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Amelioration of commercial traits in *Bombyx mori* by *Emblica officinalis* (Amla) *fruit*-based ascorbate fortification

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Abstract
A popular bivoltine silkworm hybrid (CSR2 x CSR4) larvae were orally fed with vitamin C-quantified crude methanolic extract of *Emblica officinalis* Gaertn fruit in six different doses during 5th instar at seven different application times. The dose and time-dependent effect on economic traits were observed. The best and repeatable results were recorded in 0.05% (C1) dose fed at 0 h of 5th instar once (T0) and 0 h to spinning daily once (T1) in larval weight (12.97-15.64%), pupation rate (8.50-11.24%), cocoon wt. (14.07-15.02%) and shell weight (15.11-16.55%) and there was no significant difference between the two application times (T0 & T1).

Keywords: *Emblica officinalis*, Vitamin C, *Bombyx mori* L

1. Introduction
Vitamin C (VC), also known as ascorbic acid or ascorbate is an essential nutrient involved in major biochemical processes in both plants and animals (Arrigoni and Tullio, 2002) [2]. The VC is regarded as indispensable for the growth and development of *Bombyx mori* and the latter is unable to synthesize it *de novo* (Ito and Arai 1965) [10]. In fact, VC is present in mulberry leaves (Kanafi *et al*., 2007) [13], the exclusive food for the silkworm, but gets diminished at a rate of at least 20% in 24h by preservation, even if it is protected from direct sunlight (Cappellozza *et al*., 2005) [5]. VC supplementation to mulberry leaves to improve economic traits in silkworm has been more exploited than any other vitamin (Etebari *et al*., 2004, El-Karaksy and Idriss, 1990 and Hussain and Javed, 2002) [7, 6, 9].

Emblica officinalis fruit, commonly known as Indian Gooseberry, is the richest source of VC (Jain and Khurdiya, 2004) [12]. The fruit is of great importance in Asiatic medicine, not only for its antiscorbutic actions but for its immunomodulatory, anti-inflammatory, antiulcer, hepatoprotective and anticancer properties (Tasduq *et al*., 2005 a, b) [18, 19]. Research findings show that amla is 12 times more assimilable than synthetic VC. Just 8.7mg of natural VC complex from amla is equivalent to 100 mg of the most commonly used synthetic vitamin C (Arora, 1985) [3]. The natural ascorbate present in the fruit is synergistically enhanced by the bioflavonoids and polyphenols and protected by shield of tannins from being destroyed by heat (Bajaj, 2006, Anonymous, 2001) [4, 1]. A repeated laboratory tests showed that every 100g of fresh fruit of amla provides 470 - 680mg of VC. The vitamin value of amla increased further when the juice was extracted from the fruit. The dehydrated fruit provided 2428 - 3470mg of vitamin C per 100g (Thakur, *et al.,* 1989) [20].

As per the literature survey, no report is available regarding the effect of vitamin C-quantified amla fruit extracts on silkworm, *Bombyx mori*. Therefore, the present study was conducted to explore the effect of amla-based crude extracts containing vitamin C in different doses, fed at varied application time schedules on economic traits of the silkworm, *Bombyx mori*.

2. Materials and methods
2.1 Extraction of vitamin C
Fruit pulp of amla was dried in hot air oven at 37 °C overnight and coarsely powdered. Non-polar solvent fractions were removed by centrifugation with chloroform (1:3 W/V) and ether (1:3 W/V) in succession and the resultant pallet was dried at room temperature and used for extraction in methanol using soxhlet apparatus. The methanol extract was filtered and evaporated in rotary evaporator (IKA WERKE GMBH& CO. KG. RV 06 ML, Staufen, Germany).

2.2 Estimation of vitamin C
Standard VC was procured from ICN, Biomedicals, Inc., USA. Concentration of VC in amla extract was estimated by Dinitrophenyle hydrazine (DNPH) method (Raghuramulu *et al.* 2003) [16].

2.3 Experimental insect
A popular hybrid of the silkworm, *Bombyx mori*, CSR2 x CSR4 was used for the present investigation. Rearing was conducted as per standard method (Rajan and Himantharaj, 2005) [17].

