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Extraction and characterization of three different species of silkworm pupae oil of Indian origin

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Abstract
Silkworm is generally used for producing silk yarn. Three species of silkworm such as Mulberry silkworm (*Bombyx mori*), Eri silkworm (*Philosamia ricini*), and Muga silkworm (*Antheraea assamensis*) were used to extract oil from the pupae and were characterized for their quality and fatty acid profile analysis. After reeling silk live pupae are discarded and are considered as waste material. Raw silk cocoons consist of exuvia, pupae, silk fibre, and cocoon. On average, silkworm pupae weigh about 60-70% weight of their total cocoon. The proximate composition analysis, rate of oxidation activity, colour values, and also the fatty acid composition of three different varieties of silkworm pupae revealed that they are rich in protein ranging from 50.23±0.02-45.80±12 g/100 g, fats (30.21±0.04-26.30±0.26 g/100 g) low source of CHO (12.02±0.04-10.12±0.02 g/100 g) and ash (5.30±0.03-4.60±0.03 g/100 g). The iodine value, peroxide value, and acid value of silkworm oil reveal that it has good oxidative stability and among the three species mulberry silkworm has better oil quality oil characteristics & yield were extremely compared to others. The colour values reveals that the L, a, and b values were similar to the edible oils. The fatty acid profile indicates that silkworm pupae oils are a rich source of omega 3 and omega 6 fatty acids. Especially linolenic acid and mulberry silkworms were found to have the highest value (60.71%) followed by Eri (57.9%) and Muga (54.66%) respectively. This study provides information on the quality of three species of silkworm pupae oil from Indian origin.

Keywords: Silkworm pupae, fatty acids, oil quality, linolenic acid

1. Introduction
To meet the growing demand of the population at present and for future generations and to achieve food security cheaper sources of protein and oil are essential. The theme of world food day 2022 states “Leave No One Behind” for better production, better nutrition, better environment, and better life. Alternative sources to feed the world’s population is the need of the hour. Silkworm pupae can be the best alternative source as an edible insect which is considered waste material.

Silkworm is generally used for producing silk yarn. The major four species of silkworm are Mulberry, Eri, Muga and Tassar silkworm. After reeling silk, the pupae are being considered as waste material. As per the report nearly 34,903 MT of raw silk was produced during 2021-2022 among which 25,818 MT was mulberry, 1,466 MT was tassar, 7,364 MT was Eri and 255 MT was muga silkworms (Source: Central Silk Board, Bengaluru, India). Raw silk cocoon consists of exuvia, pupae, silk fibre, and cocoon. On an average silkworm pupae weigh about 60-70% weight of their total cocoon. Silkworm pupae are rich in protein, lipids, and minerals. Sunflower oil, groundnut oil, mustard oil, and olive oil are commonly used as cooking oil. Tran’s fats and saturated fatty acids are harmful to human health. Hence alternative edible oil source that has essential fatty acids like Poly Unsaturated Fatty Acids (PUFA) and MUFA (Mono Unsaturated Fatty Acids) such as linoleic acid, linolenic acid, docosahexaenoic acid (DHA) is in greater demand. Unsaturated fatty acids has positive impact on human, reduces the heart cholesterol level and heart diseases. Longvah *et al*., 2012 [9] reported that Eri silkworm pupae have high content of alpha-linolenic content reduces serum cholesterol levels in different organs. Silkworm pupa contains good amount of carotenoids like lutein, neoxanthin and also possess good oxidative stability (Kotake-Nara, Yamamoto, Nozawa, Miyashita, & Murakami, 2002) [6]. *Bacillus subtilis* and *Staphylococcus aureus*, which are gram-positive bacteria, reacted to the antibacterial effects of silkworm (*Bombyx mori*) pupae oil as reported by Saviane *et al*., 2021 [14].

The main objective of this research is to extract and characterize silkworm pupae oil from different species of silkworms like Mulberry, Eri and Tassar grown across India and to use

silkworm pupae which are considered as waste material to utilize it as a fat replacer in future to reduce the burden on ecosystem.

2. Materials and Methods

2.1 Sample procurement

Mulberry silkworm cocoon procured from silk rearing farmer's cooperative society® Chintamani taluk, Karnataka, India. Eri and Muga silkworm cocoons received from silk-rearing farmers of Assam state in India. Live silkworm pupae were separated by cutting open of cocoons and then kept at-14 ℃ in freezing condition for slaying. Then silkworm pupae were kept in freeze drier at -80 ℃ for 48 hours for complete drying. Dried silkworm pupae pulverized for making into powder form and packed in HDPE with seal and kept in refrigeration temperature at 4 ℃ for further experimental analysis.

