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Influence of Various Concentrations of Acetone Solution of Retinol on Pattern of Chitin Deposition in the Integument of Fifth Instar Larvae of Silkworm, *Bombyx mori* (L) (PM x CSR2)

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Abstract
The ten microliters of various concentrations of acetone solution of Retinol and Fernasol Methyl Ether (FME) were used for topical application to individual larval instars of silkworm, *Bombyx mori* (L) (Race: PM x CSR2) at 48 hours after the fourth moult. The integument chitin of untreated control larvae; acetone treated control; FME treated larvae and Retinol treated larvae was estimated at 120 hours after the fourth moult. Topical application of various concentrations of acetone solutions of FME and Retinol to fifth instar larvae of silkworm, *Bombyx mori* (L) (Race: PM x CSR2) was found reflected into the reduction in the deposition of chitin in the larval body wall. The reduction in body wall chitin was found ranging from zero to hundred percent. The plot of concentrations of acetone solutions (FME and Retinol) and percent reduction in the body wall chitin was found exhibiting a characteristic sigmoid form of displacement, which herewith titled as "Punyamayee Baramati Dose Response Curve". Since the effects of juvenoids involve the inhibition of metamorphosis of insects through reduction in chitin deposition, it is possible to express the concentration (dose) applied in terms of ID50 value. The ID50 value of juvenoid contents of FME and Retinol can be defined as the specific unit (mg/ml), which enable to chitin to deposit fifty percent less in the body wall of larvae (In comparison with untreated control). Accordingly, the ID50 value calculated from the "Punyamayee Baramati Dose Response Curves" for FME was found measured 0.08 mg/ml. The ID50 value for Retinol was measured 0.095 mg/ml. Acetone soluble juvenoid content of Retinol, the Diterpene compounds may be utilized efficiently for the fortified development of fifth instars of silkworm, *Bombyx mori* (L) and thereby, the cocoon quality. Sigmoid (S-form) "Baramati Dose Response Curve" may help for quantitative estimation of juvenoid contents of various terpene compounds and terpenoids.

Keywords: FME; Diterpene; Retinol, ID50 value; Chitin, juvenoids.

1. Introduction
The terpenes are a large and diverse class of organic compounds, produced by a number of plants. The terpenes are also produced by some insects, which emit from their osmeteria. The papilionid larvae are distinguished by presence of osmeteria. The osmeterium is a defensive organ found in all Papilionid larvae, in all stages (Chattopadhyay, J. 2011) [4]. The osmeterium is situated in the prothoracic segment. It can be everted when the larva feels threatened. In everted condition, osmeterium resembles a fleshy forked tongue not unlike a snake tongue and this along with the large eye like spots on the body might be used to startle birds and small reptiles. The osmeterial organ remains inside the body in the thoracic region in an inverted position and is everted when the larva is disturbed in any way emitting a foul, disagreeable odor which serves to repel ants (Eisner, T., and Meinwald, Y.C. 1965) [8]. small spiders (Damman, H. 1986) [7]. and mantids (Chow, Y.S. and Tsai, R.S. 1989) [5]. The composition of secretion from osmeteria varies from species to species. It contains monoterpene hydrocarbons, sesquiterpenic compounds or a mixture of aliphatic acids and esters. Crossley, A.C. and Waterhouse D.F. (1969) [6]. studied the fine structure of the osmetrium of *Papilio demoleus libanius* Fruhstorfer and found to contain 3 types of specialised cells for synthesis, acid secretion. Lu, Chow-Chin; Yien Shing Chow (1991) [19]. confirmed the storage of the osmeterial secretion.

The terpenes are often strong-smelling. They may protect the plants that produce them by deterring herbivores and by attracting predators and parasites of herbivores. The biochemical actions of natural insect juvenile hormone and terpenes and terpenoid compounds are similar. That is to say, the terpenes mimics the actions of natural "Insect Juvenile Hormone".

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One of the animal forms of terpene and vitamin A is retinol. With reference to chemical structure, it is a diterpenoid and an alcohol. Many other forms of Vitamin A are possible through the interconvertion of retinol, and the retinyl ester derivative of the alcohol serves as the storage form of the vitamin in animals. Retinal form is also known as retinaldehyde and it is essential for vision. Retinoic acid is essential for skin health, teeth remineralization and bone growth. All these forms of retinol are are collectively known as retinoids, and possess the structural motif of all-*trans* retinol as a common feature in their structure. The β-ionone ring and a polyunsaturated side chain constitutes chemical structure of retinoids. The side chain of retinoids is composed of four isoprenoid units, with a series of conjugated double bonds which may exist in *trans*- or *cis*-configuration (Gropper, *et al.*, 2009). In animal body, retinol is produced from the hydrolysis of retinyl esters, and from the reduction of retinal. Retinol in turn is ingested in a precursor form; animal sources (liver and eggs) contain retinyl esters, whereas plants (carrots, spinach) contain provitamin A carotenoids (these may also be considered simply vitamin A). The Hydrolysis of retinyl esters get results in retinol. The provitamin A carotenoids can be cleaved to produce retinal by carotene dioxygenase in the intestinal mucosa. Retinal, also known as retinaldehyde, can be reversibly reduced to produce retinol or it can be *irreversibly* oxidized to produce retinoic acid, which then cannot function as the vitamin in the eye. Commercial production of retinol typically requires retinal synthesis through reduction of a pentadiene derivative and subsequent acidification/hydrolysis of the resulting isomer to produce retinol. Pure retinol is extremely sensitive to oxidization and is prepared and transported at low temperatures and oxygen free atmospheres. When prepared as a dietary supplement, retinol is stabilized as the ester derivatives retinyl acetate or retinyl palmitate.

