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Phenolic, flavonoid and antioxidant studies at different leaf stages of various mulberry cultivars

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Abstract
The present study was undertaken to quantify the total polyphenolic and flavoniod content of mulberry leaf obtained from three popular cultivars *viz*., Goshoerami (*Morus multicaulis*), Ichinose (*Morus Alba)* and Koksu-21 *(Morus Alba*) and measure their corresponding antioxidant properties/ scavenging activity. The ethanol stirrer extraction method was used for the preparation of mulberry leaf extracts. The leaf extract of Koksu-21(tender) and Ichinose (tender) recorded the highest total polyphenolic and flavoniod contents to the tone of 8.58mgGAE/g and 73.03 mgQE/g of dry weight respectively. Further, significantly higher (21.20%) scavenging activity on DPPH was observed in the Ichinose (tender) leaf extract. The study has suggested that mulberry leaf, can serve as a promising source of dietary antioxidants and further purification of these identified compounds will prove to be one of the important sources for pharmaceutical and nutraceuticals applications and would thereby add value to sericulture industry.

Keywords: Antioxidants, mulberry leaf, nutraceuticals applications and sericulture

Introduction
Mulberry is a multi-functional plant and sole food plant of the silkworm, *Bombyx mori* L. The leaves are ingested and digested by the silkworm larva to supply all kinds of essential nutrients for growth and cocoon formation. Leaves are also cultivated for dairy animal feed due to the positive effect on milk production (Gupta *et al.* 2005) [8]. Various parts of mulberry tree, including root bark, leaves and fruits, have been traditionally used for the treatment of fever, cough, hyperlipidaemia, hypertension and hyperglycemia (Chan *et al.* 2016) [3]. Reports indicate that mulberry leaves contain several bioactive compounds, including alkaloids, caroteniods, flavoniods, vitamins, minerals, proteins, carbohydrates etc. Apart from their use as animal and insect feed, they have been shown to possess excellent antioxidative activities (Sastri, 1962) [23]. Antioxidants, particularly those which are capable to prevent the effects of free radicals in the human body and deterioration of food products are gaining interest among the industries and researchers, especially antioxidants from natural sources, rather than from synthetic sources (Abdalla and Roozen, 1999) [1]. Polyphenols including flavoniods have versatile benefits for human health including effective antioxidants, anticancer, anti-bacteria and cardio protective agents (Kumar and Pandey, 2013; Chen *et al.*, 2015; Dzialo *et al.*, 2016 and Andreu, *et al.*, 2018) [14, 5, 6, 2]. Polyphenols and flavoniods are known to act as antioxidants by scavenging and neutralizing the harmful free radicals which damage the cells in the biological system. This increasing interest on using them is complimented by the increased use of analytical methods for the estimation of anti-oxidant efficiency of such substances (Schwarz, 2001 and Sanchez, 2002) [24, 21]. One of the most popular methods is based on the use of a stable free radical diphenyl picrylhydrazyl (DPPH). The scan of literature reveals scanty information with regard to antioxidant properties of mulberry leaf and therefore, the present study was undertaken to extract, quantify and analyze the antioxidant effects of polyphenols and flavoniods present in the mulberry leaf to explore its potential and possibilities for value addition to sericulture.

Materials and Methods
Experimental Material: Ethanol (analytical grade), Na_2CO_3, Folin- Ciocalteu reagent (FCR), Gallic acid, NaNO2, AlCl3, NaOH, Quercetin, DPPH (2,2-Diphenyl picrylhydrazyl) reagent, distilled water and mulberry leaf (tender, medium & coarse) of three popular cultivars *viz*.

Goshoerami, Ichinose and Koksu-21.

Collection of mulberry leaf

The leaf samples comprising of almost equal proportion of tender, medium and coarse from all the four sides of plant were taken in early morning as described by Nakashima (1931) [19].

