Indian Journal of Traditional Knowledge
Vol 20(1), January 2021, pp

Investigation of cytotoxic effect of black mulberry (*Morus nigra* L.) fruit

S Dalkiliç* , L K Dalkiliç, Ş İnci, İ Korkmaz, S Kırbağ
Firat University, Faculty of Science, Department of Biology, Elazıg, Turkey
E-mail: †sdalkilic@gmail.com

Received 27 October 2019; revised 10 September 2020

The aim of this study was to determine the cytotoxic effect of black mulberry fruit extract (*Morus nigra* L.) on human breast cancer (MDA-MB-231) and prostate cancer (PC3) cell lines. The extract prepared from mulberry (*M. nigra*) fresh fruit was applied to cancer cell lines in 5 different v/v concentrations (10%, 4%, 2%, 1.33% and 1%) for 72 h. At the end of the incubation period, cell viability was determined by MTT [3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide] assay. Results were compared with negative and positive control groups. The effect of mulberry (*M. nigra*) fruit extract on cell viability was determined depending on the concentration applied; extract showed a significant cytotoxic effect on PC3 cell line at the 10% concentration compared to the control group, but not detected any effect at 4%, 2%, 1.33% and 1% concentrations. In the MDA-MB-231 cell line, there was a high cytotoxic effect at a concentration of 10% compared to the control group, while a lower effect was observed at 4%, 2%, 1.33% and 1% concentrations. The data obtained from the study showed that extract prepared from black mulberry fruit has cytotoxic effect especially against breast cancer cells.

Keywords: Breast cancer, Cytotoxicity, *Morus nigra* L., MTT, Prostate cancer
IPC Code: Int. Cl.[21]: A61K 39/395, A61K 38/00, C12N 15/82,

Cancer is the most common pathology worldwide and has the highest mortality rate after cardiovascular diseases. It accounts for approximately 20% of all deaths worldwide[1]. According to the GLOBOCAN cancer statistics 18.1 million new cases and 9.6 million death occure every year in the world[2]. Population grow, lifestyle and some behaviors having main roles in this increasing[3]. According to data from the World Health Organization, the incidence of cancer is increasing and according to predictions 22 million new cancer cases are expected to emerge in 2030[4].

Nowadays, thanks to the advances made especially in the field of medicine, significant success has been achieved in the diagnosis and treatment of cancer. However, despite all these developments, the success achieved in some cancer types is not at the desired level[5].Since the side effects of chemotherapy agents used in cancer treatment are very high, natural and herbal treatment methods are considered as an alternative. Therefore, some natural and herbal substances have been suggested to be effective in the treatment of cancer[6].

Such practices are almost as old as the history of mankind and are still being practiced today. Intensive studies are carried out to identify new drug target molecules and to develop more effective drugs[5,6]. It is known that the leaves, bark and fruit parts of *M. nigra* belonging to Moraceae family are used as antipyretic, diuretic, expectorant, helping to protect oral mucosal health and complementary in the treatment of dysentery[7,10]. And also; has antimicrobial, antioxidative, antihelminthic, anti-inflammatory, antidiabetic and cytotoxic effects[11,16]. *M. nigra* is used in the treatment of certain diseases due to its high anthocyanin content and bioactive compounds besides being used as a food[17].

Anthocyanins are a major group of molecules called flavonoids that have strong antioxidant, antiallergic, antiapoptotic properties synthesized by phenylpropanoid[18,20] pathways. Flavonoids like as polyphenolic compounds which can show bioactivity in organism, and its anti-cancer effect is causing controversy in the scientific world[19]. Various studies indicate that flavonoids can contribute to both prevention and treatment of cancer[14,21]. Flavonoids are promising molecules in the treatment of cancer[22].

The aim of this study was to determine the cytotoxic activity of *M. nigra* fresh fruit extract on breast cancer (MDA-MB-231) and prostate cancer (PC3) cell lines.