2.4 Treatment
Bombyx mori larvae were reared up to 4th moult on normal leaf (without treatment). Freshly ecdysed 5th instar larvae were divided into 43 groups including control with each group consisting of 3 replications with 250 larvae. Quantified extract (26.24mg/g by dry weight basis) was diluted with distilled water to obtain six different doses of vitamin C *viz*., 0.10% (C1), 0.15% (C2), 0.20% (C3), 0.25% (C4), 0.30% (C5) and 0.35% (C6) and sprayed to mulberry leaves @ 60 ml/ 200g for 100 larvae. The leaves were shade dried for 15 minutes and fed to 5th instar larvae at 7 different time schedules *viz.*, treatment given only once at 0h of the fifth instar (T0) and treatments given daily

once from 0h to the onset of spinning activity (T1), 24 to the onset of spinning activity daily once (T2), 48 h to the onset of spinning activity daily once (T3), 72 h to the onset of spinning activity daily once (T4), 96 to the onset of spinning activity daily once (T5) and 120h to the onset of spinning activity daily once (T6). The above six doses (C1-C6) were taken after screening the wide range of amla-based vitamin C doses on silkworm (unpublished). Data were recorded for pupation rate, larval weight, single cocoon weight and single shell weight. Data were subjected to the Analysis of Variance (ANOVA) test to determine the significant difference between the treated and control groups.

3. Results and Discussion

The data on the effect of methanolic extract in 6 short-listed doses treated, at 7 varied application time regimes, on economic traits of a bivoltine silkworm hybrid, CSR2 x CSR4, are presented in Tables, 1-3.

3.1 Larval weight

Larval weight of CSR2 x CSR4 hybrid significantly improved only at two application times *viz*., one when treatment was given at zero hour of 5th instar only once (T0) and other when treatment was given daily once from zero hour of 5th instar till the onset of spinning (T1). Both the treatments included zero hour application. It was interesting to note that larval groups which received 0.05% (C1), 0.1% (C2) and 0.15% (C3) doses at zero hour once (T0) registered significant improvement in larval weight to the extent of 13.87, 8.64 & 3.75% respectively as compared to control group. Among the three doses, 0.50% (C1) was statistically more effective. Rest of the doses at the given application time did not produce any significant improvement over the control. 0.05% (C1), 0.10% (C2), 0.15% (C3) and 0.20% (C4), when supplemented to silkworm feed daily once from zero hour of 5th instar till spinning (T1) brought about significant improvement by 16.97, 12.79, 8.74 and 6.32% in larval weight compared to control. At this application time again 0.5% (C1) was statistically more significant than the rest of the three doses. Other doses in the T1 group did not show any significant improvement. Seemingly, the improvements produced at T1 application time appeared higher than that of the T0, but statistically there was no significant difference between the two application times. Other application times *viz*., T2, T3, T4, T5 & T6, did not produce significant improvement in any of the dose.

3.2 Pupation rate

0.05% (C1) and 0.10% (C2) doses at zero hour once (T0) registered significant improvement in pupation rate to the tone of 8.88 and 5.11% respectively and the improvement of 11.91, 9.89 and 6.76 % was shown by the 0.05% (C1), 0.10% (C2) and 0.15% (C3) doses when applied daily once (T1). 0.15% (C3) and 0.20 (C4) had no effect on pupation rate. Between the 0.05% (C1) and 0.10% (C2) doses at both T0 and T1 no statistically significant difference was observed. Other doses at both the application times did not produce any significant improvement over the control. Other application times *viz*., T2, T3, T4, T5 & T6, did not produce significant improvement in any of the dose.

3.3 Cocoon weight

As obvious from the Table 31 that 0.05% (C1), 0.10% (C2) and 0.15% (C3) VC doses were capable of bringing out significant improvement in cocoon at only two application time schedules, T0 and T1. Rest of the application time regimes were not able to produce any improvement in all the doses. Cocoon weight was improved to the extent of 15.10, 9.12 and 3.51% in T0 group and in T1 group, 16.23, 11.03 and 8.04 was observed with the same doses. However, 0.20% (C4) in T1 group also showed significant improvement of 5.06% as compared to control. 0.05% (C1) dose at both the application time regimes was statistically more significant than rest of the significant doses. Further, the two significant application times (T0 & T1) at given doses did not differ statistically with regard to cocoon weight, though effect of T1 on improvement seemed more compared to T0. Rest of the application times, T2, T3, T4, T5 & T6, did not produce significant improvement in any of the dose.

3.4 Shell weight

Shell weight improved by 16.25, 9.80 and 3.64% with 0.05% (C1), 0.10% (C2) and 0.15% (C3) in T0 and by 17.95, 12.10, 9.07 & 6.52% in T1 with the 0.05% (C1), 0.10% (C2), 0.15% (C3) and 0.20% (C4) doses. 0.050% (C1) dose was found most effective and statistically significant to improve cocoon shell weight at both the application times (T0 & T1) compared to other doses. Two significant application times (T0 & T1) at given doses did not differ statistically with regard cocoon shell weight, though effect of T1 on improvement seemed more compared to T0. Rest of the application times, T2, T3, T4, T5 & T6, did not produce significant improvement in any of the dose.

