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Research Article

Tasar silkworm pupal oil: An excellent source of edible oil for industrial and therapeutic applications

Devika Srivastava, Vandana Singh, and Venkatesh K. Ramappa*

Department of Zoology, Babasaheb Bhimrao Ambedkar University, Lucknow, India-226014

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*Address for Correspondence:

Prof. Venkatesh Kumar R, Department of Zoology, Babasaheb Bhimrao Ambedkar University, Lucknow-226014, Uttar Pradesh, India

Abstract

Polyunsaturated fatty acids are widely recognized due to their remedial properties. In our previous studies, we reported that Tasar silkworm pupal oil (TSPO) is rich in polyunsaturated fatty acids, particularly alpha-linolenic acid (ALA). Further we also extracted ALA from TSPO and reported its significant anticancer activity. In the present study, extraction of TSPO and physico-chemical characterizations including saponification, acid, peroxide, iodine pH values, and colour determination were carried out to investigate the edible properties of TSPO for commercialization as a therapeutically active functional food. Further, the anti-diabetic activity of TSPO were analyzed by α-amylase inhibition assay. The saponification (154.75 ± 0.581mg KOH/g), acid (6.24 ± 0.421 mg KOH/g), peroxide (16.20 ± 0.265 meq/kg), iodine (40.33 ± 0.577g/100 ml), pH (6.17), and colour (14 Y+1.2 R) values were within the permissible range for commercialization. In addition to this, the results of the α-amylase inhibition assay showed that TSPO exhibited an IC_{50} value of 207339 µg/ml. The results may facilitate the use of TSPO oil in therapeutic and functional food applications

Keywords: Characterization; Commercialization; Tasar pupal oil; Silkworm pupae.

1. INTRODUCTION

High-saturated fat oils are associated with several human chronic diseases, including cancer and cardiovascular disorders.[1, 2] Polyunsaturated fatty acids (PUFAs), particularly ω-3 fatty acid-based oils, have created significant progress in human health due to their therapeutic nature and have the potential to lower low-density lipoprotein (LDL) and enhance the high-density lipoprotein (HDL) cholesterol levels.[3, 4] Fish oil and a few plants are rich sources of ω-3 fatty acids, specifically eicosapentaenoic (EPA), docosahexaenoic (DHA), and alpha-linolenic acid (ALA), respectively.[5-8] Among insects, silkworm pupae are the greatest source of omega-3 fatty acids, particularly linolenic and alpha-linolenic acids. The three primary components of the total lipid recovered from silkworm pupae are triacylglycerol, phosphatidylethanolamine, and phosphatidylcholine.[9] The oils extracted from silkworm pupae have drawn tremendous attention due to their compositions of fatty acids[10], particularly polyunsaturated fatty acids that makeup around 51.64 percent of total unsaturated fatty acids in silkworm pupal oil.[11,12] The different species of mulberry and non-mulberry silkworm pupae are known sources of oil.[13] However, most of the species of silkworm are yet to be exploited as a source of oil through the extraction of omega-3 fatty acids, especially ALA. The silkworm pupal oils are witnessed to have several bio-activities, namely anti-oxidants,[14] anticancer,[15] vasculoprotective,[16] antibacterial, [17] hypercholesterolemic,[14] and antiulcerative (Long et al., 2019)[18] **(Fig. 1)**. Much work has not been done on tropical Tasar silkworm (*Antherea mylitta*) pupae when compared to mulberry, eri, and muga pupae. In our preliminary study, we reported that tasar pupal oil is a rich source of alpha-linolenic acid (ALA) (37%),[19] which is an omega-3 essential fatty acid; therefore, It may prove to be a more beneficial substitute for edible oil. For this reason, in the current effort, we have extracted and characterised tasar pupae oil for commercial and therapeutic uses.

