Anais da Academia Brasileira de Ciências (2019) 91(4): e20190337
(Annals of the Brazilian Academy of Sciences)
Printed version ISSN 0001-3765 / Online version ISSN 1678-2690
http://dx.doi.org/10.1590/0001-3765201920190337
www.scielo.br/aabc | www.fb.com/aabcjournal

# Genotoxic and Genoprotective Potential of Black Mulberry (*Morus nigra*) Fruit

**SERKAN YILMAZ[1], ASLI UÇAR[2] and BAYRAM GÖKTAŞ[1]**

[1]Ankara University Faculty of Health Sciences, Department of Midwifery,
Fatih Caddesi, 197/A, 06290 Keçiören,Ankara, Turkey
[2]Ankara University Faculty of Health Sciences, Department of Nutrition and
Dietetics, Fatih Caddesi ,197/A, 06290,Keçiören,Ankara,Turkey

*Manuscript received on January 24, 2019; accepted for publication on May 7, 2019*

**How to cite**: YILMAZ S, UÇAR A AND GÖKTAŞ B. 2019. Genotoxic and Genoprotective Potential of Black Mulberry (*Morus nigra*) Fruit. An Acad Bras Cienc 91: e20190337. DOI. 10.1590/0001-3765201920190337.

**Abstract**: Black Mulberry (*Morus nigra* L.) belongs to Moraceae family. The present study evaluated the possible genotoxic and/or protective activities of black mulberry fruit juice (BMFJ), *in vitro*, using mitomycin C (MMC) as a positive control, by chromosomal aberrations and micronucleus assays. Human lymphocytes were treated with BMFJ concentrations alone (1/1, 1/2, 1/4, 1/8 dilutions), pretreatment (49h) (0.20 μg/ml MMC+ 1/1 BMFJ, 0.20 μg/ml MMC+1/2 diluted BMFJ, 0.20 μg/ml MMC+1/4 diluted BMFJ, 0.20 μg/ml MMC+1/8 diluted BMFJ) and simultaneous-treatment (48h) (0.20 μg/ml MMC+ 1/1 BMFJ, 0.20 μg/ml MMC+1/2 diluted BMFJ, 0.20 μg/ml MMC+1/4 diluted BMFJ, 0.20 μg/ml MMC+1/8 diluted BMFJ). The *in vitro* results demonstrated that BMFJ showed no genotoxicity, but it significantly decreased chromosomal aberration and micronucleus frequency induced by MMC. Our results showed that all concentrations of BMFJ revealed no genotoxicity but protective activity against genomic changes induced by anti-tumor agent MMC in human lymphocytes. Protective effects of BMFJ on MMC induced chromosomal damages most probably due to its free radical scavenging activity.

**Key words**: Black mulberry, chromosomal aberrations, micronucleus, genotoxicity, anti-genotoxicity.

## INTRODUCTION

The relation between use of plants and health has always been widely known by mankind throughout the centuries. According to the World Health Organization nearly 80% of the world's population still relies on traditional medicinal care for better health (Farnsworth et al. 1985). Vitamins, polyphenols, terpenoids and isoflavonoids are the major bioactive compounds of plants that possess anti-oxidative, anti-mutagenic, anti-carcinogenic, and immunomodulating properties (Craig et al. 1999, Santos-Cervantes et al. 2007, Karamova et al. 2011). Since many plant components possess different pharmacological properties for the prevention or treatment of diseases (Jiménez-Medina et al. 2006), interest on anti-genotoxic, anti-carcinogenic activity of plant extracts has been risen during the past few years. Plant components are generally retard oxidative degradation of lipids and other biomolecules and/or sweep up free radicals generated by mitochondrial oxidative metabolism as well as in cellular response to xenobiotics, cytokines, and bacterial invasion in cell. Oxidative stress causes macromolecular

Correspondence to: Serkan Yilmaz
E-mail: serkanyilmaz@ankara.edu.tr
ORCid: http://orcid.org/0000-0001-8641-9475

damage and is implicated in various diseases such as atherosclerosis, diabetes, cancer, neurodegeneration and aging (Barcelos et al. 2007).