*Corresponding Author

Material and Method

Obtaining the material

M. nigra was collected from Arsuz district of Hatay (Turkey) in 2019 and fruits were extracted using blender. After the extraction of *M. nigra*, 9 mL of medium was added to 1 mL of *M. nigra* extract and 10% concentration was prepared as stock and serial dilutions (10%, 4%, 2%, 1.33% and 1%) were prepared from stock solution and stored at +4°C until use.

Supply of cell lines

Human breast cancer (MDA-MB-231) and prostate cancer (PC3) cell lines were obtained from Inönü University and Erzurum Technical University.

Cell Culture

Breast cancer (MDA-MB-231) and Prostate cancer (PC3) cells were cultured in DMEM [1% L-Glutamine, 1% Penicillin-Streptomycin and 10% FBS (Fetal Bovine Serum)] in 25 cm² flasks, at 37°C and 5% CO_2 atmospheric conditions.

Evaluation of cell Viability

The MDA-MB-231 and PC3 cell lines grown in 25 cm² flasks, after 90% of confluency the medium in the flask was removed and washed with 5ml sterile PBS solution. About 1 ml Trypsin-EDTA was added to the flasks and incubated for 2 min in an incubator under conditions at 5% CO_2 and 37°C. After the cells were separated from the surface, trypsin-EDTA was inactivated with 5 ml of culture medium. Cells were taken from the flask, centrifuged at 1200 rpm for 5 min, then the supernatant was removed and the cell pellet was thawed with 1 mL of fresh medium and counted in the microscope. Adjusted the cell number as 5000 cell in 100 µL and then seeded in 96-well plates. Only the medium was placed in the first row to be used as blank and incubated in an incubator at 37°C with 5% CO_2 for 24 h. After incubation, 100 µL of, 10%, 4%, 2%, 1.33% and 1% of each diluted mulberry extract was added in 12 replicates and incubated under condition 5% CO_2 incubator at 37°C for 72 h. MTT test was applied after incubation. Absorbance measurements were made at 570 and 540 nm wavelength. 2.5 µg/mL Doxorubicin was used as positive control and culture medium was used as negative control[23,24].

MTT Assay

MTT [3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide] assay is a method used to measure cell viability, proliferation and cytotoxicity. MTT assay is based on the fact that activated mitochondria in living cells take up electrons in MTT which can pass through cell membrane and it is converted to into purple water-insoluble formazan crystals. Then, formazan crystals are dissolved with appropriate solvents and color changes are measured by spectrophotometric methods to determine the number of viable cells[25-27].

After the incubation period with this method, 20 µL of 5 mg/mL MTT solution was added to the wells containing the cells and incubated for 4 hours at 37°C with 5% CO_2 in dark environment. After incubation, medium was removed and formazan crystals were dissolved with 100 µL DMSO (dimethyl sulfoxide). The absorbance measurements at 570 and 540 nm wavelengths were performed by ELISA plate reader[28,30].

Analysis

The absorbance values measured by ELISA plate reader device were recorded, then the measured absorbance values transformed to live cell percentage and calculated values were compared with the control groups and the data were transferred to the graphs.

Results

In this study, cytotoxic effect of *M. nigra* fruit extract on human breast cancer (MDA-MB-231) and human prostate cancer (PC3) cell lines were investigated via MTT [3- (4,5-dimethylthiazol-2-yl) -2,5 -diphenyltetrazolium bromide] assay. According to the our results, *M. nigra* fruit extract has significant cytotoxic activity on MDA-MB-231 (breast cancer) cell line at 10% concentration as shown in (Fig. 1-2). It was determined that the effect at other concentrations had a lower than 10% concentration.

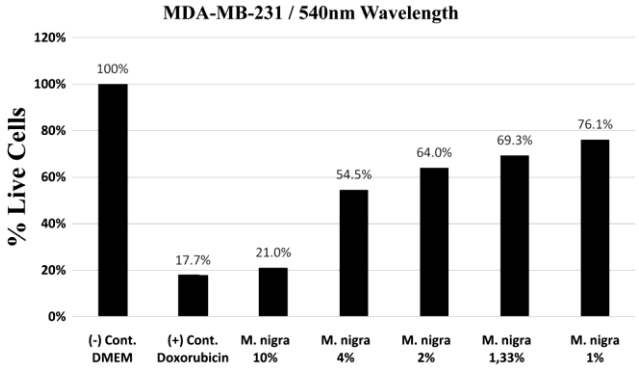

Fig. 1 — Cytotoxic effect of M. nigra on MDA-MB-231 (breast cancer) cell line.
Control: DMEM; (+) Control: Doxorubicin, *M. nigra*, 10%, 4%, 2%, 1.33% and 1%.