3.5 Shell percentage

None of the doses and application time regimes could induce significant change in the shell % and the same remained at parity with that of the control.

Table 1: Effect of *E.officinalis fruit*-methanolic extract containing VC on larval and cocoon traits of CSR2 x CSR4 hybrid.

Treat.	L. wt. (g)	P.R. (%)	C. wt. (g)	S. wt. (g)	Shell %
T0 C1	50.972*	89.89*	2.099*	0.477*	22.71
C2	48.632*	86.78*	1.990*	0.450*	22.63
C3	46.442*	85.56	1.887*	0.425*	22.52
C4	45.455	83.83	1.840	0.413	22.45
C5	44.946	82.93	1.828	0.413	22.58
C6	44.904	82.66	1.824	0.412	22.58
T1 C1	52.359*	92.39*	2.119*	0.484*	22.82
C2	50.490*	90.73*	2.024*	0.460*	22.71
C3	48.675*	88.14*	1.970*	0.447*	22.70
C4	47.591*	85.29	1.916*	0.437*	22.80
C5	45.760	84.59	1.838	0.416	22.63
C6	45.426	83.90	1.824	0.414	22.72

T2 C1	46.062	85.23	1.839	0.419	22.77
C2	45.247	84.05	1.845	0.419	22.72
C3	45.196	83.62	1.844	0.418	22.67
C4	45.146	83.57	1.839	0.414	22.58
C5	45.131	83.27	1.832	0.414	22.59
C6	45.080	83.22	1.830	0.414	22.60
T3 C1	45.550	83.34	1.824	0.414	22.71
C2	45.500	83.29	1.824	0.413	22.66
C3	45.115	83.24	1.823	0.413	22.64
C4	44.825	82.61	1.820	0.412	22.75
C5	44.810	82.56	1.817	0.412	22.67
C6	44.760	82.51	1.812	0.410	22.63
T4 C1	45.025	82.82	1.819	0.411	22.62
C2	44.838	82.74	1.820	0.410	22.55
C3	44.816	82.91	1.821	0.410	22.49
C4	44.765	82.86	1.819	0.412	22.66
C5	44.750	82.81	1.815	0.410	22.57
C6	44.700	82.76	1.810	0.408	22.53
T5 C1	45.546	83.80	1.832	0.416	22.70
C2	45.195	83.36	1.831	0.414	22.58
C3	44.917	83.26	1.829	0.413	22.56
C4	44.912	83.02	1.826	0.411	22.49
C5	44.897	82.88	1.821	0.408	22.43
C6	44.847	82.83	1.817	0.410	22.59
T6 C1	45.140	83.45	1.829	0.415	22.67
C2	44.761	83.01	1.828	0.412	22.55
C3	45.193	82.91	1.827	0.411	22.51
C4	44.756	82.66	1.827	0.409	22.41
C5	44.741	82.53	1.822	0.408	22.40
C6	44.691	82.48	1.818	0.410	22.56
Control	44.764	82.56	1.823	0.410	22.49
SE±	0.505	1.25	0.008	0.003	0.18
CD 5%	1.417	3.50	0.021	0.007	0.50

C1: 0.05%, C2: 0.10%, C3: 0.15%, C4: 0.20%, C5: 0.25% and C6; 0.30% VC dose; T0: Treatment given only once at zero hour (0 hr) of the fifth instar, T1: daily once from 0 hr-spinning, T2: daily once from 24 hr –spinning, T3: daily once from 48 hr – spinning, T4: daily once from 72 hr –spinning, T5: daily once from 96 hr –spinning, T6: daily once from 120 hr- spinning, *: significant at 5 % level

To know the individual effect of VC doses and that of application time, irrespective of each other, data were statistically analyzed which produced the data presented in Tables 02 and 03 respectively. Perusal with the table 02, it could be noted that 0.05% and 0.10% doses significantly improved larval weight (47.200 & 46.380g), pupation rate (85.84 & 85.14%), cocoon weight (1.907 & 1.881g) and cocoon shell weight (0.433 & 0.425g), irrespective of application time. 0.15 (C3) dose could also improve the pupation rate and cocoon shell weight significantly compared to control 44.764g, 82.56%, 1.820g & 0.410g). At the same time it could be noted that the improvements registered by 0.05% dose are statistically more significant than that of the 0.10% dose. 5th instar larval duration was at par with control.

