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ORIGINAL ARTICLE

In vitro Antidiabetic and Antioxidant Properties of Dioecious *Morus alba* (*Moraceae*) Extracts

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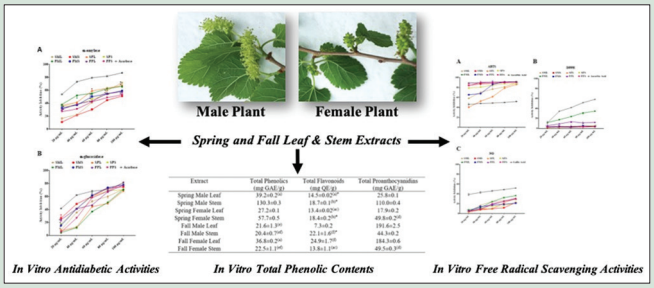
ABSTRACT

Background: Diabetes mellitus, characterized by elevated blood glucose levels, is a serious metabolic disorder affecting large populations worldwide. Mulberry food and supplement products known for their antidiabetic and antioxidant properties are commonly used in Asian and other countries. **Objectives:** The present study investigated for the first time the antidiabetic and antioxidant activities of dioecious (male and female) mulberry (*Morus alba, Moraceae*) extracts from leaf and stem tissues collected in two different seasons. **Materials and Methods:** *In vitro* α-amylase, α-glucosidase, polyphenol content analyses, and free radical scavenging activities were performed. **Results:** All extracts showed higher inhibition of α-glucosidase than α-amylase in a dose-dependent manner. Fall female stem, fall male stem, and fall female leaf extracts exhibited the highest α-glucosidase inhibitory activities. Fall male and fall female leaf extracts showed the highest ferric reducing activity. 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activities were more potent for all extracts compared to 2,2-diphenyl-1-picrylhydrazyl (DPPH) and nitric oxide (NO) scavenging activities. The highest DPPH and NO radical scavenging activities were induced by fall male leaf extracts. Spring extracts contained more phenolics and fall extracts more proanthocyanidins. Correlations between IC_{50} values of extracts used *in vitro* antidiabetic and antioxidant activities and polyphenol chemical classes were both positive and negative. In general, male extracts were more potent in their antidiabetic and antioxidant activities than female extracts. **Conclusion:** Overall, results showed that growing season, sex and organ of mulberry trees influence antidiabetic and antioxidant properties of extracts, knowledge that could be used in formulating effective dietary supplements for treating diabetes, and oxidative stress-related diseases.

Key words: Antidiabetic, antioxidant, dioecious, flavonoids, mulberry, phenolics

SUMMARY

- The present study reports the total phenolic, flavonoid, and proanthocyanidin contents, *in vitro* antidiabetic and free radical scavenging activities of male and female (dioecious) *Morus alba* leaf, and stem extracts from the tissues collected in two different seasons. To the best of our knowledge, this is the first study reporting the antidiabetic and antioxidant activities of dioecious *M. alba* extracts. The present study provides important insights into which sex, organ, and season could be more effective in formulating dietary supplements and could help in discovering new medicines for treating diabetes and oxidative stress-related diseases.



Abbreviations Used: Abs: Absorbances; ABTS: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); BHT: Butylated hydroxylated toluene; CAT: Catalase; DM: Diabetes mellitus; DNS: Dinitrosalicylic acid; DPPH: 2,2-diphenyl-1-picrylhydrazyl; DW: Dry weight; $FeCl_3$: Ferric chloride; FW: Fresh weight; GAE: Gallic acid equivalents; GSH-Px: Glutathione peroxidase; HCl: Hydrochloric acid; $K_2S_2O_8$: Potassium persulfate; $K_3Fe(CN)_6$: Potassium ferricyanide; $Na_2[Fe(CN)_5NO]$: Sodium nitroprusside; Na_2CO_3: Sodium carbonate; NaCl: Sodium chloride; NO: Nitric oxide; pNPG: *p*-nitrophenyl-α-D-glucopyranoside; QE: Quercetin equivalents; SOD: Superoxide dismutase; T1D: Type 1 diabetes; T2D: Type 2 diabetes; TCA: Trichloroacetic acid.

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INTRODUCTION

Diabetes mellitus (DM) is a general term for a group of metabolic disorders, leading to chronic hyperglycemia. DM results from either impaired insulin secretion or impaired insulin efficacy or both.[1] The DM prevalence is increasing, and by 2040, it is expected that more than 640 million adults will have the condition.[2] Type 1 diabetes (T1D), characterized by insulin deficiency, affects approximately 5%–10% of the diabetic population and can be relatively easily managed with insulin therapy.[3] Type 2 diabetes (T2D) starts with insulin resistance, in which cells do not properly respond to insulin and is more prevalent than T1D and comparatively more difficult to treat.[4]

It has been shown that oxidative stress could contribute to the pathogenesis of T2D.[5] The elevation of reactive oxygen species in DM is either due to decrease in destruction and/or increase in their production by catalase (CAT-enzymatic/non-enzymatic), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) antioxidants.[6] The variations in the level of these enzymes make the tissues susceptible to oxidative stress, leading to the development of diabetes complication.[7]

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Therefore, the reduction of intracellular free radical formation would provide a therapeutic strategy to prevent oxidative stress and related diabetic vascular complications. Antioxidants may act at different levels, inhibiting the formation of reactive oxygen species, scavenging free radicals, or increasing the scavenger activity of enzymes such as CAT, GSH-Px, glutathione transferase, peroxiredoxin, SOD, and thioredoxin.[8]