Vitamins are the organic compounds required by organism a vital nutrient in limited amounts. Supplementation of vitamins serve to orchestrate the metabolism. The larvae of silkworm, Bombyx mori (L) deserve appreciation for synthesis of silk for it's metamorphosis. Sericultural practices are serving a lot to provide the silk fibre. The silkworm, *Bombyx mori* (L) exert a significant influence on the concept of insect metamorphosis through it's simple life cycle and efficient utilization of the nutrients from the mulberry, *Morus alba* (L). Interplay of juvenile hormone and moulting hormone in the insect larval body serves to orchestrate the progression of metamorphosis from one instar to next, with moulting hormone regulating the onset and timing of moulting cycle and juvenile hormone regulating the quality of moult (Riddiford, 1985, 1994; Sehnal, 1985). [25, 26, 27]. During the last larval stadium of holometabolous insects, such as silkworm, *Bombyx mori* (L), a reduction of JH in haemolymph is the necessary step in the initiation of metamorphosis. It has been demonstrated that, haemolymph ecdysteroid and JH level undergo the developmental changes during larval - larval and larval - pupal cycles in silkworm, *Bombyx mori* (L) (Calvez *et al.*, 1976; [3]. Kiguchi and Agui, 1981). Juvenoids are well known in prolonging the larval life in the insect and have long been tried for qualitative improvement of silk (Ratnasen, 1988; Granier and Granier, 1983 and Mamatha *et al.*, 1999) [24, 10, 20]. There is considerable evidence that juvenile hormone mimics occur in plants, which occasionally leads to economically important consequences in the insect development (Slama, 1979) [32]. Juvenile hormone active compounds are found in many higher plants, exogenous application through suitable solvents of which exhibited potent activity in the insects (Prabhu and John, 1975). Efficient use of available system, the principle of

quality improvement made man to use juvenoids for pest control as well as for the silk yield. Use of juvenoids (synthetic, plant derived and animal derived) in the rearing of silkworm larvae had positive influence, especially in the silk yield (Ching *et al.*, 1972; Nihmura *et al.*, 1972 ; Muroga *et al.*, 1975; Kamada and Shimada, 1988 ; Rajashekhargouda, 1991 ; Vitthalrao *et al.*, 2002, 2003 and Vitthalrao, 2004).

Retinoic acid and insect juvenile hormone (JH) are structurally related terpenoids, which are widespread in nature and are involved in much more biological activities including morphogenesis, embryogenesis and cellular differentiation. The retinol is a diterpenoid, a terpenoid derived from a diterpene, which include the compounds with C20 skeleton of the parent diterpene, which has been rearranged or modified by the removal of one or more skeletal atoms (generally, methyl groups) (The retinoids deserve important role in the process of morphogenesis and in immune response in the insects like R. prolixus, suggesting that the molecular mechanism recognize the terpenoid backbone as one of the important structural determinant in insects (CHEBI: 23849 http://www.ebi.ac.uk/chebi/searchId.do?chebiId=CHEBI:2384 9) Angelica Nakamura, *et al.*, 2007). Terpenoid hormones seems to act as the morphogens throughout the metazoan. The regulatory activities of terpenoid hormones range from controlling metamorphosis in insects (Riddiford and Ashburner, 1990) and to determine the germ cell fate in the mammalians (Bowels, *et al.*, 2006). In the metamorphosis, the interplay of the juvenile hormone and ecdysone serve to orchestrate the progression from one instar to the next, with ecdyosteroid regulating the onset and timing of the moult and JH determining whether the moult would be larval – larval or larval – pupal (Gilbert, *et al.*, 1996; Mamatha, *et al.*, 2005). Phytophagous insects like silkworm, *Bombyx mori* (L) derive their juvenoid nutrients through the plant material available for them (Khyade, *et al.*, 2007) [17]. Retinol like vitamin nutrients may either be synthesized by the insect tissue or derived from the plant material. Nutrition with vitamins is playing important role in the improvement of growth and development in silkworm, *Bombyx mori* (L).

Juvenoids are known for disruption of normal developmental pattern leading to the deformities in the insects. Interestingly, the silkworm, *Bombyx mori* (L) is known to have a stimulatory influence on the administration of exogenous Juvenoids (JHA) in a appropriate quantities. The specific titer of juvenoids, either topical or through the food, at the specific period of the larval instars of silkworm, *Bombyx mori* (L) are positively reflected into the retention of larval features long enough enabling the larvae to consume maximum quantity of mulberry leaves and to synthesize paramount silk to be used in spinning the qualitative cocoon (Akai, *et al.*, 1990 ; Mamatha, *et al.*, 2005, 2006, 2008; Chowdhary, *et al.*, 1990; Miranda, *et al.*, 2002; Mamatha, *et al.*, 2006, 2008; Vitthalrao B. khyade and Jiwan P. sarawade. 2013) [36]. Diterpene structure, insect juvenoid activity and vitamin nature of Retinol made to plan for the efforts on it's topical application through the acetone to the fifth instar larvae of silkworm, *Bombyx mori* (L) (PM x CSR2). The attempt was carried at the laboratory level.