Black Mulberry (*Morus nigra*), belongs to the Moraceae family (Boschini 2002) and grow up in East, West and South-East Asia, South Europe, South of North America, Northwest of South America and some areas of Africa (Calín-Sánchez et al. 2013) and also in Turkey. The cultivation for fruit production of mulberry reaches nearly 80.000 tons per year (Ercişli and Orhan 2007). Fruit, leaves and even branches of black mulberry are traditionally used as antipyretic, diuretic, lowering blood pressure in various countries (Zhishen et al. 1999). The main bioactive compounds of Black Mulberry fruit have been widely studied in the literature. It is a rich source of phenolic compounds including flavonoids, anthocyanins, phenolic acids and carotenoids (Lin and Tang 2007, Du et al. 2008, Özgen et al. 2009, Kutlu et al. 2011, Fazaeli et al. 2012, Calín-Sánchez et al. 2013). Bae and Suh (2007) reported that anthocyanins are the powerful bioactive components of black mulberry. Gundogdu et al. (2011) deeply investigated the phenolic compound contents, vitamin C (ascorbic acid) content and total antioxidant capacity (Table I ). Chlorogenic acid and rutin was to be found the highest value of phenolic content.

Recently, studies have shown that the extracts of this plant have strong antioxidant activity because of high phenolic and anthocyanin contents (Gerasopoulos and Stavroulakis 1997, Zhishen et al. 1999, Kutlu et al. 2011). The extract of the black mulberry prevents cell from the lipid oxidation and bio-membranes from peroxidative damage (Naderi et al. 2004), especially flavonoids protect DNA from oxidative damage and effective on enzymes that promotes cancer development (Kris-Etherton et al. 2002). Black mulberry fruit has also possess laxative, odontalgic, antihelmintic, expectorant, hypoglycemic and antimicrobial effects (Mandal and Kumar 2002, Kone et al. 2004, Lin and Tang 2007, Ozgen et al. 2009).

There is scanty report on genotoxicity and anti-genotoxicity of BMFJ has been published in the literature. Therefore, in this study we report the genotoxic and geno-protective activity of BMFJ using cultured human peripheral blood lymphocytes. The use of CAs and MN assays provides useful tools to detect potential genotoxicity

**TABLE I**
**Phenolic compound content, vitamin C (ascorbic acid) content and total antioxidant capacity of black mulberry fruit (from Gundogdu et al. 2011).**

| **Black mulberry (*Morus nigra* L.) fruit content** | |
|---|---|
| Vitamin C (mg 100 $g^{-1}$ fw) | 11.302 ± 0.241 |
| Total Antioxidant Capacity (µmol TE $g^{-1}$ fw) | 13.999 ± 0.008 |
| Gallic acid (mg $g^{-1}$ fw) | 0.150 ± 0.003 |
| Catechin (mg $g^{-1}$ fw) | 0.075 ± 0.000 |
| Chlorogenic acid (mg $g^{-1}$ fw) | 3.106 ± 0.004 |
| Caffeic acid (mg $g^{-1}$ fw) | 0.131 ± 0.001 |
| Syringic acid (mg $g^{-1}$ fw) | 0.103 ± 0.000 |
| p-Coumaric acid (mg $g^{-1}$ fw) | 0.129 ± 0.002 |
| Ferulic acid (mg $g^{-1}$ fw) | 0.064 ± 0.001 |
| o-Coumaric acid (mg $g^{-1}$ fw) | 0.134 ± 0.001 |
| Phloridzin (mg $g^{-1}$ fw) | 0.031 ± 0.001 |
| Protocatechuic acid (mg $g^{-1}$ fw) | 0.017 ± 0.000 |
| Vanilic acid (mg $g^{-1}$ fw) | 0.036 ± 0.002 |
| Rutin (mg $g^{-1}$ fw) | 1.423 ± 0.036 |
| Quercetin (mg $g^{-1}$ fw) | 0.113 ± 0.021 |

and anti-genotoxicity of environmental mutagens and other chemicals.