One of the effective managements of DM, especially for non-insulin-dependent DM (T2D), is the retardation of glucose absorption in the digestive system. The inhibition of α-amylase, which hydrolyses alpha-linked polysaccharides such as starch and glycogen and α-glucosidase, which digests disaccharides and starch, has been associated with preventing carbohydrate digestion to absorbable glucose. Metformin, acarbose, and other α-glucosidase inhibitors currently in use have been associated with various gastrointestinal side effects such as abdominal pain, diarrhea, and flatulence in patients.[9,10] The side effects of the synthetic drugs underscore the search for more effective natural compounds. Therefore, research for alternative and combination herbal medicine–drug therapies with less side effects in the treatment of diabetes have gained much attention.[11] Huh *et al.* treated DM-induced rats with mulberry leaf extracts which reduced elimination of metformin, thus enhancing its antihyperglycemic effect.[12]

From ancient times, Ayurvedic medicine in Asia has valued *Morus alba (Moraceae*) for its pharmacological, medicinal, nutritional, and economical values.[13] Different parts of the plant, such as bark, branch, leaf, fruit, and root, also have been used in the traditional Chinese medicines for treating fever, protecting liver, improving eyesight, strengthen joints, lowering blood pressure, whitening skin, and helping with dizziness and blurred vision.[14-18] Furthermore, *M. alba* tea or dietary supplements have been used in treating diabetes.[19] The antidiabetic and antioxidant activities of *M. alba* have been reported before with no mention of the reproductive system of the plant specimens used.[20-25]

The dioecious system with separate male and female individuals is characteristic to *Moraceae* family. Mulberry can be monoecious, with both staminate and pistillate inflorescences on the same tree, but they are mostly dioecious, represented by separate male and female trees and very rarely with hermaphrodite flowers.[26] To the best of our knowledge, the present study is the first report on the *in vitro* antidiabetic and antioxidant properties of dioecious *Morus alba* (*Moraceae*) extracts. Exploring alternative and natural remedies for T2D with better efficacy and lesser side effects, this study could provide information on the potential mulberry extract candidate for the treatment and/or prevention of diabetes and other oxidative stress-related diseases.

MATERIALS AND METHODS

Plant material

Three branches of each male and female *Morus alba* plants were collected in spring (March–April) and fall (September). The plant specimens were authenticated and vouchers were deposited in Texas Woman's University Herbarium in Denton, TX, USA.

Chemicals

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), acarbose, aluminum chloride, ascorbic acid, butylated hydroxylated toluene (BHT), dinitrosalicylic acid (DNS), ethyl alcohol, ferric chloride ($FeCl_3$), Folin-Ciocalteu reagent, gallic acid, Griess reagent, hydrochloric acid (HCl), methyl alcohol, *p*-nitrophenyl-α-D-glucopyranoside (pNPG), potassium ferricyanide [$K_3Fe(CN)_6$], potassium persulfate ($K_2S_2O_8$), quercetin, sodium carbonate (Na_2CO_3), sodium chloride (NaCl), sodium nitroprusside Na_2 [$Fe(CN)_5NO$], soluble starch (extra pure), trichloroacetic acid (TCA), vanillin, α-amylase from *Aspergillus oryzae* and α-glucosidase from *Saccharomyces cerevisiae* were from Sigma-Aldrich, St Louis, USA.

Preparation of plant extracts

Leaves were separated from the young green stems for each sex and season, and each tissue batch was homogenized in a blender (Black and Decker) in 95% ethanol in a 1:4 weight/volume ratio. The homogenates were extracted at room temperature for 2 days and centrifuged at 3000 rpm for 20 min. The supernatants were filtered through Whatman's filter paper # 54 (Thomas Scientific) and the filtrates were stored at −20°C for future use.

In vitro antidiabetic activities of mulberry extracts

α-amylase inhibition assays

The assays were performed according to the method of Kwon *et al.*[27] with some modifications. Different concentrations (500 µl of 20–100 µg/mL each) of each mulberry extracts were incubated with 500 µl α-amylase solution (1 U/ml in 0.02 M sodium phosphate buffer containing 0.006 M NaCl [pH 6.9]) at 25°C for 10 min. Starch solution (500 µl of 1% starch in 0.02 M sodium phosphate buffer containing 0.006 M NaCl [pH 6.9]) was added, and the reaction mixture was incubated at 25°C for 10 min. DNS (1 ml) was added to the test tube to terminate the reaction. The reaction mixtures were incubated in a boiling water bath for 10 min and then cooled at room temperature. Distilled water (10 ml) was added to the tubes. The absorption at 540 nm of the reaction mixture was determined spectrophotometrically (Shimadzu UV Mini 1240 UV-vis). The standard was prepared using the same protocol except for substituting the mulberry extract with 500 µl of deionized water. Acarbose was used as a positive control. The percent inhibition of the mulberry extracts on α-amylase activity was calculated as:

Percentage inhibition of α-amylase activity = (Control Abs_{540} – Extract Abs_{540}/Control Abs_{540}) × 100

α-glucosidase inhibition assays

The assays were performed according to the method of Kim *et al.*[28] with some modifications. Different concentrations (50 µl of 20–100 µg/mL) of mulberry extracts at were incubated with 100 µl of α-glucosidase solution (1.0 U/ml in 0.1 M phosphate buffer [pH 6.9]) at 37°C for 10 min. Fifty microliters of 5 mM pNPG dissolved in 0.1 M phosphate buffer (pH 6.9) were added to the test tube, and the solution was incubated again for 20 min at 37°C. Two milliliters of 0.1 M Na_2CO_3 were added to terminate the reaction. The inhibitory activity was measured spectrophotometrically at 405 nm. The standard was prepared using the same protocol except for substituting the mulberry extract with 50 µl DI water. Acarbose was used as a positive control. The percent of inhibition of the mulberry extracts on α-glucosidase activity was calculated as:

Percentage inhibition of α-glucosidase activity = (Control Abs_{540} – Extract Abs_{540}/Control Abs_{540}) × 100.

