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Bio-activity of mulberry silkworm droppings in different instars

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Abstract
Silkworm droppings (SWD) are one of the major wastes generated during rearing of silkworms. However, these droppings are excellent source of manure, fish and poultry food and are utilized in nutraceutical and pharmaceutical industries because of the presence of some important bioactive compounds. Present study was undertaken to determine the chlorophyll, polyphenol, flavonoid and antioxidant activity of SWD in droppings of all the 5 instars of the silkworm, *Bombyx mori*. The ethanol stirrer extraction method was used for the preparation of silkworm dropping extracts of all the 5 instars. The total chlorophyll was found in the increasing order from 1.839mg/g to 3.609mg/g in the 1st to 4th instar SWD. However, the content of total chlorophyll decreased in the 5th instar SWD (2.628mg/g). Total polyphenol content was recorded in decreasing trend from 1st to 5th instar SWD from 3.108mgGAE/g to 0.510mgGAE/g, similar trend was observed in the total flavonoid content from 19.097mgQE/g in 1st instar to 4.07mgQE/g in 5th instar SWD. Scavenging activity shows direct correlation with the concentration. Highest scavenging activity was observed at 50mg/ml sample. Further, the scavenging activity showed decreasing trend in the SWD samples collected from 1st to 5th instar larvae from 87.46% to 76.03% respectively. From the study we conclude that silkworm droppings can serve as a promising dietary, nutraceutical and scavenging application and would thereby add value to sericulture.

Keywords: Silkworm droppings, bioactive compounds, antioxidant, polyphenol and flavonoid

Introduction
Sericulture is an agro based industry that involves cultivation of host plants to produce silk. The main domain of mulberry sericulture lies in the utilization of cocoons for textile purpose however; its byproducts generated from mulberry cultivation to cocoon production have the potential to add value to the enterprise. Serious attention is being paid to unexplored potential of sericulture byproducts. Silkworm *Bombyx mori* L. wholly depends on mulberry leaf for nutrition hence called monophagous insect. The silkworm larva digests around 40% of the ingested food and egests 60% of it in the form of droppings (Lee and Lee, 1971) [14]. Silkworm larvae convert only 6-7% of the leaf ingested into cocoon shell and rest of it is transformed into wastes like spent pupae and droppings. Silkworm droppings form major waste generated during silkworm rearing and is a rich reservoir of medicinally important components like DNJ, GABA, chlorophyll, sterols etc. (Yin *et al*., 2010) [29] and Park *et al.*, 2011) [21].
SWD are good source of polyphenols, Polyphenols including flavonoids have versatile benefits for human health exhibiting antioxidant, anticancer, anti-bacterial and cardioprotective activities (Kumar and Pandey, 2013; Chen *et al.*, 2015; Dzialo *et al*., 2016 and Andreu *et al.*, 2018) [13, 5, 6, 1]. Polyphenols and flavonoids are known to act as antioxidants by scavenging and neutralizing the harmful free radicals which damage the cells in the biological system. In addition, the SWD contain organic matter (83.77- 90.44%) and ash (16.23- 9.56%) according to the age of silkworms (Bergmann, 1934; Hiraki *et al*., 1996 and Neelagund, 2007) [4, 9, 19]. It also contains a variety of amino acids such as glycine, serine, leucine, lysine, histidine etc. and triacontanol, a very long chain fatty alcohol (VLCFA) and β-sitosterol, a phytosterol (Raju, 1996) [22]. Literature reveals scanty information regarding the biochemical characterization of SWD at different instars of larval development. Therefore a study was undertaken to analyze the biochemical characterization and properties of SWD obtained from five different instars of mulberry silkworm larvae.

Materials and Methods

Collection of silkworm droppings

The silkworm droppings (SWD) were collected from all the 5 instars during silkworm rearing. Dead/diseased silkworms and left-over mulberry leaves were manually removed. The sample was oven dried at 50 ºC and then ground into fine powder.

Preparation of SWD extract

25g of SWD powder were weighed and mixed with 500 ml of 80% ethanol in a flask. The contents were kept at room temperature for 24 hours, centrifuged at 8000 rpm for 15 minutes and supernatant was used as stock solution (50 mg/ml) for analysis. In case of chlorophyll estimation, dry samples were taken for analysis.

