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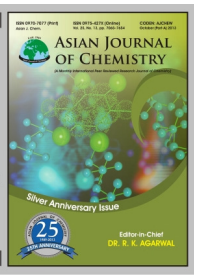

Evaluation of Phytochemical and Anti-oxidant Activity in Different Mulberry Varieties

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The aims of present study were to screen phytochemical constituents and evaluate *in vitro* anti-oxidant activity of leaves of different varieties of mulberry plant [Family Moraceae]. The methanolic extract of leaves of different mulberry varieties namely S-13, S-54, BR-2, S-36 and S-1 which belongs to *Morus alba* and *Morus indica* were subjected for the phytochemical screening. The results obtained from the HPLC analysis, revealed that the methanolic extracts of mulberry leaves samples of S-36 and S-1 varieties contain more amount of phenolics and flavonoids. The *in vitro* FRAP assay clearly indicate that S-1 mulberry leaf extract have maximum significant FRAP concentration at 800 µL/mL and 1000 µL/mL (0.408 ± 0.001 mmol $FeSO_4$/100 g dried sample and 0.410 ± 0.001 mmol $FeSO_4$/100 g dried sample, respectively) a significant source of natural anti-oxidant, which might be helpful in preventing the progress of various oxidative stresses. However, the components responsible for the antioxidative activity are morin, querectin, resveratrol, scopoletin, 7-hydroxycoumarin. Furthermore, these mulberry varieties may be utilized in epidemiological studies.

Key Words: Antioxidant, Flavonoids, *Morus alba*, Mulberry, Morin, Phenols.

INTRODUCTION

The production of traditional, complementary and alternative medicines, in particular those based on plant materials, is a global business. A recommended human diet contains significant quantities of polyphenolics, as they have long been assumed to be anti-oxidants that scavenge excessive, damaging, free radicals arising from normal metabolic processes[1]. Flavonoids and other phenolic compounds are widely distributed in foods and are important constituents of the human diet. The phenolic compounds may be protective against cardiovascular disease and have certain potential anticarcinogenic properties due to their anti-oxidant activity or other properties[2]. The content of the anti-oxidants, phenols and flavonoids in foods has been evaluated by different methods[3-5]. With the advent of the nutraceutical industry, Mulberry has become one of the most popular herbal medicines of recent years, although its use dates back many hundreds of years to ancient Chinese medicine. Polyphenols such as scopoletin, querectin, morin, hydroxycoumarin, rutin, isoquercetin, querectin 3-malynoglucoside (Q3MG) had been identified as natural antioxidants in mulberry leaves[6,7]. Scopoletin may play an important role in regulating free radical generation *via* metabolic pathways such as mitochondrial transport of long chain free fatty acids and cytochrome-p450 transport chain[8]. Mulberry leaf extracts which contain rutin, quercetin, isoquercetin and other flavonoids have been shown to inhibit oxidative modification of low density lipoprotein and may reduce atherosclerosis[5,6,9]. Quercetin has been reported to exhibit biological effects such as antioxidant[10]. At concentrations of 75-100 mmol, morin inhibits oxidation of low density lipoprotein (LDL) by free radicals[11] or Cu^{2+}. Scopoletin is reported to possess antiinflammatory, immunomodulatory and antioxidant activity[12]. In view of the various physiological activities of Mulberry leaves, the present work was undertaken to elucidate phytochemical constituents and to evaluate *in vitro* antioxidant activity of the different Mulberry varieties.

EXPERIMENTAL

Resveratrol, querecetin dihydrate, isoquerectin, kaempferol, 7-hydroxycoumarin, scopoletin were purchased from Sigma Aldrich Chemical Limited, (St. Louis, MO, USA). All organic solvents like methanol, acetonitrile, water were 98 % HPLC grade were purchased Sigma Chemicals Pvt. Limited, Bangalore. Hydrochloric acid (36.2 %) (HCl), ammonium acetate, Folin-Ciocalteau reagent, TPTZ, ferric chloride ($FeCl_3$), ferrous sulphate ($FeSO_4$), acetate buffer pH 3.6, were obtained from Merck (Darmstadt, Germany), morin, sodium carbonate, tannic acid, *n*-butanol, sodium nitrite, aluminium nitrate, sodium hydroxide, rutin from Loba Chemie Pvt. Limited, Mumbai *etc*.

