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# Influence of Treating the mulberry leaves with aqueous maceratives of seed powder of *Syzigium cumini* (L) on the activities of digestive enzyme in the fifth instar larvae of silkworm, *Bombyx mori* (L) (Race: PM x CSR2)

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**ABSTRACT**

Three different concentrations (5.0 ppm; 10.0 ppm; 20.0 ppm & 50.0 ppm) of the aqueous solution of seed powder of *Syzigium cumini* (L) were used to treat the leaves of mulberry & fed to the fifth instar larvae of polyvoltine, crossbreed silkworm, *Bombyx mori* (L) (Race : PM x CSR2 ) for first three days; second day & third day; third day (only). The larvae fed with untreated & water treated leaves were also maintained. Bioassays of proteins (S.P. & T.P.) & enzymes (protease & amylase) were carried out on fifth day through the use of mid gut homogenate. Treating the mulberry leaves with herbal preparations (*S. cumini* ) & feeding them to fifth instar larvae was found reflected into significant improvement in the levels of proteins (S.P. & T.P.) & velocities of biochemical reactions catalyzed by protease & amylase. The pattern of increase in soluble proteins & total proteins in the mid gut tissue were 32.147 to 90.074 percent & 5.657 to 39.052 percent respectively. The activities of mid gut protease & amylase were increased by 21.444 to 83.706 percent and 14.54 to 52.257 percent respectively. The nutrient contents of seed powder of *Syzigium cumini* (L) serve to improve the digestibility & exert the influence of efficient metabolism in the fifth instar larvae of silkworm, *Bombyx mori* (L).The herbal formulation like Syzigium seed powder may gear overall biochemical constituency of silkworm larvae, through the significant improvement in the velocity of mid gut enzyme catalyzed biochemical reactions.



## Introduction

Silkworm, *Bombyx mori* (L) is economically important insect, being a primary producer of silk. A silkworm's preferred food is white mulberry leaves (monophagous). Domestic silk moths are closely dependent on humans for reproduction, as a result of millennia of selective breeding. Wild silk moths are different (having not been selectively bred) to their domestic cousins; they are not commercially viable in the production of silk. Life of insect herbivores is in the orchestrate progression, which closely interlinked with plant metabolites. The biochemical constituents of plants could have been the factors of growth & metamorphosis for insects (Bowers *et al*,1966).The phytophagous insects are able to avoid poor quality food or able to select a high quanlitative food from variety available to them. The silkworm, *Bombyx mori* (L) is a monophagous insect, feeding exclusively on the leaves of mulberry *Morus alba* (L). It is therefore, essential to improve either food quality or appetite ( or both) of larval instars of silkworm for better performance in silk production. The factors responsible to influence the growth, development & subsequent physiology of insect body include: nutritional qualities of food, biochemical status of nutrients in the food, hormonal level in the body & environmental conditions (Murugan & Georgr, 1992).Elements of the insect body are primarily derived from the food source. For silkworm, the leaves of mulberry contain many stimulants ( Ito, 1960,1961; Nayar & Fraenkel, 1962; Ito, *et al*, 1964; Ito & Hyashiya, 1965). Nutrition quality in silkworm, *Bombyx mori* (L) serve to accelerate the growth, metamorphosis & forms the physiological foundation for sericulture. The leaves of mulberry are the sole source of food for larval instars of silkworm, *Bombyx mori* (L), biochemically constituted with proteins, lipids, carbohydrates (Murali, 1992) & minerals (Subramanyam Reddy, 1992). Therefore, corresponding diversity of enzymes capable of hydrolyzing the biocompounds of mulberry is exhibited by mid gut of larval instars of silkworm, *Bombyx mori* (L). The body tissues of larval instars of silkworm, *Bombyx mori* (L) especially, the fat bodies accumulates large quantity of proteins, lipids & glycogen during the development, which is nothing but the reflection of efficient consumption & utilization of nutrient biocompounds of mulberry leaves. The variation in the food consumption in phytophagous insects may be for varied biochemical processes, ultimately for successful adaptations ( Slansky, 1982). It has been suggested that, there is a functional difference between the activity of digestion by the digestive fluid in mid gut & tissue of mid gut. It has been reported by Horie, *et al* (1963) that, molecular proteins are hydrolyzed into peptides by digestive fluid content & into aminoacids with peptidases in the mid gut tissue. Likewise, the polysaccharides, are digested in the insect gut lumen by digestive fluid & disaccharides and/or trisaccharides get hydrolysed into their constituent monasaccharide sugars mainly in the gut tissue (Horie, 1967). Lipase, the lipid digesting enzyme of the insect mid gut has been reported to have analogy with pancreatic lipase of vertebrates ( Yamafugi & Yonezawa, 1935). The efforts towards the qualitative silk production through the improvement in the efficiency of consumption & utilization of food by larval instars

