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ORIGINAL PAPER

Antioxidant and antiproliferative effects of methanol extracts from raw and fermented parts of mulberry plant (*Morus alba* L.)

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Abstract Total phenolic content, phenolic acids, radical scavenging activity and antiproliferative properties of different parts of mulberry (*Morus alba* L.) were determined. The highest phenolic content was found in methanol extracts of mulberry root (117.7 ± 2.0), followed by leaves (71.4 ± 2.4), branches (49.0 ± 1.5) and fruit (11.2 ± 0.3) [mg ferulic acid equivalents (FAE) kg^{-1} dry weight (DW)] ($P < 0.05$). The highest level of total phenolics in the fractions was in roots (166.2 ± 7.5 and 160.8 ± 7.2 mg kg^{-1} DW for BuOH and EtOAC, respectively). Methanol extracts and their fractions dose dependently increased radical scavenging activity of mulberry branches, roots and leafs (more than 70%). Total phenolic content of the tested plant extracts was highly correlated with the radical scavenging activity. The antiproliferative effect of mulberry parts on human cell lines was different and connected to the concentrations of the investigated extracts. The fermentation of the mulberry leaves did increase their methanol extract antiproliferative effect only on human gastric carcinoma (SNU-601) cell line in concentration of 1,000 mg mL^{-1}. In conclusion, some plant parts of the Korean mulberry could be recommended as preventative and therapeutic agents, based on their antioxidant and antiproliferative properties.

Keywords Mulberry plant parts · Total phenolics · Phenolic acids · Radical scavenging activity · Antiproliferative effect

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Introduction

It was reported that some plants exercise various bioactivities, including antioxidant, anti-inflammatory, anticancer and anti-diabetic [1–3]. Recently, there has been a worldwide trend towards the use of various plants [4, 5]. The search for new plants has gained momentum as they are considered, rightly or wrongly, not to pose any health risk to consumers [6, 7].

One of the less studied plants is mulberry (*Morus alba* L.). Mulberry is a fast-growing deciduous plant [8]. It is valued for its foliage, which constitutes the chief feed for silkworms. The leaves are nutritious, palatable and non-toxic and are stated to improve milk yield when fed to

dairy animals [9]. Reports indicate that mulberry leaves contain proteins, carbohydrates, calcium, iron, ascorbic acid, β-carotene, vitamins B-1 and D, and folic acid [10]. Also, rutin, quercetin, isoquercetin and other flavonoids in mulberry leaves have been found [11]. Mulberry plant possesses diuretic, hypoglycemic and hypotensive medicinal properties [9, 12]. As was mentioned, this plant is less studied. Therefore, it was decided to investigate the mulberry plant.

Methanol is frequently used to extract specific bioactive ingredients from various natural products. Anti-inflammatory ingredients have been found in the methanol extraction from *Culcasia scadens* [13], antimicrobial compounds from *Ceanothus americanus* [14] and antihistaminic compounds from *Mentha spicata* [15]. Therefore, extraction with methanol side by side with other solvents was used in this investigation.

Some investigators used hexane and butanol to extract ingredients from mulberry roots with low polarity, and then investigated the specific functional compounds such as prenyflavas, glycoside, isoquercetin, and astragalin from extracted solution [16]. In addition, ethyl acetate and *n*-hexane have also been applied to extract bioactive ingredients. According to these authors, the solvent polarity would appear to be important for extracting specific functional ingredients from natural products. Therefore, a variety of solvents, pure and mixtures, have been applied to extract bioactive ingredients with various polarities [17]. It was reported that the optimal solvent polarities for the extraction of polyphenols, other antioxidants and antityrosinase ingredients were predicted with solubility parameters [$MPa^{1/2}$] as 38.5; 37.33; and 33.0, respectively [18]. Kim et al. [18] suggest that the target bioactive ingredient of polyphenols, antioxidant and antityrosinase exhibited different dependency of extraction efficiency on the alcohol solvents (methanol, ethanol, *n*-propanol and *i*-propanol) and composition of binary solvent. However, so far, no work on optimal extraction methods from mulberry plant was reported. Therefore, in the present study, we try to find the best solvent for the optimal extraction of mulberrys' total phenolics and examine its radical scavenging activity and cytotoxic effect. It was important to determine some phenolic acids and flavonoids in the different solvent fractions.