Five different mulberry varieties namely S-13, S-54, BR-2, S-36 and S-1 which belongs to *Morus alba* and *Morus indica* were selected for the present investigation. All the mulberry varieties were taxonomically authenticated from Regional Sericulture Research Station, Sahasnapur, Dehradun. About 100 g of 4th, 5th and 6th matured mulberry leaves below apical part of each mulberry variety were collected after 90 days of pruning from the mulberry garden of Babasaheb Bhimrao Ambedkar (Central University), Lucknow, India. All the mulberry leaves samples were dried in oven at 60 ºC and grinded into powdered form for extraction.

Extraction procedure: Method for extraction and hydrolysis of various phenolics was developed by Hertog *et al*.[13] was applied in the current work with minor modifications for HPLC analysis, total phenolic, total flavonoid and *in vitro* anti-oxidant activity. About 5 g of powdered mulberry leaves samples were dissolved in methanol and acidified in 0.1 % HCl. The residues were again dissolved in methanol for two times and all filtrates were pooled together and evaporated in water bath. The dried extract material was used further used for analysis.

Determination of total phenolic and total flavonoid: The content of phenolic compounds in the extracts was determined according to the reported method[14]. The extracts were dissolved in water. Aliquots of 0.5 mL samples were mixed with 2.5 mL of 10-fold-diluted Folin-Ciocalteu reagent and 2 mL of 7.5 % sodium carbonate. The mixture was allowed to stand for 0.5 h at room temperature before the absorbance was measured at 760 nm spectrophotometrically. The final results were expressed as tannic acid equivalents.

The flavonoid content of the extracts was measured using a modified colorimetric method[15]. A quantity of 0.5 g of extracts was dissolved in 10 mL water and extracted by 10 mL *n*-butanol for three times. The extracts were pooled and concentrated under vacuum at 60 ºC. The residue was re-dissolved in 5 mL of 60 % ethanol and washed twice with 5 mL of 30 % ethanol. All three parts were pooled together and filtered. The filtrate was diluted, up to 25 mL, with 30 % ethanol. A volume of 0.5 mL of the solution was transferred to a test tube containing 4.5 mL of 30 % ethanol and mixed with 0.3 mL of 5 % sodium nitrite for 5 min. Then, 0.3 mL of 10 % aluminium nitrate was added in the solution. After 6 min, the reaction was stopped by adding 2 mL of 1 M sodium hydroxide. The mixture was further diluted with 30 % ethanol up to 10 mL. The absorbance of the mixture was immediately measured at 510 nm. The flavonoid content was calculated and expressed as rutin equivalents. Three replicates were maintained for determination of total phenolic and total flavonoid content.

Phytochemical screening by HPLC method: About 1 mg of extracted material of different mulberry varieties were dissolved in 1 mL of methanol and analyzed for HPLC analysis. Different phytochemicals namely resveratrol, querecetin dihydrate, isoquerectin, kaempferol, 7-hydroxycoumarin, scopoletin and morin were dissolved in methanol was used as calibration standards. The HPLC analysis comprises of Analytical Waters HPLC system equipped with Waters 2958 Photodiode Array Detector, C_{18} column (250 mm × 4.6 mm inner diameter, 5 µm, Varian, USA), Waters 717 plus Autosampler and Waters 515 HPLC Pumps. The data was collected and analyzed on IBM computing system equipped with Empower Programming Software.