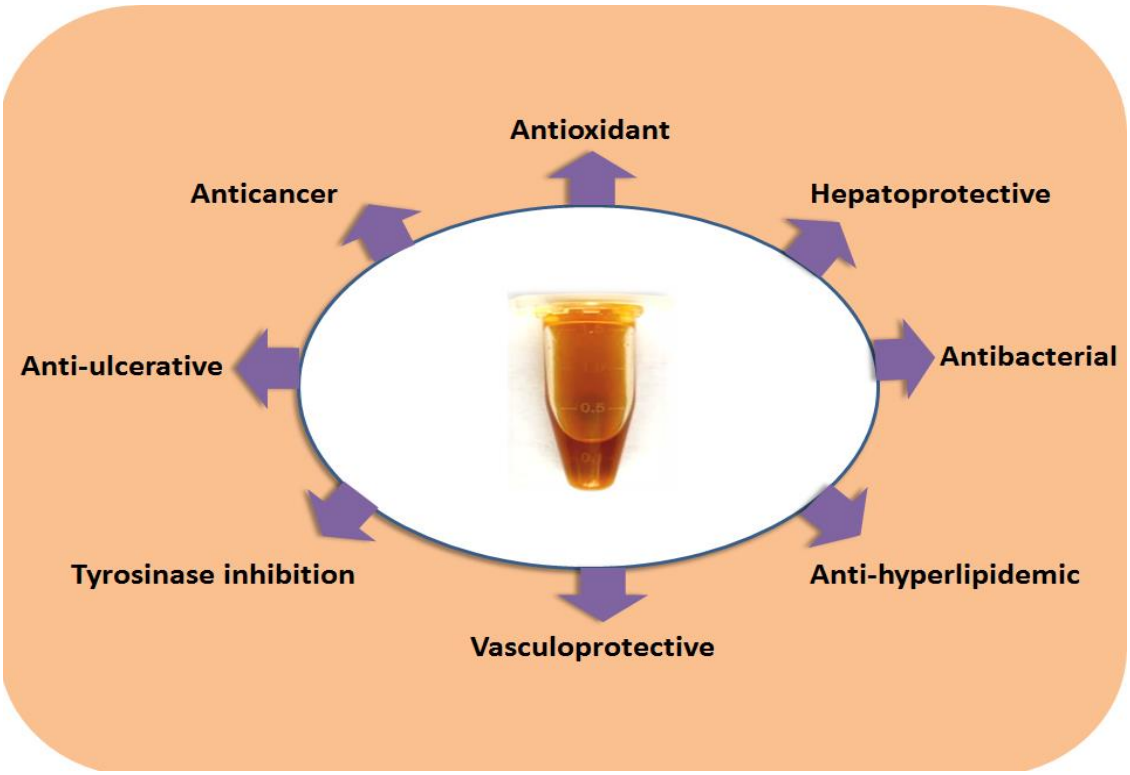

Figure 1: Multi-spectrum therapeutic activities of silkworm pupal oil

MATERIALS AND METHODS

Chemicals and reagents

Chloroform, hydrochloric acid, phenolphthalein, potassium iodide, 0.1 M sodium thiosulfate solution, and α-amylase were procured from Himedia (India). 0.1% soluble starch was purchased from Fisher Scientific (USA). Acarbose, ABTS, dichloromethane, and iodine monobromide were received from SRL Company (India). Ethyl alcohol and ascorbic acid were purchased from SD Fine Chemicals. Acetic acid and potassium hydroxide were purchased from TCI Chemicals (Japan).

Sample collection

Fresh Tasar pupae were obtained from the local farmers with the help of the State Silk Board, Kanpur, Uttar Pradesh, India.