## MATERIALS AND METHODS

Black Mulberries at full mature stage were collected from the Balıkesir province in Turkey and deposited in our Department (voucher specimen number: 2015FHS38). Chromosome medium B was obtained from Biochrome. Mitomycin C (CAS No: 200-008-6), cytochalasin B (CAS No: 14930-96-2) was obtained from Sigma. Other chemicals was obtained from Sigma (USA).

Before experiments ethical approval was obtained from local ethic committee (Reg. No: 17-195). Peripheral venous blood was collected from non-smoking healthy 2 females and 2 males adults (aged 25-26 years). Whole blood (0.2 ml) was added to 2.5 ml Chromosome Medium B. Cultures were incubated at 37 ˚C for 72h. Test substance was added after 24h and 23h of culture initiation. Human lymphocytes were exposed to BMFJ concentrations alone (1/1, 1/2, 1/4, 1/8 dilutions), pretreatment (49h) (0.20 µg/ml MMC+ 1/1 BMFJ, 0.20 µg/ml MMC+1/2 diluted BMFJ, 0.20 µg/ml MMC+1/4 diluted BMFJ, 0.20 µg/ml MMC+1/8 diluted BMFJ) and simultaneous-treatment (48h) (0.20 µg/ml MMC+ 1/1 BMFJ, 0.20 µg/ml MMC+1/2 diluted BMFJ, 0.20 µg/ml MMC+1/4 diluted BMFJ, 0.20 µg/ml MMC+1/8 diluted BMFJ). Negative and positive control (MMC, 0.20 µg/ml) were also included in the test. Colchicine was added to arrest mitosis in the metaphase stage at a concentration of 0.06 µg/ml at 70$^{th}$ h of the culture. At the end of the culture period, the cells were collected by centrifugation (1200 rpm, 10 min), re-suspended in a hypotonic solution (KCI, 0.075 M) for 30 min at 37 ˚C and fixed in cold methanol acetic acid (3:1) for 20 min at room temperature. The treatment with fixative was repeated 3 times. Finally, metaphase spreads were prepared by dropping the concentrated cell suspension onto slides. Air dried slides were stained with 5% Giemsa (pH. 6.8) prepared in Sorensen buffer solution for 20 min and mounted with DPX. A hundred well spread metaphases, totally 400 metaphases per concentration, were analyzed for the CA assays per donor.

For micronucleus analysis, human lymphocyte culture was incubated at 37ºC for 72h and 44h from the initiation; cytochalasin B (Cyt-B) at a final concentration of 5.2 µg/ml was added to block cytokinesis. Human lymphocytes were exposed to BMFJ concentrations alone (1/1, 1/2, 1/4, 1/8 dilutions), pre-treatment (49h) (0.20 µg/ml MMC+ 1/1 BMFJ, 0.20 µg/ml MMC+1/2 diluted BMFJ, 0.20 µg/ml MMC+1/4 diluted BMFJ, 0.20 µg/ml MMC+1/8 diluted BMFJ) and simultaneous-treatment (48h) (0.20 µg/ml MMC+ 1/1 BMFJ, 0.20 µg/ml MMC+1/2 diluted BMFJ, 0.20 µg/ml MMC+1/4 diluted BMFJ, 0.20 µg/ml MMC+1/8 diluted BMFJ). Negative and positive control (MMC, 0.20 µg/ml) were included. Cultures were harvested at 72$^{nd}$h. Then the cells were treated with cold hypotonic solution (0.075 M KCI for 5 min). The cells were fixed with methanol: glacial acetic acid (3:1v/v) supplemented with formaldehyde. Finally, cell spreads were prepared by dropping the concentrated cell suspension onto slides. The slides were air-dried and stained with 5% Giemsa. Micronuclei were scored from 1000 bi-nucleated cells per donor (totally 4000 bi-nucleated cells per concentration).