2. Material and Method
The experimentation was divided into seven steps, which include: Rearing of larval instars of silkworm, *Bombyx mori* (L); Daily bioassay of body wall chitin of fifth instar larvae; Preparation of acetone solutions of Retinol; Grouping the fifth instar larvae and topical application of acetone solution of Retinol; Bioassay of body wall chitin at 120 hours after the fourth moult; Statistical analysis of the data and Plotting the

"Punyamayee Baramati Dose Response Curves" for FME and Retinol used for topical application.

(A). Rearing of larval instars of silkworm, *Bombyx mori* (L): The disease free layings (DFL) of polyvoltine, crossbreed race (PM x CSR2) of silkworm, *Bombyx mori* (L) were procured from sericulture unit at the farm of Agriculture Development Trust, Malegaon (Baramati). They were processed for incubation through black boxing for 48 hours. The larvae were reared in laboratory condition on the leaves of mulberry (M-5 variety). Standard Methods of rearing (Krishnaswami, *et al.*, 1978 and Vitthalrao B. Khyade, 2004). [18, 15].

(B). Daily bioassay of body wall chitin of fifth instar larvae: The chitin content of body wall was estimated at zero (soon after the fourth moult) ; 24; 48; 72; 96 and 120 hours after the fourth moult. The method followed for chitin estimation was volumetric (Baishya and Hazarika, 1996; Vitthalrao Khyade, *et al.*, 2006) [2]. Twenty larvae for each time were selected randomly and anaesthetized with little quantity of chloroform soaked cotton pad. They were dissected in insect saline. The abdominal fat bodies and visceral organs were removed carefully. After removing all the organ systems, trachae and adhering fat bodies the part remained was designated as integument. The integument of each larva was blotted and weighed on electronic balance. The integument piece of individual larva was placed in separate test tube containing 50 ml. of 30 percent potassium hydroxide (KOH) solution. All the test tubes in a group were placed in separate water bath. The contents of test tube were allowed for boiling for thirty minutes. After treating the integument with boiling potassium hydroxide solution, it was subsequently washed with distilled water; two times in ninety six percent ethanol and two times in ether. Treated pieces of integument (body wall) were weighed accurately on electronic balance. The weight of integument (body wall) after potassium hydroxide treatment corresponds to the quantity of chitin (mg/gm).

(C). Preparation of acetone solutions of Retinol and FME: Retinol and FME were procured through the local chemical suppliers. Based on preliminary studies, known quantity of FME was dissolved in known volume of acetone so as to get desired concentration. Various concentrations of acetone solution of FME include: 00.000 to 00.160 mg/ml. Likewise, Retinol was dissolved in acetone to get desired concentrations (00.000 to 00.175 mg/ml). FME was used as a "standard Insect Compound responsible for reduction in chitin deposition in lnsects" for comparison.

(D). Grouping the fifth instar larvae and topical application of acetone solution of Retinol and FME: Soon after the fourth moult, the larvae of fifth instar were grouped into control (Untreated and acetone treated, each one) groups and experimental groups (2 x 35), each with fifty individuals. Ten microliters of each concentration of acetone extractives of FME (as a standard Insect JHA) and Retinol were topically applied with micropipette separately to the individual fifth instar larvae at 48 hours after the fourth moult. The larvae of all groups were maintained according to usual schedule. (E). Bioassay of body wall chitin at 120 hours after the fourth moult:

Body wall chitin contents of fifth instar larvae (Untreated Control group; Acetone Treated Control group; FME Treated and Retinol Treated groups) was carried out at 120 hours after the fourth moult. The method followed for chitin estimation was volumetric (Baishya and Hazarika, 1996; Vitthalrao Khyade, *et al.*, 2006) [2]. Twenty larvae from each group were selected randomly and anaesthetized with little quantity of chloroform soaked cotton pad. They were dissected in insect saline. The abdominal fat bodies and visceral organs were

removed carefully. After removing all the organ systems, trachae and adhering fat bodies the part remained was designated as integument. The integument (body wall) of each larva was blotted and weighed on electronic balance. The integument (body wall) piece of individual larva was placed in separate test tube containing 50 ml of 30 percent potassium hydroxide (KOH) solution. All the test tubes in a group were placed in separate water bath. The contents of test tube were allowed for boiling for thirty minutes. After treating the integument with boiling potassium hydroxide solution, it was subsequently washed with distilled water; two times in ninety six percent ethanol and two times in ether. Treated pieces of integument were weighed accurately on electronic balance. The weight of integument after potassium hydroxide treatment corresponds to the quantity of chitin (mg/gm).

(F). Statistical analysis of the data: The experimentations were repeated for three times for the consistency in the results. Data was collected and subjected for statistical analysis (mean, standard deviation and student "t" test for knowing the significant level of treatment) (Norman and Baily, 1955) [21]. Soon after the fourth moult (zero hour) and 120 hours after the fourth moult were considered as initial and final quantity of chitin respectively. Subtraction of initial quantity from final quantity give the quantity of chitin deposited in body wall of the fifth instar larvae for 120 hours after the fourth moult (5 days of fifth instar larvae). Quantity of chitin (mg/gm) deposited in the treated group was subtracted from the quantity of chitin deposited in the control group. This figure was divided by quantity of chitin deposited in control group. The quotient, thus obtained was multiplied by hundred to know percent reduction in the chitin in the integument of larvae of treated groups.