In order to receive reliable data of the radical scavenging activity, DPPH assay was chosen [12].

The cytotoxic effect of different natural products was described [2, 19, 20]. It was of interest to know if mulberry plant also possesses such properties. Therefore, also the cytotoxic effect of mulberry plant was studied.

As far as we know, there are no published results of such investigations.

Materials and methods

Chemicals

Folin-Ciocalteu reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) were obtained from Sigma Chemical Co., St. Louis, MO, USA. All reagents were of analytical grade. Deionized and distilled water were used throughout. The cell lines (Calu-6, human pulmonary carcinoma; MCF-7, human breast adenocarcinoma; HCT-116, human colon carcinoma; SNU-601, human gastric carcinoma) were purchased from Korean Cell Line Bank (KCLB).

Plant material

Whole parts of Korean mulberry plants (*M. alba*) grown in a mountain area of the Suncheon City, Korea, were harvested on June 2008. The samples were separated into leaves, fermented mulberry leaves, branches, roots and fruits, immediately freeze-dried at −40 °C for 5 days, ground with a Wiley mill to pass a 1-mm screen, and stored in a refrigerator at 2 °C until use. The samples were three times extracted with 95% methanol in a shaker at 24-h interval for 3 days at room temperature. Then the extracts were filtered through a Whatman No. 1 filter paper. The collected filtrate was evaporated to dryness under vacuum at 40 °C using a rotary evaporator (N-1000V-W, Eyela, Japan). After evaporation, the yield of dried methanol extract was about 10% of the original plant sample. For fractionation, the evaporated methanol extracts were mixed with distilled water and hexane to collect hexane extracts, were shaken for 2 h. After hexane collection, the distilled water fractions were added to ethyl acetate (EtOAc) to obtain EtOAc extracts in the same way. The same procedure was used in preparing butanol and water extraction. The fractions were again taken to dryness on a rotary evaporator at 40–50 °C. The methanol extracts and dried hexane, EtOAc, butanol, and water fractions were redissolved in methanol and used for determination of the total phenolic content and DPPH radical scavenging activity.

Total phenolic content

The content of total phenolics (TPs) was measured using the classical Folin-Ciocalteu assay [21]. 5 mL of Nanopure water, 0.5–1.0 mL of sample, and 1.0 mL of Folin-Ciocalteu reagent were added to a 25 mL volumetric flask. The above-mentioned substances were mixed and allowed to stand for 5–8 min at room temperature. Next, 10 mL of

a 7% sodium carbonate solution was added, followed by the addition of Nanopure water filled to volume. Solutions were mixed and allowed to stand at room temperature for 2 h. Sample aliquots were filtered through a Whatman 0.45 m poly(tetrafluoroethylene) filter prior to the determination of TP content using a UV-1650 spectrophotometer (Shimadzu, Japan) monitoring 640 nm. TPs were standardized against ferulic acid and expressed as mg of ferulic acid equivalents (FAE) kg^{-1}. The linearity range for this assay was determined as 0.5–5.0 mg L^{-1} FAE ($R^2 = 0.9990$).

Phenolic acids in different solvent fractions

For high-performance liquid chromatography (HPLC) analysis, the dried methanol extracts were prepared from different solvent fractions from plant parts of mulberry. The extracts were again dissolved in HPLC grade MeOH to give 1,000 ppm. The standard phenol compounds used for HPLC analysis were chlorogenic, caffeic, syringic, *p*-coumaric and ferulic acids, naringin (flavonoid) and coumarin (Aldrich Co., CA, USA). All of the chemicals were purchased as high-purity standards and the used solvents were HPLC spectral grade. All solvents and distilled water were degassed before use. All solvent ratios were based on volume.

