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# Enhancement of polyphenols, flavonoids and antioxidant activities in water extract of mulberry (*Morus alba* L.) root bark by steam treatment

Kamidi Rahul[1,2], HaeYong Kweon[1], Hyun-Bok Kim[1], Ji Hae Lee[1,*], and Pooja Makwana[2]

[1]*Industrial Insect and Sericulture Division, National Institute of Agricultural Sciences, RDA, Wanju-gun 55365, Republic of Korea*
[2]*Central Sericultural Research & Training Institute, Central Silk Board, Ministry of Textiles: Govt. of India, Berhampore - 742101, Murshidabad, West Bengal, India*

## Abstract

Different parts of the mulberry plant are described to be potential sources of polyphenolics exhibiting strong antioxidant activity. In this study, we prepared various aqueous extracts of mulberry root bark by subjecting to steam at different temperatures and time intervals (45°C, 15 h; 70°C, 15 h; 95°C, 6 h and 95°C, 15 h) followed by extracting at 80°C for 1 h. The total polyphenolic content ranged from 66.82-101.20 mg gallic acid equivalent (GE)/g of extract whereas the flavonoids were in the range of 13.03-25.23 mg catechin equivalent (CE)/g of extract. The extracts also exhibited strong antioxidant activities (0.99-1.66 mg trolox equivalent (TE)/g of extract in DDPH assay and 10.65-16.26 mg TE/g of extract in ABTS assay). This study clearly showed an improvement in the antioxidant activity of the water extract of mulberry root bark by the steam treatment, which can be used as a tea or health-promoting materials.


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## Introduction

Mulberry (*Morus alba* L.), an ecologically and economically important plant species of the family Moraceae is widely cultivated across many countries of the globe (Polumackanycz *et al*., 2019). The family 'Moraceae' encompasses twenty four species, one subspecies and approximately more than a hundred identified varieties among which *Morus alba*, *Morus nigra* and *Morus rubra* are prominent (Jan *et al*., 2021). *Morus* spp. are perennial, fast growing, woody, monoecious or dioecious, highly adaptable to diverse climatic zones and display good growth characteristics across different soils (Rohela *et al*., 2020).

From time immemorial, mulberry was only considered as a dietary source to the monophagous, domesticated and economically important Lepidopteran insect, *Bombyx mori* L. which produces silk (Vijayan *et al*., 1998). Reports also claim consumption of mulberry fruits by humans and leaves by livestock from ancient times (Rohela *et al*., 2020). However, the phytochemical constituents of mulberry and their biological properties were ignored and under explored for a long time. This scenario has transformed and the past two decades has witnessed a tremendous surge in research related to delineating the pharmacological significance of diverse parts of mulberry. Solvent/aqueous extracts of different parts (fruit, leaf, root, stem and twig) of *Morus* spp. were reported to exhibit anti-atherosclerotic, anti-cancerous, anti-diabetic, anti-hyperlipidemic, anti-inflammatory, antimicrobial, anti-obese, and antioxidant properties (Lim and Choi, 2019).

The major bioactive compounds reported from different parts of the mulberry plant include 1-deoxynojirmycin, carotenoids,

***Corresponding author.**
Ji Hae Lee, Ph.D.
Division of Industrial Insect and Sericulture, National Institute of Agricultural Sciences, RDA, Wanju-gun 55365, Republic of Korea
Tel: +82-63-238-2844 / FAX: +82-63-238-3833
E-mail: jihae@korea.kr

caffeic acid, coumaric acid, fagomine, flavonoids, kaempferol-3,7- glucopyranoside, lectin, morin, phenolics, polyphenols, quercetin, vitamins, γ -aminobutyric acid from leaves (Andallu *et al*., 2009, Ansari *et al*., 2009, Chen *et al*., 2013, Deepa *et al*., 2011, Eva *et al*., 2015, Fang *et al*., 2005, Gundogdu *et al*., 2017, Ionica *et al*., 2017, Kiran *et al*., 2019, Kojima *et al*., 2010, Li *et al*., 2005, Zeni *et al*., 2017, Zheng *et al*., 2014); 1- deoxynojirmycin, albanin from stem bark (Kiran *et al*., 2019, Shirata *et al*., 1983); anthocyanins, cyanidin-3-glucoside, flavonoids, isobavachalcone, kaempferol-3-O-glucoside, melatonin, morachalcone, odisolane, pyrrole alkaloids from fruits (Chen *et al*., 2016, Du *et al*., 2008, Kim *et al*., 2010, Kim *et al*., 2013, Lee *et al*., 2016, Wang *et al*., 2013,Wang *et al*., 2016, Wei *et al*., 2016 a, Xiao *et al*., 2017 a, b) and 1-deoxynojirmycin, 2-arylbenzofuran, 5-2′-dihydroxiflavanone-7,4′-di-OD-glucose, albanol B, leachianone G, mulberrofuran G, phenolics from root bark (Du *et al*., 2003, Kiran *et al*., 2019, Singab *et al*., 2005, Sohn *et al*., 2004, Zhang *et al*., 2009 a, b). Most of the above mentioned bioactive substances were reported to be isolated and characterized from leaves and fruits of mulberry. However, reports on the same from stem and root bark are comparatively less.