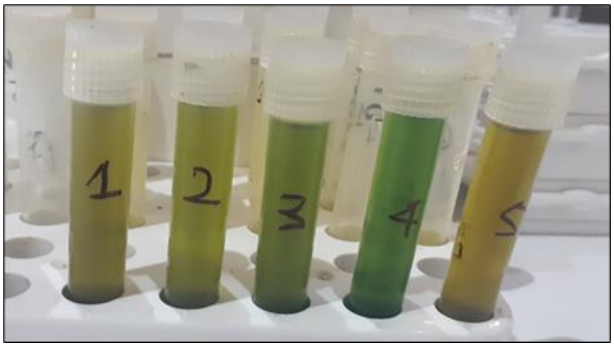

Fig 1: Silkworm droppings extract

Determination of Chlorophyll

The chlorophyll content in silkworm droppings was estimated by the method of Arnon (1949) [2]. Using the absorption coefficients, the amount of chlorophyll was calculated. 1 g of SWD powder was weighed and mixed thoroughly with 20 ml of 80% acetone. The solution was centrifuged at 5000 rpm for 5 minutes and the supernatant was transferred to 100ml volumetric flask. The procedure was repeated until the residue was colorless. The mortar and pestle were washed thoroughly with 80% acetone and the washings were collected in the volumetric flask. The volume was made up to 100 ml with 80% acetone. The absorbance of the solution was recorded at 645, and 663 nm against the solvent (80% acetone) blank. The amount of chlorophyll present in the extract mg chlorophyll per g tissue was calculated using the following equations.

Mg chlorophyll a/g = 12.7 (A_{663}) – 2.69 (A_{645}) x V/1000xW
Mg chlorophyll b/g = 22.9 (A_{645}) – 4.68 (A_{663}) x V/1000xW
Mg total chlorophyll = 20.2 (A_{645}) + 8.02 (A_{663}) x V/1000xW

Where
A= Absorbance at specific wavelength
V= Final volume of chlorophyll extract 80% acetone
W= Fresh weight of tissue extracted

Determination of total polyphenolic content (TPC)

TPC of SWD was determined by using the Folin–denis colorimetric method described by Kim *et al*., (2009). About 100µl of the silkworm droppings extract was mixed with 2ml of 2% Na_2CO_3 and 100µl of 50% Folin- Ciocalteu reagent (FCR). The mixture was left for 30min to react at room temperature and absorbance was measured at 720 nm using Hitachi U-1800 UV-VIS spectrophotometer. A calibration curve of standard reference was established using Gallic acid. TPC was revealed as Gallic acid equivalents in milligrams per gram of dry weight (mgGAE/g DW).

Determination of total flavonoid content (TFC)

TFC of SWD was determined by aluminum chloride colorimetric assay by Atanassova *et al.*, (2011) [3]. 1 ml of silkworm droppings extract (50mg/ml) was added with 4ml of distilled water in test tubes. 0.3ml of 5% $NaNO_2$ was added and the mixture was allowed to react for 5 min at room temperature followed by addition of 0.3ml of 10% $AlCl_3$. The mixture was again left to react for 6 minutes at room temperature. Further, 2ml of 1M NaOH was added and final volume was made up to 10 ml by distilled water. Absorbance was taken at 510nm using Hitachi U-1800 UV-VIS spectrophotometer. The total flavonoid content was measured from the standard Quercetin curve (0-1mg/ml). TFC was revealed as quercetin equivalent in milligram per gram dry weight (mgQE/g DW).

Determination of DPPH radial scavenging activity

The DPPH free radical scavenging activity was analyzed by the method described by Nithiananthian *et al*., (2011) [20] with slight modification. Different concentrations (6.25, 12.5, 25 and 50 mg/ml) of silkworm droppings extract were taken for analysis. 2 ml of DPPH (0.004%) solution was added to 2ml of extracts of each concentration. In case of control, 2ml of silkworm droppings extract was replaced by 80% ethanol. All the mixtures were mixed and left to incubate in dark for 30 minutes at room temperature. Absorbance was measured at 517nm on UV/VIS spectrophotometer (Hitachi U-1800) and scavenging activity percentage was calculated as follows: -

I % = [(Ao – As)/Ao] x 100

Where,
Ao represent the absorbance value of the control reaction.
As represent the absorbance value of the sample extract.
I% represent the percentage inhibition.

Statistical analysis

Each experiment was carried out in triplicate. The data was analyzed by O.P Stat software and expressed as means ± standard deviation (SD). Results were considered significant at $p< 0.05$.