The phenolic compounds were identified by a HPLC system (SPP 10AVP, Shimadzu, Japan) with a flow rate of 1 mL min^{-1}; the column was CAPCELL PAK C18 SG120 (4.6 × 250 mm); an autoinjector with a 10 μL sample loop was employed. The mobile phase consisted of water, methanol and acetic acid in the ratio of 12:15:1 volume, respectively. The UV detector wavelength was set at 275 nm. Standard compounds were chromatographed alone and as mixtures. Retention times for the standard compounds and the major peaks in the extract were recorded. Phenolic compounds from each fraction were identified by retention times or standard addition, and their amounts were calculated by comparing peak area with those of standards.

Radical scavenging activity

Free radical scavenging activity of the methanol extracts and their fractions was determined as previously described [20, 22].

Antiproliferative effect

Antiproliferative effect of methanol extracts from mulberry plant parts on human cancer cell lines (Calu-6 for human pulmonary carcinoma; SMU-601 for human gastric carcinoma, MCF-7 for human breast adenocarcinoma and HCT-116 for human colon carcinoma) were measured using MTT assay. The cell lines were purchased from Korean Cell Line Bank for MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The treatment of the cell lines was done according to Chon et al. [20]. Controls and samples were assayed in duplicate for each concentration and replicated three times for each cell line. The cytotoxicity was obtained by comparing the absorbance between the samples and the control. The values were then used to iteratively calculate the concentration of plant extracts required to cause a 50% reduction (IC_{50}) in growth (cell number) for each cell lines.

Statistical analysis

To verify the statistical significance, mean ± SD of three independent measurements was calculated. Differences between groups were tested by two-way ANOVA. In the assessment of the antioxidant potential, Spearman correlation coefficients (R) were used. Linear regressions were also calculated. The P values of <0.05 were considered significant.

Results and discussion

Total phenolic content

The contents of total phenolics in methanol extracts were 117.7 ± 2.0, 71.4 ± 2.4, 49.0 ± 1.5 and 11.2 ± 0.3 [mg ferulic acid equivalents (FAE) kg^{-1} DW] for roots, leaves, branch and fruits, respectively (Fig. 1). The differences in the phenolic content were significant ($P < 0.05$). Also, the content of total phenolics in the fractions differed significantly: the highest content of total phenolics in leaf was

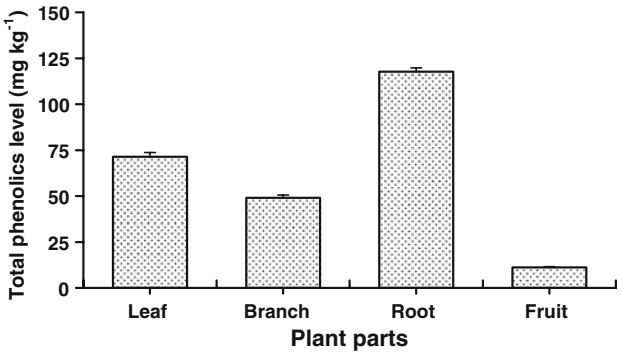

Fig. 1 Total phenolic amount [mg ferulic acid equivalents (FAE) kg^{-1} dry weight (DW)] of methanol extracts from the mulberry plant parts. The content of total phenolics (TPs) was measured using Folin-Ciocalteu assay. TP was standardized against ferulic acid and expressed as mg of ferulic acid equivalents (FAE) kg^{-1}. The content was the highest in the root part and followed by leaf, branch, and fruit

recorded in hexane and ethyl acetate fractions (154.3 ± 7.1 and 152.2 ± 7.0 mg kg^{-1} DW, respectively), while in roots, 97.4 ± 4.3 and 160.8 ± 7.2 mg kg^{-1} DW, respectively (Table 1). The result was highly consistent with the finding of DPPH radical scavenging activity [20]. Also, Zhou and Yu [23] reported that total phenolic content of the tested vegetable extracts was correlated with the DPPH radical scavenging activity, suggesting that total phenolic is playing a major role in the antioxidant activity of plant materials.

