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Effect of inclusion of *Morus alba* (Mulberry) leaves at varying levels in concentrate mixtures on total phenols, non-tannin phenols, tannins content and DPPH radical scavenging activity

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Abstract
A study was conducted to evaluate the effect of inclusion of mulberry leaves at varying levels in concentrate mixture on total phenols and antioxidant activity. The antioxidant capacity of mulberry (MBL) leaves and their inclusion in the concentrate mixtures at varying levels were evaluated *in vitro*. The control concentrate mixture was prepared with maize grain, de-oiled rice bran, cotton seed cake and soybean meal as major ingredients. The other 7 iso-nitrogenous (20% CP) concentrate mixtures were prepared by partially replacing de-oiled rice bran and cotton seed cake with MBL at varying levels (5, 10, 15, 20, 25, 30 and 35%). The total phenolic content (TPC) gradually increased ($P<0.01$) from 3.87 to 7.68 mg of gallic acid equivalent (GAE) per g and from 4.13 to 11.06 mg of tannic acid equivalent (TAE) per g with inclusion of MBL leaves from 0 to 35% in concentrate mixtures. The non-tannin phenols (NTAP) and tannins (TA) increased ($P<0.01$) from 2.79 to 4.86 mg of TAE per g and 1.34 to 6.21 mg of tannins per g. Similarly, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (%) increased ($P<0.01$) from 0 to 30% (7.29 to 73.22) with inclusion of MBL leaves in concentrate mixtures and the activity at 35% (72.90) inclusion was comparable with that of 30% (73.22). The increase in TPC, NTAP, TA and DPPH activity in concentrate mixtures was due to higher content of these components in MBL leaves.

Keywords: Mulberry leaves, total phenolic content, tannins, non-tannin phenols, DPPH activity

Introduction
Free radicals produced during stress are unstable and cause cellular damage. Synthetic antioxidants like butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT) are used commercially to reduce oxidative stress in animals. Due to the potential adverse effects of synthetic antioxidants like BHA and BHT, whose inclusion in foods is currently limited due to their possible carcinogenicity, there has been an increase in interest in substituting natural ingredients for commercial antioxidants (Madhavi *et al.*, 1995) [14]. Natural antioxidants are also thought to be risk-free and safe for the environment, and they are crucial in reducing stress in animals. Antioxidant-rich plants and herbs are becoming more popular right now. Mulberry is one such plant that has considerable amounts of protein and antioxidants, which can be used as a substitute for other sources of protein in animal and poultry feed. Mulberry (*Morus alba*) leaves, belonging to Moraceous plant, is commonly used as the sole food source for silkworms. Mulberry for sericulture production, is practiced mainly in five states in India namely, Karnataka (42.24%), Andhra Pradesh (30.71%), West Bengal (11.67%), Tamil Nadu (8.99%) and Jammu and Kashmir (4.43%), which collectively account for about 98 per cent of the total mulberry silk production in the country (Kumar *et al.*, 2019) [12]. Mulberry leaf also has highly palatable and digestible (70–90%) macronutrients for herbivorous animals, especially for its relative high protein content (15–28%) and good essential amino acid profiles (Doran *et al.*, 2007) [6].
Moreover, mulberry leaf has antioxidant, antibacterial and immune enhancing properties due to its abundant bioactive phytochemicals, such as polysaccharides, flavonoids, phenolic acid and alkaloids (Butt *et al.*, 2008, Zhao *et al.*, 2015 and Lin *et al.*, 2017) [3, 18, 13]. Mulberry leaf can be used as a functional feed source or feed supplement in the diets of ruminants and monogastric animal not only because of their abundant resources and exceptional nutritional

value but also for their biological activity role (Zhao *et al.*, 2015 and Cai *et al.*, 2019) [18, 4]. Mulberry leaves also have radical scavenging activity (Arabshahi-Delouee and Urooj 2007 and Iqbal *et al.*, 2012) [2, 11] thus prevents damage at cellular level during stress conditions. Hence, in order to determine the total phenols, tannins and antioxidant activity in concentrate mixtures with increased inclusion of mulberry leaves at various levels, the current research was carried out.

Materials and Methods

The effect of inclusion of *Morus alba* (mulberry) leaves at 0, 5, 10, 15, 20, 25, 30 and 35% in the concentre mixtures were taken for *in vitro* antioxidant assay. The concentrate mixtures with various inclusion levels of mulberry leaves were prepared in triplicate for each diet. A basal concentrate mixture (control, CON) was formulated with maize, de-oiled rice bran, cotton seed cake and soybean meal as major ingredients. The remaining 7 concentrate mixtures were formulated with *Morus alba* leaves (MBL). The ingredient composition of the 8 concentrate mixtures containing *Morus alba* (Mulberry) leaves (MBL) at various inclusion levels is given in Table 1.