Results and Discussion

Table 1: Chlorophyll A, B and Total chlorophyll content in different instars of SWD

Instar droppings	Chlorophyll A (mg/g)	Chlorophyll B (mg/g)	Total Chlorophyll (mg/g)
1st Instar	1.397	0.442	1.839
2nd Instar	1.855	0.538	2.393
3rd Instar	1.802	0.626	2.428
4th Instar	2.608	1.001	3.609
5th Instar	1.92	0.708	2.628
CD	0.002	0.002	0.002
SE(d)	0.001	0.001	0.001
SE(m)	0.001	0.001	0.001
CV	0.053	0.170	0.038

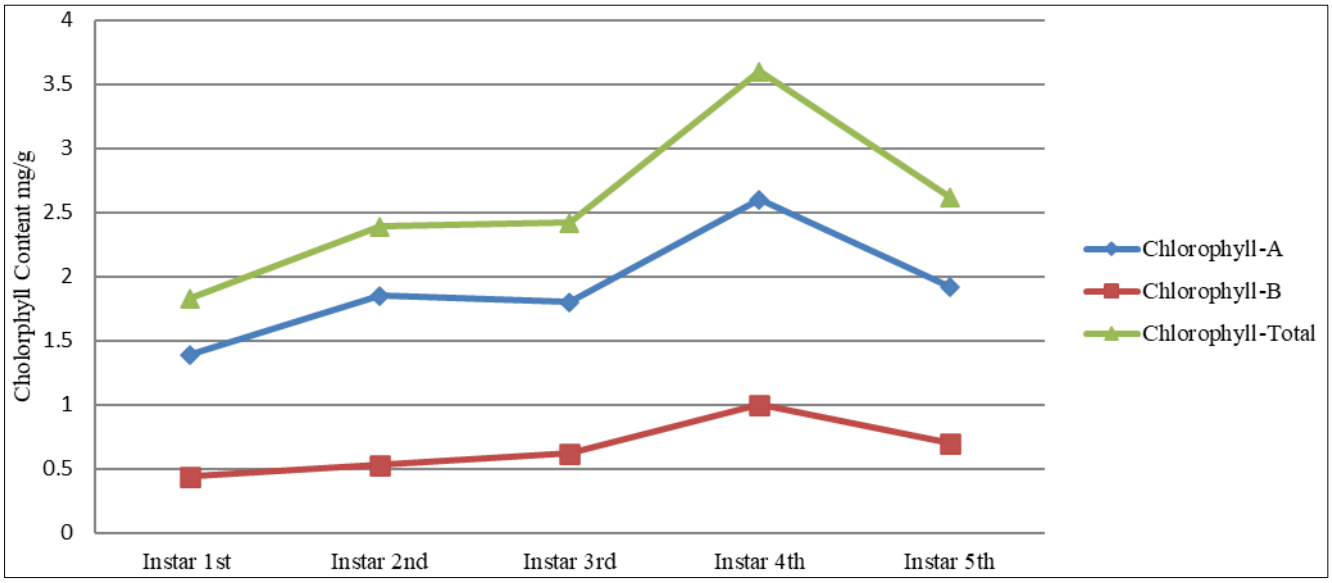

Fig 2: Graphical representation of Chlorophyll content in different instars of SWD.

Results with regard to Chlorophyll content estimated in the SWD of 5 different larval stages are presented in table 1 and graphically represented in figure 2. The Chlorophyll content (a, b and total) showed an increasing trend from 1st to 4th instar SWD in the range from 1.397 to 2.608, 0.442 to 1.001 and 1.839 to 3.609mg/g DW respectively. Where as in 5th instar the Chlorophyll content (a, b and total) showed slight decreasing trend in range of 1.92, 0.708 and 2.628 mg/g DW respectively. Chlorophyll content in SWD of different instars is surmised to correspond with the type of leaf (coarse, medium or tender) fed to the silkworms. Murthy *et al.*, (2013) reported that medium leaf contains highest chlorophyll followed by coarse and tender.

Table 2: Polyphenol and Flavonoid content in different instars of SWD

Instar droppings	Concentration 50mg/g	
	Polyphenol (mgGAE/g)	Flavonoid (mgQE/g)
1st Instar	3.108	19.097
2nd Instar	2.13	7.151
3rd Instar	1.399	7.036
4th Instar	0.599	6.835
5th Instar	0.510	4.07
CD	0.013	0.002
SE(d)	0.006	0.001
SE(m)	0.004	0.001
CV	0.378	0.010