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of silkworm, *Bombyx mori* (L) include: improvement in the quality of mulberry leaves & supplementation of nutrient biocompounds like soya protein; potassium iodide, copper sulphate, other mineral salts, herbal products ( or drugs) like digoxin (Vitthalrao & Kulkarni, 2011) & kho-go ( Desai, *et al*, 2011). *Syzygium cumini* (L) jambul, jambolan, jamblang, or jamun, is an evergreen tropical tree in the flowering plant family Myrtaceae. *Syzygium cumini* (L) is native to Bangladesh, India, Nepal, Pakistan, Sri Lanka, Malaysia, the Philippines, and Indonesia. The name of the fruit is sometimes mistranslated as blackberry, which is a different fruit in an unrelated family. The tree was introduced to Florida, USA in 1911 by the USDA, and is also now commonly grown in Suriname and Trinidad and Tobago. In Brazil, where it was introduced from India during Portuguese colonization, it has dispersed spontaneously in the wild in some places, as its fruits are eagerly sought by various native birds such as thrushes, tanagers and the Great Kiskadee. This species is considered an invasive in Hawaii, USA. *Syzygium cumini* L. (Bengali name - Jam; Family - *Myrtaceae*) is a large evergreen tree, reaching approximately 30 m in height, and is found throughout the Indian subcontinent.1 *S. cumini* is a medicinal plant, various parts of which have been pharmacologically proven to possess hypoglycaemic, antibacterial and anti-HIV activities. The bark of the tree is employed in folk medicine for treatment of inflammation. flavonol glycosides have been isolated from the roots of this plant. Acylated flavonol glycosides, including mearnsetin (3-*O*-(4″-*O*-acetyl)- α-L-rhamnopyranoside) and myricetin (3-*O*-(4″-*O*-acetyl-2″-*O*-galloyl)-α-Lrhamnopyranoside), have been isolated from the leaves of *S. cumini*.5 The seeds of the tree have also been reported as a rich source of polyphenols, gallic acid and ellagic acid derivatives, corillagin and related ellagitannins, 3,6- hexahydroxydiphenoyl-glucose, 4,6-hexahydroxydiphenoyl-glucose, 1-galloyl glucose, 3-galloyl glucose and quercetin. There are no reports on treating the mulberry leaves with aqueous solution of seed powder of *Syzigium cumini* (L), herbal formulation and feeding the larval instars of silkworm, *Bombyx mori* (L) for the midgut enzyme activities. For the purpose to screen correct dosage for treating the mulberry leaves & appropriate time of feeding mulberry leaves treated with aqueous solution of powder of seeds of *Syzigium cumini* (L), herbal formulation, the present study has been planned.