For the analysis, mobile phase A was water solution of 5 mM ammonium acetate and mobile phase B was 100 % HPLC grade acetonitrile. The elution conditions were as follows: 90 % A, 0-1 min; 40 % A, 1-15 min; 20 % A, 15-30 min; 90 % A, 30-40 min with flow rate 1.0 mL min^{-1}. Column oven temperature was set at 25 ºC and 10 µL of the standards and samples injected in to HPLC system. PDA spectra were recorded in wavelength range from 200-450 nm (detection wavelength was 254 and 320 nm).

***in Vitro* anti-oxidant activity by FRAP method:** The FRAP procedure described by Benzie and Strain[3] was followed to measure total antioxidant activity. Briefly, the FRAP reagent contained 2.5 mL of 10 mM TPTZ solution in 40 mM HCl plus 2.5 mL of 20 mM $FeCl_3$ and 25 mL of 0.3 M acetate buffer, pH 3.6 and was freshly prepared and warmed at 37 ºC prior to use. Six different concentrations between 100-1000 µL/mL of each mulberry extract and standard were mixed with 1 mL distilled water. Aliquots of 40 µL diluted mulberry sample and standard solution were mixed with 0.2 mL distilled water and 1.8 mL FRAP reagent. The absorbance of reaction mixture at 593 nm was measure spectrophotometrically in UV-spectrophotometer after incubation at 37 ºC for 10 min. The 1 mM $FeSO_4$ was used as the standard solution. The final result was expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1 mM $FeSO_4$.

Statistical analysis: The data were expressed as mean ± SD. Two-way analysis of variance (ANOVA) followed by Tukey's multiple range test was used to assess the presence of significant differences ($p > 0.01$) between the mulberry varieties and concentrations.

RESULTS AND DISCUSSION

Determination of total phenolic and total flavonoid content: Total phenolic content showed significant differences ($p > 0.01$ level) among all mulberry varieties. Highest concentration of total phenolic content was found in S-1 mulberry variety (103.814 ± 5.032 µg/g dried leaves) while BR-2 mulberry variety (43.722 ± 2.854 µg/g dried leaves) recorded least concentration when compared to rest of the mulberry varieties. S-1 variety recorded maximum total phenolic content followed by S-13 (85.533 ± 4.271 µg/g dried leaves), S-54 (85.142 ± 4.842 µg/g dried leaves) and S-36 (68.621 ± 4.242 µg/g dried leaves) (Table-1).

TABLE-1
TOTAL PHENOLIC AND FLAVONOID CONTENT IN DRIED LEAVES OF DIFFERENT MULBERRY VARIETIES

Mulberry varieties	Total phenolic content (µg/g dried leaves)	Total flavonoid content (µg/g dried leaves)
S-13	85.533 ± 4.271	40.712 ± 13.064
S-54	85.142 ± 4.842	104.012 ± 4.321
BR-2	43.722± 2.854	103.342 ± 5.063
S-36	68.621 ± 4.242	150.424 ± 0.601
S-1	103.814 ± 5.032	165.554 ± 2.874
Inference	HS	HS
CD 5 %	0.007	0.011
CD 1 %	0.009	0.015

Note: All the data are expressed as mean ± SD (n = 3).

For total flavonoid content, the maximum content was recorded in S-1 mulberry variety (165.554 ± 2.874 µg/g dried leaves) whereas least level was recorded in S-13 mulberry variety (40.712 ± 13.064 µg/g dried leaves) when compared to other mulberry varieties. The flavonoid content of S-36, S-54 and BR-2 was found to be 150.424 ± 0.601 µg/g dried leaves, 104.012 ± 4.321µg/g dried leaves, 103.342 ± 5.063 µg/g dried leaves, respectively (Table-1).