1.1 Extraction of tasar silkworm pupal oil

The pupae **(Fig. 2)** were taken from tasar silkworm cocoons and dried at 70°C until the complete moisture was lost. Further, a powdered form of pupae was prepared in order to extract oil by the soxhlet method using N-hexane as a solvent. Following extraction, the solvent was eliminated using a vacuum evaporator, and the oil was kept in a refrigerator for additional testing.[11]

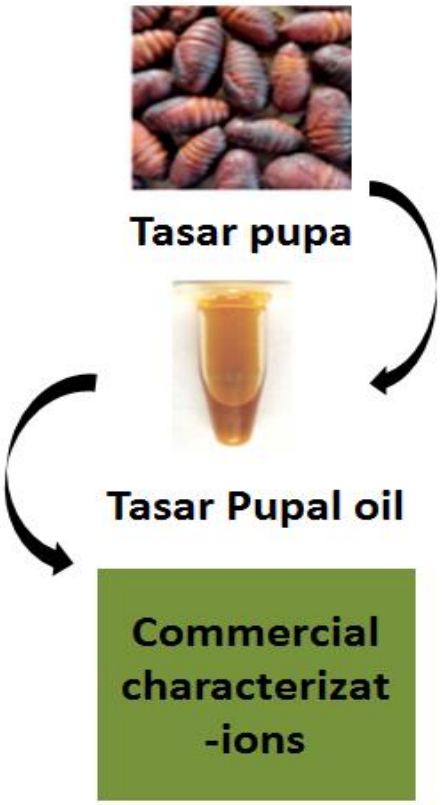

Figure 2: Experimental overview of the study

2.2 Chemical characterization of tasar pupal oil

The chemical characterization of TSPO was determined through the test method IS 548 (P1): 1964 (RA 2015) [20] to find out the values of saponification, acid, and peroxide. In addition, the iodine value was determined according to the recommendations of Odoom and Edusei [21] (2015).

Saponification Value (SV)

2 mL of TSPO was put into a 250 mL Erlenmeyer flask, followed by 25 mL of alcoholic potassium hydroxide pipetted into the flask. The blank determination was carried out concurrently with the sample (TSPO). The sample and blank flasks were then connected to air condensers and placed in a water bath. They were left to slowly and gradually boil until saponification was complete, as evidenced by the lack of any greasy particles and the appearance of a clear solution. The flask and condenser were then allowed to cool. The inside of the condenser was cleaned with 10 mL of hot ethyl alcohol, neutral to phenolphthalein. Titration with 0.5N hydrochloric acid and a 1.0 mL phenolphthalein indicator was used to detect the excess potassium hydroxide. The following formula was used to calculate SV:

$$SV = 56.1 (B-S) N/W$$

Where

B = volume in ml of standard hydrochloric acid required for the blank,

S = volume in ml of standard hydrochloric acid required for the sample,

N = normality of the standard hydrochloric acid, and

W = weight in g of the material taken for the test.

Acid value (AV)

10 ml of cooled TSPO was added in a conical flask, followed by 50 ml of freshly neutralised hot ethyl alcohol and about one millilitre of phenolphthalein indicator solution. The mixture was boiled for about five minutes and titrated while as hot as possible with a standard aqueous alkali solution, shaking vigorously during titration. The acid value was calculated by applying the following formula:

$$AV= 56.1VN/W$$

Where

V= volume in ml of standard potassium hydroxide or sodium

Hydroxide solution used,

N=normality of standard potassium hydroxide or sodium

hydroxide solution, and

W= weight in g of the material taken for the test.

Peroxide value (PV)

For PV analysis, 5 ml of both TSPO samples were taken individually and mixed with a 30 mL acetic acid chloroform solvent mixture in a stoppered conical flask mixture, which was then allowed to dissolve by swirling. After that, 0.5 mL of saturated potassium iodide solution was added with the help of a Mohrs pipette. The mixture was allowed to stand for one minute in the dark with occasional shaking, and then about 30 mL of water was added. Further, the liberated iodine was slowly titrated with a 0.1 N sodium thiosulfate solution with vigorous shaking until the yellow colour was almost gone. 0.5 ml of starch solution was added as an indicator. A blank determination was conducted. The peroxide value was calculated by the following formula:

$$PV= (S-B) X N X 1000/g$$

S = volume in ml of sodium thiosulphate solution used up by

the sample,

B = volume in ml of the sodium thiolulphate solution used up

in the blank determination.