For the statistical analysis of the results, z-test for percentage of abnormal cell, CA/cell and MN were used. Dose-response relationships were determined from the correlation and regression coefficients for the percentage of abnormal cell, CA/cell and mean MN.

## RESULTS

No significant difference in the induction of chromosomal aberrations and micronuclei

was observed between the groups treated with the concentrations of BMFJ and the negative control. Beyond, the treatment of the cells with BMFJ plus MMC (pre-treatment and simultaneous treatment) showed a significant reduction of chromosomal aberrations when compared to treatment with MMC only (Table II).

The results also showed that BMFJ significantly reduced the frequency of micronuclei (pre-treatment and simultaneous treatment) induced by MMC at the three concentrations (1/1, 1/2 and 1/4 dilutions) tested. Only lowest concentration in simultaneous treatment was not significant (Table III). Our results exhibited that all concentrations of BMFJ showed no genotoxicity but protective activity against genomic changes induced by anti-tumor agent MMC in human lymphocytes.

## DISCUSSION

Mitomycin C (MMC) is an antitumor agent that has been used for a treatment of variety of tumors. However, it has bind covalently to DNA (Tomasz 1995, Paz et al. 2012), and has been shown to alkylate other cellular nucleophiles in addition to DNA, such as glutathione (GSH) (Sharma and Tomasz 1994, Sharma et al. 1994, Paz et al. 2012). Depending on the biotransformation pathway, metabolism of MMC may generate Reactive Oxygen Species (ROS) (Gustafson and Pritsos 1992, Ortega-Gutiérrez et al. 2009). When ROS interact with cells and exceed endogenous antioxidant systems, there is indiscriminate damage to biological macromolecules such as nucleic acids, proteins, and lipids (Offord et al. 2000, Ortega-Gutiérrez et al. 2009).

**TABLE II**
**Protective role of black mulberry fruit juice (BMFJ) against MMC induced chromosomal damages *in vitro*.**

| Test substance | Treatment | | Aberrations | | | | | | | Abnormal cell ± SE (%) | CA/Cell ± SE |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Period (hour) | Dose (µg/ml+ dilution) | ctb | csb | f | scu | dic | cte | p | | |
| Negative Control | | 0.00 | 3 | 1 | 2 | 2 | - | - | - | 2.00±0.70 | 0.002±0.002 |
| MMC | 48 | 0.20 | 68 | 21 | 12 | 7 | 14 | 16 | - | 30.00±2.29 | 0.345±0.023 |
| BMFJ | 48 | 1/1 | 2 | 2 | 3 | 5 | 1 | - | 1 | 3.50±0.92 | 0.035±0.009 |
| | 48 | 1/2 | 4 | - | 1 | 3 | - | - | - | 2.00±0.70 | 0.020±0.007 |
| | | 1/4 | 1 | 1 | 2 | 1 | - | - | - | 1.25±0.56 | 0.013±0.006 |
| | | 1/8 | 2 | 2 | - | 3 | - | - | - | 1.75±0.66 | 0.018±0.007 |
| MMC+BMFJ (pre-treatment) | 49 | 0.20+1/1 | 23 | 7 | - | 8 | 5 | 6 | 1 | 11.75±1.61** | 0.125±0.017** |
| | | 0.20+1/2 | 29 | 11 | 3 | 7 | 8 | 5 | - | 15.00±1.79** | 0.158±0.018** |
| | | 0.20+1/4 | 42 | 10 | 8 | - | 6 | 9 | - | 17.50±1.90** | 0.188±0.020** |
| | | 0.20+1/8 | 53 | 6 | 5 | 6 | 7 | 11 | 1 | 21.25±2.05** | 0.223±0.021** |
| MMC+BMFJ (simultaneous-treatment) | 48 | 0.20+1/1 | 31 | 11 | 6 | - | 3 | 9 | - | 15.00±1.79** | 0.150±0.018** |
| | | 0.20+1/2 | 26 | 6 | 7 | 12 | 5 | 12 | 2 | 17.50±1.90** | 0.175±0.019** |
| | | 0.20+1/4 | 44 | 9 | 9 | 19 | 7 | 9 | - | 22.75±2.10* | 0.243±0.021* |
| | | 0.20+1/8 | 49 | 12 | 9 | 11 | 8 | 15 | - | 24.50±2.15* | 0.260±0.022* |