It has been recognized that flavonoids show antioxidant activity and their effects on human nutrition and health are considerable. The mechanisms of action of flavonoids are through scavenging or chelating process[15]. Phenolic compounds are a class of antioxidant agents which act as free radical terminators[16]. Data presented by other authors had reported that total phenolic is playing a major role in the antioxidant activity of plant materials[17] *i.e.*, the more the concentration of phenolics content the more is the anti-oxidant activity. The total phenolic content of all mulberry varieties showed significant results ($p > 0.01$) level. The phenolic content of S-1 mulberry variety (103.814 ± 5.032 µg/g) showed significant highest total phenolic content when compared to other mulberry varieties in the current study.

The total flavonoid content of all mulberry varieties showed significant results ($p > 0.01$) level. As the formation of flavonoids is light dependent, flavonoids occur predominantly in the leaves. The total flavonoid content of S-1 mulberry variety (*M. alba*) showed highest value in the current study. In a similar study by Radojkovic *et al.*[18], found maximum total flavonoid content in *M. nigra* leaves when compared to *M. alba* leaves and fruits.

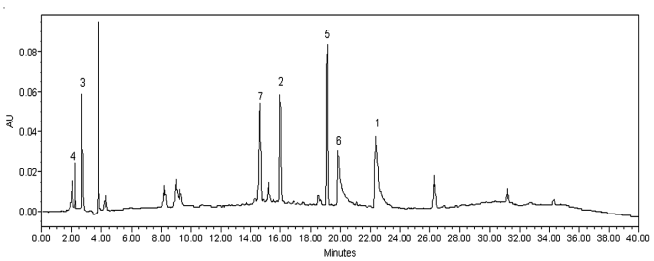
Phytochemical screening of different mulberry varieties: In order to separate and determine individual phenolic compounds present in leaves extracts of different mulberry varieties, HPLC method was applied for the analysis. The different phenolic compounds were identified on the basis of retention time of standard compounds peaks (Table-2). Based on HPLC chromatograms profiles, the quercetin, resveratrol, morin, scopoletin and 7-hydroxycoumarin were identified in all mulberry varieties. Kaempferol was identified in all mulberry varieties except in S-36 variety. However, Isoquercetin was identified only in two mulberry varieties namely S-13 and S-54 out of five varieties (Table-3 and Fig. 1).

TABLE-2
HPLC RETENTION TIMES OF DIFFERENT PHYTOCHEMICALS

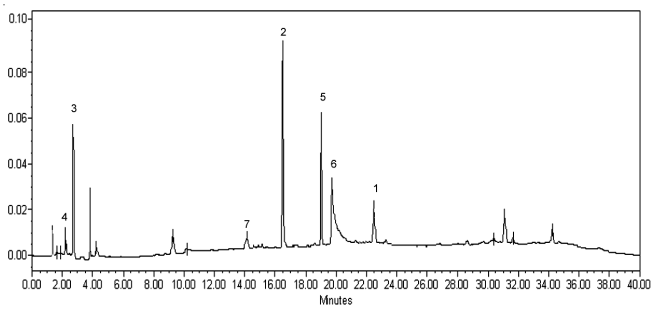
Phytochemical	Retention time (min)
Quercetin	23.02 ± 0.02
Resveratrol	15.97 ± 0.05
Morin	2.69 ± 0.09
Kaempferol	2.24 ± 0.07
Scopoletin	19.80 ± 0.04
7-Hydroxycoumarin	19.96 ± 0.02
Isoquerectin	14.93 ± 0.02

Note: All the data are expressed as mean ± standard deviation (n = 3).

Quercetin was highest recorded in S-36 mulberry variety *i.e.*, 12.917 ± 5.965 µg/g while recorded lowest in S-54 (0.113 ± 0.025 µg/g) variety. Secondly highest value of quercetin content was found in S-1 (2.750 ±1.152 µg/g), BR-2 (0.553 ± 0.123 µg/g) followed by S-13 (0.247 ± 0.070 µg/g) (Table-3). Quercetin is one of the potent sources for antioxidant activity[19].



