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## Phytochemical profile and biological potential of mulberry teas (*Morus nigra* L.)

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**Abstract:** Black mulberry is the most medicinally important plant of the genus *Morus*. This study examines the chemical composition, antioxidant, antimicrobial and cytotoxic activity of mulberry leaves tea. The tea drinks were prepared in water (70, 80, 90 ° C and boiling temperature). This study has shown that mulberry teas have high antioxidant, antimicrobial and cytotoxic activity. Experimental investigation has shown that the best conditions for preparing mulberry tea are with boiling water. Mulberry teas could be regarded as a promising source of bioactive natural compounds, which can be used both as a food supplement and herbal remedy.

**Key words:** *Morus nigra*, tea, phytochemical profile, biological potential.

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### Introduction

From the ancient times up to the present day medicinal plants have been used as an essential resource of health benefit compounds (Vidović et al., 2013). Eastern countries have been using herbal remedies to treat infections, ailments and diseases. Herbal remedies are often consumed in the form of tea, i.e. an

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infusion of dried plant parts steeped in boiling water (Chan et al., 2010; Piljac-Žegarac et al., 2013; Samec et al., 2010). They do not have any particular nutritional value; also constitute an important source of antioxidants (Warren, 1999). On the other hand, herbal infusions and teas could be taken as a good complement of the antioxidants intake in the human diet (Alarcon et al., 2008). Antioxidants are capable of stabilizing, or deactivating free radicals before the latter attack cells and biological targets. They are therefore critical for maintaining optimal cellular and systemic health and well-being (Percival, 1998; Atoui et al., 2005). Today tea is used as a beverage and as a source of health benefit compounds. Due to the awareness of health benefit compounds, consumption of tea is becoming more and more popular in all world countries, including Serbia (Vidović et al., 2013). Many therapeutic properties such as neuroprotective, cardioprotective, chemoprotective, anticarcinogenic, hepatoprotective and anti-inflammatory have been attributed to herbal preparations (Campanella et al., 2003; Visioli et al., 2000), including teas. In Serbia the production of teas and other herbal products have been increased at a rate of 10% annually (Vidović et al., 2013).

*Morus* genus is composed of 24 species and more than 100 known variations (Ahmet et al., 2011). Mulberry possesses a long usage history in folk medicine, while almost all parts of the plant exhibit certain level of biological activity. Many biological components such as moranolin, albafrican, albanol, morusin, kuwanol, calystegin and hydroxymorcin have been isolated from mulberry plant and possess significant role in the pharmaceutical industry (Bose, 1989). Due to high content of polyphenolic compounds and bio-potential, this plant becomes more and more interesting and significant for scientific world (Ercisli et al., 2010; Radojković, 2012). Leaves of black mulberry have been used as insect feed in the silk industry in Asian countries. Preparations obtained from leaves of this plant was used for thousands of years for cough suppression, treatment of high sugar level in the blood, different inflammatory treatment, high blood pressure treatment and cancer treatment (Ahmed et al., 2011; Radojković, 2012). This plant is widespread in Serbia, but insufficiently exploited natural resource.

In order to estimate biological activity of black mulberry tea, prepared samples were tested using DPPH assay and assays of antimicrobial and cytotoxic activity (MTT assay). Total phenolics and flavonoids contents were determined, while influence of temperature of water on their content was also estimated.

## **Materials and methods**

### Chemicals and microorganisms

1,1-Diphenyl-2-picryl-hydrazyl-hydrate (DPPH), Folin-Ciocalteu reagent and (±)-catechin were purchased from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). Gallic acid was purchased from Sigma (St. Louis, MO, USA). All

other chemicals and reagents were of analytical reagent grade. The following bacteria: *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* ATCC 13883, *Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 13315, *Proteus mirabilis* ATCC 14153, *Bacillus subtilis* ATCC 6633, and fungi; *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404. The fungi were cultured on potato-glucose agar for 7 days at room temperature of 20 °C under alternating light and dark conditions. They were recultured on a new potato-glucose substrate for another 7 days. The culturing procedure was performed four times until pure culture was obtained. The identification of the test microorganisms was confirmed by the Laboratory of Mycology, Department of Microbiology, Torlak Institute, Belgrade, Serbia.

#### Plant material

In this research dried plant material of the commercially available *Morus nigra* L. leaves was used (Adonis D.O.O., Sokobanja). The samples of mulberry leaves were dried naturally (in the shade, on draft) during one month and grounded in a blender before the extraction. Mean particle size was determined using sieve sets (Erweka, Germany). The mean particle size of the plant samples was  $1.26 \pm 0.07$  mm.