Processing of leaf samples

The fresh leaf samples after collection were first washed with running tap water to decontaminate leaves from dust and other foreign materials followed by washing with distilled water. The samples were air dried on filter papers and then oven dried at 60-65 ºC (Chapman, 1964) [4] till constant weight was obtained. The samples were crushed in stainless steel blender and sieved through 2 mm mesh sieve and were stored in labeled paper envelops for subsequent analysis.

Preparation of leaf extracts

For preparation of the leaf extract, 0.5g from each sample were dissolved in 10ml of 80% ethanol and kept at room temperature for 24 hours. Sample concentrations of 50mg/ml were diluted with 80% ethanol to make 25, 50, 100 and 200µg/ml concentrations.

Determination of total polyphenolic content (TPC)

TPC of mulberry leaf was determined by using the Folin–denis colorimetric method described by Kim *et al.*, (2009). About 100µl of the mulberry leaf extracts (tender, medium and coarse of all the three cultivars) from different concentrations (i.e., 6.25, 12.50, 25 and 50 mg/ml) were taken and mixed with 2ml of 2% Na_2CO_3 and100µl of 50% Folin-Ciocalteu reagent (FCR). The mixture was left for 30min to react at room temperature and absorbance was measured at 720 nm using Hitachi U-1800 UV-Vis spectrophotometer. A calibration curve of standard reference was established using gallic acid (range of concentration from 0 to 500µg/ml). TPC was revealed as gallic acid equivalents in milligrams per 100g of dry weight (mg GAE/100g DW).

Determination of total flavonoids content (TFC)

TFC of mulberry leaf was determined by aluminum chloride colorimetric assay. 1 ml of sample (different concentrations of 0.25, 0.50, 0.75, 1mg/ml) from each variety and 4ml of distilled water were taken in test tubes, 0.3ml of 5% NaNO2 was added and the mixture was allowed to react for 5 min at room temperature followed by addition of 0.3ml of 10% AlCl3, the mixture was again left to react for 5-6 minutes at room temperature. Further, 2ml of 1M NaOH and 2.4ml distilled water was added to each test tube to make the final volume of 10ml. Absorbance was taken at 510nm using Hitachi U-1800 UV-Vis spectrophotometer. The total flavonoid content was measured from the standard Quercetin curve (0-1mg/ml). TFC was revealed as quercetin equivalent in milligram per gram dry weight (mgQE/g DW).

Determination of DPPH radial scavenging activity

The DPPH free radical scavenging activity was analyzed by the method described by Nithiananthian *et al.*, (2011) with slight modification. Different concentrations (25, 50, 100 and 200µg/ml) of sample extracts were taken for analysis. 2ml of DPPH (0.1mM/ml or 0.004%) solution was added to 2ml of sample extracts of each concentration. In case of control, 2ml of sample extract was replaced by 80% ethanol. All the mixtures were mixed and left to incubate in dark for 30 minutes at room temperature. Absorbance was measured at 517nm on UV/VIS spectrophotometer (Hitachi U-1800) and scavenging activity percentage was calculated as follows:-

$$I\ \% = [(Ao - As)/Ao] \times 100$$

Where,

Ao represent the absorbance value of the control reaction.

As represent the absorbance value of the sample extract.

I% represent the percentage inhibition.

Statistical Analysis

Each experiment was carried out in triplicate. The data was analyzed by O.P Stat software and expressed as Mean ± Standard Deviation (SD). Data were considered significant at $p \leq 0.05$.

