hydrate.*

2. Material and Method

2.1. Cells and Chemicals

Human colorectal adenocarcinoma cell line HT-29 were cultured in McCoy's 5A Modified Medium (Biochrom, Berlin) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 0.1 mM non-essential amino acid and Gentamicin at 37°C, 5% CO_2 in humidified air.

Cetuximab was purchased from Merck Serono SA (Bari, Italy); 5 Fluorouracil (5-FU) was purchased from Ebewe Pharma (Unterach, Austria); Chalcone 4 hydrate was purchased from Sigma-Aldrich Chemical Co. Inc. (St, Louis, MO); Morniga G was purchased from EY Laboratories (San Mateo, CA).

2.2. Preparation of the Morus Nigra Extract

M. nigra fruits were obtained as frozen. 50 g of M.nigra was homogenized with 200 ml 80% v/v methanol. The homogenate was centrifuged at 4°C and 5000 rpm for 5 min. The supernatant was concentrated by drying at 50°C under low pressure via using rotary evaporator. The extract was resuspended with DMSO to prepare stock solution [11].

2.3. Evaluation of Cell Viability

Cell impedances were measured for 72 hours by using xCELLigence RTCA SP Instrument (ACEA Biosciences, Inc. San Diego, USA). 96 well E-plate that can measure cell index depending on impedance by its electrodes found under each well was used for measurement. Background impedance signal was measured with 100µl cell culture medium per well. The final volume was completed to 200 µl/well by adding 100 µl cell suspension providing 10^4 cells/well. Cell impedance was measured for 24 hours at every 60 min. After 24 hours of cell seeding, wells are separated into groups and exposed to M. nigra extract (10%, 1%, 0.1%), MorG (0.5, 5, 50 µM) and Chalcone 4 hydrate (0.5, 5, 50 µM) at different concentrations with or without cetuximab application (200 µg/ml). 5-FU (40 µM) was used as cytotoxic agent to compare the antiproliferative effects of M. nigra extract, MorG and Chalcone 4 hydrate. After applying chemicals and drugs, cell impedance was measured for 48 hours at every 60 min and cell index was evaluated. All experiments were repeated 4 times for each chemical concentration as triplicate.

2.4. Evaluation of the Effects of M.nigra Extract and Its Active Compounds on Cell Migration

Cells were seeded on 6-well plates to provide $25x10^4$ cells/well and incubated till they became confluent. After confluency, wells were scratched with 200 µL tips, and medium in the wells were exchanged with M. nigra extract (0.1%, 1%, and 10%), *MorG (*0.5, 5, 50 µM), Chalcone 4 hydrate (0.5, 5, 50 µM) and 5-FU supplemented complete cell culture media. Photos of the wounds were taken by JuLI Br, live cell movie analyzer (NanoEnTek, JuLI Br04, Korea) at every 12 hours for 48 hours. The range of gaps of the wounds was measured by ImageJ software 1.49. Changes in areas in each plate were evaluated according to the rate of increase or decrease of wound by use of percentage change calculation method.

2.5. Evaluation of the Interaction of M.nigra Extract and Its Active Compounds with Targeted Drug

After 24 hours of cell seeding, HT-29 colorectal adenocancinoma cell line was exposed to M. nigra extract (100%, 10%, 1%), MorG (0.5, 5, 50 µm) and Chalcone 4 hydrate (0.5, 5, 50 µm) with Cetuximab (200 µg/ml). The effect of Cetuximab on cell line and the role of chemicals on the effect of Cetuximab were evaluated with real time cell analyser. Cell viability was determined according to cell index. IC_{50} dose was determined via RTCA Software (ACEA Biosciences, Inc. San Diego, USA). The concentration at which cytostatic and cytotoxic effects observed were calculated with Ti=Tz and [(Ti-Tz)/Tz] ×100 = (-50) formulations respectively. (Tz: Time zero, C: Control growth Ti: Test growth in the presence of drug at different concentration levels).

2.6. Statistical Analysis

Cell viability was calculated in percentage terms compared

to control group. The significance between different chemicals and their effects was determined by using Mann-Whitney U Analysis. All analyses were performed via SPSS (Version 15.0; SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. The Effects of M.nigra Extract, MorG and Chalcone 4 Hydrate on Cell Proliferation

The in vitro cytotoxic effects of Morus nigra extract, Morniga G and Chalcone 4 hydrate at different concentrations on HT-29 cell viability were shown in Figure 2. After 48 hours exposure, 50 µM Chalcone 4 hydrate increased cell viability 14.76%. 5 and 0.5 µM Chalcone 4 hydrate concentrations reduced cell viability 4.43% and 31.26% respectively. After 48 hours exposure of 50, 5 and 0.5 µM MorG concentrations cell viability decreased 73.68%, 31.04% and 49.98% respectively. After 48 hours exposure of 10%, 1% and 0.1% M. nigra extract concentrations cell viability decreased 74.56%, 15.93% and 5.44% respectively.