DPPH (2,2-diphenyl-1-picrylhydrazyl) Radical Scavenging Activity: The DPPH radical scavenging activity was determined according to the method described by Hossain and Shah (2015) with slight modifications. The samples (1 g) were extracted in 20 ml cold solution of 3% oxalic acid in 8% glacial acetic acid and centrifuged at 15000 rpm at 4ºC for 10 minutes and supernatant was collected and used for assay. The DPPH (Sigma Aldrich) solution (0.2 mM) was prepared using 95% ethanol. Samples were analyzed using a microtitre plate using blank, positive, negative and test samples. The blank, positive, negative and test samples consists of 25 µl of distilled water, L-ascorbic acid, di-methyl sulphoxide and extract from concentrate mixture along with 250 µl of DPPH (0.2 mM). The samples were incubated in dark for 30 minutes and absorbance was measured at 517 nm using ELISA reader - µQuant (BioTek instruments). L-ascorbic acid (17.6 mg in 100 ml distilled water) was used as positive control and di-methyl sulphoxide as negative control.

Absorbance of control = Postive control OD – Negative control OD

The scavenging activity was calculated using the following formula and expressed in percent of radical scavenging activity. The determination of DPPH radical scavenging activity was carried out in triplicate and results were averaged.

$$\text{DPPH Radical scavenging activity (\%)} = \frac{\text{(Abs. of control – Abs. of sample)}}{\text{Abs. of control}} \text{ X } 100$$

Total Phenolic Content – Gallic Acid Equivalent

The total phenolic content (TPC) was determined by the Folin–Ciocalteu method (Hossain and Shah, 2015) [10] with some modifications. About 200 mg of sample was treated with 10 ml diethyl ether containing 1% acetic acid, vortexed and centrifuged at 3000 rpm for 5 minutes to remove any pigments. The supernatant was carefully discarded. To the pellet, 10 ml of 70% aqueous acetone was added and incubated for 2 h at 30ºC using shaker. A standard curve was prepared using 0.00, 0.02, 0.04, 0.06, 0.08 and 0.10 ml of 0.1 mg/ml gallic acid stock solution and volume was made up to 0.5 ml with distilled water. Then transferred 0.5 ml of extracts to test tubes, later 0.25 ml of 1N Folin–Ciocalteu reagent was added to samples and standards followed by 1.25 ml of 20% sodium carbonate solution. The test tubes were vortexed and incubated at room temperature for 40 minutes and absorbance was recorded using spectrophotometer (UV-61 PCS, Metstar) at 650 nm. Total phenols as gallic acid equivalent (GAE mg/g) was calculated from calibration curve and expressed on DM basis.

Table 1: Ingredient composition (%) of concentrate mixtures containing varying levels of *Morus alba* leaves (MBL)

Ingredient	CON[2]	MBL5[3]	MBL10[3]	MBL15[3]	MBL20[3]	MBL25[3]	MBL30[3]	MBL35[3]
Maize	25.00	25.00	25.00	25.00	25.00	25.00	25.00	25.00
Soybean meal	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00
De-oiled rice bran	37.00	33.00	28.50	24.00	19.50	15.50	11.00	6.50
Cotton seed cake	21.00	20.00	19.50	19.00	18.50	17.50	17.00	16.50
Morus alba leaves	0.00	5.00	10.00	15.00	20.00	25.00	30.00	35.00
Mineral and vitamin mixture[1]	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Calcite powder	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80
Salt	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

[1]Mineral and vitamin mixture provided per kg diet: Calcium 2.5 g, Phosphorus 1.275 g, Magnesium 0.065 g, Iron 0.0175 g, Sulphur 0.092 g, Zinc 0.096 g, Copper 0.042g, Manganese 0.015 g, Potassium 1.5 mg, Sodium 0.2 mg, Iodine 3.5 mg, Cobalt 1.5 mg, Vitamin B_6 0.2 mg, Vitamin A 7500 IU, Vitamin D_3 750 IU, Vitamin E 3 mg, Niacinamide 0.012 g.
[2]Control diet
[3]*Morus alba* (Mulberry) leaves included at 5, 10, 15, 20, 25, 30 and 35% in the concentrate mixture partially replacing de-oiled rice bran and cotton seed cake.