Results and Discussion

Total polyphenolic and flavonoids content

The results with respect to TPC are presented in table 1 and figure 01. In general, TPC in all the three mulberry cultivars exhibited decreasing trend from tender followed by medium and coarse textured leaves. TPC ranged from 4.57mgGAE/g in coarse textured leaf of Goshoerami to 8.58mg GAE/g in tender leaf of Koksu-21 mulberry variety. Sanchez-Salcedo *et al.*, (2015) [22] recorded TPC of 12-15mgGAE/g and 13-16mg GAE/g dry weight of white and black mulberry leaves respectively. Memon *et al.*, (2010) [17] found TPC of 8.33mg GAE/g dry weight in white mulberry leaves. Total phenolic content of 5.98mgGAE/g dry wt. of *Morus nigra* leaves, 8.64mg GAE/g dry wt. of *Morus Alba* leaves and 9.94mgGAE/g dry weight of *Morus rubra* leaves were recorded by Thabti *et al.*, 2011 [28]. Higher phenolic content exhibits good antioxidant and antibacterial activities. Phenolic compounds have redox properties and the properties allow them acting as antioxidants (Soobrattee *et al.*, 2015 and Shoib & Shahid, 2015) [27, 25].

The results with respect to TFC are presented in table 2 and figure 02. TFC also followed the decreasing trend like TPC with highest being in tender leaf extracts followed by medium and coarse leaf extracts of all the three tested cultivars. The TFC in the mulberry leaf extracts ranged from 37.03mgQE/g in coarse textured leaf of Goshoerami to 73.03mgQE/g in tender leaf of Ichinose. The present results are in conformity with the results obtained by Ganzon *et al.*, (2017) [7] who reported that the flavonoids accumulate in apical leaves at significantly higher levels than in lower levels. Hu *et al.*, (2021) were of the opinion that flavonoid accumulation differs throughout the mulberry growth period. Thabti *et al.*, (2011) [28] recorded the total flavonoids content of 4.40 to 7.89mg RE/g DW of mulberry leaf. Katsube *et al.*, (2006) [11] reported flavonoids {rutin, quercetin 3-(6-malonyglucisidase), isoquercitrin and flavonol glycosides} as major antioxidants in *Morus Alba* leaves. These flavonoids are well known to have multiple-bioactivities, including antimicrobial, antio-xidant and α-amylase inhibition activity. Li *et al.*, 2009 reported that flavonoids extracted from mulberry leaves have anti-fatigue activity in mice. Many flavonoids have ability to prevent coronary heart disease and exhibit hepatoprotective, anti- inflammatory and anti-cancer effects (Kumar and Pandey, 2013) [14] in addition to have anti-diabetic properties (Yang, *et al.*, 2012) [29].

Scavenging activity on DPPH radical

DPPH free radical method is a widely used antioxidant assay used in biological materials which works on the principle based on discoloration of the DPPH free radical upon reacting with hydrogen donating species i.e., antioxidants present in the plant extract (Krishnaiah *et al.*, 2011) [13].

The results of scavenging activity of the leaf extracts of all the three mulberry cultivars are presented in table-03 and figure-03. Among the 04 concentrations tested (25, 50, 100 and 200µg/ml) significantly higher scavenging activity was observed in the highest concentrations tested (200µg/ml and 100µg/ml) whereas the other concentrations (50 and 25µg/ml) show low scavenging activity in all the extracts. So increase in concentration shows promising DPPH inhibition. Further, tender leaves showed higher scavenging activity, followed by medium and coarse leaves in all the three tested cultivars. The experimental results of Hu, *et al.*, (2021) are in accordance with the results obtained in present study. They reported that young mulberry leaves had better antioxidant properties than older leaves. Higher antioxidant activity in the ethanolic extract of tender leaves is surmised to be due to presence of higher TPC and TFC as compared to medium and coarse textured leaves of all the cultivars tested. Mustafa, *et al.*, 2010 and Sim *et al.*, 2010 [26] reported that the capacity of the antioxidant is highly associated with the total flavonoids content and total phenolic compounds of the plant leaf crude extract. Flavonoids contain active hydrogen, which can terminate the chain reaction of oxygen radicals, scavenge free radicals and eliminate the toxic effect of radicals

Table 1: Total polyphenolic content of leaf extracts of different mulberries cultivars (mgGAE/g).