2.2 Proximate analysis

Protein content was determined by using nitrogen conversion factor (6.25 x N) from AOAC Kjeldahl method (984.13), moisture content, ash, fat, crude fibre of silkworm pupae powder determined using AOAC methods 934.01, 942.05, 920.39, 962.09 respectively.

2.2 Extraction of oil using the soxhlet method

Three species of dried silkworm pupae powder (100 g) were sealed in extracting paper thimble and placed into extraction chamber with round bottom flask then 300 ml of N-hexane (99.9% purity used as solvent and closed with condenser connected to inlet and outlet water supply. Whole setup kept in heating mantle and temperature set at 70 ℃ then ran for 6 hours. After that N-hexane was evaporated in water bath at 90 ℃ then oil was filtered in Whatman filter paper and sealed in 30 ml glass bottle and kept refrigerated condition for further analysis.

2.3 Acid value

Acid value was determined using 5g of oil dissolved in ether and ethanol (2:1, v/v) with alcoholic potassium hydroxide (KOH) then added 100µL of 1% phenolphthalein as an indicator.

$$\text{Acid value(mgKOH/g)} = \frac{\text{Titre value} \times \text{Normality of KOH} \times 56.1}{\text{Weight of the sample(g)}}$$

2.4 Iodine value

Iodine concentration was determined by adding 25ml of Wijs iodine solution to 0.5g of oil that has been dissolved chloroform (10ml). The mixture was combined with 20 ml of potassium iodide 20% solution and distilled water (100ml) after being kept in the dark for 30 minutes. As an indication, it was titrated against 0.1N sodium thiosulfate and 1% starch.

$$\text{Iodine number} = \frac{(B-S) \times N \times 12.69}{\text{g sample}}$$

Where,
B: Blank (ml thiosulfate).
S: Sample (ml thiosulfate).
N: Thiosulphate normality.

2.5 Peroxide value

To determine peroxide values, 2g of oil were dissolved in 25 ml of chloroform and acetic acid in a 2:3 by volume ratio, together with 1 ml of a potassium iodide solution which is saturated and left in the dark for ten minutes. Then 30 ml of distilled water added with 1% starch solution, then the mixture titrated with sodium thiosulfate (0.01N) until the blue colour disappeared.

2.6 Saponification value

Saponification value of pupae oil was determined by mixing 5 g of an oil sample with 50 ml of alcoholic KOH, then dissolving it and connected the flask to air condenser, gently boiled about 1hr. Oil was cooled and condensed and 1 ml of 1% phenolphthalein indictor was added and titrated against 0.5N Hydrochloric acid (HCl) till the pink colour disappears and same was done for blank also.

$$\text{SV} = \frac{28.05 \times (\text{titrate value of blank} - \text{titrate value of sample})}{\text{weight of the sample(g)}} \times 100$$

2.7 Antioxidant activity, specific gravity and refractive index

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) used to determine the antioxidant activity of the extracted oil which stable radicle with absorbance of 517 nm that can be simply lowered by an antioxidant molecule. It is frequently used to examine the ability of hydrophilic and lipophilic substances to scavenge radicals. (Bakhshabadi *et al*., 2017) [16]. About 0.8 g of sp in 25 ml of the test tube with 4.0 ml of 0.4 g/L 2-diphenyl-2-picrylhydrazyl was added. The oil sample was then vortexed and incubated for 15 minutes in the dark at 30±2 °C ambient temperature. The sample data were obtained in a UV spectrophotometer at 517 nm absorbance. Instead of the oil sample, 0.82 ml of ethyl acetate was utilised for the blank readings (Naik *et al*., 2021) [17].
The refractive index of the extracted SPO was determined using an Abbe refractometer and the specific gravity was determined in a 50 ml Pycnometer bottle. It should be noted that measurements were made at room temperature or 25 °C.

2.8 Colour value

The silkworm pupae powder colour parameters (L, a, b) were calculated using a Hunter colour flex model. It works on the principle of collecting the light and measures energy from sample reflected across the entire visible spectrum. Each time the equipment was standardized with standards like white and black. Samples were scanned to determine the L*, a* and b* where L* indicates lightness and darkness, a* indicates red (+a) and green (-a), b* indicates blue (+b) and yellow (-b).