Table 2: Effect of *E.officinalis fruit*-based VC dose irrespective of application time on larval and cocoon traits of CSR2 x CSR4 hybrid.

Treat.	L. wt. (g)	P.R. (%)	C. wt. (g)	S. wt. (g)	Shell %
C1	47.200*	85.84*	1.907*	0.433*	22.69
C2	46.380*	85.14*	1.881*	0.425*	22.62
C3	45.711	84.24*	1.857	0.419*	22.58
C4	45.350	83.46	1.842	0.416	22.60
C5	45.005	83.08	1.825	0.411	22.55
C6	44.915	82.91	1.819	0.411	22.60
Control	44.764	82.56	1.820	0.410	22.53
SE±	0.360	0.58	0.014	0.003	0.066
CD 5%	0.999	1.62	0.037	0.009	0.183

Table 03, reveals the data on the effect of 7 application time schedules on larval and cocoon traits regardless of VC dose. It is clear that larval weight (47.849 & 48.384g), pupation rate (85.97 & 87.51%), cocoon weight (1.918 & 1.949g), cocoon shell weight (0.436 & 0.443g) and cocoon shell % (22.70 & 22.73%) of CSR2 x CSR4 hybrid has improved significantly only at two application times *viz*., T0 and T1 as compared to control (44.764g, 82.56%, 1.820g & 0.410g). Rest of the application time regimes were not significant. Further, it was strictly noted that among the improvements produced by two application times there is no statistically significant differentiation.

Table 3: Effect of application time irrespective of plant-based VC dose on larval and cocoon traits of CSR2 x CSR4 hybrid.

Treat.	L. wt. (g)	P.R. (%)	C. wt. (g)	S. wt. (g)	Shell %
T0	47.849*	85.97*	1.918*	0.436*	22.70*
T1	48.384*	87.51*	1.949*	0.443*	22.73*
T2	45.310	83.83	1.842	0.417	22.64
T3	45.030	82.93	1.820	0.413	22.68
T4	44.816	82.82	1.817	0.410	22.57
T5	45.052	83.19	1.826	0.412	22.56
T6	44.880	82.84	1.825	0.411	22.52
Control	44.764	82.56	1.823	0.410	22.49
SE±	0.330	0.567	0.012	0.003	0.073
CD 5%	0.914	1.570	0.034	0.008	0.203

In the present study improvement was recorded at considerably lower doses compared to the doses of synthetic ascorbic acid used by earlier workers. Improvement in economic traits was observed at 2-3% synthetic ascorbic acid doses by EI-Karaksy and Idriss (1990) [6] and Prasad, (2004) [15] which if compared to the effective dose of the present study (0.05%) is almost 9 times higher. The present investigation confirms the fact of "enhanced efficacy" of the amla based vitamin C compared to synthetic vitamin C on silkworm. It can be assumed that the improvement in silkworm at considerably lower doses could possibly be due to synergetic activity of VC with other compounds in the vitamin C-complex which increase its bioavailability and efficacy. Vitamin C extracted from amla has been reported effective at 12 times lower doses when compared to synthetic VC in higher animals (Arora, 1985) [3]. Bioflavonoids often found in vitamin C-rich fruits, especially citrus fruits have been reported to increase the bioavailability or efficacy of vitamin C (Johnston and Luo, 1994) [11]. Khopde *et al*., (2001) [14] demonstrated in their experiment on rats that the natural formulations of amla extract containing vitamin C show much superior antioxidant activity compared to their equivalent amounts in pure isolated form. Natural and synthetic L-ascorbic acids are chemically identical and there are no known differences in their biological activities or bioavailability (Gregory, 1993) [8].

The present study reveals that the 0h application of amla-based crude extract is effective to bring a marked change in silkworm. It is interesting to note that 0h treatment with 0.05% of amla based vitamin C alone or included in other treatment as in the treatment with 0.05% given daily once from 0h till spinning of 5th instar could produce significant improvements in silkworm as compared to control. Although, maximum improvement seemed to be recorded when treatment was given daily once from 0h of 5th instar till spinning (T1C1), statistically the difference was not significant compared to T0C1 (applied only once). In both the treatments 0h is common. 0 hour is a crucial time when larvae, after the 4th moult with empty stomach, are fed with amla based vitamin C supplemented mulberry leaf. At this point of time the treatment works more efficiently by boosting the larval weight which subsequently results in improvement in economic traits of silkworm. In the present study it is established that 0.05% VC dose of *Emblica officinalis* fruit pulp extracted in methanol given only once at 0h of 5th instar is economically viable and can be recommended for the improvement of economic traits in silkworm, *Bombyx mori*.

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