[A]



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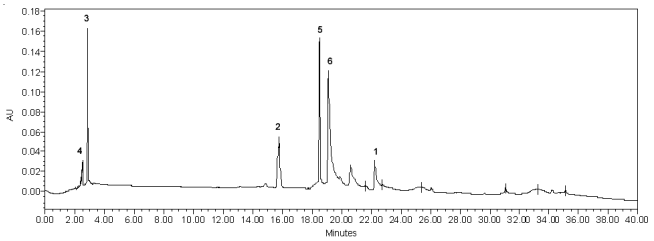

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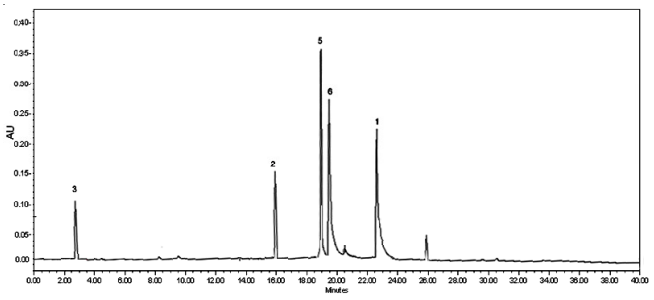

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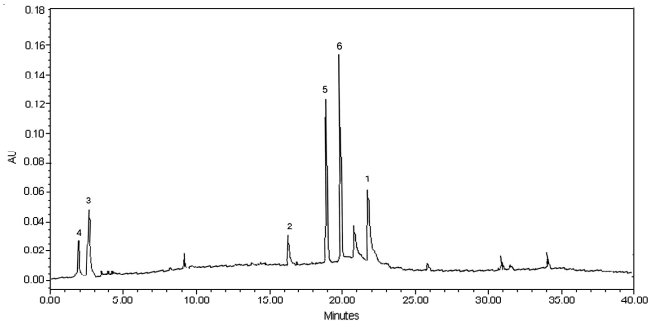

[E]

Fig. 1. HPLC chromatograms of leaves extract [A] S-13 [B] S-54 [C] BR-2 [D] S-36 [E] S-1 mulberry varieties, quercetin (1), resveratrol (2), morin (3), kaempferol (4), scopoletin (5), 7-hydroxycoumarin (6) and isoquercetin (7)

TABLE-3
PHYTOCHEMICALS CONTENT (µg/g DRY WEIGHT BASIS) IN DIFFERENT MULBERRY VARIETIES

Mulberry varieties	Quercetin (µg/g)	Resveratrol (µg/g)	Morin (µg/g)	Kaempferol (µg/g)	Scopoletin (µg/g)	7-Hydroxy-coumarin (µg/g)	Isoquerectin (µg/g)
S-13	0.247 ± 0.070	0.170 ± 0.080	42.407 ± 3.936	14.702 ± 3.298	19.060 ± 6.520	1.101 ± 0.104	2.715 ± 0.864
S-54	0.113 ± 0.025	1.138 ± 0.176	39.077 ± 10.058	19.310 ± 4.458	1.332 ± 0.358	1.188 ± 0.265	2.030 ± 0.210
BR-2	0.553 ± 0.123	1.071 ± 0.104	138.707 ± 26.83	47.193 ± 15.510	7.203 ± 3.587	1.071 ± 0.113	ND
S-36	12.917 ± 5.965	2.427 ± 0.436	16.907 ± 6.161	ND	8.660 ± 3.354	6.887 ± 3.673	ND
S-1	2.750 ±1.152	1.277 ± 0.331	36.357 ± 12.353	21.237 ± 9.890	2.035 ± 1.115	1.403 ± 0.560	ND
Inference				HS			
CD 5 %				23.030			
CD 1 %				31.494			

Note: All the data are expressed as mean ± SD, ND: Not detected (n = 3).

Han *et al.*[6] identified quercetin in *Morus multicaulis* whereas it has been identified in the current study in *Morus alba* and *Morus indica*. Quercetin is one of the main effective compounds of mulberry leaves with the functions of controlling the increase of fat in serum and controlling the formation of arteriosclerosis[20].