Polyphenol content analyses of mulberry extracts

Total phenolic content

Total phenolic content of the mulberry extracts was determined by Folin–Ciocalteu method.[29] Each extract (400 µl each), 7.5% sodium carbonate dissolved in deionized water (1.6 ml), and Folin–Ciocalteu reagent diluted 10 times in DI water (2 ml) were incubated at room temperature for 1 h and the absorbances (Abs) were measured at 765 nm. The total phenolic content of mulberry extracts was calculated using gallic acid standard curve, and the results were expressed as mg gallic acid equivalents (GAE)/g of tissue fresh weight (FW).

Total flavonoid content

Total flavonoid content of the mulberry extracts was determined by the method of Ordoñez *et al.*[30] Each extract (500 µl each) and 2% aluminum chloride (500 µl) prepared in ethanol were mixed. The reaction mixtures were incubated at the room temperature for 1 h and the Abs were measured at 430 nm. The total flavonoid content of the extracts was calculated using a quercetin standard curve, and the results were expressed as mg quercetin equivalents (QE)/g of tissue FW.

Total proanthocyanidin content

Total proanthocyanidin content of the mulberry extracts was determined by the method of Aiyegoro and Okoh.[31] Each extract (500 µl each), 4% (v/v) vanillin methanol (3 ml), and HCl (1.5 ml) were added, and the reaction mixtures were vortexed thoroughly. The resulting mixtures were allowed to react at room temperature for 15 min and the Abs were measured at 500 nm. Total proanthocyanidin content was calculated using a gallic acid standard curve, and the results were expressed as mg GAE/g of tissue FW.

In vitro antioxidant and free radical scavenging activities of mulberry extracts

Ferric reducing power

Ferric reducing power of the mulberry extracts was determined by the method of Oyaizu.[32] Each extract (20–100 µg/mL) (1 ml), 0.2 M phosphate buffer (pH 6.6) (2.5 ml), and 1% (w/v) $K_3Fe(CN)_6$ (2.5 ml) were incubated at 50°C for 20 min. After addition of 10% (w/v) TCA (2.5 ml), the reaction mixtures were centrifuged at 3000 rpm for 10 min. The supernatants (2.5 ml) were mixed with distilled water (2.5 ml) and 0.1% (w/v) $FeCl_3$ (0.5 ml). The results were presented as Abs measured at 700 nm.

2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity

The ABTS radical scavenging activity of the mulberry extracts was determined by the method of Re *et al.*[33] ABTS solution was prepared by mixing 7 mM ABTS and 2.4 mM $K_2S_2O_8$ in equal amounts, and the mixture was allowed to react in the dark at room temperature for 12–16 h. At the end of the reaction, 1 ml of ABTS radical cation solution was mixed with 60 ml of methanol to obtain an Abs of 0.76 ± 0.001 at 734 nm. Each extract (1 ml of 20–100 µg/mL) and ABTS cation solution (1 ml) were combined and Abs were read at 734 nm after 7 min. Ascorbic acid was used as a standard. The percentage of ABTS radical scavenging activity was calculated according to the following equation:

Percentage of ABTS radical scavenging activity = (Control Abs_{734} – Extract Abs_{734}/Control Abs_{734}) × 100

2,2-diphenyl-1-picrylhydrazyl radical scavenging activity

The DPPH radical scavenging activity of the mulberry extracts was determined by the method of Gülçin *et al.*[34] with some modifications. Each extract (50 µl of 20–100 µg/mL) and DPPH (2.95 ml of 0.1 mM) in methanol were mixed and vortexed thoroughly. The mixture was kept in dark at room temperature for 30 min and the Abs were read at 517 nm. Ascorbic acid was used as a standard. The percentage of DPPH radical scavenging activity was calculated according to the following equation:

Percentage of DPPH radical scavenging activity = (Control Abs_{517} – Extract Abs_{517}/Control Abs_{517}) × 100.

Nitric oxide scavenging activity

The nitric oxide (NO) radical scavenging activity of the mulberry extracts was performed by the modified method of Balakrishnan *et al.*[35] Each extract (1 ml of 20–100 µg/mL) and $Na_2[Fe(CN)_5NO]$ (2 ml of 10 mM) in phosphate-buffered saline were mixed and the reaction mixtures were incubated at 25°C for 150 min. After incubation, the reaction solution (0.5 ml) was mixed with Griess reagent. Mixtures were allowed to react at the room temperature for 30 min, and Abs were read at 540 nm. Gallic acid was used as a standard. The percentage of NO radical scavenging was calculated according to the following equation:

Percentage of NO radical scavenging activity = (Control Abs_{540} – Extract Abs_{540}/Control Abs_{540}) × 100.

IC_{50} and correlation

IC_{50} values, concentrations required to inhibit 50% of the free radicals, were calculated based on a linear regression analysis. The Pearson's correlation analysis was performed between IC_{50} values of inhibitory activities and polyphenol contents.

Statistical analysis

All experiments with two replicates each were repeated three times. Means and standard deviations are calculated. One-way ANOVA followed by Tukey's *post hoc* test was performed to determine the significant differences among means.

RESULTS AND DISCUSSION

In vitro antidiabetic activities of mulberry extracts

The *in vitro* antidiabetic activities of dioecious mulberry extracts were determined through α-amylase and α-glucosidase inhibitory assays. It is known that *in vivo*, inhibition of these carbohydrate hydrolyzing enzymes will lead to retardation of absorption of glucose in the blood stream, which could be one of the most effective ways to control diabetes.[36-38] Figure 1 summarizes the *in vitro* α-amylase [Figure 1a] and α-glucosidase [Figure 1b] inhibitory activities of white mulberry extracts. Three variables were introduced in the experiments: mulberry sex (male and female), organ (leaf and young branch), and the season when the tissues were collected (spring and fall).