#### Preparation of tea samples

Leaves of black mulberry (2 g) were topped with water (200 mL) at different temperatures (70, 80, 90 °C and boiled water). After 10 minutes the beverages were filtrated. Obtained extracts were used for further analysis.

#### Determination of total phenolics and flavonoids content

Total phenolics content (TPC) in tea and extracts were determined using the Folin–Ciocalteu reagent (Singelton i Rosi, 1965). The reaction mixture was prepared by mixing 0.1 mL of methanolic solution (concentration 50 mg/mL) of extract, 7.9 mL of distilled water, 0.5 mL of the Folin–Ciocalteu's reagent and 1.5 mL of 20% sodium carbonate. After 2 h, the absorbance at 750 nm (VIS spectrophotometer, Janwey 6300, Germany) was measured against the blank solution which had been prepared in a similar manner only with replacing the extract with distilled water. The total phenolic content, expressed as mg of chlorogenic acid equivalents per g dry mulberry extract (mg CAE/g) and per 200 mL of tea, was recalculated using calibration curve of chlorogenic acid as standard.

Total flavonoids content (TFC) in tea and extracts were estimated according to previously described method (Markham, 1989). Flavonoids from *Morus* extracts (0.2 g) were extracted with 2 mL of extraction medium (70% [v/v]

methanol, 5% [v/v] acetic acid and 25% [v/v] distilled water) at room temperature for 60 min. The resulting solution was filtered through Whatman paper No. 4 followed by filtrate volume adjustment to 10 mL. The mixtures were prepared by mixing 5 ml of extract, 1 ml of distilled water and 2.5 mL of AlCl<sub>3</sub> solution (26.6 mg AlCl<sub>3</sub> x 6H<sub>2</sub>O and 80 mg CH<sub>3</sub>COONa dissolved in 20 mL distilled water). A blank solution was prepared by replacing the extract sample with distilled water. The absorbance of probes and blank probe were measured immediately at 430 nm. Total flavonoid content, expressed as mg of rutin equivalents per g dry extract (mg RE/g) and per 200 mL of tea, was calculated from a calibration curve using rutin as standard.

#### Determination of biological activity of tea samples

The free radical scavenging activity of mulberry extracts was determined according the previously described method (Espin et al., 2000). Radical scavenging capacity (%RSC) was calculated using the equation 1, where A<sub>s</sub> is the absorbance of sample solution and A<sub>b</sub> is the absorbance of a blank sample.

$$\%RSC = 100 - (A_s \times 100) / A_b \quad (1)$$

This activity was also expressed as IC<sub>50</sub>; which is the concentration of the solution tested required to obtain 50% of radical scavenging capacity.

Antimicrobial activity was determined according the method described by Satyajit et al. (2007). Minimum inhibitory concentrations (MIC) of the extract and cirsimarin against the test bacteria were determined by microdilution method in 96 multi-well microtiter plates. All tests were performed in Muller–Hinton broth (MHB). A volume of 100 µL stock solutions of samples (in methanol, 200 µL/mL) and cirsimarin (in 10% DMSO, 2 mg/mL) was pipetted into the first row of the plate. 50 µL of Mueller Hinton or Sabouraud dextrose broth (supplemented with Tween 80 at a final concentration of 0.5% (v/v) for analysis of samples) was added to the other wells. A volume of 50 µL from the first test wells was pipetted into the second well of each microtiter line, and then 50 µL of scalar dilution was transferred from the second to the twelfth well. 10 µL of resazurin indicator solution (prepared by dissolution of a 270-mg tablet in 40 mL of sterile distilled water) and 30 µL of nutrient broth were added to each well. Finally, 10 µL of bacterial suspension and fungi suspension was added to each well. For each strain, the growth conditions and the sterility of the medium were checked. Standard antibiotic amracin was used to control the sensitivity of the tested bacteria, whereas ketoconazole was used as control against the tested fungi. Plates were wrapped loosely with cling film to ensure that bacteria did not become dehydrated and prepared in triplicate, and then they were placed in an incubator at 37 °C for 24 h for the bacteria and at 28 °C for 48 h for the yeast. Subsequently, color change was assessed visually. Any color change from purple

to pink or colorless was recorded as positive. The lowest concentration at which color change occurred was taken as the MIC value. The average of 3 values was calculated, and the obtained value was taken as the MIC for the tested compounds and standard drug.