(G). Plotting the "Punyamayee Baramati Dose Response Curves" for the compounds used for topical application: Dose response curve for each compound was plotted (Fig. 1). The scale for plotting the graph, for X- axis was 1 = 00.010 mg/ml concentration of acetone solution. And that for Y- axis, the scale was 1 = 5 percent. Dose response curve for each compound was plotted (Fig. 1). The x- co-ordinate that corresponds to the value of fifty on y-axis in dose response curve was designated as ID50 value for given compound. Thus, ID50 value for FME and Retinol was calculated through the use of respective dose response curve. The plot of dosages of acetone solution of selected compounds (FME and Retinol) and percent change in the body wall chitin of larval instars of silkworm, *Bombyx mori* (L) is to be recognized as "Punyamayee Baramati Dose Response Curve".

3. Results and Discussion
The results pertaining the screening of acetone solution of Fernasol Methyl Ether (FME) and Retinol for JH activity through the changes in pattern of chitin deposition in the integument of fifth instar larvae of silkworm, *Bombyx mori* (L) are summerised in Table – 1 to 3 and Fig.1. The amount of chitin(mg/ gm) deposited in the body wall of the fifth instar larvae at 0.00; 48; 72; 96 and 120 hours after the fourth moult were found measured as: 19.774 (±1.087); 19.779 (±1.143); 19.786 (±2.057); 20.679(±1.789); 26.823(±3.018) and 38.186(±3.632) units respectively. In the untreated and acetone treated groups, the body wall chitin at 120 hours after the fourth moult was 38.186 (±3.632) and at 48 hours after the fourth moult was 19.786 (±2.057). Subtraction of chitin content at 48 from 120 hours gives the amount of chitin deposited during the experimental period (38.186–19.786=18. 400).

Table 1: Chitin content in the body wall of the fifth instar larvae of silkworm, Bombyx mori (L) (Race: PM x CSR2).

Serial No.	Hour After the Fourth Moult	Body Wall Chitin Content (mg/Gm)
1	000.000	19.774 (± 1.087)
2	024.000	19.779 (± 1.143)
3	048.000	19.786 (± 2.057)
4	072.000	20.679 (± 1.789)
5	096.000	26.823 (± 3.018)
6	120.000	38.186 (± 3.632)

Each figure is the mean of three replications. 2. Figures with ± sign in parentheses are the standard deviations. 3. Chitin Deposition for Untreated Control Larvae = Chitin content at 120 hours after the fourth moult-Chitin content at 48 hours after the fourth moult (18.4 = 38.186 – 19.786).
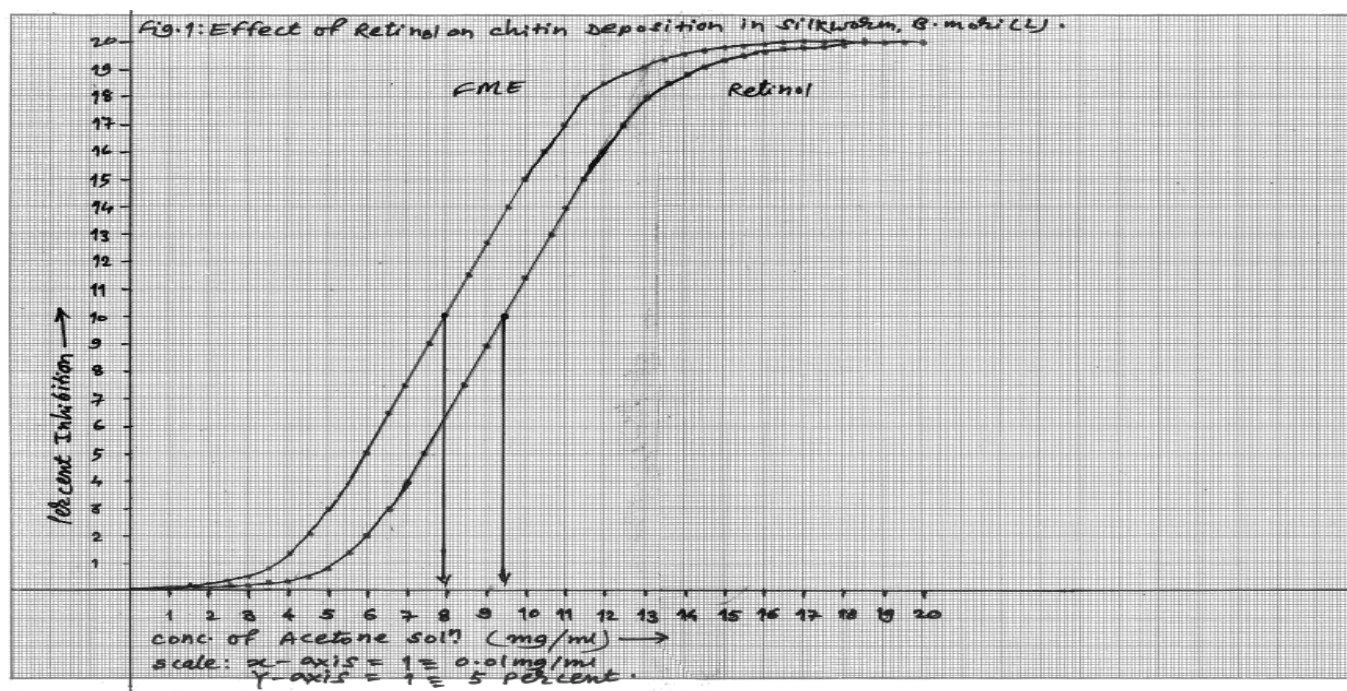
Table-2: Chitin content of the body wall of the fifth instar larvae of silkworm, *Bombyx mori* (L) (Race: PM x CSR2) recipient of topical application of various concentration of acetone solution of Fernasol Methyl Ether (FME) at 48 hours after the fourth moult.