Discovery of numerous pharmacological compounds of significance from diverse plant parts of *Morus* spp., triggered usage of the same in development of different value added products for human consumption which included biscuits, bread, jams, jellies, syrups, tea, vinegar, wine and yogurt. Apart from serving as a source of functional foods, mulberry products are also used as medicines to treat numerous disorders in humans owing to their immense therapeutic potential (Jan *et al*., 2021).

Our previous study indicated that the polyphenolic and flavonoid contents as well as the antioxidant activities exhibited by the aqueous extracts of the mulberry root bark were comparatively less in comparison to the ethanolic extracts (Lee *et al*., 2020). The extraction protocol was modified in the present study wherein the mulberry root bark was subjected to steam at different temperatures and time intervals in order to soften the cell wall of root bark which helps in better elution of internal polyphenols and thereby an enhanced antioxidant activity. Hence, the objective of this study was to quantify the polyphenols, flavonoids and determine the antioxidant activities of mulberry (*Morus alba* L.) root bark extracts (aqueous) of Cheongil cultivar, a mulberry variety native to the Republic of Korea by following the aforementioned procedure.

## Materials and Methods

### Plant material and Extract preparation

The root bark of *Morus alba* L. (Cheongil cultivar) experimented in the present study was collected from the mulberry farm at the National Institute of Agricultural Sciences, Wanju-gun, Republic of Korea. Upon collection, the root bark was cleaned, coarsely grinded to small pieces and subjected to steam in a boiling plate apparatus (Ocoo, OC-S1170S, Anyang, Korea) at different temperatures and time intervals (T1: 45°C, 15 h; T2: 70°C, 15 h; T3: 95°C, 6 h and T4: 95°C, 15 h). A control batch (C) wherein the root bark was not subjected to steam was also maintained.

The aqueous extracts of the root bark samples were obtained by subjecting 20 g each of the above treated and control samples to boiling in 40 mL of distilled water at 80°C for 1h followed by filtration using Miracloth (Merck, Burlington, MA, USA). The extracts thus obtained were lyophilized and reconstituted in distilled water to obtain a stock solution of 10 mg/mL for use in further experiments.

### Determination of total polyphenolics content

The total polyphenolics in the extracts were determined according to the method as delineated by Lee *et al.* (2018). Extracted samples (10 µL) were mixed with 200 µL of 2% $Na_2CO_3$ in 96 well-plates and incubated at room temperature for 3 min to which 10 µL of 50% Folin-Ciocalteu (Sigma-Aldrich, St. Louis, MO, USA) was added and allowed to stand for another 3 min. The absorbance was measured spectrophotometrically at 750 nm (Multiskan™ GO Microplate Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA). The total polyphenolic contents in the extracts were expressed as mg gallic acid equivalents (GE) /g of extract.

### Determination of flavonoids content

20 µL of extracted samples were mixed with distilled water (100 µL) and 5% $NaNO_2$ (6 µL). After 5 min, 10% $AlCl_3 \cdot 6H_2O$ solution (12 µL) was added and allowed to stand for another 5 min. NaOH (1M) was added (500 µL) to the reaction mixture and absorbance was measured after 10 min in a spectrophotometer at 510 nm. The results were expressed as mg catechin equivalents (CE)/g of extract (Lee *et al*., 2018).

### Radical scavenging activity using 2, 2-diphenyl-1-picrylhydrazyl (DPPH)

DPPH radical scavenging activity was determined according

to Dudonne *et al*., 2009 with minor modifications. DPPH reagent (Sigma Aldrich, USA) was dissolved in ethanol to arrive at a concentration of 0.2 mM and the same was used for the assay. 10 µL of the extracts pertaining to different treatments and control were dispensed into 96 well-plates and 200 µL of DPPH solution was added. Upon 10 min of incubation at room temperature, absorbance was recorded at 520 nm in a spectrophotometer. The data were transformed into Trolox standard curve values and expressed.