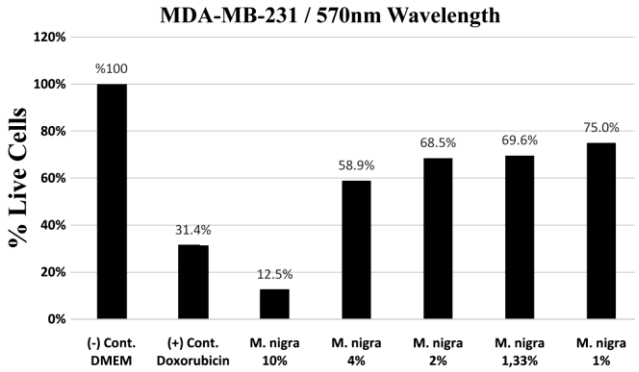

Fig. 2 — Cytotoxic effect of *M. nigra* on MDA-MB-231 (breast cancer) cell line.
Control: DMEM; (+) Control: Doxorubicin, *M. nigra*, 10%, 4%, 2%, 1.33% and 1%

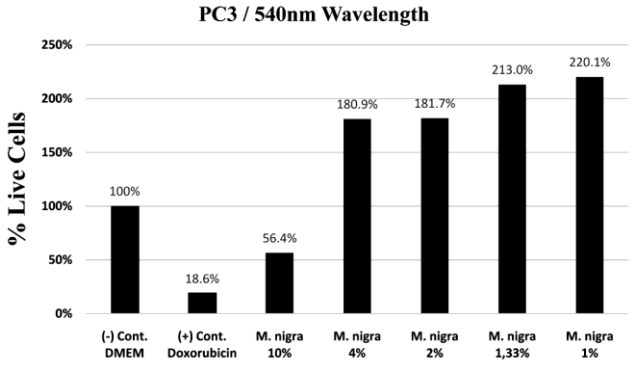

Fig. 3 — Cytotoxic effect of *M. nigra* on PC3 (prostate cancer) cell line.
Control: DMEM; (+) Control: Doxorubicin; *M. nigra* 10%, 4%, 2%, 1.33% and 1%

The effect on PC3 (prostate cancer) cell line showed significant cytotoxic activity at 10% concentration as shown in (Fig. 3-4), while no cytotoxic effect on cells was observed at other concentrations. *M. nigra* extract was shown concentration-dependent cytotoxic activity on the MDA-MB-231 cell line compared to the PC3 cell line.

Our results have shown that *M. nigra* extract has cytotoxic effect against to breast cancer cell line. The most effective cytotoxicity has determined in the concentration of 10%. Total live cell percentage has determined as 21% in this concentration at the 540 nm wavelength. The lowest cytotoxic effect has observed as 76.1% in the concentration of 1%. Cytotoxic effect has decreasing as a dose dependent manner. (Fig. 1) has shown cytotoxic effect of *M. nigra* against the MDA-MB-231 (breast cancer) cell line.

The maximum cytotoxic effect has observed in concentration of 10% on MDA-MB-231 cell line in