2.9 Fatty acid profile

To study the fatty acid composition, oil was first transformed into FAME (Fatty acid methyl esters). A sample of 500 µl silkworm pupae oil was taken and 2 ml of dichloro methane (DCM) and sodium methoxide were added. The sample was then vortexed for 2 min, and it was then kept in a water bath at 40 °C until it evaporated up to 95%. The sample is then cooled, 5 ml of distilled water is added then vortexed for another 2 to 3 minutes. The top layer supernatant is then collected and the sample is then injected into the GC unit. GC Column Rtx-5MS equipment 8890GC/5977B GC/MSD-Agilent. Carrier gas 1ml/min with single quadrupole mass spectrometer detector used then 2 µl sample injected with Oven temperature-110 °C and raised up to 200 °C at the rate

of 10 °C/min-no hold up to 280 °C. The mass spectrometer programme was established using the NIST Version 2020 library. The electron energy was 70 eV, the source temperature was 250 °C, the inlet line temperature was 290 °C and the entire run time was around 40 minutes.

2.10 Statistical analysis
Using ANOVA and grouping data from the Turkey test, all the raw data were entered into the Minitab programme to establish the significance of the differences between three samples was done using a boxplot and an interval plot, respectively.

3. Results and Discussion
3.1 Proximate composition of three different silkworm pupae
Proximate composition of the three different silkworm pupae were analysed using AOAC method and the results are presented in Fig. 1. Among the three silkworm pupae (Mulberry, Eri and Muga) Muga was found to have maximum protein content of 50.23±0.02 g per 100 g followed by Eri 49.42±0.14 g/100 g and Mulberry 45.80±12 g/100 g respectively. Mulberry silkworm pupae has good amount of fat 30.21±0.04 g/100 g followed by eri 27.30±0.34 g/100 g and muga 26.30±0.26 g/100 g respectively. Fat composition indicates that pupae may has very good profile of fatty acid profile which can also include omega 3 and omega 6 fatty acids. There is no significant difference found in moisture and ash content of silkworm pupae i.e., 6.56±0.03% (Mulberry), 7.03±0.06% (Eri) and 6.92±0.01% (Muga) respectively. The ash content was found maximum in Eri 5.30±0.03 g/100 g followed by Muga 4.83±0.04 g/100 g and Mulberry 4.60±0.03 g/100 g respectively. For the mulberry 12.02±0.04 g/100 g, eri 10.12±0.02 g/100 g and muga 10.80±0.02 g/100 g were found to be low in carbohydrates and also crude fibre present in minimum quantity in muga 0.92±0.01 g/100 g followed by eri 0.83±0.02 g/100 g and mulberry 0.808±0.01 g/100 g respectively. The study conducted by (Longvah *et al*., 2011) [10] is creditable with our study.

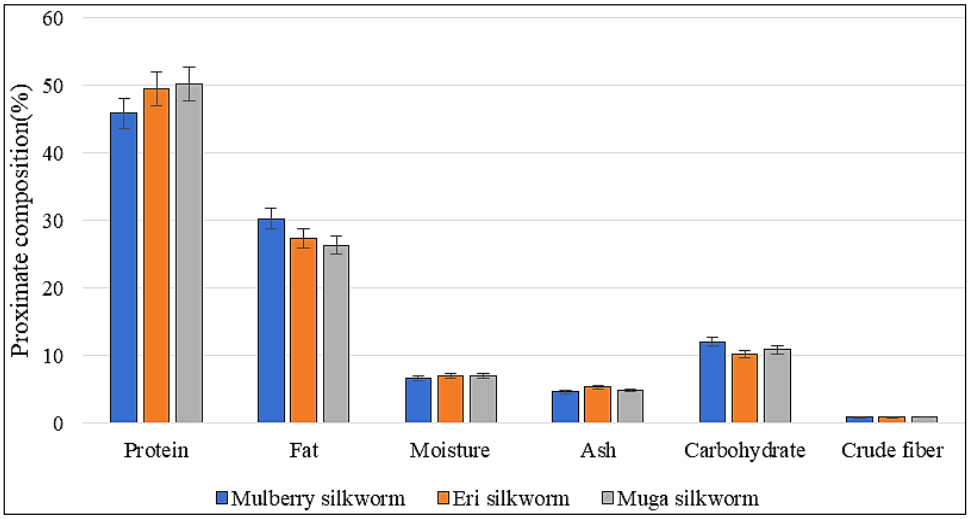

Fig 1: Proximate of silkworm pupae powder (Mulberry, Eri and Mug

3.2 Oil quality characterization for different species of silkworm pupae
The extraction yield (%) of oil from all the three varieties of silk worm was analysed. The acid value, iodine value, peroxide value, saponification value, antioxidant activity, specific gravity and refractive index for three different silkworm pupae is the indicator of the rancidity of the samples are shown in Table 1.