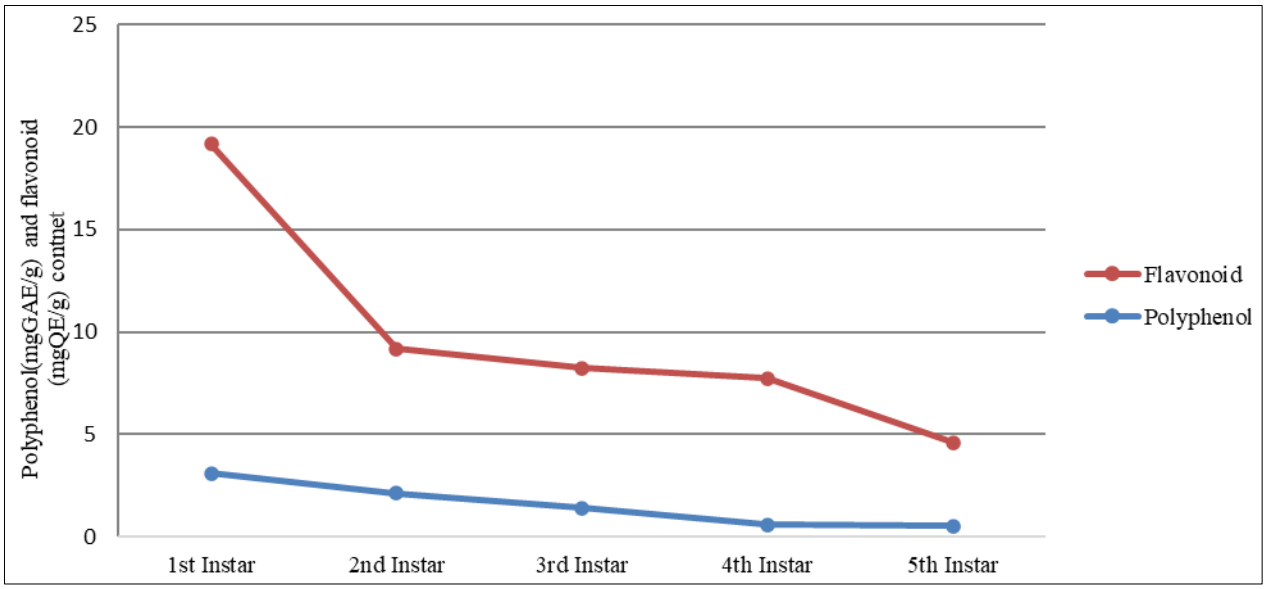

Fig 3: Graphical representation of Polyphenol and Flavonoid content in different instars of SWD.

Polyphenols and flavonoids have good pharmaceutical value. SWD have good amount of this content as 60% of mulberry leaves are egested with little transformation. Results with regard to TPC and TFC in SWD are presented in table 2 and figure 3. The results for TPC were derived from a calibration curve ($y = 0.0033x + 0.2405$, $R^2 = 0.9949$) of gallic acid (0–500µg/ml) and expressed in gallic acid equivalents (GAE) per gram dry extract weight. Whereas, in case of TFC determination the results were derived from the calibration curve ($y = 0.0031x + 0.0223$, $R^2 = 0.9984$) of quercetin (0–100 mg/L) and expressed in quercetin equivalents (QE) per gram dry weight.

In the present study the TPC and TFC showed decreasing trend in the SWD collected from 1st to 5th instar larvae from

3.10 to 0.51 mgGAE/g and 19.09 to 4.07mgQE/g of DW. Phenolic compounds have redox properties and the properties which allow them acting as antioxidants (Soobrattee *et al.*, 2005 and Shoib and Shahid 2015) [25, 23]. Presence of polyphenolic compounds such as chlorogenic acid, caffeic acid, rutin and quercetin etc. strengthens its anti-diabetic property (Manaharan *et al.*, 2012) [16].

TFC in SWD also depends on the type of feed given to the silkworms. TFC vary from variety to variety. SWD are rich in flavonoids as reported by Liu *et al.*, (2007) [15], in the range of 15.025 ± 0.050mg/g in ethanol extract. Our results are in agreement with the results obtained by Park *et al.*, (2011) [21]. They put forth increased expression of heme-oxygenase in HepG2 cells owing to the presence of flavonoids in SWD extract. Thabti *et al.*, (2011) [26] recorded the total flavonoid content of 440.5 to 789.7 mg RE/100g DM of mulberry leaf. Katsube *et al.*, (2006) [11] reported that plants of the genus morus are known to be rich in flavonoids. Furthermore, flavonoids have shown to decrease blood glucose levels (Yang *et al.*, 2012) [28]. Flavonoid rich products can have good pharmaceutical use.