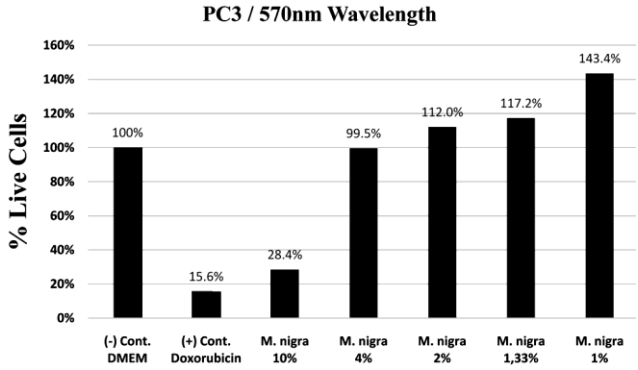

Fig. 4 — Cytotoxic effect of *M. nigra* on PC3 (prostate cancer) cell line.
Control: DMEM; (+) Control: Doxorubicin, *M. nigra* 10%, 4%, 2%, 1.33% and 1%

the 570 nm wavelength. This effect has better than doxorubicin effect on cancer cell in this concentration (Fig. 2).

(Fig. 3) and Figure 4 has shown observed cytotoxicity of *M. nigra* extract on PC3 prostate cancer cell line. The most effective cytotoxicity has observed as 28.4% at the concentration of 10% in the 570 nm wavelength. We have not observed any significant cytotoxicity in other concentrations on PC3 cell line. Maximum cytotoxic effect has determined as 56.4% at the 10% concentration in the 540 nm wavelength. There has not observed any significant cytotoxic effect in other concentrations on PC3 cell line.

Discussion and Conclusion

M. nigra has been observed to have apoptotic and antiproliferative effects on PC3 cell line in previous studies[31]. Leaf extract of the same species has been reported to show no significant cytotoxic activity against L-929 (fibroblast), B16F10 (melanoma), HeCat (keratinocyte) cell lines[32]. It has been reported that *M. nigra* exhibits strong antibacterial and antioxidant activity and weak cytotoxic effect[33]. The differences in the results obtained in the aforementioned studies and the study may be due to the different cell lines used or the difference in the experimental and extraction procedure.

The fruit of *M. nigra* is rich in antioxidant flavonoids[18,20]. Flavonoids are known to inhibit carcinogenic and harmful oxidant agents such as superoxide anion radicals and nitric oxide[14-16,34]. In the light of these studies, it is understood that black mulberry (*M. nigra*) species is rich with antioxidants but it has low cytotoxic effect against some cancer cell lines.

In the review of Lim and Choi, *M. nigra* particular fruit and leaf parts have various pharmacological properties such as antinociceptive, anti-inflammatory, antimicrobial, melanogenic, antidiabetic, anti-obesity, anti-hyperlipidemic and anticancer activities, as well as central nervous system, liver, and kidney protective and therapeutic effects on the gastrointestinal tract and the female reproductive system, and most of these properties are based on *M. nigra* antioxidant compounds such as polyphenols, flavonoids and anthocyanins[35].

Eo have stated that the root bark of *Morus alba* L. (white mulberry) has anti-inflammatory effect as well as anticancer effect and this effect is antiproliferative and apoptosis-stimulating in nature[36]. As pointed out in this study, the effect of *M. nigra* and *M. alba* species on cancer cells shows that not only the fruits but also parts of leaves, roots, barks, stem can have a potential effect. Fallah et al. applied antioxidants obtained from the leaves of *M. alba* on cancer in mice and observed a significant increase in the survival of mice[37]. These studies have shown that *M. nigra* and *M. alba* have anticancer effect and increase the survival of mice[37].

More comprehensive studies should be performed for detection of cytotoxic and antiproliferative effects of mulberry (*M. nigra*) and white mulberry (*M. alba*) fruit extract and also should test against other cancer cell lines. Further studies are needed to determine whether there is any cytotoxic effect on healthy cell lines. Likewise, more studies should perform on the mechanism of action and determination of active substances on cancer cells.

Acknowledgement

We thank to Firat University Faculty of Medicine Department of Parasitology, Prof. Dr. Mustafa Kaplan which contributed to the laboratory environment in the conduct of this study.

Conflict of Interests

Authors declare no conflict of interest

Author Contributions

S D and L K D designed the experiments and MTT assay

L K D and İ K collected and prepared fruit and extract concentration for the experiment

S D, L K D, S K, Ş İ and İ K Interpreted of the results.