3.2.1 Extraction yield
Silk worm pupae oil extraction yield ranged from (25%) to (29%), doesn't show significantly (p>0.05) differences between the three different varieties of silk worm pupae oil samples. Mulberry silkworm pupae oil has more extraction yield about 29.00±1.15^{a} followed by 27.51±0.71^{b} eri and 25.50±0.57^{b} muga.

3.2.2 Specific gravity
Specific gravity of all the three varieties of silk worm had no much significant difference. Specific gravity values were 0.93±0.00^{b} for eri followed by 0.92±0.00^{a} mulberry and 0.89±00^{b} muga silkworm pupae oil. According to reports, the average specific gravity values for some vegetable oils were 0.9193 for soybean oil, 0.9188 for corn oil and 0.9073 for rapeseed oil (Noureddini *et al*., 1992) [11]. Apparently, the percentage of USFA (unsaturated fatty acids) and SCFA (short chain fatty acids) in oil frequently affects its specific gravity. (Han *et al*., 1991) [4]

3.2.3 Saponification and Iodine value
The amount of potassium hydroxide needed to saponify 1 g of oil is described as saponification value. High levels of saponification indicate the presence of short-or medium-chain fatty acids (Predojević, 2008) [13]. The saponification value of all the three varieties of silkworm pupae are as follows Eri (210.69±0.25^{a}) followed by Muga (205±0.12^{b}) and Mulberry (191.3±0.3^{c}) there was slight differences in the saponification value. When compared to other edible oils, the saponification values of all three types of silkworm pupae oil were quite comparable with other oils. Safflower seed oil and palm oil have a respective saponification value of 190.23 mg/g and 205.00 mg/g. (Gopinath *et al*., 2009) [3]. The iodine value of all the three varieties of silkworm pupae were 118.26±0.78^{c} for eri followed by 113.93±0.55^{b} for mulberry and 90.90±0.36^{a} for muga. Sunflower seed oil showed a similar tendency, which can be attributed to a decrease in unsaturated

areas as a result of oxidation and polymerization. (Anjum *et al*., 2006) [1].

3.2.4 Acid value and peroxide value

The acid value had no much significant difference in all the three samples *i.e.*, Muga (3.83±0.17b) followed by Eri (3.21±0.21b) and Mulberry (2.93±0.15a) respectively. The acid value in oil is an extremely important indicator for edible oils that assess the amount of free fatty acids present in the oil (Skiera *et al*., 2012) [15]. Oils were extracted from three different varieties silkworm pupae that were freeze dried. Hydrolysis of oil might doesn't occur due to a less water (Kim *et al*., 2015) [5] the peroxide value indicates the rate of rancidity of the oil. The peroxide value of all the three samples of silkworm pupae oil were Eri (11.89±0.45b) followed by Muga (11.83±0.25b) and Mulberry (11.36±0.20a), respectively. Low peroxide value indicates that the oil was free from rancidity as reported by (Läubli & Bruttel, 1986) [7].

3.2.5 Antioxidant activity

There are no significant differences between antioxidant activity of three different silkworm pupal oil (µmol/g) which are tabulated as Eri (23.63±0.15b) followed by Mulberry (22.56±0.25a) and Muga (21.6±0.15c) respectively. According to (Liawsakul *et al*., 2002) [8], based on his study he reported that, the antioxidant tocopherol is present in the silkworm pupae oil. Additionally, as antioxidants in the oils, phospholipids and tocopherol found in silkworm pupae oil may also have significant role in preventing oxidation of lipids, particularly linoleic acid (Kotake-Nara, Yamamoto, Nozawa, Miyashita, & MURAKAMI, 2002) [6].

3.2.6 Refractive Index

It is the optical parameter to estimate the light rays traverse through the medium of material. Refractive index depicts the chances of development of rancidity of the oil. Higher the values of refractive index, higher will be the spoilage chances of oil due to oxidation. The refractive index analysed for all the three varieties of the silkworm pupae oil indicated similar values with no much deviation and the average value is 1.474 ± 0.00^{c} which was similar to sunflower and rapeseed oil 1.45 (Pereira *et al*., 2003) [12].