Mulberry cultivars		Concentration mgGAE /g				
		6.25 (mg/ml)	12.5 (mg/ml)	25 (mg/ml)	50 (mg/ml)	Mean
Goshoerami	Tender	1.84	7.08	8.55	9.16	6.66
	Medium	1.30	5.47	7.51	8.27	5.64
	Coarse	0.23	4.40	6.87	6.80	4.57
Sub mean		1.12	5.65	7.64	8.08	5.62
Ichinose	Tender	2.30	9.76	10.60	11.54	8.55
	Medium	1.30	5.47	7.51	8.40	5.67
	Coarse	1.78	3.97	6.74	8.02	5.13
Sub mean		1.80	6.40	8.29	9.32	6.45
Koksu-21	Tender	3.85	8.15	10.60	11.74	8.58
	Medium	2.91	4.94	9.70	11.28	7.21
	Coarse	1.78	8.60	9.70	8.58	7.17
Sub mean		2.85	7.23	10.00	10.53	7.65

C.D (P≤0.05)
Goshoerami: 0.006
Ichinose: 0.006
Koksu-21: 0.007
Goshoerami × Ichinose × Koksu-21: 0.020

Table 2: Total flavonoid content of leaf extracts of different mulberries cultivars (mg QE/g)

Mulberry cultivars		Concentration (mg QE/g)				
		0.25 (mg/ml)	0.50 (mg/ml)	0.75 (mg/ml)	1.00 (mg/ml)	Mean
Goshoerami	Tender	43.46	63.49	83.51	97.82	72.07
	Medium	44.89	53.48	69.21	89.23	64.20
	Coarse	27.74	33.46	39.18	47.75	37.03
Sub mean		38.69	50.14	63.97	78.27	57.77
Ichinose	Tender	44.14	72.67	82.65	92.64	73.03
	Medium	39.86	58.40	72.67	99.77	67.67
	Coarse	25.60	41.29	65.53	95.49	56.98
Sub mean		36.53	57.45	73.62	95.97	65.89
Koksu -21	Tender	35.58	56.98	78.73	106.90	69.55
	Medium	29.88	49.85	66.97	82.65	57.34
	Coarse	69.82	54.12	37.01	22.75	45.92

C.D (P≤0.05)
Goshoerami: 0.007
Ichinose: 0.007
Koksu-21: 0.008
Goshoerami × Ichinose × Koksu-21: 0.023

Table 3: DPPH radial scavenging activity of leaf extracts of different mulberry cultivars (%)

Mulberry cultivars		Scavenging (%)				
		25 (µg/ml)	50 (µg/ml)	100 (µg/ml)	200 (µg/ml)	Mean
Goshoerami	Tender	4.12	7.34	21.50	47.67	20.16
	Medium	2.23	5.89	15.77	39.24	15.78
	Coarse	2.21	5.33	11.69	30.21	12.36
Sub mean		2.86	6.19	16.32	39.04	16.10
Ichinose	Tender	7.21	11.36	24.30	41.93	21.20
	Medium	5.21	9.51	19.48	38.28	18.12
	Coarse	3.70	8.70	18.57	36.29	16.82
Sub mean		5.37	9.86	20.79	38.83	18.71
Koksu -21	Tender	2.69	6.80	16.77	24.32	12.65
	Medium	1.17	3.80	12.75	23.32	10.26
	Coarse	1.09	3.05	11.77	22.87	9.69
Sub mean		1.65	4.55	13.76	23.50	10.87

C.D (P≤0.05)
Goshoerami: 0.005
Ichinose: 0.005
Koksu-21: 0.006
Goshoerami × Ichinose × Koksu-21: 0.018