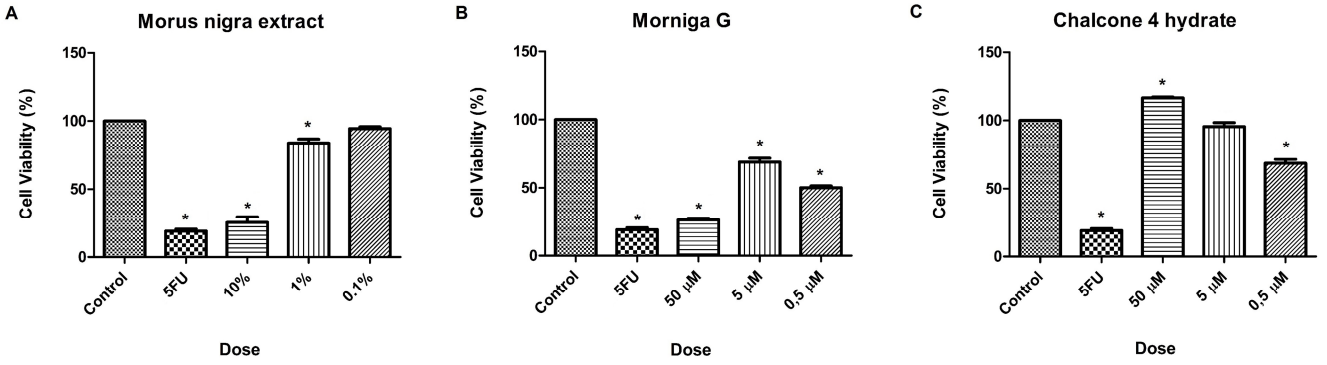

Figure 2. *Dose-dependent effect of (A) M.nigra extract, (B) MorG and (C) Chalcone 4 hydrate on HT-29 cell proliferation after 48 hours exposure. *: p<0.05 vs. control. The percent viable cells were calculated in comparison to untreated cells taken as 100%. Values were expressed as mean ± SD and the experiment was performed in triplicate.*

3.2. The Effects of M.nigra Extract and Chalcone 4 Hydrate on Cell Migration

After 48 hours, control group exhibited 7.1% closure. The closure of cells treated with 50 µM Chalcone 4 hydrate and 0.1% and M.nigra extract were 0.3% and 0.1% respectively. The wound opened up 2.8% at 10% M.nigra extract concentration (Figure 3).

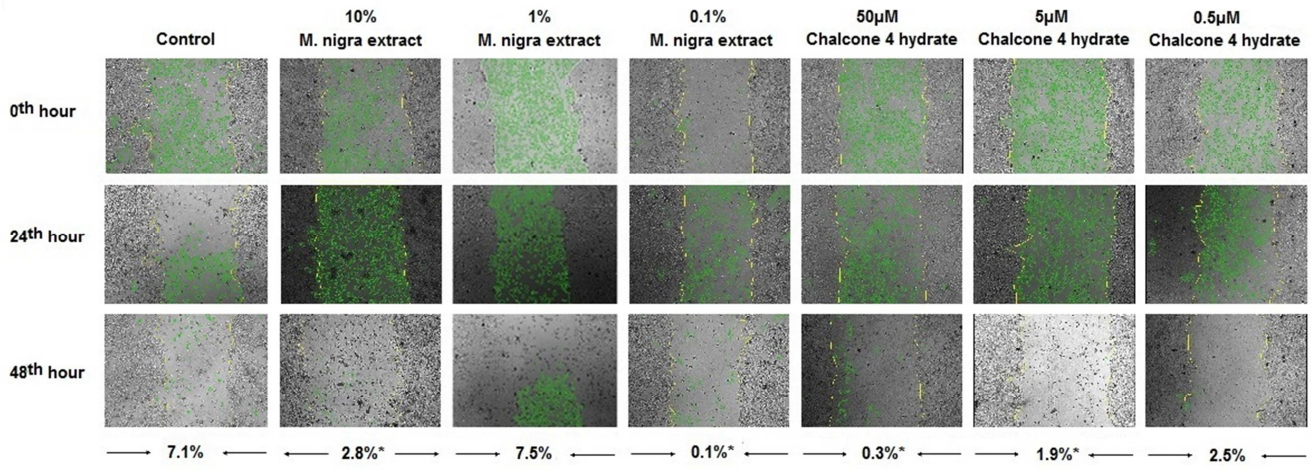

Figure 3. *Effect of M. nigra extract and Chalcone 4 hydrate concentrations on HT-29 cell migration after 48 hours exposure. *: p<0.05.*

3.3. Antiproliferative Effect of Cetuximab Co-treated with M.nigra Extract and Its Active Compounds on Proliferation of HT-29 Cells

Effect of Cetuximab on proliferation of HT-29 cells when co-treatment with M. nigra extract, MorG and Chalcone 4 hydrate was shown in Figure 4. After 48 hours of exposure, cell viability was 73.15% at 200 µg/ml Cetuximab concentration. After 48 hours of 200 µg/ml Cetuximab exposure with 50, 5 and 0.5 µM Chalcone 4 hydrate, cell viability decreased 14.11%, 11.08% and 2.35% respectively. After 48 hours exposure of 200 µg/ml Cetuximab with 50, 5 and 0.5 µM MorG concentrations cell viability decreased 78.01%, 63.29% and 46.25% respectively. After 48 hours of 200 µg/ml Cetuximab exposure with 10%, 1% and 0.1% M. nigra concentrations cell viability decreased 88.08%, 57.16% and 35.49% respectively.