N =normality of the sodium thiolulphate solution and

g = weight in g of the sample.

Iodine Value (IV)

For this process, 0.25 g of TSPO oil was taken in a volumetric flask, and to that, 20 ml of dichloromethane or chloroform was added. Then, to the flask, 25 ml of iodine monobromide solution was added, shaken vigorously, and then incubated for 30 minutes in the dark. Similarly, a blank was also prepared without oil (only solvent) and was also incubated in the dark for 30 minutes. After incubation, 10 ml of a 15% potassium iodide solution was added to the sample and blank flask, and then 0.5 ml of a 1% starch indicator was added. Then the sample and blank were both titrated against a 0.1M sodium thiosulfate solution to the endpoint colourless from purple. The initial and final readings for the titrant were noted, and each titration of every sample and blank was performed in triplicate. Then, to estimate the iodine value, the below-given formula was used:

$$IV=(B-S)X\ M\ x\ 12.69\ x100/Weight\ of\ sample\ 1000$$

Where,

B = mL thiosulphate for blank

S= mL thiosulphate for sample

M= Morality of thiosulphate solution.

2.3 pH measurement

A WTW pH-720 digital pH metre (U.K) was used to measure the pH of TSPO.[22] After calibrating with phosphate-buffer saline, the electrode was dipped in TSPO and the pH was recorded.

2.4 Colour determination

The color determination of TSPO was done using tintometer (Lovibond, UK). The oil colour was reported in terms of Lovibond units as follows: Colour reading = (a Y + 5 b R) in (* cell). Where, a = sum total of the various yellow slides (Y) used b = sum total of the various red (R) slides used Y + 5R is the mode of expressing the colour of light colored oils (ISI, 1984; IS 548 (Part 1)-1964). [23]

2.5 α-Amylase-inhibition assay

TSPO dilutions (of 0-2500 µg/ml) in sodium phosphate buffer was prepared. Enzyme solution (10 µl) containing 20mg/ml α-amylase was placed in defined well of a 96-well plate. TSPO (10 µl) were added and mixture was incubated for 10 minutes. The reaction was initiated by adding 50 µl substrate (0.1% Soluble Starch) and mixture was further incubated for 15 minutes. Finally, after 15 minutes 100µl GOD-POD Reagent was added to the mixture and then plate was incubated at room temperature for 10 minutes and absorbance was taken at 490 nm using a micro plate reader (iMark, BioRad, USA). Inhibitor, Acarbose (50 µg/ml final Concentration) was used

as a positive control. IC_{50} was calculated using Software Graph Pad Prism 6.[24]

Statistical analysis

Standard deviation was calculated for the experiments repeated in triplicates.

3. RESULTS

3.1 Extraction of oil from tasar silkworm pupae

A significant quantity of oil was extracted from the tasar silkworm pupae **(Fig. 3)**. The total yield was achieved. 14.5% (approximately 90 ml) of oil was extracted from 255 grams of Tasar pupae powder.

Figure 3: Extracted tasar pupal oil

3.2 Chemical characterization of silkworm pupal oil

The results of the chemical characterization of TSPO are presented in **Table 1**.

The saponification number (Majidi and Bader, 2015) represents the typical length of the fatty acid chain in a lipid. For instance, a sample with a high SV has a shorter and lighter fatty acid chain, while a sample with a low SV has a longer and heavier chain. The reason for this inverse relationship is that fatty acid chains are longer on average when there are fewer carboxylic groups per unit mass (Food Analysis- FScN 4312W). According to the SV (154.75 ± 0.581mg KOH/g), long-chain fatty acids are present in TSPO. The acid valueindicates how many milligrams of KOH are required to neutralise the free fatty acids in one gram of oil.TSPO had an AV value of 6.24 ± 0.421 mg KOH/g oil. The PV value indicates how many hydroperoxides are present in the oil. It provides an estimate of oil quality, expressed in meq/kg (milliequivalent oxygen per kilogram of oil). The peroxide value of TSPO was 16.20 ± 0.265 meq/kg.The IV determines how unsaturated the fatty acids in edible oils are. It is expressed in g iodine/100 g oil. It is used to measure the degree of oil oxidation (Chebet *et al.,* 2016). TSPO had an IV of 40.33 ± 0.577g/100 ml in this study.