S D, L K D, Ş İ, İ K and S K wrote the manuscript

Reference

1 Turkish Statistical Institute (TÜİK), Cause of Death Statistics, http://www.tuik.gov.tr/PreHaberBultenleri.do?id=24572 (2017) Date of Access: 12.10.2019.

2 Ferlay J, Colombet M, Soerjomataram I, Mathers C, Parkin DM, Piñeros M, Znaor A & Bray F, Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods, *Int J Cancer*, 144 (8) (2019) 1941-1953.

3 Torre LA, Siegel RL, Ward EM & Jemal A, Global cancer incidence and mortality rates and trends—an update, *Cancer Epidemiol Biomarkers Prev*, 25 (1) (2016) 16-27.

4 World Health Organization, Latest Global Cancer Data, https://www.who.int/cancer/PRGlobocanFinal.pdf Press Release No: 263, (2018), Date of Access: 12.10.2019.

5 Baykara O, Current Modalities In Treatment Of Cancer, *BAUN Sağ Bil Derg*, ISSN:2146-9601, (2016) 154-165.

6 Özgüç S, Kayalar H & Zeybek U, Medicinal Plants And Their Secondary Metabolites Effective Against Breast Cancer, *J. Fac. Pharm. Ankara*, 42 (2): (2018) 42-62.

7 Baytop T, Türkiyede bitkilerle tedavi, (İstanbul Eczacılık Fakültesi Yayınları, İstanbul), 1999, pp.444.

8 Çubukçu N Ü & Çinar S, Can oral mucositis be prevented in patients receiving chemotherapy?. *Clin Exp Health Sci*, *2* (4) (2012) 155.

9 Lin J Y & Tang C Y, Determination of total phenolic and flavonoid contents in selected fruits and vegetable as wellas their stimulatory effects on Mouse splenocyte proliferation, *J.Agric. Food Chem*., 101, (2007) 140-147.

10 Ozgen M, Serce S & Kaya C, Phytochemical and antioxidant properties of anthocyanin rich Morus nigra and Morus rubra fruits, *Sci. Hortic*,119, (2009) 275-279.

11 Asono N, Yamashita T, Yasuda K, Ikeda K, Kizu H & Kameda Y, Polyhydroxylated alkoloids isolated from mulberry trees and silkworms. *J Agric. Food Chem*., 49, (2001) 4280–4213.

12 Kim S Y, Park K J & Lee W C, Antiinflamatory and antioxidative effects of Morus spp. fruit extracts, *Korean J Med. Crop Sci*., 41, (1998) 123–128.

13 Kim H B, Bang H S, Lee H W, Seuk Y S & Sung G B, Chemical characteristics of mulberry syncarp, *Korean J Med. Crop Sci*., 47 (1999) 3206– 3209.

14 Muhsiroğlu Ö, Flavonols Cardiovascular/Cerebrovascular Diseases And Cancer, *Bes. Diy. Derg*, 45 (2): (2017) 178-184.

15 Chun O K, Kim D O & Lee C Y, Superoxide Radical Scavenging Activity Of The Major Polyphenols in Fresh Plums, *J Agric Food Chem*, 51: (2003) 8067-8072.

16 Pehluvan M & Güleryüz M, The Blackberries For Human Health, *Bahçe*, 33 (1-2): (2004) 51-57.

17 Gerasopoulos D & Stavorulakis G, Quality characteristics of four mulberry (Morus sp.) cultivars in the area of China, *J. Sci. Food Agric*.73, (1997) 261–264.

18 Nondave M, Ojha S K & Arya D S, Protective Role Of Flavonoids in Cardiovascular Diseases, *Nat. Prod. Radiance*, 4: (2005) 166-176.

19 Birman H, Bioactivites Of Plant Flavonoids And The Possible Action Mechanisms, *J. Ist. Faculty Med*., 75:3, (2012) 46-49.

20 Çimen M B Y, Flavonoids And Their Antioxidant Properties, *T Klin J Med Sci*, 19: (1999) 296-304.