**MATERIAL & METHODS**

The experimentation was carried in the laboratory of K. G. Kataria College, Daund. The disease free layings (DFL) of multivoltine, crossbreed race: PM x $CSR_2$ of silkworm, *Bombyx mori* (L) were procured through the sericulture unit of Agriculture Development Trust, Malegaon (Baramati). They were processed for incubation (through black boxing); transfer of hatched larvae on the rearing bed of mulberry leaves and reared through the methods prescribed by Krishnaswami, *et al* (1978) ; explained by Khyade (2004) with modifications suggested by Sharad G. Jagtap (2014). Soon after the fourth moult, the fifth instar larvae were divided into various groups like untreated control(1), water treated control(3) & the treated (12), each with 100 individuals. Four feedings were followed (6 am, 11 am, 4 pm & 10 pm).100 grams of fresh mulberry leaves were used to feed the group of 100 larvae, for each feeding. The seed powder of *Syzigium cumini* (L) was procured from local herbal store. The Known quantity of seed powder was mixed in known volume of distilled water to prepare aqueous solution of desired strength. The stock solutions of seed powder were of strength: 05 ppm; 10 ppm; 20 ppm & 50 ppm. The stock solutions were prepared freshly before the feeding. 400 ml of aqueous solution was used to soak 100 grams of fresh mulberry leaves. The soaking was carried out for half an hour before feeding. The soaked/ treated mulberry leaves were drained off completely & then fed to the fifth instar larvae of silkworm, *Bombyx mori* (L). Hundred grams of mulberry leaves were utilized for feeding each time, for the group of hundred larvae. The untreated control group of larvae were supplied with untreated leaves of mulberry. Water treated group of larvae were supplied with water treated leaves of mulberry. For each concentration (ppm), three groups of larvae were made, out of which the first group was fed with treated leaves of mulberry for the first three days; second group for second day & third day. Third group of larvae for each concentration was supplied with treated leaves for only third day (Table-1). For remaining days, the larvae were fed with untreated leaves of mulberry. The bioassay of activity of mid gut protease & amylase was carried out on fifth day of fifth instar. Twenty larvae from each group were selected randomly; anaesthetized with chloroform soaked cotton pads & dissected for mid gut in chilled saline ( 0.9 percent NaCl). The larvae were opened from dorsal side; the entire alimentary canal was removed from each larva; flushed with ice cold saline so as to remove the debris of mulberry leaf & washed with ice cold saline. The mid gut from alimentary canal was separated; washed with saline; blotted & weighed accurately on electronic balance. The mid gut tissue was fragmented & then homogenized in chilled saline. Homogenate was centrifuged at $40^0$C for 15 min. at 10000 rpm. The supernatant was equalized to the volume, aliquots of which contain 10 mg per ml & used as assay sample. Half the volume of assay sample was utilized for bioassay of soluble proteins & another half for mid gut enzymes (protease & amylase).

Bioassay of soluble proteins was carried out through the methods of Lowery, *et al*, (1951). For each assay sample ( of each group), bioassay was carried in the triplicate set. One ml of assay sample was added in each test tube. The blank test tube was also prepared simultaneously, in which the assay sample was replaced with distilled water. Addition of 5 ml Lowery's "C" solution was made in each test tube, mixed well & kept for 15 minutes for the purpose to form the copper-protein complex. After fifteen minutes; 0.5 ml Folin's phenol reagent was added in each test tube & mixed well. The content in each test tube was allowed to develop colour. Then the optical density of content of each test tube was recorded at 660 nm on spectrophotometer. The concentration of soluble proteins of each assay sample was calculated through the reference of optical density assay sample & standard proteins (BSA) (the plot of optical density against conc. of BSA). The experimentations were repeated for thrice to obtain consistent results.

The content of soluble proteins in each assay sample was expressed in the unit as microgram protein per mg tissue.

For the purpose to determine total protein contents of tissue, another set of twenty larvae was selected randomly from each group. They were anaesthetized with chloroform soaked cotton pads & dissected for mid gut tissue.

The mid gut tissue was homogenized in chilled distilled water by using clean & sterilized morter & pestle in one normal (1.0 N) solution of sodium hydroxide & kept at $37^0$C for 24 hours. Then it was precipitated with equal volume of ten percent solution of TCA & centrifuged at 10000 rpm for 10 minutes. The precipitate was dissolved in 1.0 NaOH & used as assay sample for total proteins. Further methods of determination of contents of total proteins are similar as described for soluble proteins.