### Radical scavenging activity using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid, ABTS) radical scavenging activity

ABTS reagent was prepared by dissolving 7 mM ABTS (Sigma Aldrich, USA) and 2.5 mM potassium persulfate (Sigma Aldrich) in distilled water. The solution was incubated in dark for 24 h, and diluted to obtain an absorbance value of 1 at 734 nm with distilled water prior to use in the assay. Extracts (10 µL) were dispensed into 96 well-plates and ABTS reagent (200 µL) was added. Upon 10 min of incubation at room temperature, absorbance was measured in a spectrophotometer at 734 nm. Standard curves were expressed using trolox, and the data were converted as mg trolox equivalents (TE)/g of sample (Dudonne *et al*., 2009).

### Statistical analysis

All the results recorded from the aforesaid assays performed in triplicates were expressed as the mean ± standard deviation. Significant differences among mean values were determined by adapting Duncan's Oneway-ANOVA analysis (Software Package SAS 7.1). Results were deemed to be significant at $p < 0.05$.

## Results and Discussion

The extraction conditions *viz*., time, temperature, medium (solvent/water) play an indispensible role in attaining the desired levels of phytochemical constituents in the extraction medium and also determines their functional applicability. Needless to mention that the aforesaid conditions vary depending on the sample composition and target compounds to be extracted. In the present study, water is used as a medium of extraction considering that it is safe, in expensive and numerous components (carbohydrates, proteins, glycosides, phenolic compounds) soluble in water can be easily obtained (Chang *et al*., 2021). Moreover, the main intention of the present study is to provide a base for designing and developing mulberry root bark tea (permissions for sensory evaluation under progress; unpublished) rich in phytochemical components and hence aqueous extract is considered safe over solvents and was chosen.

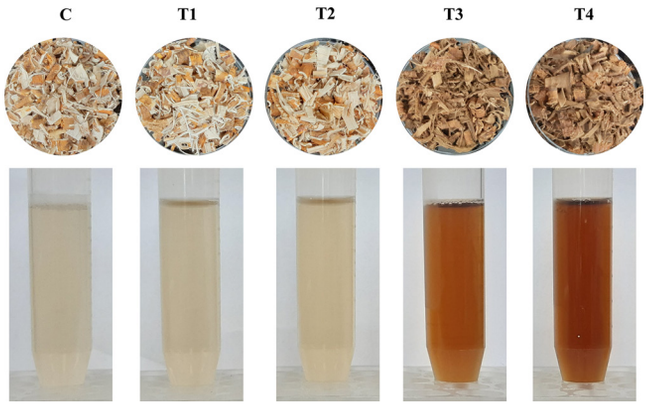


**Fig. 1. Steamed mulberry root bark samples at various conditions and their aqueous extracts**
Mulberry root bark samples subjected to steam at different temperatures and time intervals were shown in top row. Extracts obtained by boiling the steamed root bark samples in distilled water at 80°C for 1 h followed by filtration were shown in bottom row. Abbreviations were Control-devoid of treatment (C), pretreatment at 45°C for 15 h (T1) 70°C for 15 h (T2), 95°C for 6 h (T4), 95°C for 15 h (T5).

The extracts of the root bark samples subjected to steam at different temperatures and time intervals followed by boiling in water exhibited various colored views. The solutions (T1, T2) were turbid and the color was faded white which is similar to control (C). T3 and T4 extracts were dark brown in color indicating the imperative role of higher temperature treatments in extraction processes of phytochemical constituents (Fig. 1).

Polyphenols, the secondary metabolites synthesized by plants diversified into different classes which majorly include phenolic acids, flavonoids (anthocyanidins, flavanols, flavonols, isoflavons, flavons, flavanones), stilbenes and lignans are compounds of enormous interest to the researchers in view of the immense beneficial effects to the humans (Pandey and Rizvi, 2009; Abbas *et al*., 2017).

The total polyphenolic and flavonoid contents (mean values) ranged in between 66.82-101.20 mg GE/g of extract and 13.03-25.23 mg CE/g of extract, respectively across different treatments in comparison to the control (65.43 mg GE/g of extract and 12.98 mg CE/g of extract). The polyphenolic constituents