X	Concentration of Acetone Solution (ppm)	Body Wall Chitin (mg / gm)	Chitin Deposition (mg / gm)	Percent Reduction	Y
00.000	00.000	38.186 (± 4.673)	18.400	000.000	000.000
00.500	00.005	38.002 * (± 4.651)	18.216	01.000	000.200
01.000	00.010	37.910 * (± 4.397)	18.124	01.500	000.300
01.500	00.015	37.823* (± 4.089)	18.037	02.000	000.400
02.000	00.020	37.726* (± 3.391)	17.940	02.5000	000.500
02.500	00.025	37.634* (± 3.906)	17.848	03.000	000.600
03.000	00.030	37.542* (± 4.289)	17.756	03.500	000.700
03.500	00.035	37.266* (± 3.258)	17.483	05.000	001.000
04.000	00.040	36.990 * (± 4.078)	17.204	06.500	01.300
04.500	00.045	36.346 * (± 3.966)	16.560	10.000	02.000
05.000	00.050	35.610* (± 4.023)	15.824	14.000	02.800
05.400	00.054	34.966* * (± 3.843)	15.180	17.500	03.500
06.000	00.060	35.586 * * (± 4.143)	13.800	25.000	05.000
07.000	00.070	31.286 * * (± 4.518)	11.500	37.500	07.000
08.000	00.080	28.986 * * (± 3.513)	09.200	50.000	10.000
09.000	00.090	26.686* * (± 3.795)	06.900	62.500	12.500
10.000	00.100	24.386* * (± 3.786)	04.600	75.000	15.000
10.500	00.105	23.236* ** (± 3.897)	03.450	81.250	16.250
11.000	00.110	22.362* * * (± 3.841)	02.576	86.000	17.200
11.500	00.115	21.718* * * (± 4.948)	01.932	89.500	17.900
12.000	00.120	21.258* * * (± 4.013)	01.472	92.000	18.400
12.500	00.125	20.798* * * (± 3.427)	01.012	94.500	18.900
13.000	00.130	20.522* * * (± 3.734)	00.736	96.000	19.200
13.500	00.135	20.246* * * (± 3.964)	00.460	97.000	19.500
14.000	00.140	20.062* * * (± 3.687)	00.276	98.500	19.700
14.500	00.145	19.878* * * (± 3.789)	00.092	99.500	19.900
15.000	00.150	19.786 * * * (± 3.881)	00.000	100.00	20.000
15.500	00.155	19.786 * * * (± 3.963)	00.000	100.00	20.000
16.000	00.160	19.786* * * (± 3.794)	00.000	100.000	20.000

1. Each figure is the mean of three replications;
2. Figures in parenthesis with ± sign are the standard deviations.
3. * = P < 0.005; * * = P < 0.01 and * * * = P < 0.001

Table 3: Chitin content of the body wall of the fifth instar larvae of silkworm, *Bombyx mori* (L) (Race: PM x CSR2) recipient of topical application of various concentration of acetone solution of Retinol at 48 hours after the fourth moult.

X	Concentration of Acetone Solution (ppm)	Body wall Chitin (mg/Gm)	Chitin Deposition (mg/Gm)	Percent Reduction	Y
00.000	00.000	38.186* (± 4.975)	18.4	00.000	00.000
00.500	00.005	38.186* (± 5.087)	18.4	00.000	00.000
01.000	00.010	38.186* (± 4.039)	18.4	00.000	00.000
01.500	00.015	38.186* (± 4.536)	18.4	00.000	00.000
02.000	00.020	38.186* (± 4.743)	18.4	00.000	00.000
02.500	00.025	38.186* (± 4.678)	18.4	00.000	00.000
03.000	00.030	38.002* (± 6.019)	18.216	01.000	00.200
03.500	00.035	37.818* (± 4.235)	18.032	02.000	00.400
04.000	00.040	37.542* (± 6.117)	17.756	03.500	00.700
04.500	00.045	37.358* (± 5.067)	17.572	04.500	00.900
05.000	00.050	37.082 (± 6.269)	17.296	06.000	01.200
05.500	00.055	36.622* (± 6.168)	16.836	08.500	01.700
06.000	00.060	36.050* (± 5.084)	16.264	11.500	02.300
06.500	00.065	35.426 (± 5.626)	15.640	15.000	03.000
07.000	00.070	35.598* (± 6.786)	14.812	19.500	03.900
07.500	00.075	33.586* (± 6.219)	13.800	25.000	05.000
08.000	00.080	32.390** (± 6.317)	12.604	31.500	06.250
08.500	00.085	31.286** (± 5.769)	11.500	37.500	07.500
09.000	00.090	30.136** (± 5.987)	10.350	43.750	08.750
09.500	00.095	28.986** (± 4.994)	09.200	50.000	10.000
10.000	00.100	27.836** (± 5.742)	08.050	56.250	11.250
10.500	00.105	26.686** (± 5.553)	06.900	62.500	12.500
11.000	00.110	25.536** (± 6.364)	05.750	68.750	13.750
11.500	00.115	24.386** (± 4.481)	04.600	75.000	15.000
12.000	00.120	23.282*** (± 4.493)	03.496	81.000	16.200
12.500	00.125	22.362*** (± 5.741)	02.576	86.000	17.200
13.000	00.130	21.810 *** (± 6.213)	02.024	89.000	17.800
13.500	00.135	21.166 *** (± 5.863)	01.380	92.500	18.500
14.000	00.140	20.798*** (± 6.087)	01.012	94.500	18.900
14.500	00.145	20.430*** (± 5.975)	00.644	96.500	19.300
15.000	00.150	20.154*** (± 4.788)	00.368	98.000	19.600
15.500	00.155	20.062*** (± 5.383)	00.276	98.500	19.700
16.000	00.160	19.970*** (± 4.978)	00.184	99.000	19.800
16.500	00.165	19.878*** (± 5.975)	00.092	99.500	19.900
17.000	00.170	19.786*** (± 5.179)	00.000	100.00	20.000
17.500	00.175	19.786 *** (± 5.882)	00.000	100.00	20.000

Fig.1: Effect of Retinol on chitin Deposition in silkworm, B. mori (L).