**Table 1. Schedule of treating the mulberry leaves with aqueous solution of seed powder of *Syzigium cumuni* (L) & feeding to the fifth instar larvae of silkworm, *Bombyx mori* (L) (Race: PM x $CSR_2$)**

| Group | Day for feeding → / concentration of Syzigium (ppm) ↓ | 1 | 2 | 3 |
|---|---|---|---|---|
| 0-0 | Untreated control | - | - | - |
| 0-I | Water treated control | ✓ | ✓ | ✓ |
| 0-II | Water treated control | - | ✓ | ✓ |
| 0-III | Water treated control | - | - | ✓ |
| A-1 | 5 ppm | + | + | + |
| A-2 | 5 ppm | - | + | + |
| A-3 | 5 ppm | - | - | + |
| B-1 | 10 ppm | + | + | + |
| B-2 | 10 ppm | - | + | + |
| B-3 | 10 ppm | - | - | + |
| C-1 | 20 ppm | + | + | + |
| C-2 | 20 ppm | - | + | + |
| C-3 | 20 ppm | - | - | + |
| D-1 | 50 ppm | + | + | + |
| D-2 | 50 ppm | - | + | + |
| D-3 | 50 ppm | - | - | + |

- = Untreated mulberry leaves.
✓ = Water treated mulberry leaves
\+ = Syzigium treated mulberry leaves.

**Table 2. Contents of proteins & activity of enzymes in the mid gut tissue of the fifth instar larvae of silkworm, *Bombyx mori* (L) fed with the leaves of mulberry, *Morus alba* (L) (M-5: variety) treated with aqueous solution of seed powder of *Syzigium cumuni* (L).**

| Sr.No. | Moiety | Soluble proteins | Total Proteins | Protease activity | Amylase activity |
|---|---|---|---|---|---|
| | Group | | | | |
| 1. | Untreated control(0-0) | 138.83 (± 49.851) | 579.43 (± 126.51) | 1.786 (± 0.154) | 3.817 (± 0.229) |
| 2 | Water treated control(0-I) | 134.11 (± 61.486) | 566.14 (+ 159.38) | 1.797 (+ 0.468) | 3.839 (+ 0.897) |
| 3. | Water treated control(0-II) | 136.07 (± 55.668) | 567.89 (± 143.23) | 1.786 (± 0.521) | 3.839 (± 0.897) |
| 4. | Water treated control(0-III) | 136.69 (± 52.579) | 571.51 (+ 139.28) | 1.791 (± 0.815) | 3.851 (± 0.914) |
| 5. | A-1 | 183.46** (± 52.409) 32.147 | 612.21* (± 166.35) 5.657 | 2.169*** (± 0.043) 21.444 | 4.372* (± 0.631) 14.54 |
| 6. | A-2 | 185.29** (± 59.931) 33.465 | 619.16* (± 143.78) 6.856 | 2.236** (± 0.147) 25.195 | 4.465* (± 0.871) 16.976 |
| 7. | A-3 | 185.47** (± 59.126) 33.595 | 621.09* (± 151.71) 7.185 | 2.274** (± 0.263) 27.323 | 4.478* (± 0.889) 17.317 |
| 8. | B-1 | 219.78** (± 98.213) 98.306 | 683.27* (± 298.68) 17.921 | 2.313*** (± 0.279) 29.507 | 4.559* (± 0.914) 19.439 |
| 9. | B-2 | 238.01** (± 77.324) 71.439 | 758.58* (± 121.29) 30.918 | 2.889*** (± 0.348) 61.758 | 4.842* (± 0.929) 26.853 |
| 10. | B-3 | 241.79** (± 68.763) 74.162 | 758.61** (± 189.73) 30.923 | 2.989*** (± 0.348) 67.357 | 4.913** (± 0.782) 28.713 |
| 11. | C-1 | 242.47** (± 76.296) 74.652 | 789.43** (± 134.18) 36.242 | 3.026*** (± 0.312) 69.428 | 5.483** (± 0.859) 43.646 |
| 12. | C-2 | 242.51** (±79.012) 74.681 | 789.59 (±247.26) 36.270 | 3.083*** (±0.617) 72.62 | 5.489** (±1.012) 43.804 |
| 13. | C-3 | 242.57** (± 88.136) 74.724 | 789.63** (± 313.13) 36.277 | 3.087*** (± 0.983) 72.844 | 5.588** (± 1.132) 46.397 |

- Each figure is the mean & three replications.
- Figure in parenthesis with ± sign is the standard deviation.
- Figure below parenthesis is percent change.