Total Phenolic Content – Tannic Acid Equivalent

The total phenolic content (TPC) was determined by the Folin–Ciocalteu method (Makkar, 2003) [15] with some modifications. About 200 mg of samples were treated with 10 ml diethyl ether containing 1% acetic acid, vortexed and centrifuged at 3000 rpm for 5 minutes to remove any pigments. The supernatant was carefully discarded. To the pellet, 10 ml of 70% aqueous acetone was added and incubated for 2 h at 30ºC using shaker. A standard curve was prepared using 0.00, 0.02, 0.04, 0.06, 0.08 and 0.10 ml of 0.1 mg/ml tannic acid stock solution and volume was made up to 0.5 ml with distilled water. Then transferred 0.5 ml of extracts to test tubes, 0.25 ml of 1N Folin–Ciocalteu reagent was added to samples and standards followed by 1.25 ml of 20% sodium carbonate solution. The contents were vortexed and incubated at room temperature for 40 minutes and absorbance

was recorded using spectrophotometer (UV-61 PCS, Metstar) at 725 nm. Total phenols as tannic acid equivalent (TAE mg/g) was calculated form calibration curve and expressed on DM basis.

Non-tannin Phenolic Content and Tannins

The non-tannin phenols was determined by the Folin–Ciocalteu method (Makkar, 2003) [15] with some modifications. Polyvinyl polypyrolidone (PVPP) binds tannins present in extract. Weighed 100 mg of PVPP (100 mg of PVPP is sufficient to bind 2 mg of tannin phenols) and transferred to test tube, to this 1 ml distilled water and 1 ml of tannin extract was added. Standard curve (tannic acid) as mentioned earlier using tannic acid and extracts were treated similarly as that of total phenols estimation for tannic acid equivalent and expressed as non-tannin phenols.

Tannins in samples were estimated using the formula

Total tannins (mg of TAE/g) = (Total tannin phenols) – (non-tannin phenols)

The data obtained were subjected to statistical analysis using software (SPSS, Version 17). One way analysis of variance through generalized linear model was used to analyse all the results. The treatment means were ranked using Duncan's multiple range test with a significance at $P<0.05$ (Duncan, 1955) [7]. All the statistical procedures were done as per Snedecor and Cochran (1994) [17].

Results and Discussion

The total phenolic content (GAE and TAE mg/g), non-tannin phenols (mg TAE/g), and tannins (mg/g) differed significantly ($P<0.01$) among concentrate mixtures containing varying levels of mulberry leaves in concentrate mixtures (Table 2). The total phenolic content in terms of gallic acid equivalent (GAE) (mg/g GAE) was highest ($P<0.01$) in MBL30 (7.34) and MBL35 (7.68) and lowest was observed in CON (3.87) concentrate mixtures. The GAE in MBL25 (6.26), MBL20 (5.63), MBL15 (5.44), MBL10 (4.83) and MBL5 (4.11) concentrate mixtures was intermediate between CON and MBL35. The total phenolic content as GAE increased ($P<0.01$) gradually and linearly from MBL5 to MBL30, except for no difference between MBL15 and MBL20. No difference was observed between MBL30 and MBL35. Similarly, the total phenolic content in terms of tannic acid equivalent (TAE) (mg/g TAE) was highest ($P<0.01$) in MBL35 (11.06) and lowest was observed in CON (4.13) and MBL5 (4.62) concentrate mixtures. The TAE equivalent of total phenolics in remaining concentrate mixtures (MBL 10 to MBL30) was intermediate of above concentrate mixtures and were in the order of MBL30 (9.59) > MBL25 (7.15) ≥ MBL20 (6.79) ≥ MBL15 (6.41) > MBL10 (5.43) concentrate mixtures.

The non-tannin phenols and tannins also showed similar trend as that of total phenols in concentrate mixtures with varying levels of mulberry leaves inclusion. The results showed (Table 2) that the mulberry leaves inclusion had increased tannin content in concentrate mixtures. The non-tannin phenols (NTAP) (mg TAE/g) was highest ($P<0.01$) in MBL35 (4.86) and lowest was observed in MBL5 (1.08) concentrate mixtures (Table 2). The NTAP content with mulberry leaves inclusion decreased from 0 (CON) (2.79) to 5% (MBL5) (1.08), increased at 10% (MBL10) (2.74) and 15% MBL15 (2.97) and remained comparable to 0% (CON), later significantly increased with 20 (MBL20) (3.73) and 25% (MBL25) (3.97) and further increased at 30% MBL30 (4.32) and then at 35% MBL35 (4.86). No difference in NTAP was observed between CON, MBL10 and MBL15 and similarly between MBL20 and MBL25. The tannins (TA) (mg/g) was highest ($P<0.01$) in MBL35 (6.21) and lowest was observed in CON (1.34) concentrate mixtures. The TA in MBL30 (5.27), MBL25 (3.17), MBL20 (3.06), MBL15 (3.44), MBL10 (2.69) and MBL5 (3.54) concentrate mixtures was intermediate when compared with CON and MBL35. The tannin content though increased with mulberry leaves inclusion, it was comparable among 5-25% inclusion levels and increased significantly at 30% and further at 35% inclusion of mulberry leaves in concentrate mixtures.