Obtained *Morus* extracts were evaluated for their cytotoxic activity through their influence on growth of malignantly transformed cell lines using the MTT assay. Malignantly cell lines used in this assay were cell line derived from human rhabdomyosarcoma (RD cell line), cell line derived from human cervix carcinoma (Hep2c cell line) and cell line derived from murine fibroblast (L2OB cell line). Cells were seeded (104 cell/mL; 100  $\mu$ L/well) in 96-well cell culture plates (NUNC) in nutrient medium (MEM Eagle supplemented with 5% (for Hep2c) or 10% (for RD and L2OB) and grown at 37 °C in humidified atmosphere for 24 h. Then, corresponding extract (stock solution: 5 mg of extract dissolved in 1 mL of absolute ethanol) and control (absolute ethanol) diluted with nutrient medium to desired concentrations were added (100  $\mu$ L/well) and cells were incubated at 37 °C in humidified atmosphere for 48 h. Pure nutrient medium (100  $\mu$ L) represented positive control for each cell line. After incubation period, supernatants were discarded and 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) (dissolved in D-MEM (Dulbecco's modification of Eagle's medium) in concentration of 500  $\mu$ g/mL) was added in each well (100  $\mu$ L/well). After addition all wells were incubated at 37°C in humidified atmosphere for 4h. Reactions were halted by addition of 100  $\mu$ L of sodium dodecyl sulfate (SDS) (10% in 10 mM HCl). After overnight incubation at 37°C, absorbance was measured at 580 nm using a spectrophotometer. The number of viable cells per well (NVC) was calculated from a standard curve plotted as cell numbers against absorbance at 580 nm. Corresponding cells (grown in flasks), after cell count by haemocytometer, were used as standards. Standard suspensions were plated in serial dilution, centrifuged at 800 rpm for 10 min and then treated with MTT/D-MEM and 10% SDS/10 mM HCl solutions in the same way as the experimental wells (*ut supra*). The number of viable cells in each well was proportional to the intensity of the absorbed light, which was then read in an ELISA plate reader at 580 nm. Absorbance (A) at 580 nm was measured 24 h later. Cell survival (%) was calculated by dividing the absorbance of a sample with cells grown in the presence of various concentrations of the investigated extracts with control optical density (the A of control cells grown only in nutrient medium), and multiplying by 100. The blank absorbance was always subtracted from the absorbance of the corresponding sample with target cells. IC50 concentration was defined as the concentration of an agent inhibiting cell survival by 50%, compared with a vehicle-treated control. The results of the measurements are expressed as the percentage of positive control growth taking the cis-diamminedichloroplatinum (Cis-DDP) determined in positive control wells as 100% growth (Mosmann 1983; Baviskar i sar. 2012). All experiments were done in triplicate.

## Results and Discussion

Total phenolics and flavonoids contents were investigated in prepared teas from black mulberry leaves together with antioxidant, cytotoxic and antimicrobial activities. In dependence of the used temperature, the TPC was in the range of 21.66-31.77 mg CAE/g of plant material or 45.40-63.40 mg CAE/200 mL of prepared tea (Table 1). From the obtained results it could be noticed that total phenolics content rising with applied temperature. The highest TPC was achieved applying the boiling water. Previous research conducted by Hong et al. (2013) showed that the best temperature range for tea preparation was 90-100 °C which was in accordance with the results obtained in this study. Obtained results in the case of TFC showed that TFC exhibited different tendency than TPC (Table 1). In the beginning, TFC was increasing with temperature until certain value and then started to decrease. This indicated that at the certain value of temperature decomposition of flavonoid occurred (Lee et al., 2005; Kim et al., 2006).

Table 1. Total phenolic content (TPC) and total flavonoid content (TFC) of mulberry teas

Temperature (°C)	TPC (mg CAE/g)	TPC (mg CAE/200 mL)	TFC (mg RE/g)	TFC (mg RE/200 mL)
70	21.66 ± 2.03	45.40 ± 2.03	24.46 ± 0.40	48.80 ± 0.40
80	26.55 ± 0.84	53.20 ± 0.84	27.25 ± 0.45	54.40 ± 0.45
90	29.22 ± 2.54	58.40 ± 2.54	22.02 ± 0.38	44.00 ± 0.38
Boiling temperature	31.77 ± 2.52	63.40 ± 2.52	20.58 ± 0.90	41.20 ± 0.90

Antioxidant activity obtained by DPPH assay was presented in Table 2. The tea sample prepared using the boiling water exhibited the highest activity (lowest IC<sub>50</sub> value) of 0.0724 mg/mL. From the obtained results it could be noticed that with decreasing in temperature antioxidant activity also decrease. Gazzani et al. (1998) concluded that antioxidant activity of fruit juices were stabilized with boiling. Castenmiller et al. (2002) claimed that antioxidant activity of teas depended on thermal processes and plant material.