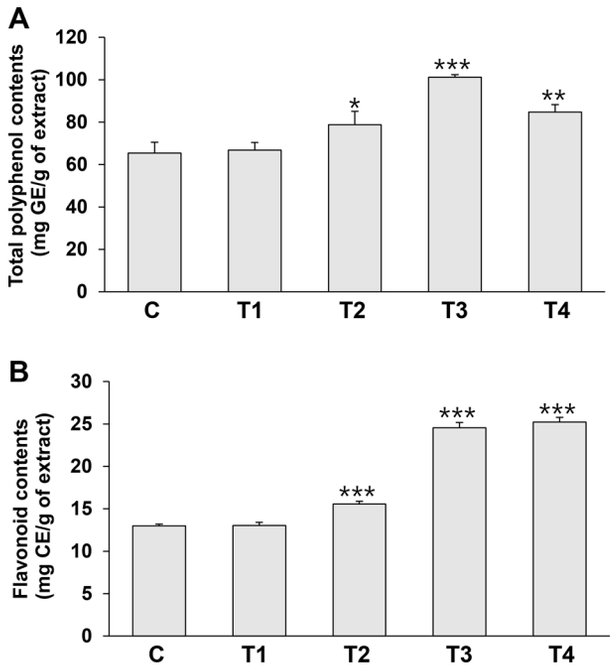


**Fig. 2. Total polyphenol (A) and flavonoid (B) contents in extracts of mulberry root bark samples treated under different conditions**

Values are expressed as mean ± standard deviation ($n$ = 3). *, ** and *** indicates a significant difference in comparison to control at $p$<0.05, $p$<0.01 and $p$<0.001, respectively. Abbreviations were gallic acid equivalent (GE), catechin equivalent (CE).

were found to be highest in T3 whereas flavonoids were extracted better in T4 (Fig. 2). The extraction conditions of both the aforementioned treatments were similar except mulberry root bark was steamed for nine hours more at the same temperature of 95°C in T4 prior to extraction. There was a 2.12-54.66% and 0.38-94.37% increase in polyphenolic and flavonoid contents, respectively across different treatments employed in this study, over control. Our previous study indicated that the contents of polyphenols and flavonoids in mulberry root bark by water and ethanol (30, 50, 70 and 100%) extraction at different temperatures (room temperature, 50, 70, 100°C) ranged in between 12.13-20.37 mg/g of extract and 4.36-18.87 mg/g of extract, respectively. It was observed that the polyphenols and flavonoids were better extracted with 100% ethanol at room temperature (Lee *et al*., 2020). The extraction procedure employed in this study resulted in a significant surge in the polyphenolic and flavonoid contents in comparison to the yields of the same obtained by aqueous extraction in our previous study (Lee *et al*., 2020). The total polyphenols and flavonoids extracted by using methanol from root bark of *Morus alba* L. were reported to be 313.73 ± 8.08 mg/g (Phenolics: 165.27 ± 3.28 mg GE/g; flavonoids: 12.59 ± 2.96 mg CE/g; flavonols: 132.54 ± 1.77 mg quercetin equivalent (QE)/mg; proanthocyanidins: 3.33 ± 0.07 mg CE/g) and 12.59 ± 2.96 mg CE/g, respectively (Khan *et al*., 2013). Hexane, ethanol and aqueous extracts of twelve (natural and hybrid) varieties of *M. alba* root samples were evaluated for their polyphenolic and flavonoid contents. Ethanolic extracts were found to be possessing highest quantity of phenolics (9.85 mg GE/g) and flavonoids (0.8 mg QE/g) (Sande *et al*., 2016).

It is evident that the antioxidant ability of natural foods is governed by numerous constituents and their diverse mechanisms. As a result, a variety of methodologies dealing with various mechanisms should be employed to assess this potential (Pérez-Jiménez *et al*., 2008). In this study, the ability of the extracts to stabilize free radicals was evaluated by performing DPPH and ABTS radical scavenger assays. Mean values of different treatments varied from 0.99-1.66 mg TE/g in DDPH assay (Control: 1.01 mg TE/g) whereas, it is 10.65-16.26 mg TE/g in ABTS assay (Control: 13.90 mg TE/g). Based on ABTS assay, it was observed that T2 and T3 exhibited significantly higher antioxidant activity in comparison to control whereas it was otherwise with T1 and T4. However, the observations of DPPH assay specified that T3 displayed strong antioxidant activity and the activity of other treatments in comparison to control is insignificant (Fig. 3). The disparities in observations of both the assays may be attributable to the differing role of each polyphenolic component to the antioxidant response in a particular assay (Mena *et al*., 2011). Other researchers have also reported the potential of mulberry root extracts in combating oxidative stress. The antioxidant activities of aqueous and 70% ethanol extracts of *M. alba* root bark were evaluated and found to be 65.8% and 87.0% (DPPH assay); 89.3% and 77.1%, respectively at 1,000 µg/ml (ABTS assay) (Jee, 2009). Our previous study depicted that 100% EtOH extracts of mulberry root bark at different temperatures (room temperature, 50, 70, 100°C) exhibited better antioxidant activities based on DPPH assay in comparison to aqueous extractions at same temperatures. The $IC_{50}$ values reported for water extractions were 16.75, 14.48, 11.46, and 12.78 µg/mL at room temperature, 50, 70, and 100°C, respectively whereas for 100% EtOH extractions, it was 15.08, 6.97, 10.03, and 8.31 µg/mL (Lee *et al*., 2020). Chang *et al*., (2011) demonstrated that extracts of *M. alba* twigs displayed better