Phenolic acids and flavonoids

The phenolic acids and flavonoids in leaf were found in the following solvent fractions: hexane [chlorogenic (3.669 ± 0.18^b), *p*-coumaric (10.334 ± 0.51^b) and ferulic (0.844 ± 0.12^a) acids, naringin (28.817 ± 1.4^b)]; ethyl acetate [chlorogenic (2.003 ± 0.11^a), *p*-coumaric (1.005 ± 0.11^a) and ferulic (1.567 ± 0.11^b)] acids; butanol [chlorogenic (44.151 ± 2.12^d), caffeic (2.391 ± 0.11^a), *p*-coumaric (1.009 ± 0.11^a) and ferulic (3.215 ± 0.21^c) acids, naringin (6.061 ± 0.32^a)]; water [chlorogenic acid (14.254 ± 0.71^c)].

As can be seen, the most active solvents of the leaf extracts were butanol for chlorogenic and ferulic acids and hexane for *p*-coumaric acid and naringin ($P < 0.05$ in both cases). However, methanol solvent was completely not effective.

The phenolic acids and flavonoids in branch were found in the following solvent fractions: hexane [chlorogenic (2.727 ± 0.12^b), *p*-coumaric (3.641 ± 0.22^c) and ferulic (11.282 ± 0.12^b) acids, naringin (4.096 ± 0.21^a)]; ethyl acetate [chlorogenic (66.342 ± 3.44^d), caffeic acid (3.939 ± 0.21^b), *p*-coumaric (1.035 ± 0.01^b) and ferulic (3.667 ± 0.11^a)] acids; butanol [chlorogenic acid (12.162 ± 0.62^b); water [chlorogenic acid (27.671 ± 1.32^c), caffeic acid (0.352 ± 0.01^a) and *p*-coumaric (0.371 ± 0.01^a) acids, naringin (5.261 ± 0.3^b)]. In the investigation of branch as in the investigation of leaf extracts, methanol solvent was completely not effective. However, ethyl acetate for chlorogenic and caffeic acids and hexane for *p*-coumaric and ferulic acids were significantly effective ($P < 0.05$).

The phenolic acids and flavonoids in roots were found in the following solvent fractions: methanol [caffeic (4.366 ± 0.01^c) acid, naringin (3.625 ± 0.72^b)]; ethyl acetate [caffeic (0.734 ± 0.031^a), syringic (1.267 ± 0.11^a), ferulic (0.105 ± 0.01)] acids, naringin (15.297 ± 0.78^b), coumarin (4.293 ± 0.21)]; butanol [chlorogenic (9.011 ± 0.12^b); syringic (5.358 ± 0.31^b)], water [chlorogenic (5.487 ± 0.12^a) and caffeic (1.596 ± 0.08^b) acids]. In the investigation of root extracts, the significantly effective solvent was butanol ($P < 0.05$) for chlorogenic and syringic acids and methanol for caffeic acid.

The phenolic acids and flavonoids in fruit were found in the following solvent fractions: ethyl acetate [chlorogenic acid (4.032 ± 0.11^a), naringin (0.927 ± 0.11)], butanol [chlorogenic (5.27 ± 0.12^b)]. In the investigation of fruit, most of the used solvents were not effective. Only butanol solvent was effective in three of the four studied parts of mulberry (leaf, root and fruit).

Radical scavenging activity

Table 2 shows that mulberry fruits exhibited the highest radical scavenging activity ($P < 0.05$) with IC_{50} values (8151 ± 91, 752 ± 28, 1312 ± 37 and 3251 ± 81 mg kg^{-1}, with hexane, EtOAc, BuOH and water solvents, respectively).

Figure 2 summarized the results of the determination of DPPH radical scavenging activity in the methanol extracts of mulberry parts. According to this figure, the maximal activity (more than 70%) was registered for branches and roots at the concentration of 1,000 mg kg^{-1} followed by leafs. The lowest DPPH radical scavenging activity in the methanol extracts of fruits is 35%. However, the DPPH radical scavenging activity values of synthetic antioxidants vitamin E and BHT with IC_{50} values are higher with 31.0 and 113.0 mg kg^{-1}, respectively. Fractions of mulberry root parts exhibited the highest DPPH radical scavenging, with IC_{50} values of 646.4 mg kg^{-1}, followed by leaves and branches. DPPH radical scavenging activity was relatively