* : P<0.05
** : P<0.01
*** : P<0.001

The activity of mid gut protease was carried out according to the method of Brik, et al, (1962) with modifications suggested by Isshaya, et al, (1971) & outlined by Chougale (1992) & Khyade (2004). The mid gut protease activity was determined in triplicate set along with the blank. The mixture of incubation consisted of substrate ( one ml of ten percent casein solution) ; source of enzyme (0.5 ml assay sample) & 0.5 ml of 0.2M Trisbuffer (pH= 8.4). For the blank, assay sample was replaced by distilled water. The incubation was carried out in water bath at $30^0$C for 20 minutes with constant shaking. Addition of 6 ml of 2 percent trichloroacitic acid was made. The content was centrifuged at 8000 rpm for 15 minutes. The supernatant was used to read the optical density at 280 nm on spectrophotometer. Amount of tyrosine liberated from the casein due to action of mid gut protease was calculated through the use of optical density readings for assay sample; tyrosine ( from standard graph) & predetermined soluble protein contents of each assay sample. The activity of mid gut protease was expressed in terms of specific activity: microgram tyrosine liberated per mg protein per minute.

The activity of mid gut amylase was determined according to the methods of Bernfeld (1955); explained by Ishaaya & Swirski (1970), with modifications suggested by Gaikwad (1998) & outlined by Khyade (2004) & Desai, *et al*, (2011).

For the purpose to determine the activity of mid gut amylase, 20 larvae were selected randomly & processed for assay sample preparation as described for soluble proteins. Mid gut amylase was determined in triplicate set along with blank. The incubation mixture consisted of one ml of one percent starch solution ( as substrate), phosphate buffer (pH=9.2) & 0.5 ml of assay sample. For the blank, assay sample was replaced by distilled water. The process of incubation was carried out in water bath at $30^0$C for 20 minutes. After incubation the termination of activity of enzyme was made by addition of 2 ml DNSA & 2 ml distilled water. The contents were heated in boiling water bath exactly for five minutes, cooled immediately & the optical density of content was read at 540 nm on spectrophotometer.

For the purpose to calculate the mid gut amylase activity; the optical density readings for each assay sample; standard solution of maltase ( from graph) and soluble proteins were utilized. The enzyme activity was expressed in specific activity: micrograms of maltose liberated per mg protein per minute.

The experimentations were repeated for thrice for the purpose to obtain consistency in the results. The collected data was subjected for statistical analysis (mean, standard deviation, percent change & significance through student t – test) by the methods of Norman & Baily (1955).

**Results & Discussion**

The results on the biochemical response of the mid gut tissue in the fifth instar larvae of polyvoltine, crossbreed, silkworm, *Bombyx mori* (L) to the aqueous solution of Syzigium seed powder treated leaves of mulberry *Morus alba* (L) (M-5: variety) are summarized in table 2 & presented in Table: 2

Treating the mulberry leaves with various concentrations of aqueous solution of Syzigium seed powder & feeding them to the fifth instar larvae of silkworm, *Bombyx mori* (L) for first three days; second & third days & for third day (only) was found variously reflected in the levels of contents of proteins (soluble & total) & activity of enzymes (protease & amylase) in the mid gut tissue homogenate. The soluble proteins of untreated control (0-0) & water treated ( for first three days) (0-I) control were found measured 138.83 ( + 49.851) & 134.11( + 61.486) units respectively. The water treated control groups for second day & third day(0-II) & for only third day were exhibited 136.07 (+ 55.668) & 136.69 (+52.579) units of soluble proteins in their mid gut homogenate. The contents of total proteins in the mid gut tissue of fifth instar larvae fed with untreated mulberry leaves were measured 579.43 (+ 126.51)units. The group of larvae fed with water treated mulberry leaves for first three days; second day & third day, only for third day were found 566.14 ( + 159.38); 567.89 ( + 143.19) & 571.51 ( + 139.28) units of total proteins respectively in their mid gut tissue homogenate. Treating the mulberry leaves with water may affect either digestibility in the mid gut lumen or absorption of digested matter by epithelial surface of mid gut in fifth instar larvae of silkworm, *Bombyx mori* (L) (Vitthalrao Khyade & Jyoti Kulkarni, 2011 & Desai, *et al*, 2011). To allow the water treated mulberry leaves for complete draining or shade drying may help for the larval efficiency.