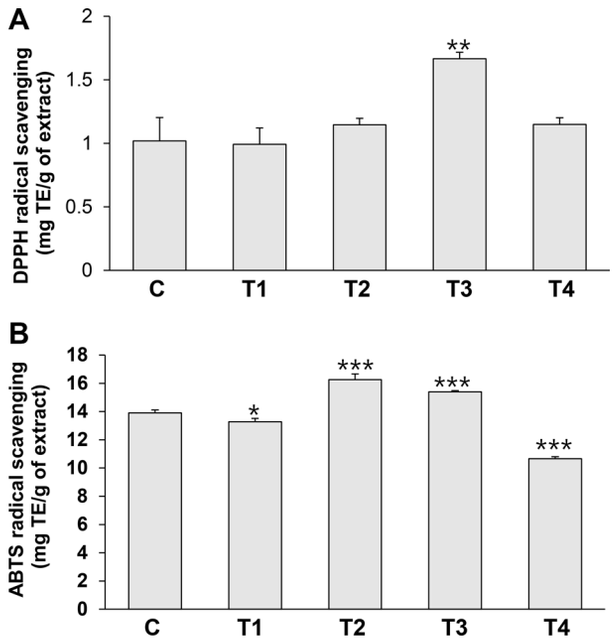


**Fig. 3. DPPH (A) and ABTS (B) radical scavenging activity exhibited by extracts of mulberry root bark samples treated under different conditions**

Values are expressed as mean ± standard deviation ($n$ = 3). *, ** and *** indicates a significant difference in comparison to control at $p$<0.05, $p$<0.01 and $p$<0.001, respectively. Abbreviation was trolox equivalent (TE).

antioxidant potential that root bark extracts. The antioxidant activities of methanolic extracts of *M. alba* stem bark was found to be superior followed by root bark, fruits and leaves (Khan *et al*., 2013). The antioxidant activities of *M. alba* root extracts (Hexane, etahnol and aqueous) of 12 diverse varieties were reported by Sande *et al*. (2016). All these studies including the present indicates the potential of mulberry root bark extracts in possessing rich amounts of bioactive compounds and their potential antioxidant activities.

Among the different plant parts of *Morus* spp., root bark is known to encompass diverse bioactive compounds in significant quantum and hence our interest to evaluate the phytochemical constituents and antioxidant properties of the same. It is estimated that over 110 bioactive substances have been isolated from the mulberry root bark employing varied extraction protocols. The bioactive substances are known to exhibit remarkable pharmacological potential and are used to treat a variety of medical anomalies way long back from 500 BC (Memete *et al*., 2022; Wei *et al*., 2016 b). Despite the fact that composition and extent of phytochemical components vary across different plant species, *Morus* has undoubtedly gained prominence considering the wide range of bioactive compounds it comprises (Memete *et al*., 2022).

Numerous research studies signify the antioxidative potential of natural foods is attributed to the presence of diverse phenolic compounds and their contents. However, the contents significantly vary in different plant parts synthesized as a part of natural metabolism and depend on a variety of factors; prominent among them includes genotype and growing conditions (Chepel *et al*., 2020). The extraction protocols also play a considerable role in recovery of bioactive substances. The results based on the present study and from the modest data available for comparison as well as taking into consideration that the extraction protocols employed were entirely diverse, flavonoids were better extracted from mulberry root bark by the procedure employed in the present work whereas methanol extraction was found to be superior in terms of polyphenols extraction. But considering the pros of aqueous over solvent extractions, the polyphenolic quantum extracted by the present process is also significant. The antioxidant activities displayed by the mulberry root bark extracts in the present work also seems to be promising.

## Conclusion

In conclusion, it is apparent that the extraction approach employed in the present study, which is in contrast to other methods previously reported, i.e. steam of mulberry root bark at varied temperatures and time intervals, resulted in improved recovery of internal polyphenols by softening the cell wall of root bark, and therefore resulted in higher antioxidant activities. Finally, the findings of this study once again signified that mulberry root bark water extract with the steam treatment is a promising source of phytochemicals with significant biological activity and the same can be utilized in fortification of numerous foods as well as in pharmaceuticals.

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