Table 1: Oil quality characterization for different species of silkworm pupae

Paraments	Mulberry silkworm	Eri silkworm	Muga silkworm
Extraction yield (g)	29.00 ± 1.15^{a}	27.51 ± 0.71^{b}	25.50 ± 0.57^{b}
Acid value (mg KOH/g)	2.93 ± 0.15^{a}	3.21 ± 0.21^{b}	3.83 ± 0.17^{b}
Iodine value	113.93 ± 0.55^{b}	118.26 ± 0.78^{c}	90.90 ± 0.36^{a}
Peroxide value (mEq/kg)	11.36 ± 0.20^{a}	11.89 ± 0.45^{b}	11.83 ± 0.25^{b}
Saponification value	191.3 ± 0.3^{c}	210.69 ± 0.25^{a}	205 ± 0.12^{b}
Antioxidant activity (µmol/g)	22.56 ± 0.25^{a}	23.63 ± 0.15^{b}	21.6 ± 0.15^{c}
Specific gravity (g/ml)	0.92 ± 0.00^{a}	0.93 ± 0.00^{b}	0.89 ± 00^{b}
Refractive index	1.473 ± 0.00^{c}	1.479 ± 0.00^{a}	1.475 ± 0.00^{b}

All values are mean ± standard deviation of three replicates in case of yield.
Letters in the column (yield) represents statistically significant differences ($p<0.05$).

3.3 Colour values

The silkworm pupae powder colour parameters (L, a, b) were calculated using a Hunter colour flex model. It operates on the theory of absorbing light and measuring energy from a sample that has been reflected throughout the whole visible spectrum. Each time the equipment was standardized with standards like white and black. Samples were scanned to determine the L*, a* and b* where L* indicates lightness and darkness, a* indicates red (+a) and green (-a), b* indicates blue (+b) and yellow (-b). The colour values assessed and tabulated in Table 2. shows that the oil with L value was light in colour in all the three silkworm varieties (Muga: 8.81, Eri: 8.21 and Mulberry: 7.86) followed by a parameter indicated by slightly negative value (Eri: -0.33, Muga: -0.32 and Mulberry: -0.26) resulting in slightly green tinge and b parameter having positive values (Eri: 4.23, Muga: 3.89 and Mulberry: 3.66) indicates as the oil is yellow in colour. Overall, the colour assessment reveals that the oil was of good quality.

Table 2: Colour values of three different silkworm pupae powder

Colour value	L	a	b
Mulberry	7.86	-0.26	3.66
Eri	8.21	-0.33	4.23
Muga	8.81	-0.32	3.89

3.5 Fatty acid profile of the three different silkworm pupae oil

The fatty acid profile of the three different varieties of silkworm pupae oil is tabulated in Table 3 reveals that the analysed samples are good source of omega 3 and omega 6 fatty acids with are unsaturated fatty acids. Mulberry has very good source of linolenic acid (60.71%) followed by Eri (57.9%) and Muga (54.66%) respectively. It also contains Hexadecenoic acid Palmitic acid which is a reflection of good source of Vitamin A due to the presence of carotenoids as it acts as an antioxidant, as a righteous agent to scavenge oxidative activity. All three varieties had no significant difference in Hexadecanoic acid-Palmitic acid value (Muga: 27.75%, Eri: 26.27% and Mulberry: 25.57%). Silkworm pupae oil were quite rich in linolenic acid when compared to sunflower oil (38.41%) and soya (54.21%) (Fu *et al*., 2016). The linolenic acid has the therapeutic properties to cure coronary heart diseases (Han *et al*., 1991) [4].

Table 3: Fatty acid profile of the three different silkworm pupae oil

Sl. No.	Fatty acid compounds	Formula	Peak area % Mulberry silkworm	Peak area % Eri silkworm	Peak area % Muga silkworm
1.	Hexadecenoic acid-Palmitoleic acid	$C_{17}H_{32}O_2$	1.02	1.63	1.87
2.	Hexadecanoic acid-Palmitic acid	$C_{17}H_{34}O_2$	25.57	26.27	27.75
3.	Octadecadienoic acid-Linoleic acid*	$C_{19}H_{34}O_2$	8.63	5.9	5.23
4.	Octadecatrienoic acid-Linolenic acid**	$C_{19}H_{32}O_2$	60.71	57.9	54.66
5.	Methyl stearate	$C_{19}H_{38}O_2$	7.07	8.3	9.49

4. Conclusion

Among three silkworm's mulberry silkworm pupae oil contains high oil quality characteristics followed by eri and muga silkworm. Mulberry silkworm pupae constitutes about 75% (PUFA and MUFA) which is considered as healthy edible oil. This can be an alternate fat replacer for many edible oils and also fat based processed products like junk foods that are considered as unhealthy. Inclusion in silkworm oil in regular diet may combat many cardiovascular disease and diabetes. Silkworm pupae oil helps to combat obesity as it is having high density lipoprotein it is considered as good fat. Since silkworm pupae are already being used in pharmaceutical and cosmetic industries but correspondingly it has edible oil characteristics so it can be used as an alternate source of edible oil/fat in future which generally produced as the byproduct of silkworm. It is possible to produce superior quality of oil from silkworm pupae which is also considered as healthy oil.

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