The inhibition of α-amylase activity by 100 µg/mL mulberry extracts ranged from 51.1 ± 2.2% to 71.9 ± 1.4% and of α-glucosidase from 68.7 ± 1.4% to 81.4 ± 1.1%. The increase in enzyme inhibition activity

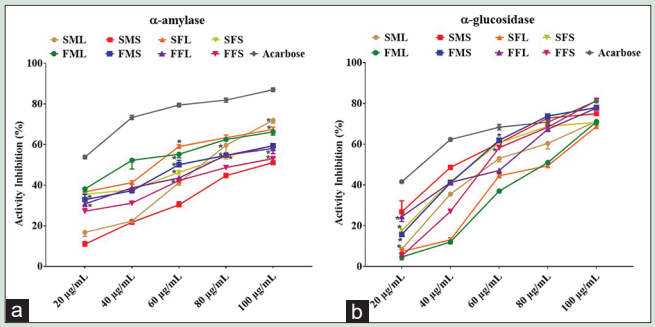

Figure 1: Inhibition of *in vitro* (a) α-amylase and (b) α-glucosidase activities of dioeciuos *Morus alba* extracts. Results represent means ± standard deviation of three experiments. In each graph, *Mean values with are significantly different from each other at $P \leq 0.05$ (Tukey's test). SML: Spring male leaf; SMS: Spring male stem; SFL: Spring female leaf; SFS: Spring female stem; FML: Fall male leaf; FMS: Fall male stem; FFL: Fall female leaf; FFS: Fall female stem

was dose-dependent for each extract. Spring male leaf extract induced the highest inhibition (71.9 ± 1.4% at 100 μg/mL) of α-amylase activity with an IC_{50} value of 70.3 ± 1.1 μg/mL. In fact, among all extracts, leaf extracts irrespective of sex and season showed the highest inhibition of α-amylase activity. Although the fall male leaf extract (100 μg/mL) inhibited α-amylase significantly lower than the spring male leaf, its IC_{50} value (45.1 ± 4.0 μg/mL) was also much lower, which was the closest to the IC_{50} of acarbose (33.2 ± 0.23 μg/mL). At 100 μg/mL, spring female leaf induced 67.5% inhibition of α-amylase with an IC_{50} of 51.3 ± 1.3 μg/mL.

In general, all extracts showed a higher inhibition of α-glucosidase than α-amylase. The highest α-glucosidase inhibitory activity was induced by fall female stem (81.4 ± 1.1%), fall male stem (78 ± 0.7%), and fall female leaf (77.7 ± 0.5%) extracts (100 μg/mL), which were not significant different from the acarbose inhibition activity (81.2 ± 0.9%) at the same concentration. However, the IC_{50} of fall female stem (62.0 ± 0.1%) was 2.4X of acarbose IC_{50} (26.4 ± 0.7%). The IC_{50} values of α-glucosidase inhibitory activities of mulberry extracts ranged from 48.6 ± 2.4 to 77.3 ± 0.4 μg/mL. The lowest IC_{50} of all extracts inhibiting α-glucosidase was that of spring male stem (48.6 ± 2.4 μg/mL), which inhibited α-glucosidase by 74.9 ± 0.2%.

Other studies reported the *in vitro* antidiabetic activity of mulberry extracts.[39-44] However, there is no mention of the plant sex, and almost all studies were performed with leaves. Since mulberry species are mostly dioecious, we consider that it is important to study the sexual dimorphism of the plant, which could help in finding the more effective tissues for at least formulations of antidiabetic supplements. Most other studies employed cultivars or clones of *M. alba* with no mention of plant sex.[44,45] There are also very few mentions in the literature about the season when mulberry tissues were collected. Secondary metabolites in plants are synthesized by biosynthetic pathways that could change with seasons and environmental stresses. One mention of season is by Nickavar and Mosazadeh who used *M. alba* leaf extract from plants collected in May and reported the highest α-amylase inhibitory activity of 75.58 ± 1.1% at 23 mg/ml of extract with an IC_{50} values of 17.60 mg/mL.[40] As far as studying other mulberry tissues than leaf tissues, Qiu *et al.* used extracts from bark peeled from branches of *M. alba* var. *multicaulis* (HuSang 32 cultivar) for treating diabetes in mice.[38] For the above reasons, we could not compare our study with previous studies on the antidiabetic properties of mulberry extracts.

Sudha *et al.* reported the pancreatic α-amylase inhibitory activity of *M. alba* isopropanol and methanol leaf extracts. The isopropanol leaf extract showed 60.5% inhibition at 1.8 mg/ml and the methanol leaf extract showed 15.5% inhibition at 3.9 mg/ml.[42] The above-mentioned results were lower than the results of the present study, where the α-amylase inhibitory activity of the male and female leaf extracts collected in spring and fall ranged from 58.1 to 71.9% at 100 μg/mL. A study by Adisakwattana *et al.* reported IC_{50} values of *M. alba* leaves as 0.59 ± 0.06 mg/mL against intestinal maltase and 0.94 ± 0.11 mg/mL against intestinal sucrase, two α-glucosidase enzymes.[43] The present study reports the lowest IC_{50} value as 48.6 ± 2.4 μg/mL by spring male stem. In above studies, the effective inhibitory concentrations of mulberry extracts were much higher than the ones in our study. Other differences between the present and previous studies could be attributed to the use of different methods of extraction (ethanol vs. isopropanol/methanol/water) and the season of plant collection.

Polyphenol content analyses of mulberry extracts

Polyphenol contents of dioecious *M. alba* leaf and stem extracts collected in spring and fall were estimated by quantifying total phenolics, flavonoids, and proanthocyanidins. Table 1 summarizes the polyphenol contents of mulberry extracts. In general, total flavonoid content in the mulberry extracts, ranging from 7.3 ± 0.2 to 24.9 ± 1.7 mg QE/g, was lower than total phenolic (20.4 ± 0.7–130.3 ± 0.3 mg GAE/g) and total proanthocyanidin (17.9 ± 0.2–191.6 ± 2.5 mg GAE/g) contents for all extracts. Total proanthocyanidin contents were much higher than those of phenolics and flavonoids, especially in fall extracts. Spring male stem extract had the highest total phenolic content (130.3 ± 0.3 mg GAE/g). The highest total flavonoid content was quantified in the fall male stem (22.1 ± 1.6 mg QE/g) and fall female leaf (24.9 ± 1.7 mg QE/g) extracts and the highest total proanthocyanidin contents were identified in spring male stem (110.0 ± 0.4 mg GAE/g), fall male leaf (191.6 ± 2.5 mg GAE/g), and fall female leaf (184.3 ± 0.6 mg GAE/g) extracts.