Table 1 Content of total phenolics in the different solvent fractions of mulberry plant parts

Plant parts	Total phenolic content, mg kg^{-1}				
	Hexane	EtOAc	BuOH	Water	Total
Leaf	154.3 ± 7.1^a	152.2 ± 7.0^a	121.8 ± 5.9^b	28.4 ± 1.7^a	456.7
Branch	107.8 ± 5.2^b	77.9 ± 3.8^c	87.6 ± 4.2^c	4.5 ± 0.3^b	277.8
Root	97.4 ± 4.3^b	160.8 ± 7.2^a	166.2 ± 7.5^a	32.2 ± 1.9^a	456.6
Fruit	81.3 ± 3.9^c	102.6 ± 4.4^b	25.1 ± 1.6^d	4.5 ± 0.3^b	213.5
Total	440.8	493.5	400.7	69.6	1,404.6

Values are means ± SD of three measurements. Values in columns with different superscript letters are significantly different ($P < 0.05$)

Table 2 RC_{50} values of DPPH radical scavenging activity from the different solvent fractions of mulberry plant parts

Plant parts	DPPH radical scavenging activity, RC_{50}^{a}				
	Hexane	EtOAc	BuOH	Water	Mean
Leaf	1,593 ± 52[b]	122 ± 6[d]	104 ± 5[d]	1,442 ± 43[c]	815.25
Branch	1,450 ± 43[b]	399 ± 19[b]	432 ± 19[b]	2,927 ± 79[b]	1,302.4
Root	480 ± 23[c]	227 ± 11[c]	347 ± 19[c]	1,532 ± 51[a]	646.4
Fruit	8,151 ± 91[a]	752 ± 28[a]	1,312 ± 37[a]	3,251 ± 81[c]	3,366.3
Mean	2,918.5	375.0	548.75	2,288	1,532.6

Values are means ± SD of three measurements. Values in columns with different superscript letters are significantly different ($P < 0.05$)

[a] Extract concentrations, which show 50% activity of DPPH radical scavenging, were determined by interpolation

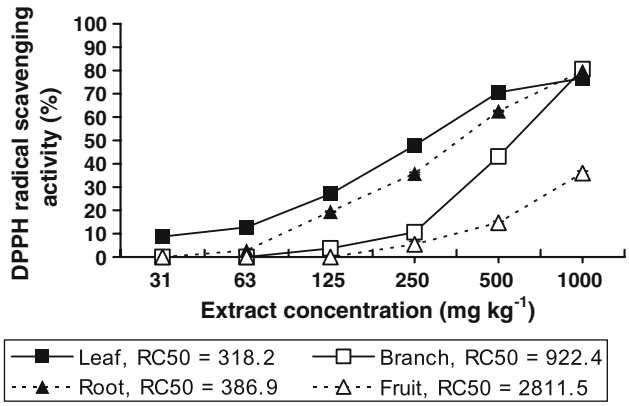

Fig. 2 Radical scavenging activity of methanol extracts from the mulberry plant parts. Free radical scavenging activity of the methanol extracts was determined using 1,1-diphenyl-2-picrylhydrazyl method (DPPH). The highest activity (70%) was registered for leaves, branches and roots at the concentration of 1,000 mg kg^{-1}. The lowest DPPH radical scavenging activity in the methanol extracts was in fruits (35%). The antioxidant activity of plant extracts was partially expressed as IC_{50}, which was defined as the concentration (mg kg^{-1}) of extract required to inhibit the formation of DPPH radicals by 50%

high in ethyl acetate and butanol fractions, with IC_{50} values of 375.2 and 548.8 mg kg^{-1}, respectively.