Topical application of ten microlitres of different concentrations of FME and Retinol was found reduction in chitin deposition in the body wall (integument) of the fifth instar larvae of silkworm, *Bombyx mori* (L). And the pattern was exhibiting significant response with reference to chitin deposition pattern in the body wall of fifth instar larvae of silkworm, *Bombyx mori* (L) (Race: PM x CSR2). The reduction in body wall chitin was found ranging from zero to hundred percent. The plot of concentrations of acetone solutions of compounds in the study (FME and Retinol) and percent reduction in the body wall chitin exhibiting a characteristic Sigmoid form of displacement, which herewith titled as "Punyamayee Baramati Dose Response Curve". The sigmoid curve of pattern of percent reduction in chitin deposition and concentrations of acetone solutions of FME topically applied at 48 hours after the fourth moult to the larval instars of silkworm, *Bombyx mori* (L) (Race: PM x CSR2) in the study seems to reflect three groups of concentration of acetone solutions, which include: Non-significant; Significant and the most significant. The concentrations of FME namely, 00.000 to 00.060 ppm (mg/ml) of FME were found with non-significant reduction in chitin deposition. The concentrations of FME namely, 00.065 to 00.100 ppm (mg/ml) of FME were found with significant reduction in chitin deposition. And The concentrations of FME namely, 00.105 to 00.160 ppm (mg/ml) of FME were found with the most significant reduction in chitin deposition.

The sigmoid dose response curve for Retinol also exhibited three groups of concentrations of it's acetone solutions. The concentrations of Retinol namely, 00.000 to 00.075 ppm (mg/ml) were found with non-significant reduction in chitin deposition. The concentrations of FME namely, 00.080 to 00.115 ppm (mg/ml) of Retinol were found with significant reduction in chitin deposition. And the concentrations of Retinol namely, 00.120 to 00.175 ppm (mg/ml) of FME were found with the most significant reduction in chitin deposition.

The concentrations (mg/ml) of acetone solutions of FME and Retinol in the study that inhibit the fifty percent chitin deposition in the body wall of larvae can be calculated by the use of "Punyamayee Baramati Dose Response Curves". This concentration of acetone solution responsible for fifty percent reduction in chitin deposition in the body wall (integument) of fifth instar larvae of silkworm, *Bombyx mori* (L) is herewith termed as ID_{50} value. Accordingly, the ID_{50} values for FME and Retinol were found calculated 00.080 and 00.095 units (mg/ml) respectively. Ten microlitres out of thousand microlitres of each acetone solution is utilized for topical application on individual larva in each group. This study may co-relates the "Finding the efficacy of acetone soluble compound for it's juvenoid activity through reduction in chitin deposition in the of fifth instar larvae of silkworm, *Bombyx mori* (L).

The "Punyamayee Baramati Dose Response Curves" in the study may form baseline platform for estimation of ID50 values of any compounds (plant derived; animal derived and synthetic compounds). The present study tried its best to establish preliminary work on screening the acetone solutions of FME and Retinol for juvenoid activity in the fifth instar larvae of silkworm, *Bomby xmori* (L) (Race: PM x CSR₂). Farnasol Methyl Ether (FME) or acetone like solvents may serve the purpose to know intensity of juvenoids in any compound. The diterpene retinol deserve many more cellular and molecular activities that could potentially underlie it's juvenomimetic index with reference to the phytophagous insects like, silkworm, *Bombyx mori* (L). The present attempt is going to help to establish maximum tolerated dose of

Retinol to be used for future trials in which the efficacy of retinol will be tested for qualitative improvement of silk spinned by mature fifth instar larvae of silkworm, *Bombyx mori* (L). If the efficacy is seen in larval developmental setting, it will likely trigger future development and testing the monoterpenes for the fortified health of larval instars, that could spin the qualitative silky cocoon. The monoterpenes are thus an example of the development of agents that will bridge the areas of sericulture. The Baramati attempt of use of terpenes for topical application to the larval instars of silkworm, *Bombyx mori* (L) hope more efficiently benefitting the areas of both the areas of sericulture and juvenoid research. And the "Punyamayee Baramati Dose Response Curves" in the present attempt may open a new avenue in the field of Juvenoid research.