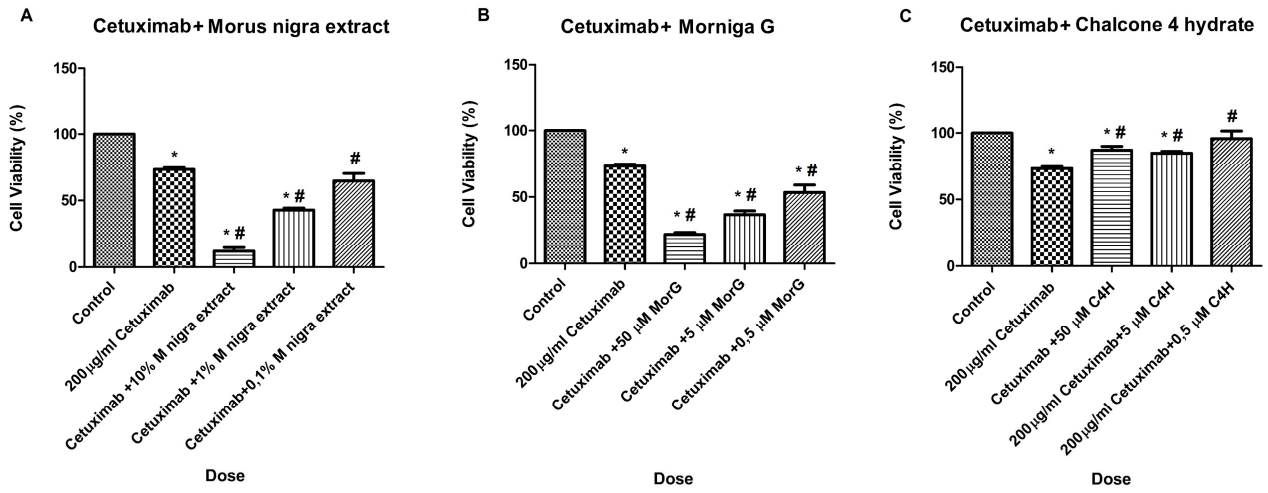

Figure 4. *Effect of Cetuximab on proliferation of HT-29 cells when co-treatment with (A) M.nigra extract, (B)MorG and (C) Chalcone 4 hydrate after 48 hours exposure. *: p<0.05 vs. control, #: p<0.05 vs. Cetuximab. The percent viable cells were calculated in comparison to untreated cells taken as 100%. Values were expressed as mean ± SD and the experiment was performed in triplicate.*

4. Discussion

M. nigra is one of the phytochemicals that have been reported to have anticancer effect [12] and its lectin MorG have been demonstrated to show high affinity to Tn-positive Jurkat leukemia cells and enhance efficiency of therapy [13]. Therefore, we investigated effects of M. nigra extract and its active components MorG and Chalcone 4 hydrate and on HT-29 colorectal adnocarcinoma cell line. According to the results we obtain Chalcone 4 hydrate, a chemokine inhibitor, didn't demonstrate significant anti-proliferative effect only at 0,5 µM concentration and it reduced anti-proliferative effect of Cetuximab on HT-29 cells. On the contrary, MorG, a lectin og M. nigra, demonstrated significant anti-proliferative effect and increased anti-proliferative effect of Cetuximab both in a dose-dependent manner. M. nigra extract showed similar effect with lectin. In the study of Qi Z. et al. a synthetic chalcone derivative 4,3',4',5'-tetramethoxychalcone (TMOC) has shown to has anti-proliferative effect on A2780, A2780/CDDP and SKOV3 ovary cancer cell lines and to supress invasion and migration of A2780 cells [7]. In the study of Takahashi M. et al. (2012) 2,4,2',4'-hydroxychalcone analogue isolated from methanol extract of Morus australis has shown to reduce viability of B16 murine melanoma cells [14]. Also in the study of Liu B. et al. (2009) a mannose or glucose specific legume lectin Concanavalin-A has shown to induce apoptosis in A375 human melanoma cells [15].

5. Conclusion

We showed effects of M. nigra extract, MorG and Chalcone 4 hydrate on HT-29 cell proliferation and migration; and their their synergistic effect in combination with cetuximab application on colorectal adenocarcinoma cell line HT-29 for first time. When we evaluate our results in conjunction with other work done with Chalcone 4 hydrate our results were contrary to expectations. These kind of differences can be based on chemical structures of chemical derivatives. According to our results, MorG can be suggested as a potantial conjugate for targeted drug. However, futher studies are required to fully understand its mechanisms of action.

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