Resveratrol has been shown to suppress proliferation of a wide variety of tumor cells, including lymphoid and myeloid cancers; breast, colon, pancreas, stomach, prostate, head and neck, ovary, liver, lung and cervical cancers; melanoma and muscles[21], antiinflammatory, blood-sugar-lowering[22]. The resveratrol content was recorded least in S-13 mulberry variety (0.170 ± 0.080 µg/g). Similarly, Song *et al.*[23] found highest resveratrol content in Da 10 (*Morus atropurpurea* Roxb). Morin is a natural compound found abundantly found in the twigs and leaves of genus *Morus*, which is documented in traditional Chinese medicine as herb used in the treatment of conditions akin to gouty arthritis in modern medicine[24]. Recent studies have demonstrated that morin has nephroprotective action against organic anion transporters (OAT-mediated) nephrotoxicity[25], antioxidant properties[26] and inhibit rat brain phosphatidylinositolphosphate kinase activity *in vitro* and *in vivo*[27] and for the first time this compound has been quantified in different mulberry leaves extracts. Morin was found maximum in BR-2 mulberry variety (138.707 ± 26.836 µg/g) (*Morus alba*) and minimum in S-36 mulberry variety (16.907 ± 6.161 µg/g).

Kaempferol and its derivatives like kaempferol-3-O-glucoside and kaempferol-3-O-(6-malonyl) glucoside, are antiamyloidogenic substances which were extracted from the methanol extract of mulberry leaves and helps in treatment of Alzheimer's disease. Mulberry leaf extract inhibits the amyloid beta-peptide (1-42) fibril formation by both the thioflavin T fluorescence assay and atomic force microscopy. Furthermore, mulberry leaves extract protected hippocampal neurons against amyloid β-peptide (1-42) induced cell death in a concentration-dependent manner and further it induced neurotoxicity[28]. The kaempferol content in the current study was identified all varieties except in S-36 mulberry variety and recorded maximum in BR-2 (47.193 ± 15.510 µg/g). The variations in phenolic constituents is mainly attributed to climatic conditions and farming practices[29], cultivar or variety[30]. In the current study, Kaempferol was identified *Morus alba* and *Morus indica* whereas Han *et al.*[6] identified this compound in *Morus multicaulis*. In the present study scopoletin was identified in all mulberry varieties which belong to *M. alba* and *M. indica*. Previously, Dugo *et al.*[7] and Han *et al.*[6] reported the presence of scopoletin in *Morus alba* and *Morus multicaulis* as well. Scopoletin content in leaves of different mulberry varieties content in the current study ranged from 1.332 ± 0.358-19.060 ± 6.520 µg/g (which is approximately 0.0133-0.1906 %). In a similar study at China, recorded scopoletin content in range 0.002-0.017 %[31]. The results are not in accordance with the current findings. 7-Hydroxycoumarin content in the current study was found maximum in S-36 mulberry variety (6.887 ± 3.673 µg/g) (*M. indica*). Umbelliferone (7-hydroxycoumarin) had been used in folk medicine for the treatment of inflammation and arthritis[32] and the cytokin macrophage migration inhibitory factor has been recently identified as a possible target for these herbal antirheumatic agents[33].

Isoquerectin was not detected in BR-2, S-36 and S-1 mulberry varieties whereas it was found only in S-13 (2.715 ± 0.864 µg/g) and S-54 (2.030 ± 0.210 µg/g) which is due to climatic conditions and farming practices[29], cultivar or variety[30].