The contents of soluble & total proteins in the mid gut tissue homogenate of larvae fed with 5 ppm Syzigium seed powder treated mulberry leaves for first three days; second day & third day & third day (only) were found increased by 32.147 to 33.595 & 5.657 to 7.185 percent respectively. The mid gut protease activity & amylase activity in these groups were found elevated from 21.444 to 27.323 and from 14.54 to 17.317 percent respectively. Increase in the concentration of Syzigium, herbal formulation for treating mulberry leaves & feeding as per the schedule was found reflected into significant improvement in the levels of proteins ( soluble & total) & the activity of enzymes in the mid gut tissue homogenate. Soluble proteins seems to increase significantly in their level ( up to 90 percent) & total proteins (up to 39 percent).

The mid gut protease activity in all the groups of Syzigium, herbal formulation was found increased significantly. The levels of significance for the improvement in the mid gut amylase activity were found similar for the groups: 5, 6, 7, 8 & 9. The mid gut amylase activity in the groups: 10-16 was found with higher level of significance.

Increase in the levels of proteins (S.P. & T.P.) in the fifth instar larvae of silkworm, *Bombyx mori* (L) fed with mulberry leaves treated with various concentrations of aqueous solution of seeds of Syzigium, herbal formulation may be explained away as due to enhanced break down of contents of mulberry leaves. The contents of seeds of *Syzigium cumini* (L) improve appetite & digestion. Some of the herbal powders contain insect juvenoids (like eugenol) which are known to increase the capability of consumption & utilization of food by insects like silkworm. In phytophagous insects, the exogeneous compounds through herbal feed mimic the action of natural juvenile hormone, which enhance the synthesis of poly (A) RNA for major silk protein (Sen, 1988).Most significant response for herbal drug treatment in the study seems to be the levels of soluble proteins & activities of mid gut protease & mid gut amylase. The soluble proteins contribute in the tissue metabolism through enzymes. According to Applebaum (1985), continuous feeding in insects get reflect into advancement of production of mid gut enzymes, which improve the enzyme efficiencies. Most significant improvement in the protease activity in the treated group of study may be concerned with contents of specific plants. Individual plant extractive treatment may screen out the plant responsible for improved protease activity. Likewise the amylase enhancing herbal constituents of herbal formulations should be screened

The final larval instars of lepidopteran insects have four phases of growth which include: preparatory ( first two days);

Accumulation phase (third & fifth days); Regression phase (sixth day) & Degeneration phase (day of spinning). The initial preparatory phase is characterized by high rate of DNA synthesis, high rate of digestion, moderate RNA synthesis & low protein synthesis. This phase seems to be juvenile hormone dependent. Accumulation phase, regression phase & degeneration phase are concerned mainly with silk glands. Improvement in the levels of mid gut proteins (S.P. & T.P.); efficiency of mid gut protease & amylase in the group of larvae fed with mulberry leaves treated with aqueous solution of seeds of Syzigium, herbal formulation in the present study seems to be affecting the growth phases of larva. Treating the mulberry leaves with aqueous solution of seeds of Syzigium, herbal formulation & feeding them to the fifth instar larvae of silkworm, *Bombyx mori* (L) for first three days, seems to be significant in comparison with others (2). Feeding treated leaves for first three days possibly availing the herbal nutrients, which affect digestibility of larvae & may contribute phyto-juvenoids or other compounds of growth & development. The study should be extended for screening juvenoid activity of herbal drug. Larval parameters & commercial parameters in sericulture, which may fortify the concept & exert significant and applicable influence.

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