Table 3: DPPH radial scavenging activity (%) in different instars of SWD

Instar Droppings	Scavenging (%)				
	6.25 (mg/ml)	12.50 (mg/ml)	25 (mg/ml)	50 (mg/ml)	Mean
1st Instar	49.64	70.94	82.05	87.46	72.52
2nd Instar	49.01	64.95	80.34	85.47	69.94
3rd Instar	48.43	63.81	77.77	79.77	67.44
4th Instar	37.03	62.96	75.48	78.77	63.56
5th Instar	32.47	57.83	72.77	76.03	59.77
Mean	43.31	64.09	77.68	81.50	
Factors			C.D.	SE(d)	SE(m)
Instar (A)			0.0130	0.0065	0.0046
Concentration(B)			0.0117	0.0058	0.0041
Instar x Concentration (A x B)			0.026	0.0129	0.0091
Superscript (a, ab, b) signify levels of significance by DMRT (1955).					

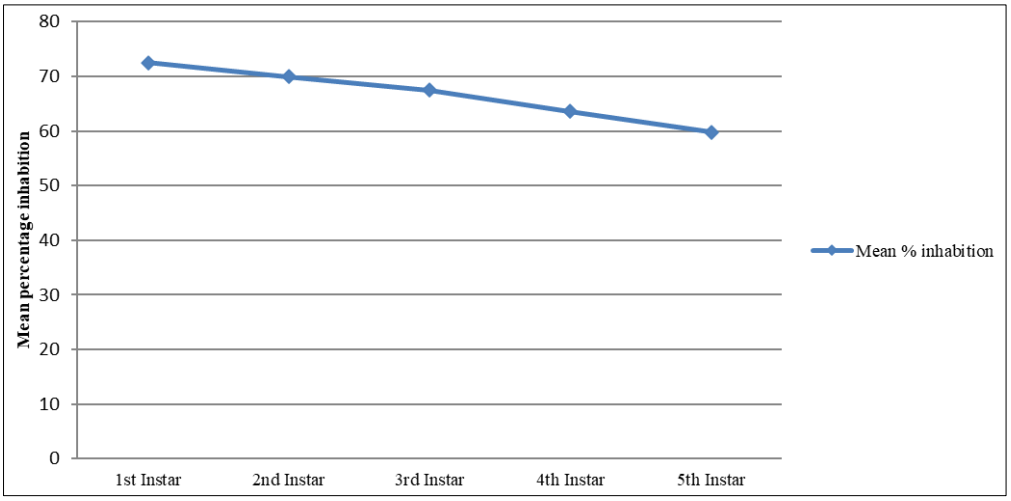

Fig 4: Graphical representation of Mean % Inhibition in different instars of SWD.

Results with regard to scavenging activity of SWD are presented in table 3 and figure 4. Scavenging activity was directly proportional to the concentration of the sample taken. Highest scavenging activity was observed at 50mg/ml sample. Further, the scavenging activity showed decreasing trend in the SWD samples collected from 1st to 5th instar larvae from 87.46% to 76.03% respectively. Antioxidant free radical scavenging activity works on the principle on one-electron reduction activity of antioxidants. Antioxidant activity has the directly proportional relationship with the TPC and TFC. Flavonoids are known to have potential antioxidant properties and probable roles in preventing oxidative stress associated diseases (Haminiuk *et al.*, 2012) [7]. Literature shows capacity of the antioxidant is highly associated with the total flavonoid content and total phenolic compounds of the plant leaves crude extract (Sim *et al.*, 2010; Mustafa *et al.*, 2010 and Hassanbaglou *et al.*, 2012) [24, 18, 8]. Ju *et al.*, (2014) [10] observed that the capability of scavenging DPPH free radical of silkworm droppings extracts from different solvents was the highest (80%) in water ultrasonification extraction (WUE). DPPH free radical scavenging of aqueous and ethanol extracts of SWD upto 80% was reported by Xu *et al.*, (2014) [27].

Conclusion

This study observed the instar specific contents of bioactive ingredients and their corresponding activities. From the study, 1st instar droppings shows high concentration of TPC and TFC, hence shows more antioxidant activity as compared to other four instars. Chlorophyll content of chlorophyll-a, chlorophyll-b and total chlorophyll was found to be maximum in 4th instar SWD. It is because of feeding the fully grown chlorophylls leaves to the silkworms. SWD apart of being used as manure, fish food, filtered spirit drinks, poultry and animal feed can serve as a good source of dietary antioxidant. It contains promising amount of phenols, flavonoids and therefore can become promising material for the pharmaceutical and nutraceutical industries.

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