The mulberry leaves contain phenols, tannins, rutin, quercetin, isoquercetin and other flavonoids which possess antioxidant activity (Arabshahi-Delouee and Urooj, 2007, Iqbal *et al.*, 2012, Andallu *et al.*, 2014, Simbaya *et al.*, 2020 and He *et al.*, 2020) [2, 11, 1, 16, 9]. The total phenolic content (GAE and TAE mg/g), non-tannin phenols (mg/g) and tannins (mg/g) in mulberry leaves were 15.55, 17.39, 6.39 and 11.01, respectively (Table 2). Iqbal *et al.* (2012) [11], reported 16.21 mg of GAE/g total phenolic content and He *et al.* (2020) [9] reported 11.49 to 30.03 mg of GAE/g total phenolic content in different varieties of mulberry leaves and was comparable with our results. Similarly, Arabshahi-Delouee and Urooj (2007) [2] reported 93.2 mg of GAE/g dry weight of mulberry leaf methanolic extract (12.35% yield). While, Andallu *et al.* (2014) [1] and Simbaya *et al.* (2020) [16] reported 28.8 mg of GAE/g and 27.2 mg of TAE/g of total phenolic content in mulberry leaves which was higher than our findings. Cheema *et al.* (2011) [5] reported 12.7 mg/g of tannin content on DM basis in mulberry leaves, comparable to our results. Further, Gonzalez *et al.* (2010) [8] also reported 20.4 and 27.5 mg/g of tannins in mulberry leaves on DM basis in two different mulberry varieties, which was higher than our findings. The total phenolic content in concentrate mixtures increased gradually with increase in inclusion of mulberry leaves due to higher total phenolic content in mulberry leaves compared to other feed ingredients in concentrate mixtures and corroborated with that of Arabshahi-Delouee and Urooj (2007) [2], Iqbal *et al.* (2012) [11], Andallu *et al.* (2014) [1], Simbaya *et al.* (2020) [16] and He *et al.* (2020) [9].

The DPPH assay has been widely used to evaluate the free radical scavenging ability of various plant extracts. The DPPH radical scavenging activity (%) was highest ($P<0.01$) in MBL30 (73.22) and MBL35 (72.90) and lowest was observed in CON (7.29) concentrate mixtures (Table 3). The DPPH activity (%) of MBL5, MBL10, MBL15, MBL20 and MBL25 was intermediate between CON and MBL30 and the activity was in the order of MBL25 (67.13) > MBL20 (64.03) > MBL15 (45.21) > MBL10 (37.21) > MBL5 (23.89) concentrate mixtures. In present study, the DPPH radical scavenging activity gradually increased ($P<0.01$) with increase in inclusion of mulberry leaves in concentrate mixture up to 30% inclusion and the activity between 30 and 35% inclusion levels was comparable, while DPPH radical scavenging activity (%) was 80.39 in the mulberry leaves. Similar to our results Arabshahi-Delouee and Urooj (2007) [2], Andallu *et al.* (2014) [1] and He *et al.* (2020) [9] reported concentration dependent increase in DPPH radical scavenging activity (%) with increasing concentration of mulberry leaf extract which was in agreement with present results. The high

DPPH activity in the concentrate mixtures with higher inclusion levels of mulberry could be due to increase in total phenols, non-tannins phenols and tannin content in these concentrate mixtures. The antioxidant plant activities of mulberry leaves and concentrate mixtures containing mulberry leaves at increasing levels correlate with presence of total polyphenols (Arabshahi-Delouee and Urooj, 2007, Iqbal *et al.*, 2012 and Andallu *et al.*, 2014) [2, 11, 1], flavonoids, ascorbic acid (Iqbal *et al.*, 2012) [11], total tannins (Gonzalez *et al.*, 2010 and Cheema *et al.*, 2011) [8, 5], rutin and quercetin (He *et al.*, 2020) [9] in these tree leaves. The variations observed with regard to the antioxidant activity of mulberry leaves with present and previous studies could be due to factors such as mulberry genotype, growing environment and sample extraction procedures.