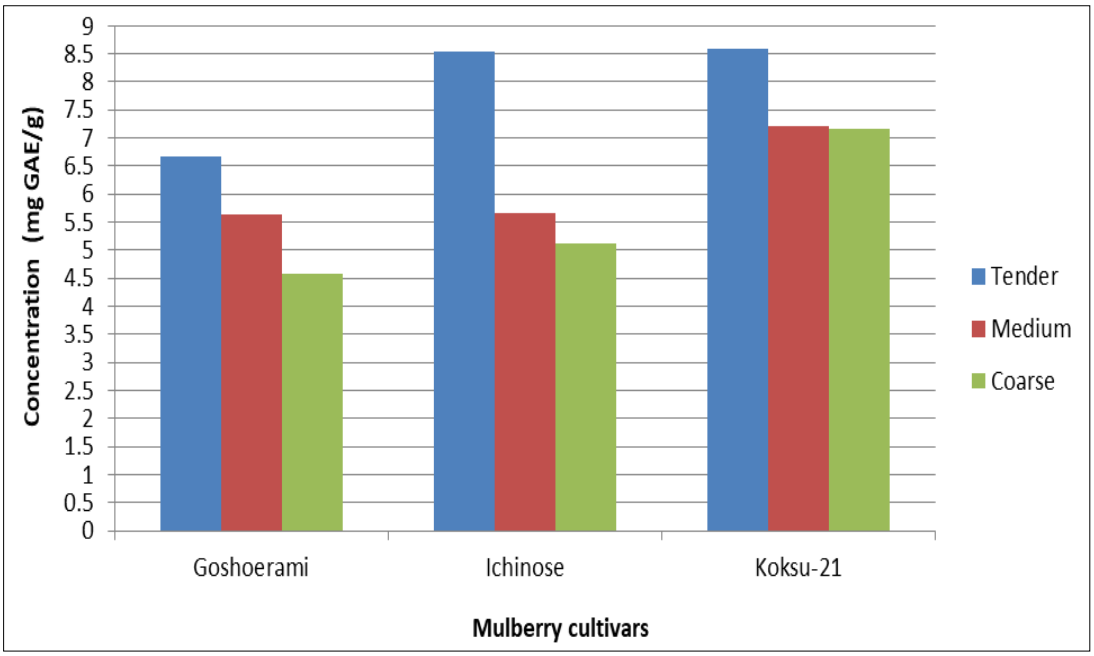

Fig 1: Graphical representation of total polyphenolic content of mulberry leaf extracts

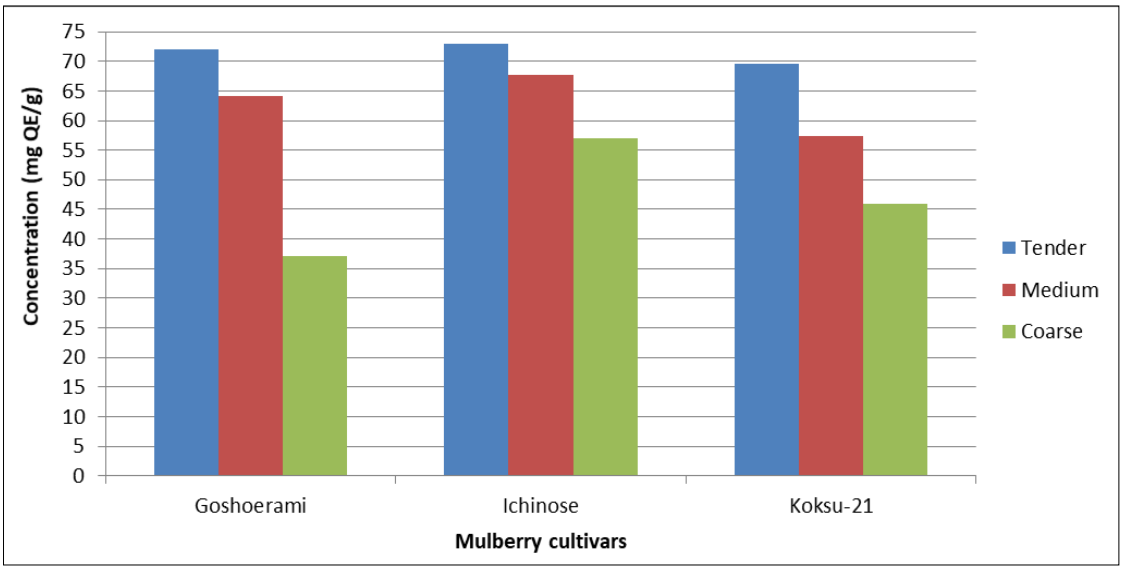

Fig 2: Graphical representation of total flavonoid content of mulberry leaf extracts