It is known that plants have seasonal changes in the phenyl-propanoid biosynthetic pathway products as a response to different stresses during their growth and development.[46-50] The mulberry spring leaf extracts are very much enriched in phenolics since the leaves and stem are very young, still growing, and simple phenols are synthesized earlier in the phenyl-propanoid pathway. The spring stem extracts contained more phenolics and proanthocyanidins than flavonoids. All fall extracts are very much enriched in proanthocyanidins than phenolics and flavonoids, representing the end products of the phenyl-propanoid pathway in older tissues.

Comparing the male and female extracts, a sexual dimorphism influenced by season and plant organ was noticed. Both spring male leaf and stem extracts have significantly higher levels of phenolics and proanthocyanidins than the corresponding female extracts, although flavonoid levels were the same. However, the fall male leaf extract has lower levels of both phenolics and flavonoids than the female leaf extract.

Khan *et al.* reported total phenolic contents of methanolic *M. alba* leaf, fruit, stem, and root bark extracts ranging from 52.71 ± 3.17 (leaf) to 285.62 ± 2.54 (stem bark) mg GAE/g dry extract (DW),[51] which were higher than the results of the present study, where the total phenolic contents of leaf extracts were 21.6 ± 1.3–39.2 ± 0.2 and those of stem extracts were 20.4 ± 0.7–130.3 ± 0.3 mg GAE/g FW. The differences are attributed to the use of different solvents (methanol vs. ethanol), plant tissues (stem bark vs. stem), and reporting data (DW vs. FW). Sánchez-Salcedo *et al.* showed that total phenolic contents of ethanolic leaf extract of *M. alba* clones collected in June were 12.81–15.50 mg GAE/g DW,[45] which is lower than in the present study. Kim *et al.* reported the lowest polyphenol content (28.2 ± 1.7 mg GAE/g) in extracts from mulberry leaves collected in September.[52] Another study conducted by Sývacý and Sökmen reported the highest total phenolic content of extracts of *M. alba* stem collected in October.[46] None of these studies mention the sex of the mulberry plants.

According to Zhishen *et al.*, the total flavonoid content of leaves of the same mulberry variety reached the highest values in spring rather than autumn, while the present study reports the highest flavonoid contents in fall extracts.[53] They explained their results by the fact that the spring leaves were mature, whereas autumn leaves were senescent. The fall leaves in our study were not senescent. In addition, the differences in flavonoid content among studies could be due to the use of different standards and other factors such as geographical location and climate, levels of pollution, UV light, and pathogen attacks, as suggested by Thabti *et al.*[21]

Not many earlier studies have reported total proanthocyanidin content of the mulberry extracts. However, mulberry fruit is a rich source of anthocyanins, natural fruit pigments, and their profiles and antioxidant activities were studied recently in 12 genotypes of mulberry fruits and syrup.[54] A study conducted by Khan *et al.* reported that total proanthocyanidin content of *M. alba* leaf extract as 2.36 ± 0.04 mg of CAE/g.[51] The proanthocyanidin contents reported in the present study are much higher and are reported as mg GAE/g.

Table 1: Total phenolic, flavonoid and proanthocyanidin contents of dioeciuos *Morus alba* extracts

Extract	Total phenolics (mg GAE/g)	Total flavonoids (mg QE/g)	Total proanthocyanidins (mg GAE/g)
Spring male leaf	39.2±0.2[a]	14.5±0.02[a,*]	25.8±0.1
Spring male stem	130.3±0.3	18.7±0.1[b,*]	110.0±0.4
Spring female leaf	27.2±0.1	13.4±0.02[a,c]	17.9±0.2
Spring female stem	57.7±0.5	18.4±0.2[b,*]	49.8±0.2[d]
Fall male leaf	21.6±1.3[e]	7.3±0.2	191.6±2.5
Fall male stem	20.4±0.7[e,f]	22.1±1.6[f,*]	44.3±0.2
Fall female leaf	36.8±0.2[a]	24.9±1.7[f]	184.3±0.6
Fall female stem	22.5±1.1[e,f]	13.8±1.1[a,c]	49.5±0.3[d]

*Mean values are significantly different from each other at $P \leq 0.05$; Mean values with same superscript letters are not significantly different (Tukey's test). Results represent means±SD of three experiments. In each column, mean values with no superscript letters are significantly different from each other at $P \leq 0.01$. GAE: Gallic acid equivalents, QE: Quercetin equivalents, SD: Standard deviation

In vitro antioxidant and free radical scavenging activities of mulberry extracts

In vitro assays were performed to determine the antioxidant and free radical scavenging activities of mulberry extracts. The ferric reducing power and all free radical scavenging activities of mulberry extracts increased in a dose-dependent manner. Table 2 summarizes the ferric reducing power, and Figure 2 summarizes the free radical scavenging activities of mulberry extracts. Table 3 summarizes the IC_{50} values of free radical scavenging activities of mulberry extracts.

A dose-dependent increase in ferric reducing power, although not significant among different concentrations of extracts, was observed with all extracts. The ferric reducing power ranged from 0.128 ± 0.002 to 1.861 ± 0.067 at 100 µg/mL. Both fall male (Abs_{700} = 1.813 ± 0.084) and female leaf (Abs_{700} = 1.861 ± 0.067) extracts induced the highest ferric reducing power at 100 µg/mL. In general, higher reducing power was observed with fall extracts than spring extracts. At higher concentrations (80–100 µg/mL), spring male extracts induced a significantly higher reducing power than the female extracts, while fall extracts showed same reducing power.