ctb: chromatid break, csb: chromosome break, f: fragment, scu: sister chromatid union, dic: dicentric, cte: chromatid exchange, p: polyploidy. 400 metaphases were scored for each treatment.

* Significantly different from the MMC $P < 0.01$.

** Significantly different from the MMC $P < 0.001$.

**TABLE III**
**Protective role of black mulberry fruit juice (BMFJ) against MMC induced micronuclei *in vitro*.**

| Test substance | Treatment | | Binucleated cells (BN) scored | Distribution of BN cells according to the no. of MN | | | | MN (%) |
|---|---|---|---|---|---|---|---|---|
| | Period (hour) | Dose (µg/ml+ dilution) | | (1) | (2) | (3) | (4) | |
| Negative Control | 48 | 0.00 | 4000 | 10 | - | - | - | 0.25±0.0070 |
| MMC | 48 | 0.20 | 4000 | 43 | 6 | 2 | 1 | 1.63±0.0003 |
| BMFJ | 48 | 1/1 | 4000 | 7 | - | - | - | 0.18±0.0060 |
| | 48 | 1/2 | 4000 | 8 | - | - | - | 0.20±0.0063 |
| | 48 | 1/4 | 4000 | 7 | 1 | - | - | 0.23±0.0067 |
| | 48 | 1/8 | 4000 | 11 | 1 | - | - | 0.33±0.0074 |
| MMC+BMFJ (pre-treatment) | 49 | 0.20+1/1 | 4000 | 16 | - | - | - | 0.40±0.0077*** |
| | 49 | 0.20+1/2 | 4000 | 23 | - | - | - | 0.58±0.0078*** |
| | 49 | 0.20+1/4 | 4000 | 20 | 2 | - | - | 0.60±0.0077*** |
| | 49 | 0.20+1/8 | 4000 | 32 | 1 | 1 | - | 0.93±0.0040* |
| MMC+BMFJ (simultaneous-treatment) | 48 | 0.20+1/1 | 4000 | 16 | 1 | - | - | 0.45±0.0078*** |
| | 48 | 0.20+1/2 | 4000 | 22 | 1 | - | - | 0.60±0.0077*** |
| | 48 | 0.20+1/4 | 4000 | 28 | 1 | 1 | - | 0.83±0.0060** |
| | 48 | 0.20+1/8 | 4000 | 40 | - | 1 | 1 | 1.18±0.0073 |

* Significantly different from the MMC $P < 0.05$.
** Significantly different from the MMC $P < 0.01$.
*** Significantly different from the MMC $P < 0.001$.

Black mulberry (*Morus nigra*) fruit has a high nutrient content, especially of antioxidants (Naderi et al. 2004). It has the highest acidity, reduced ascorbic acid, Fe++, total flavonoids, and total monomeric anthocyanins (especially Cyanidin) (Jiang and Nie 2015, Veberic et al. 2015). Extracts of mulberry fruits were found to possess free radical scavenging activity, anti-hyperlipidemia, anti-atherogenic properties and neuroprotective effects and anti-cancer effects (human lung cancer cell line A549) (Chen et al. 2005, 2006, Kang et al. 2006, Bae and Suh 2007, Jiang and Nie 2015).