All samples of mulberry plant parts proved that DPPH radical scavenging activity is dose dependent. The results show that antioxidant activity compounds could be of different amount in various plant species. It was reported that antioxidant activity of plants is higher than that of synthetic antioxidants [24], and this is in accordance with our obtained results. The above-mentioned authors investigated methanol extracts of nine medicinal plants traditionally used in Chinese medicine versus resveratrol. They found relatively high levels of DPPH radical scavenging activity in extracts of *Areca catechu* var. *dulcissima*, *Paeonia suffruticosa* and *Cinnamomun cassia* ($IC_{50} < 6.0$ μg mL^{-1}). The extracts of *A. catechu* var. *dulcissima* showed higher antioxidant activity than resveratrol in all experiments. In some investigations, applied solvents were with different polarities for polyphenols extraction and these solvents had significant effect on polyphenol content and antioxidant activity [25–27]. Others have shown that the polyphenols and the antioxidant capacities in water extracts were higher than in methanol. The correlation coefficients between polyphenols and antioxidant capacities of Prolipid with 1,1-diphenyl-2-picrylhydrazyl radical assay were about 0.97 [25]. It was also shown that 50% of ethanol extract from mate tea had the greatest antioxidant activity [25–27].

The methanol extract of *Ulmus davidiana* exhibited strong antioxidant activity in the tested model systems [27]. *U. davidiana* extracts may be exploited as biopreservatives in food applications as well as for health supplements of functional food, to alleviate oxidative stress. Our results also showed high phenolic content and antioxidant activity in the methanol extracts from leaf and root parts. Correlation coefficient between the extracted polyphenols with ethanol and antioxidant activity was about 0.88. Relatively high correlation between above-mentioned variables in the studied extracts was compared with tea, Prolipid and *U. davidiana* extracts [25–27] (Fig. 3).

Our results are in correspondence with others [28], where polar and non-polar extracts of some tropical green leafy vegetables were investigated. The antioxidant activity depended on the amount of bioactive compounds such as total phenols and flavonoids in water and hexane extracts.

Antiproliferative effect

The antiproliferative effect of the methanol extracts of mulberry leafs on the cell lines Calu-6 (human pulmonary carcinoma), MCF-7 (human breast adenocarcinoma) and HCT-116 (human colon carcinoma) are shown in Fig. 4. In Fig. 5, the results of the cytotoxic effect of methanol extracts of different parts from *M. alba* on human colon carcinoma (HCT-116) are summarized. In Fig. 6, cytotoxic effect of methanol extract of different parts from *M. alba* on human gastric carcinoma (SNU-601) is summarized. As can be seen, the cytotoxic effect of mulberry parts on human cell lines is different and connected to the extract's concentrations.

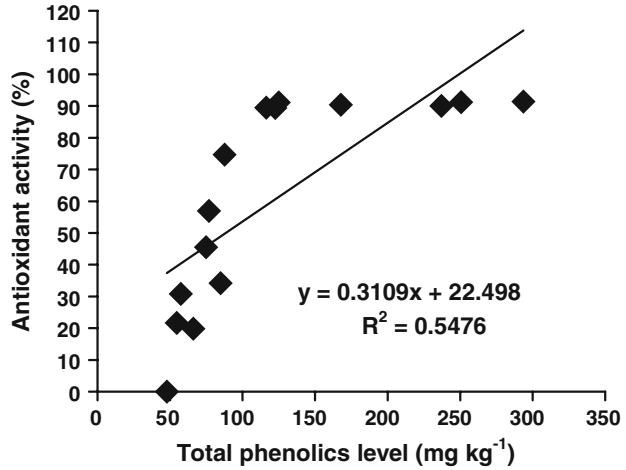

Fig. 3 Correlation between the polyphenols amount [mg ferulic acid equivalents (FAE) kg^{-1} dry weight (DW)] and antioxidant potential (%) in mulberry plant parts. In the assessment of the antioxidant potential, Spearman correlation coefficients (R) were used and linear regressions were also calculated. The P values of <0.05 were considered significant. Total phenolic level was highly correlated with the free radical scavenging activity ($r^2 = 0.5476$)