Table 2. Radical scavenging activity of mulberry teas

Temperature (°C)	IC <sub>50</sub> ± SD (mg/mL)
70	0.1060 ± 0.004
80	0.0860 ± 0.004
90	0.0810 ± 0.002
Boiling temperature	0.0724 ± 0.001

Results obtained for antimicrobial activity were presented in Table 3. Presented values showed that prepared tea samples inhibited growth of all tested microorganisms. Minimal inhibitory concentrations (MIC) were in the range of 39.100-156.250  $\mu\text{g/mL}$  for bacteria and 19.53-78.125  $\mu\text{g/mL}$  for fungi (Table 3). The highest activity was exhibited against *Candida albicans* (19.530  $\mu\text{g/mL}$ ) and *Klebsiella pneumoniae* (39.100  $\mu\text{g/mL}$ ). Obtained results showed significant activity against *Staphylococcus aureus* (78.125  $\mu\text{g/mL}$ ). Exhibited activity was very significant for general characterization of biological activity of mulberry tea samples, taken into account that *Staphylococcus aureus* showed high resistance against antibiotics especially against  $\beta$ -lactams and macrolides which is still using in the therapy against bacteria (Garrity et al., 2005). Fukai et al. (2005) showed that chalconoracin isolated from mulberry leaves exhibited very high antibacterial activity against *Staphylococcus aureus* (0.78  $\mu\text{g/mL}$ ).

Table 3. Minimum inhibitory concentration (MIC) of mulberry teas

Microbial strains	MIC ( $\mu\text{g/mL}$ )		
	Tea	Amracin (standard)	Ketokonazol (standard)
<i>Staphylococcus aureus</i> ATCC 25923	78.125	0.970	/
<i>Klebsiella pneumoniae</i> ATCC 13883	39.100	0.490	/
<i>Escherichia coli</i> ATCC 25922	78.125	0.970	/
<i>Proteus vulgaris</i> ATCC 13315	156.250	0.490	/
<i>Proteus mirabilis</i> ATCC 14153	156.250	0.490	/
<i>Bacillus subtilis</i> ATCC 6633	78.125	0.240	/
<i>Candida albicans</i> ATCC 10231	19.530	/	1.950
<i>Aspergillus niger</i> ATCC 16404	78.125	/	0.970

Results of cytotoxic activity obtained using MTT test were presented in Table 4. Three cell lines were used (*Hep2c*, *RD* and *L2OB* cell lines) and tested *in vitro*. Obtained results were compared with *cis*-diamminodichloroplatinum (*cis*-DPP) activity as standard. From the presented results it might be noticed that prepared tea samples showed certain diversity in capability of inhibition of cell

lines growth. Exhibited activity was in the range of 22.25-101.25  $\mu\text{g/mL}$ . sensitivity of tested cell lines decreased in following order *Hep2c cells* > *RD cells* > *L2OB cells*.

Table 4. IC<sub>50</sub> values of mulberry teas

Sample	IC <sub>50</sub> ± SD ( $\mu\text{g/mL}$ )		
	<i>Hep2 cells</i>	<i>RD cells</i>	<i>L2OB cells</i>
<i>Tea</i>	22.25 ± 1.17	30.07 ± 0.63	101.25 ± 0.14
<i>cis</i> - diamminedichloroplatinum (Cis-DDP)	0.94 ± 0.55	1.40 ± 0.97	0.72 ± 0.64

### Conclusion

Obtained results showed that prepared tea samples of black mulberry leaves possessed high content of polyphenolic compounds. It was also showed that tea samples exhibited high antioxidant, antimicrobial and cytotoxic activity. Proposed water temperature for preparation of the tea was near boiling point. It may be concluded that tea of mulberry leaves, as simplest form of phytopreparation, should be recommended for usage and further examination due to placement of mulberry products in the market as food supplement.

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## FITHEMIJSKI PROFIL I BIOLOŠKI POTENCIJAL ČAJNOG NAPITKA DUDA (*MORUS NIGRA* L.)

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### Rezime

Crni dud (*Morus nigra* L.) je medicinski važna biljka iz porodice *Morus*. U ovom radu ispitan je sadržaj ukupnih fenola i flavonoida kao i antioksidativna, antimikrobna i citotoksična aktivnost čajnih napitaka lista crnog duda. Čajni napitci su pripremani korišćenjem vode različite temperature (70, 80, 90°C i ključala voda). Ispitivanja koja su urađena su pokazala da čajni napitci poseduju visoku antioksidativnu, antimikrobnu i citotoksičnu aktivnost. Najbolje osobine pokazali su napitci pripremani sa ključalom vodom. Čajni napitci lista duda se mogu smatrati odgovarajućim izvorom prirodnih bioaktivnih komponenti, koji mogu naći primenu u vidu dodataka ishrani ili herbalnih lekova.

**Ključne riječi:** *Morus nigra*, dud, čajni napitak, fitohemijski profil, biološka aktivnost.