Aydın et al. (2015) reported that no statistically significant difference was observed in the hepatic lipid fractions of rats containing *M. nigra* fruit extract. However, the level of lipid peroxidation were found to be very low in *M. nigra* treated group (Aydın et al. 2015). Anticancer activity of *M. nigra* on human breast cancer cell line (MCF-7) was investigated by Ahmed et al. (2016). Black mulberry (BM) extracts administration gave significant morphological evidence of apoptosis and increased cell death after 48 hours more than that of 24 hours-treated MCF-7 cells among fresh and dry extract groups. DNA analysis after 24h of BM extracts treated groups has no fragmentation while apoptosis is generated after 48h showing more fragmentation in fresh BM groups. The mitotic index records were significantly decreased in dose and time dependent manner showing the better anti-proliferative effect with fresh extract treatments. DNA single strand breaks were also increased among the treatment groups at dose and time dependent manner with the best results with fresh extract.

Ghasemnezhad Targhi et al. (2017) investigated the radio protective effect of black mulberry extract on liver tissue and bone marrow cells in the rat. 200

mg/kg extract significantly reduced the frequencies of micronucleated polychromatic erythrocytes and micronucleated normochromatic erythrocytes, it also decreased the level of malondialdehyde and superoxide dismutase, as well as enhanced the total thiol content and catalase activity in rat's liver. Authors concluded that black mulberry extract reduced the genotoxicity and cytotoxicity induced by gamma irradiation in bone marrow cells and liver in the rat. Turan et al. (2017) evaluated the antiproliferative and apoptotic effect of *M. nigra* extract on human prostate cancer cells. Authors reported that *M. nigra* extract arrested the cell cycle of PC-3 cells at the G1 phase, induced apoptosis via increased caspase activity and reduced mitochondrial membrane potential. In another study statistically no significant difference was observed in the hepatic lipid fractions of rats containing *M. nigra* fruit extract. However, the level of lipid peroxidation were found to be very low in *M. nigra* treated group (Aydın et al. 2015). Volpato et al. (2011) reported that *M. nigra* leaf aqueous extract had antioxidant effect, contributing to reduce incidence of internal anomalies in offspring from diabetic rat dams. Kutlu et al. (2011) informed that acidified extract of black mulberry was higher in β-carotene prevention and DPPH radical scavenging activity than non-acidified extract. However, non-acidified extract represented a higher reducing power and metal chelating activity, and a higher content of total phenolics.

As shown in the Table II no significant difference in the induction of chromosomal aberrations was observed between the groups treated with the concentrations of BMFJ and the negative control. Beyond, the treatment of the cells with BMFJ plus MMC (pre-treatment and simultaneous treatment) showed a significant reduction of chromosomal aberrations when compared to treatment with MMC only (Table II). A relationship between chromosomal damage and cancer development has been suggested since the beginning of the 20$^{th}$ century (Ribbert et al. 1914, Bonassi et al. 2000), and now we clearly know that increased frequency of chromosomal aberrations cause cancer development because of genomic imbalance.

BMFJ has also significantly reduced the frequency of micronuclei (pretreatment and simultaneous treatment) induced by MMC at the three concentrations (1/1, 1/2 and 1/4 dilutions) tested. The *in vitro* micronucleus test detects genotoxic effects of xenobiotics in cells and predicts cancer development (Bonassi et al. 2011). Our results showed that all concentrations of BMFJ indicated no genotoxicity but protective activity against genomic changes induced by anti-tumor agent MMC in human lymphocytes. Protective effects of BMFJ on MMC induced chromosomal damages most probably due to its free radical scavenging activity.

## ACKNOWLEDGMENTS

The authors have thanks to the Recep Özbudak and Fehmi Doğançay who provides black mulberry fruits.

## AUTHOR CONTRIBUTIONS

SY supervised the research, designed and performed experiments, analyzed data and co-wrote the paper. AU performed experiments and co-wrote the paper. BG collected samples, analyzed data and co-wrote the paper.

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