21 Kahraman A, Serteser M & Köken T, Flavonoids, *The Medical Journal Of Kocatepe*, 3, (2002) 01-08.

22 Karakaya S & El S N, Flavonoidler ve Sağlık, *J. Nutr and Diet*, 26 (2): (1997) 54-60

23 Van Meerloo J, Kaspers G J & Cloos J, Cell Sensitivity Assays: The MTT Assay. In Cancer Cell Culture, *Humana Press*, (2011) (pp. 237-245).

24 Tekin S, Beytur A, Çakır M, Tekin Ç & Sandal S, Saksagliptin ve Sitagliptinin Farklı Tip İnsan Kanser Hücre Canlılığı Üzerine Etkilerinin Araştırılması, *F.Ü. Med. J. Health. Sci.*, 31 (3), (2017) 111-116.

25 Mosmann T, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, *J Immunol Methods*, 65 (1-2), (1983) 55-63.

26 Gerlier D & Thomasset N, Use Of MTT Colorimetric Assay To Measure Cell Activation, *J Immunol Methods*, 94 (1-2), (1986) 57-63.

27 Altman F P, Tetrazolium salts and formazans, *Prog histochem cytochem*, 9 (3), (1976) III-51.

28 Riss T L & Moravec R A, Use of multiple assay endpoints to investigate the effects of incubation time, dose of toxin, and plating density in cell-based cytotoxicity assays, *Assay Drug Dev Technol*, 2 (1), (2004) 51-62.

29 Freimoser F M, Jakob C A, Aebi M & Tuor U, The MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay is a fast and reliable method for colorimetric determination of fungal cell densities, *Appl. Environ. Microbiol*, 65 (8), (1999) 3727-3729.

30 Van Tonder A, Joubert A M & Cromarty A D, Limitations of the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay when compared to three commonly used cell enumeration assays, *BMC Res Notes*, 8 (1), (2015) 47.

31 Turan İ, Demir S, Kılınç K, Burnaz N A, Yaman S Ö, Akbulut K, Menteşe A, Aliyazıcıoğlu Y & Değer O, Antiproliferative And Apoptotic Effect Of Morus Nigra Extract On Human Prostate Cancer Cells, *Saudi Pharm J*, 25, 2: (2017) 241-248.

32 Freitas D M M, Fontes P R, Souza P M, Fagg C W, Guerra E N S, Nobrega Y K M, Silveira D, Bazzzo Y F, Simeoni L A, Mello M H & Magalhaes P O, Extracts Of Morus Nigra L. Leaves Standordized In Chlorogenic Acid Rutin And Isoquercitrin Tyrosinase Inhibition And Cytotozicity, *PloS One*, (2016), 11.9e0163130.

33 Souza G R, Olıveıra-Junıor R G, Dınız T C, Branco A, Lıma-Saraıva S R G, Guimaraes A L, Olıveıra A P, Pacheco A G M, Sılva M G, Moraes-Fılho M O, Costa M P, Pessoa C O & Almeıda J P G S, Assessment Of The Antibacterial Citotoxic And Antioxidant Activities Of Morus Nigra L.(Moraceae), *Braz J Microbiol*, 78.2: (2018) 248-254.

34 İkizler M, Erkasap N, Dernek S, Kural T & Kaygısız Z, Dietary Polyphenol Qercetin Protects Rats Hearts During Reperfusion: Enhanced Antioxidant Capacity With Chronic Treatment, *Anadolu Kardiol Derg*, 7: (2007) 404-410.

35 Lim S H, & Choi C I, Pharmacological Properties of Morus nigra L.(Black Mulberry) as A Promising Nutraceutical Resource, *Nutrients*, 11 (2), (2019) 437.

36 Eo H J, Park J H, Park G H, Lee M H, Lee J R, Koo J S, & Jeong J B, Anti-inflammatory and anti-cancer activity of mulberry (Morus alba L.) root bark, *BMC Complement Altern Med*, 14 (1), (2014) 200.

37 Fallah S, Hajihassan Z, Zarkar N, Chadegani A R, Mohammadnejad J & Hajimirzamohammad M, Evaluation of anticancer activity of extracted flavonoids from morus alba leaves and its interaction with DNA, *Braz Arch Biol Technol*, (2018) 61.