Furthermore, at high concentrations (80–100 µg/mL), the ferric reducing power of all extracts was higher than the positive control, BHT. Thabti *et al*. reported the increasing reducing power activities with increasing concentrations of aqueous and hydromethanolic extracts from different varieties of mulberry leaves and stem barks.[23] The high reducing power of the leaf extracts in the present study indicates that the fall leaves contain high level of antioxidants. Other studies reported the reducing power of mulberry extracts with no mention of plant sex.[51,55] Overall, these results demonstrate that the reducing power of mulberry extracts is affected by seasonal changes and the use of different solvents for extraction.

All extracts at all concentrations induced higher ABTS-free radical scavenging activities than ascorbic acid with lower IC_{50s} (11.9 ± 0.3–45.8 ± 0.4 µg/mL) than that of ascorbic acid of 74 ± 1.7 µg/mL. All the extracts scavenged ABTS-free radical more potently (86.4 ± 0.5% to 91.8 ± 0.4% at 100 µg/mL) than ascorbic acid (52.1 ± 0.5%) at 100 µg/mL.

The DPPH radical scavenging activities of mulberry extracts ranged from 3.0 ± 0.1% to 34.6 ± 0.5% at 100 µg/mL. Both male and female fall leaf extracts induced the highest DPPH scavenging activities at all concentrations with significantly higher activities for fall male leaf than female leaf extract. All extracts induced much lower *in vitro* scavenging activities in the DPPH and NO assays than in the ABTS assay.

Iqbal *et al*. also reported that Trolox equivalent values calculated for DPPH radical scavenging activity were 3–4-fold lower than those calculated for ABTS radical scavenging activity of *M. alba* leaf methanol extract.[56] One explanation could be that ABTS assay is more suitable for hydrophilic antioxidants, while DPPH assay is more applicable to hydrophobic antioxidants.[57] Mulberry extracts could contain more hydrophilic than hydrophobic antioxidants.

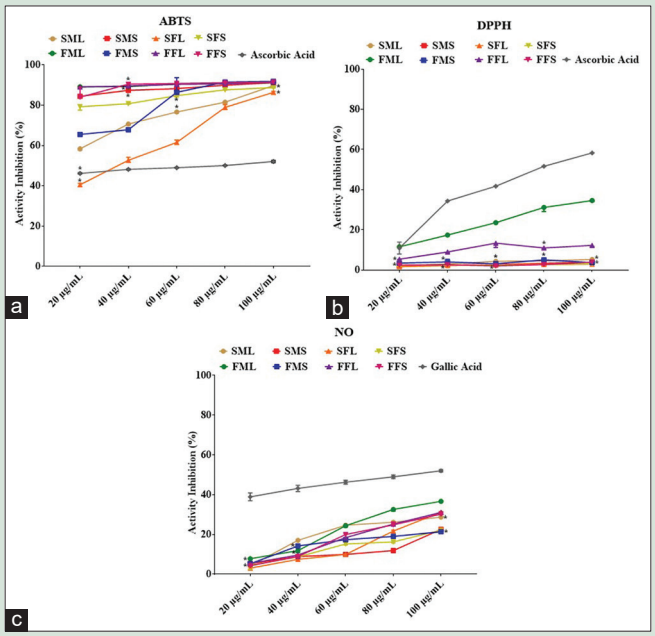

Figure 2: (a-c) *In vitro* radical scavenging activities of dioeciuos *Morus alba* extracts. Results represent means ± standard deviation of three experiments (a) ABTS, (b) DPPH and (c) NO. In each graph, *Mean values are significantly different from each other at $P \leq 0.05$ (Tukey's test). SML: Spring male leaf; SMS: Spring male stem; SFL: Spring female leaf; SFS: Spring female stem; FML: Fall male leaf; FMS: Fall male stem; FFL: Fall female leaf; FFS: Fall female stem

Both spring and fall male leaf extracts induced the highest NO radical scavenging activity at all concentrations. The NO radical scavenging activities by mulberry extracts ranged from 21.3 ± 0.6% to 36.7 ± 0.5% at 100 µg/mL, with the highest activity being induced by the fall male leaf extract. Deepa *et al*. reported that *M. alba* extract from leaves collected in March–April significantly reduced the production of NO in human colon (HCT-15) and breast (MCF-7) cancer cell lines by downregulating iNOS and inducing apoptosis in the above-mentioned cell lines.[58] The findings from the present and other studies suggest that the *M. alba* leaf and stem extracts are potent in inhibiting the production of NO *in vitro* and in inflammatory conditions.

Table 2: Ferric reducing power of dioeciuos *Morus alba* extracts

	Ferric reducing (Abs at 700 nm)				
Extract	**20 (µg/mL)**	**40 (µg/mL)**	**60 (µg/mL)**	**80 (µg/mL)**	**100 (µg/mL)**
Spring male leaf	0.122±0.001a	0.137±0.002[a]	0.153±0.001[a,*]	0.185±0.004[a]	0.234±0.004[a,*]
Spring male stem	0.103±0.004[a,b]	0.116±0.002[a,b]	0.162±0.002[a,b,*]	0.172±0.002[a]	0.180±0.001[a,b]
Spring female leaf	0.096±0.001[a,b,c*]	0.105±0.003[a,b,c]	0.111±0.001[a,b,c]	0.123±0.002[c,*]	0.128±0.002[b,c,*]
Spring female stem	0.113±0.001[a,b,c,d]	0.117±0.002[a,b,c,d]	0.120±0.002[a,b,c,d]	0.125±0.002[c,*]	0.136±0.001[a,b,c,d,*]
Fall male leaf	0.382±0.022	1.069±0.023	1.190±0.010	1.493±0.009	1.813±0.084[e]
Fall male stem	0.169±0.003[a,b,c,d,f]	0.244±0.014[f]	0.247±0.007[f,*]	0.256±0.008[f]	0.281±0.010[a,b,f,*]
Fall female leaf	0.900±0.089	0.991±0.052	1.564±0.073	1.644±0.015	1.861±0.067[e]
Fall female stem	0.191±0.009[a,b,d,h,f,*]	0.217±0.012[f]	0.228±0.009[b,f,*]	0.241±0.006[f]	0.257±0.004[a,b,f,h,*]
BHT	0.124±0.001[a,b,c,d,h,f]	0.134±0.003[a,b,c,d]	0.137±0.002[a,b,c,d]	0.146±0.002*	0.164±0.001[a,b,c,d,h,*]