During the early age (up to 48 hours) of fifth instar larvae of silkworm, *Bombyx mori* (L), the titer of juvenile hormone (JH) in the haemolymph is maintained at significant detectable level. Rate of chitin deposition during this period seems to be non-significant. Thereafter, the juvenile hormone (JH) in the larval haemolymph get decreased rapidly. The most possible reason for this include accelerative rate activity of esterase after 48 hours after the fourth moult Ajami & Riddiford (1973) [1]; Khyade, (2004). The present study demonstrate to decrease in chitin deposition in the body wall of fifth instar larvae of silkworm, *Bombyx mori* (L) (Race : PM x CSR₂) recipient of the exogenous juvenoid material in the form of acetone extractives of selected plants. The significant feature of exogenous juvenoids is to slow down the rate of chitin synthesis in the body of insects. The appreciable sclerotization before spinning seems to be prerequisite for metamorphosis to proceed Omana Joy (1983) [22]. The titer of juvenile hormone in the haemolymph of fifth instar larva in late age (last three days) is to be maintained at insignificant, undetectable level for the purpose to proceed metamorphosis through accelerate rate of metabolism including chitin deposition. Delay in the maturation for spinning in the larvae treated with FME and terpenes (let us label them "Silkworm Juvenoids"), as observed in the present study, may be to resume normal rate of chitin deposition.

The present study demonstrate the titer of exogenous juvenoid material get reflect into various conditions of juvenility (in the form of decreased amount of chitin in the body wall) of fifth instar larvae of silkworm, *Bombyx mori* (L) (Race : PM x CSR₂). Reduction in the deposition of chitin in body wall of treated larvae (irrespective of acetone solution of FME and retinol; and their concentrations too) recorded in the study, establish a positive effect, which seems to be in agreement with results obtained through the use of Juvenoids compounds in silkworm larvae (Akai and Kobayashi, 1971; Sharad Jagatap, 2007; Vitthalrao Khyade, 2009) [35]. Selected doses of selected of monoterpenes may be utilized for the purpose to sustain the larval age, which is essential to uplift the time required for eating mulberry leaves and amount of mulberry leaves eaten.

If the maximum possible juvenoid effect in the form of reduction in body wall chitin in the fifth instar larvae of silkworm considered as hundred percent reduction in the chitin content, it has been found that, successive percent reduction from zero to hundred appear to be proportional to the topically applied concentration (dosage) within some narrow range. The relationship between titer (concentration) of exogenous juvenoid material (acetone solutions of selected FME and Retinol) & intensity of chitin deposition in the body wall of larvae appear to be in the form sigmoid curve, which, herewith entitled as "Punyamayee Baramati Dose Response Curve".

These curves seems to exhibit a characteristic S-form (sigmoid) displacement across the scale of concentration (mg/ml) of FME and Retinol. The change from zero to hundred percent effect commonly exhibited over 10-50 fold change in the dose topically applied. The concentrations (dosages) of acetone solutions of FME and retinol in the study, on steeper slope of curves, seems to be most significant in the percent reduction in the body wall chitin. Therefore, the dosages of acetone solutions of FME and Retinol on the steeper slope of "Punyamayee Baramati Dose Response Curve" may be called as effective dosages. The effects of juvenoids involve inhibition of insect metamorphosis, significantly through reduction in chitin deposition Slama (1974) [30]. It has been proposed to express the concentration (dosage) of acetone solution or extractives (Juvenoid) topically applied in terms of ID_{50} value. According to Slama *et al.* (1974) [30]. the ID_{50} unit of juvenoid material (in microgram), which deposit fifty percent chitin in the body wall of insect larvae.

The present attempt is going to help to establish maximum tolerated dose of retinol to be used for future trials in which the efficacy of retinol will be tested for qualitative improvement of silk spinned by mature fifth instar larvae of silkworm, *Bombyx mori* (L). If the efficacy is seen in larval developmental setting, it will likely trigger future development and testing the retinol for the fortified health of larval instars that could spin the qualitative silky cocoon. The retinol is thus an example of diterpene compounds, which may bridge the areas of sericulture. The Baramati attempt of use of Retinol for topical application to the larval instars of silkworm, *Bombyx mori* (L) hope more efficiently benefitting both the areas of sericulture and juvenoid research. And the "Punyamayee Baramati Dose Response Curves" in the present attempt may open a new avenue in the field of Juvenoid research.