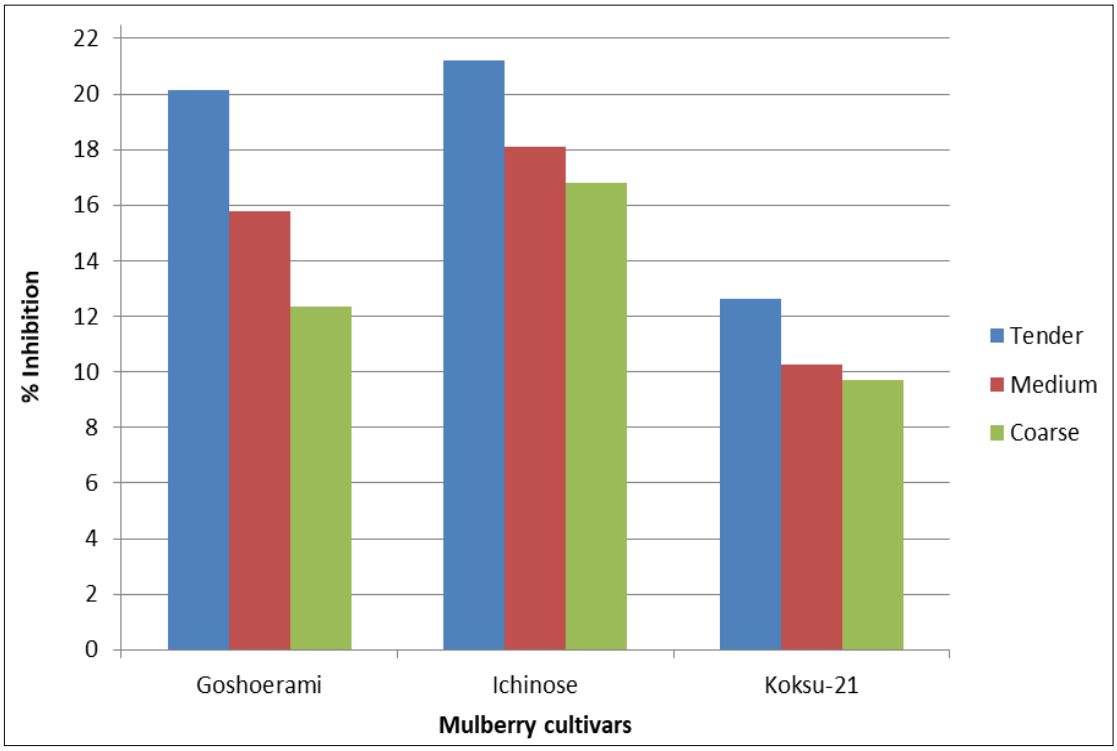

Fig 3: Graphical representation of DPPH radial scavenging activity of mulberry leaf extracts

Conclusion

There were significant variations in the TPC and TFC and antioxidant activity of leaves from three mulberry cultivars, highest being in tender textured leaves of Koksu-21 and Ichinose. Hence, tender mulberry leaves from the cultivars tested could apart from being used as food for silkworms, can be used a potential source of dietary antioxidants which can serve as bioactive agents in biomedical sector to prevent and cure various diseases. Further purification of these compounds will prove to be one of the important sources for pharmaceutical and nutraceuticals applications and would thereby add value to sericulture industry. Overall, the present study demonstrated the high phenolic composition and antioxidant potential of the leaves of three mulberry cultivars, can serve as raw material for preparation of antidiabetic tea infusion or by incorporating mulberry leaf extracts in antioxidant herbal formulations.

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