*Mean values are significantly different from each other at $P \leq 0.05$; Mean values with same superscript letters are not significantly different (Tukey's test). Results represent means±SD of three experiments. In each column, mean values with no superscript letters are significantly different from each other at $P \leq 0.01$. BHT: Butylated hydroxy toluene, SD: Standard deviation

Table 3: IC_{50} values of α-amylase, α-glucosidase and *in vitro* radical scavenging activities of dioeciuos *Morus alba* extracts

	IC_{50} (µg/mL)				
Extract	**α-amylase**	**α-glucosidase**	**ABTS**	**DPPH**	**NO**
Spring male leaf	70.3±1.1	65.7±1.0	32.9±0.2	1195.3±1.0	162.7±0.9
Spring male stem	95.3±1.8	48.6±2.4	15.2±0.2	1884.2±15.0	264.3±3.7
Spring female leaf	51.3±1.3	76.8±0.3	45.8±0.4	2896.3±94.3	160.3±1.1
Spring female stem	70.4±0.7	57.2±0.1	20.1±0.57	4298.2±2.2	232.2±12.2
Fall male leaf	45.1±4.0	77.3±0.4	11.9±0.3	148.3±5.0	129.8±1.5
Fall male stem	68.8±1.0	54.8±0.3	28.4±0.8	2739.9±48.4	247.4±10.2
Fall female leaf	73.6±2.9	57.6±0.9	11.9±0.4	433.5±14.0	155.9±2.8
Fall female stem	87.3±1.3	62.0±0.1	14.0±2.1	1639.3±52.8	155.4±2.6
Standard	33.2±0.23[a]	26.4±0.7[a]	74±1.7[b]	79±0.1[b]	86.2±4.5[c]

Results represent means±SD of three experiments. In each column, mean values with no superscript letters are significantly different from each other at $P \leq 0.01$ (Tukey's test). The last row of the table contains the corresponding values for standards as follows: [a]Acarbose, [b]Ascorbic acid, [c]Gallic acid. SD: Standard deviation, ABTS: ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), DPPH: 2,2-diphenyl-1-picrylhydrazyl, NO: Nitric oxide

Correlation of polyphenol contents with antidiabetic and antioxidant activities of mulberry extracts

The goal of estimating the polyphenol contents in the mulberry extracts was to determine how the three group of polyphenols correlate with *in vitro* antidiabetic and antioxidant activities. Table 4 shows the correlation between IC_{50} of antidiabetic and antioxidant activities of dioeciuos *M. alba* extracts and their phenolic, flavonoid, and proanthocyanidin contents. The results show both positive and negative correlations.

In general, total phenolic contents showed positive correlations with α-amylase inhibitory activity except for spring male and female leaf and fall female stem extracts. Phenolic contents in spring male stem (R^2 = 0.929), spring female stem (R^2 = 0.972), fall male stem (R^2 = 0.925), and fall female leaf (R^2 = 0.919) extracts exhibited significant positive correlations with α-amylase inhibitory activity.

Total flavonoid contents of spring female leaf extract showed a significantly positive correlation (R^2 = 0.999) with α-amylase inhibitory activity. Proanthocyanidin content in the fall female stem extract correlated with α-amylase inhibitory activity (R^2 = 0.971) and those of spring male and female stem extracts correlated with α-glucosidase inhibitory activity (R^2 = 0.835 and R^2 = 1.000, respectively). Correlation does not necessarily imply causation. Therefore, we cannot conclude from the results in Table 4 that a certain polyphenolic group in a certain extract was responsible for α-amylase or α-glucosidase inhibitory activity. However, studies with certain purified phenolics and flavonoids showed that they account for antidiabetic activity *in vitro*[59-61] and *in vivo*.[62]

The ABTS radical scavenging activity positively and strongly correlated with phenolics in fall female leaf extract (R^2 = 0.919) and flavonoids in spring female leaf (R^2 = 0.987) extracts. The DPPH scavenging activity positively and strongly correlated with phenolics in spring female leaf (R^2 = 0.933) and fall female stem (R^2 = 0.984), flavonoids in fall male leaf (R^2 = 0.956), and proanthocyanidins in spring female stem (R^2 = 0.868) extracts. Other studies found positive correlations between total phenolic content and ABTS and DPPH radical scavenging activities (R^2 = 0.973 and R^2 = 0.537, respectively) of *M. alba* leaf extract.[56] Khan *et al.* reported that total phenolic contents in *M. alba* leaf and stem bark extracts showed significant and positive correlations with DPPH-free radical scavenging activity, suggesting a probable role of phenolics in scavenging.[51] Iqbal *et al.* reported negative correlation of flavonoids with DPPH scavenging activity (R^2 = −0.235) of *M. alba* leaf extract.[56] Thabti *et al.* also reported a high negative correlation between flavonoid content and DPPH radical scavenging activity (R^2 = −0.963) of mulberry leaf extract.[21]

The NO radical scavenging activity positively and strongly correlated with phenolics in spring male leaf (R^2 = 0.999) and fall female leaf (R^2 = 0.998), flavonoids in spring male leaf (R^2 = 0.922), and spring female stem (R^2 = 0.900). Proanthocyanidin content did not show good correlation with NO radical scavenging activity. No earlier studies have reported correlation of proanthocyanidin content in mulberry extracts with α-amylase and α-glucosidase inhibitory activities or ABTS, DPPH, and NO radical scavenging activities.