Table 2: Effect of inclusion of *Morus alba* (mulberry) leaves at varying levels in concentrate mixture on total phenolic content (GAE and TAE), non-tannin phenols and tannins assessed *in vitro*

Diet	Total phenolic content (TPC) mg GAE / g	Total phenolic content (TPC) mg TAE / g	Non-tannin phenols (NTAP) mg TAE / g	Tannins mg / g
CON[1]	3.87^{e} ±0.01	4.13^{f} ±0.03	2.79^{d} ±0.03	1.34^{e} ±0.06
MBL5[2]	4.11^{e} ±0.04	4.62^{f} ±0.21	1.08^{e} ±0.02	3.54^{c} ±0.19
MBL10[2]	4.83^{d} ±0.08	5.43^{e} ±0.04	2.74^{d} ±0.05	2.69^{d} ±0.09
MBL15[2]	5.44^{c} ±0.16	6.41^{d} ±0.07	2.97^{d} ±0.09	3.44^{c} ±0.11
MBL20[2]	5.63^{c} ±0.13	6.79^{cd} ±0.18	3.73^{c} ±0.02	3.06^{cd} ±0.17
MBL25[2]	6.26^{b} ±0.05	7.15^{c} ±0.07	3.97^{c} ±0.09	3.17^{cd} ±0.04
MBL30[2]	7.34^{a} ±0.13	9.59^{b} ±0.19	4.32^{b} ±0.06	5.27^{b} ±0.14
MBL35[2]	7.68^{a} ±0.21	11.06^{a} ±0.51	4.86^{a} ±0.22	6.21^{a} ±0.30
SEM	0.274	0.470	0.231	0.299
P value	0.0001	0.0001	0.0001	0.0001
MBL[3]	15.55 ± 0.68	17.39 ± 0.30	6.39 ± 0.16	11.01 ± 0.45

Each value is an average of three observations
[abcd]Means with different superscripts in a column differ significantly: P≤0.01
GAE- Gallic acid equivalent, TAE- Tannic acid equivalent
[1]Control diet (0% mulberry leaves)
[2]*Morus alba* (mulberry) leaves included at 5, 10, 15, 20, 25, 30 and 35% in the concentrate mixture partially replacing de-oiled rice bran and cotton seed cake.
[3]*Morus alba* (mulberry) leaves
SEM: Standard Error Mean; P value: Probability value.

Table 3: Effect of inclusion of *Morus alba* (mulberry) leaves at varying levels in concentrate mixture on DPPH radical scavenging activity assessed *in vitro*

Diet	DPPH radical scavenging activity (%)
CON[1]	7.29^{g} ±0.71
MBL5[2]	23.89^{f} ±0.10
MBL10[2]	37.21^{e} ±0.66
MBL15[2]	45.21^{d} ±0.20
MBL20[2]	64.03^{c} ±1.06
MBL25[2]	67.13^{b} ±0.25
MBL30[2]	73.22^{a} ±0.12
MBL35[2]	72.90^{a} ±0.65
SEM	4.801
P value	0.0001
MBL[3]	80.39 ±3.25

Each value is an average of three observations
[abcd]Means with different superscripts in a column differ significantly: P≤0.01
DPPH- 2,2-diphenyl-1-picrylhydrazyl
[1]Control diet (0% mulberry leaves)
[2]*Morus alba* (mulberry) leaves included at 5, 10, 15, 20, 25, 30 and 35% in the concentrate mixture partially replacing de-oiled rice bran and cotton seed cake.
[3]*Morus alba* (mulberry) leaves
SEM: Standard Error Mean; P value: Probability value.

Conclusions

Mulberry leaves are included in concentrate mixtures at varying amounts, and *in vitro* testing of these levels for their impact on total phenols, non-tannin phenols, and tannin phenols indicates that these contents rise with higher mulberry leaf inclusion. Similar to this, as mulberry leaves were added to concentrate mixtures at higher rates, the DPPH radical scavenging activity (%) also increased with increase in inclusion levels. Mulberry leaves can assist improve the antioxidant status of feed when designing animal rations by having higher amounts of TPC, NTPC, tannins, and DPPH radical scavenging activity (%). This can also help with stress management. Mulberry leaves are a potential replacement for the traditional feed ingredients in the animal feeding system and can reduce the cost of feeding livestock.

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