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5. References

1. Ajami AM, Riddiford LM. Comparative metabolism of the cecropia juvenile hormone. J Insect Physiol. 1973; 19:635-646.
2. Baishya RL, Hazarika LR. Effect of methoprene and diflubenzuron on water, lipid, protein and chitin of Dicladispa armigera (Coleoptera: Chrysomelidae). Entomon 1996; 21-91(1):7-11.
3. Calvez B, Hirn M, Reggi M. Progress of development programme during the last larval instar of Bombyx mori (L). Relationship with food intake, ecdyosteroids and juvenile hormone. Journal of Insect Physiology. 1976; 24(4):233-239.
4. Chattopadhyay J. The structure and defensive efficacy of glandular secretion of the larval osmeterium in Graphium agamemnon agamemnon Linnaeus, 1758 (Lepidoptera: Papilionidae), 2011.
5. Chow YS, Tsai RS. Protective chemicals in caterpillar survival. Experientia (Basel) 1989; 45(4):390-392.
6. Crossley AC, Waterhouse DF. The infrastructure of osmeterium and the nature of its secretion in Papilio larvae (Lepidoptera; Papilionidae). Tissue & Cell 1969; 1:525-554.
7. Damman H. The osmeterial glands of the swallowtail butterfly Eurytides Marcellus as a defense against natural enemies. Ecol. Entomol 1986; 11:261-265.
8. Eisner T, Meinwald YC. The defensive secretions of a caterpillar (Papilio). Science, N.Y 1965; 150:1733-1735.
9. Gopakuma B, Ambika B, Prabhu VKK. Juvenmimetic activity in some south Indian plants and their probable cause of this activity in Morus alba (L). Entomon 1977; 2:259-261.
10. Grenier Grenier. Fenoxycarb a fairlynew growth regulator: a review of its effects on insects. Ann. App. Biol 1983; 122:369-403.
11. Jadhav G, Kallapur VL. Contribution of tissue protein to the cocoon shell in the fifth instar silk worm, Bombyx mori (L), 1989.
12. Kamimura M, Kiguchi M. Effect of juvenile hormone analogue on fifth stadium larvae of silk worm, Bombyx mori (L) (Lepidoptera: Bombycidae). Appl. Entomol. Zool. 1980; 33(2):333-338.
13. Khyade VB, Patil SB, Khyade SV, Bhawane GP. Influence of acetone maceratives of Vitis vinifera on the larval parameters of silk worm, Bombyx mori (L). Indian Journal of Comparative Animal Physiology. 2002; 20:14-18.
14. Khyade VB, Patil SB, Khyade SV, Bhawane GP. Influence of acetone maceratives of Vitis vinifera on the economic parameters of silk worm, Bombyx mori (L). Indian Journal of Comparative Animal Physiology. 2003; 21:28-32.
15. Khyade VB. Influence of juvenoids on silk worm, Bombyx mori (L). Ph.D. Thesis, Shivaji University, Kolhapur, India, 2004.
16. Khyade VB, Ganga V. Mhamane Vividh Vanaspati Arkancha Tuti Reshim Kitak Sangopanasathi Upyojana. Krishi Vdnyan. 2005; 4:18-22.
17. Khyade VB, Poonam B, Patil M, Jaybhay Rasika R, Gaikwad Ghantaloo US, Vandana D. Use of digoxin for improvement of economic parameters in silk worm, Bombyx mori (L). Bioinfrmatics (Zoological Society of India), 2007.
18. Krishnaswami S, Narasimhana MN, Suryanarayana SK, Kumaraj S. Sericulture Manual –ll: Silk worm Rearing. F A O, United Nation's Rome, 1978, 131.
19. Lu Chow-Chin, Yien Shing Chow. Fine structure of the larval osmeterium of Papilio demoleus libanius (Lepidoptera: papilionidae). Ann. Entomol. Soc Am. 1991; 84(3):294-302.
20. Mamatha DN, Nagalakshmma K, Rajeshwara Rao M. Impact of selected Juvenile Hormone Mimics on the organic constituents of silk worm, Bombyx mori (L), 1999.
21. Norman Baily TJ. Statistical Methods. 2nd ed., Halsted Press, John Wiley & Co., New York, 1955, 216. http://deepblue.lib.umich.edu/bitstream/handle/2027.42/24020/0000269.pdf?sequence=1&isAllowed=y
22. Omana Joy, Shyamala MB. Non-spinning syndrome in silk worm: Occurrence and Pathology. Research paper presented for National Seminar on silk research and development, Banglore, India, 1983.
23. Prabhu VKK, John M, Ambika B. Juvenile hormone activity in some south Indian plants. Current Science 1973; 42:72-726.

24. Ratnasen. How does juvenile hormone cause more silk yield. Indian Silk, 1988, 21-22.
25. Riddiford LM. Hormone action at cellular level. In: Comprehensive Insect Physiology, Biochemistry and Pharmacology. G. A. Kerkut and L. I. Gilbert (Eds.) (Pergamon press, Oxford) 1985; 8:37-64.
26. Riddiford LM. Cellular and molecular actions of Juvenile hormone: General consideration and premetamorphic actions. J Adv. Insect Physiology. 1994; 24:213-214.
27. Sehnal F, Rambold H. Brain stimulation and juvenile hormone production in insect larvae. Experentia 1985; 44:684-685.
28. Slama K. Plant as source material with insect hormone activity. Ent. Exp. Appl 1969; 12:721-728.
29. Slama K. Insect juvenile hormone analogues. Ann. Rev. Biochem 1971; 4o:1079-1102.
30. Slama K, Romanuk M, Sorm F. Insect hormones and Bioanalogues. Springer Verlag, Wein and New York.
31. Slama, K. (1985). Pharmacology of Insect Juvenile Hormones. In: Comprehensive Insect Physiology, Biochemistry and Pharmacology. (Eds. G. A. Kerkut and L. I. Gilbert). Pergamon Press, Oxford, New York. 1974; 11:357-394
32. Slama K. Insect Hormones and anti-hormones in plants. Herbivores, their interaction with secondary plant metabolites. (Eds. G. A. Rosenthal and D. H. Janzen; Academic Press, New York), 1979, 683-700.
33. Williams CM. The Juvenile Hormone of Insects. Nature. 1956; 178:212-213.
34. Zaoral M, Slama K. Peptides with juvenile hormone activity. Science 1970; 170:92-93.
35. Jagtap, Sharad G. Effect of plant juvenoids on consumption and utilization of mulberry leaves by silkworm, Bombyx mori (L). M. Phil. Dissertation, Alagappa University, Karaikudi-630003 (India). International Journal of Advanced Life Sciences, 2007.
36. Vitthalrao B. Khyade, Jiwan P. Sarwade Utilization of Retinol through the topical application to the fifth instar larvae of the silkworm, *Bombyx mori* (L) (Race: PM x CSR2) for qualitative improvement of the economic parameters. International Journal of Advanced Life Sciences. 2013; 6(5):532-537. http://unitedlifejournals.com/ms_files/ijals/MS19-IJALS-Iss-5-Nov-2013-_Utilisation-of-Retinol-through.pdf