The correlation results of this study also indicate that, in most cases, there could be a nonlinear relationship between polyphenolic groups and enzyme inhibitory or radical scavenging activities. The antidiabetic and antioxidant activities of the mulberry extracts assayed in the present study

Table 4: Correlation of polyphenol content with the antidiabetic and antioxidant activities of dioeciuos *Morus alba* extracts

	Correlation (R^2)		
	Phenolics	Flavonoids	Proanthocyanidins
α-amylase inhibitory activity			
Spring male leaf	−0.328	0.046	−0.997*
Spring male stem	0.929*	−0.561	0.145
Spring female leaf	−0.737	0.999*	−0.332
Spring female stem	0.972*	−0.948	−0.332
Fall male leaf	0.882	−0.433	0.108
Fall male stem	0.925*	−0.525	−0.791
Fall female leaf	0.919*	0.697	0.363
Fall female stem	−0.766	0.107	0.971*
α-glucosidase inhibitory activity			
Spring male leaf	−0.598	−0.852	0.616
Spring male stem	−0.058	0.987*	0.835
Spring female leaf	−0.995*	0.814	0.303
Spring female stem	−0.389	0.308	1.000
Fall male leaf	−0.895	0.985*	0.747
Fall male stem	0.951*	−0.950*	−0.209
Fall female leaf	0.887	0.749	0.433
Fall female stem	0.975*	−0.855	−0.400
ABTS radical scavenging activity			
Spring male leaf	−0.714	−0.403	−0.863
Spring male stem	−0.945*	−0.113	−0.755
Spring female leaf	−0.942*	0.931*	0.062
Spring female stem	0.739	−0.795	0.324
Fall male leaf	0.054	−0.632	−0.946*
Fall male stem	0.143	−0.698	0.785
Fall female leaf	0.919*	0.697	0.363
Fall female stem	0.333	0.412	−0.958*
DPPH radical scavenging activity			
Spring male leaf	−0.756	−0.945*	0.434
Spring male stem	0.852	−0.696	−0.027
Spring female leaf	0.933*	−0.941*	−0.036
Spring female stem	0.111	−0.196	0.868
Fall male leaf	−0.945*	0.956*	0.657
Fall male stem	−0.069	0.642	−0.829
Fall female leaf	−0.880	0.127	0.503
Fall female stem	0.984*	−0.587	−0.724
NO radical scavenging activity			
Spring male leaf	0.999*	0.922*	0.280
Spring male stem	−0.880	0.654	−0.029
Spring female leaf	−0.985*	0.855	0.232
Spring female stem	−0.859	0.900	−0.128
Fall male leaf	−0.292	0.043	0.565
Fall male stem	−0.292	0.799	−0.681
Fall female leaf	0.998*	0.410	0.023
Fall female stem	0.180	−0.812	0.687

The coefficient values with asterisks indicate significant differences at $P \leq 0.01$. ABTS: 2,2-diphenyl-1-picrylhydrazyl, DPPH: 2,2-diphenyl-1-picrylhydrazyl, NO: Nitric oxide

may not only be attributed to phenolic, flavonoid, and proanthocyanidin contents. Other phytochemicals, such as polyhydroxylated alkaloids, stilbenes, 2-arylbenzofuran flavonoids, and polysaccharides, present in the mulberry extracts could correlate and even contribute to the activities assayed in this study. Rivière *et al*. found resveratrol (stilbene) and moracin M (2-arylbenzofuran flavonoid) in the *M. alba* stem extract to be the most active NO inhibitory compounds.[63] Song *et al*. reported 1-deoxynojirimycin (1-DNJ), a polyhydroxylated piperidine alkaloid, in extracts of *M. alba* juice, fruit, and leaves.[41] 1-DNJ was a potent α-amylase inhibitor.[64] Other studies have shown that 1-DNJ in mulberry leaves and stem bark is also a potent inhibitor of mammalian α-glucosidase.[65,66] A strong positive Pearson's correlation (R^2 = 0.811) between 1-DNJ content and α-glucosidase inhibitory activity of *M. alba* leaf extract was reported by Yatsunami *et al*.[64] A clinical study reported that a single administration of 0.8 and 1.2 g of 1-DNJ-enriched powder resulted in a significant suppression of elevated postprandial blood glucose and secretion of glucose. This finding also indicates that DNJ-enriched mulberry powder can be used as a dietary supplement to treat DM,[66] and there were attempts to develop high DNJ content mulberry tea to provide effective doses for the inhibition of ∝-glucosidase.[67]

CONCLUSION

The present study reports the total phenolic, flavonoid, and proanthocyanidin contents, *in vitro* antidiabetic and *in vitro*-free radical scavenging activities of dioecious *Morus alba* leaf and stem extracts from tissues collected in two seasons. To the best of our knowledge, this is the first study reporting the antidiabetic and antioxidant activities of male and female *M. alba* extracts from leaf and stems collected in spring and

fall. In general, the male extracts are more potent than female extracts, and the extracts collected in fall are shown to be more potent than spring extracts, highlighting that gender and growing season of mulberry specimens could influence their antidiabetic and antioxidant properties. Despite the discrepancies between the current and previously reported studies, mainly attributed to methodology, geographical location, cultivar, sex, season, and plant organ differences, the results of the present study will add to the current knowledge of medicinal properties of *M. alba*. Overall, the male mulberry plant and male and female leaves and branches collected in fall could be good candidates for formulating more potent antidiabetic and antioxidant dietary supplements. The present study provides important insights into which sex and season could be more effective as far as antidiabetic and antioxidant properties of mulberry trees.

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Conflicts of interest

There are no conflicts of interest.

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