Assessment of anti-oxidant activity by FRAP assay: FRAP is a simple direct test for measuring antioxidative contents. This method was initially developed to assay plasma antioxidant capacity, but can be used with plant extracts too[34]. The ferric reducing antioxidant power assay measures the reducing ability of antioxidants against oxidative effects of reactive oxygen species. Electron donating antioxidants can be described as reductants and inactivation of oxidants by reductants can be described as redox reactions. This assay is based on the ability of antioxidants to reduce Fe^{2+} to Fe^{3+} in the presence of tripyridyltriazine [TPTZ] forming an Fe^{2+}-TPTZ complex[3] with an absorbance maximum at 593 nm. Increasing absorbance indicates an intense blue Fe^{2+}-TPTZ *i.e.*, increase in reductive ability[35]. A concentration dependent ferric reducing capacity was found in all the mulberry varieties. In S-13 mulberry variety, the concentration 1000 µL/mL (0.289 ± 0.007 mmol of $FeSO_4$/100 g dried sample) showed the highest ($p > 0.01$) ferric reducing capacity while 100 µL/mL (0.117 ± 0.002 mmol of $FeSO_4$/100 g dried sample) recorded lowest ferric reducing capacity (Table-4). In the current study, S-1 mulberry varieties had recorded maximum FRAP value at concentrations 800 and 1000 µL/mL. The interaction between variety and concentrations was significantly shown in S-1 mulberry varieties at concentrations 800 and 1000 µL/mL and thereby increasing the antioxidant power.

Mulberry is potential source of phytochemicals belonging to phenolics. The major phenolic compounds namely querecetin, resveratrol, morin, scopoletin, 7-hydroxycoumarin have been quantified for the first time in different Indian

TABLE-4
IN VITRO ANTI-OXIDANT ACTIVITY OF DIFFERENT VARIETIES OF MULBERRY LEAVESMEASURED BY FRAP METHOD (mmol OF $FeSO_4$/100 g DRIED SAMPLE) AT DIFFERENT CONCENTRATIONS

Mulberry varieties	Concentrations					
	100 µL/mL	200 µL/mL	400 µL/mL	600 µL/mL	800 µL/mL	1000 µL/mL
S-13	0.117 ± 0.002	0.147 ± 0.001	0.219 ± 0.002	0.244 ± 0.001	0.274 ± 0.001	0.289 ± 0.007
S-54	0.149 ± 0.001	0.172 ± 0.001	0.173 ± 0.001	0.197 ± 0.001	0.259 ± 0.001	0.288 ± 0.002
BR-2	0.155 ± 0.001	0.174 ± 0.001	0.178 ± 0.001	0.228 ± 0.001	0.297 ± 0.001	0.366 ± 0.002
S-36	0.255 ± 0.126	0.278 ± 0.134	0.309 ± 0.144	0.343 ± 0.153	0.352 ± 0.155	0.357 ± 0.002
S-1	0.134 ± 0.001	0.151 ± 0.001	0.209 ± 0.001	0.240 ± 0.001	0.373 ± 0.002	0.408 ± 0.001
Inference	HS					
CD 5%	0.1543					
CD 1%	0.1679					

Data expressed in mean ± SD (n = 5), Note: HS- Highly significant, CD 5 %- Critical difference at 5 % level, CD 1 %- Critical difference at 1 % level.

mulberry varieties which also serve as source of antioxidant activity. Compelling evidence indicates that increased consumption of dietary antioxidants or fruits and vegetables with antioxidant properties may contribute to the improvement in quality of life by delaying onset and reducing the risk of degenerative diseases associated with aging. On the basis of results obtained from the HPLC analysis, methanolic extracts of mulberry leaves samples of AR-12, S-36 and S-1 varieties contain more amounts of phenolics and flavonoids exhibiting high antioxidant activity. It also chelates iron and has reducing power. The components responsible for the antioxidative activity are morin, querectin, resveratrol, scopoletin, 7-hydroxycoumarin. Furthermore, these mulberry varieties may be utilized in epidemiological studies. It can also be used to test antioxidant effects and synergy in experimental animal and cell line studies or in human clinical trials. The ultimate goal of this research is to combine these strategies in order to understand the role of dietary phytochemical antioxidants in the prevention of cancer, cardiovascular diseases, diabetes and other chronic diseases related to oxidative stress.

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