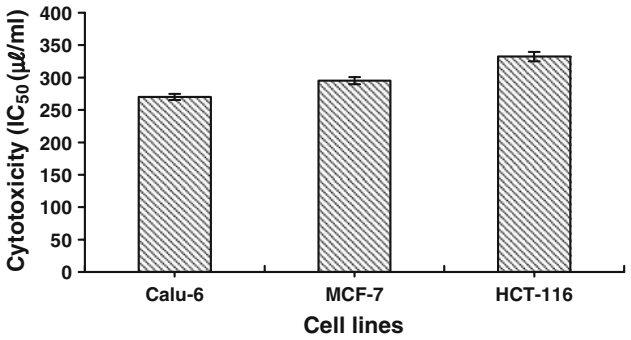

Fig. 4 The antiproliferative effect of the methanol extracts of mulberry leaves on the cell lines Calu-6 (human pulmonary carcinoma), MCF-7 (human breast adenocarcinoma) and HCT-116 (human colon carcinoma) was investigated using MTT assay. A dose-dependent inhibition of cell proliferation was observed for most of methanol extracts. The methanol extracts exhibited more inhibition on Calu-6 cell line, showing the lowest IC_{50} value, than of MCF-7 or HCT-116

Also, others and ours previous publications showed that the cytotoxic effect of different plants on human cell lines is different and connected to the extract's concentrations [2, 19, 20]. The fermentation of the mulberry leaves did increase their methanol extract cytotoxic effect only on human gastric carcinoma (SNU-601) cell line in concentration of 1,000 μg mL^{-1}.

In conclusion, (a) the content of total phenolics in mulberry plant parts differs: the significantly highest contents were found in leaves and roots, (b) the methanol extracts as well as fractions of the same plant parts were characterized by the highest DPPH radical scavenging activity, (c) the plant parts dose dependently increased the free radical scavenging activity, (d) the total phenolic level is highly correlated with the free radical scavenging activity, (e) the cytotoxic effect of mulberry parts on human cell lines was different and connected to the extract's concentrations, (f) some plant parts of the Korean mulberry could be recommended as preventative or/and therapeutic agents in addition to proper prescribed drugs.

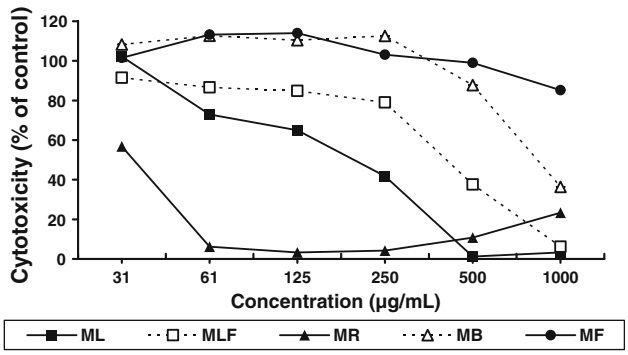

Fig. 5 The antiproliferative effect of methanol extracts of different parts from *Morus alba* on human colon carcinoma (HCT-116). *ML* mulberry leaves, *MLF* mulberry leaves fermented, *MR* mulberry roots, *MB* mulberry branches, *MF* mulberry fruits. Methanol extracts from mulberry roots exhibited the highest anticancer activity on SNU-601 tumor cell lines, and followed by leaves, fermented leaves, branches and fruits (the lowest)

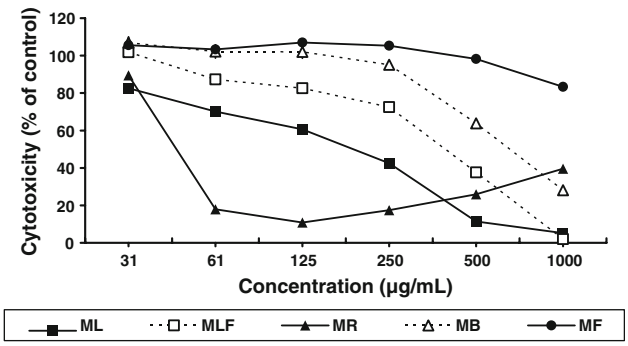

Fig. 6 Cytotoxic effect of methanol extract of different parts from *Morus alba* on human gastric carcinoma (SNU-601). *ML* mulberry leaves, *MLF* mulberry leaves fermented, *MR* mulberry roots, *MB* mulberry branches, *MF* mulberry fruits. Methanol extracts from mulberry roots exhibited the highest anticancer activity on SNU-601 tumor cell lines, and from mulberry leaves (the lowest)

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