Table 1: Values of Chemical characterization of TSPO

S. No.	Values	Mean ± SD
1.	Saponification	154.75 ± 0.581
2.	Acid	6.24 ± 0.421
3.	Peroxide	16.20 ± 0.265
4.	Iodine	40.33 ± 0.577

3.3 pH determination of pupal oil

The pH reading was measured for TSPO. The recorded pH was 6.17, which is correlated with other pH analysis data of edible oils.[25]

3.4 Colour determination

The colour of tasar pupal oil was determined by the Lovibond tintometer, and the colour of tasar pupal oil was found to be orange (14 Y + 1.2 R).

3.5 α-Amylase-inhibition assay

The results of the α-Amylase-inhibition assay are given in Figure 4. The IC 50 value of TSPO was 207339µg/ML. As shown in Figure 4, the TSPO dose-dependently inhibits the α-amylase activity. As the concentration of TSPO increases, the inhibition activity increases accordingly. The acarbose was used as a standard and showed an IC_{50} value of 1.513 µg/ML **(Fig. 4)**.

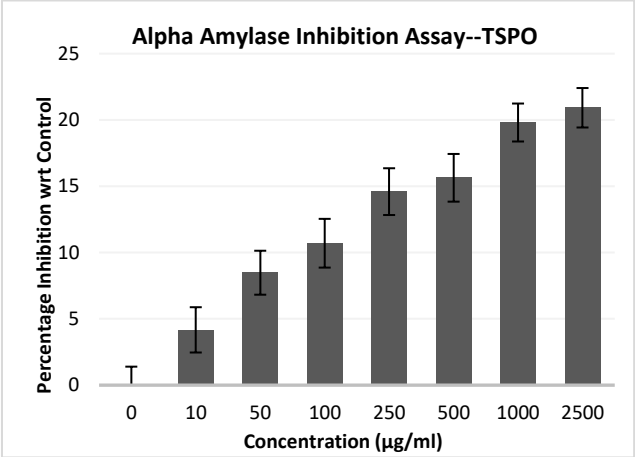

Figure 4: Dose dependent inhibition of alpha-amylase by TSPO

4. DISCUSSIONS

In this study, the soxhlet extraction method with solvent hexane was used to extract oil from Tasar silkworm pupae, which provided satisfactory oil yield. Previous studies have reported that the Soxhlet method gives a better yield than other methods. Winitchai et al.[27] reported that the soxhlet extraction method gave better and higher results than the maceration extraction method while extracting oil from the pupa of native Thai varieties of silkworm *(Bombyx mori).* A similar trend has been observed in the studies on different oils, such as flaxseed oil [27] and jatropha seed,[28] when the soxhlet extraction technique was used for the extraction of oil. Apart from extraction techniques, silkworm pupae variety, developmental stage, and sex also play a crucial role in the fat content. The aforementioned factors were recently researched in four varieties of Korean silkworm pupae: Baegokjam (BG), Golden Silk (GS), Juhwangjam (JH),

andYeonNokjam (YN). According to the authors, males had significant levels of fat, which rose in YN and GS throughout the late stage but fell in BG. Additionally, the prevalence of fatty acids varied according to the variety. Oleic acid concentrations were high in BG, linoleic and linolenic acid concentrations in GS, and palmitic and stearic acid concentrations in JH.[29]

One of the studies reveals the SV value of mulberry silkworm *(Bombyx mori)* is 182.43 mg KOH/g.[30] Similarly, SVs values of 196 and 195 mgKOH/gwere reported for castor-fed eri silkworm pupal oil and tapioca-fed silkworm oil, respectively (Ravinder *et al.,* 2015). The SV of TSPO in this study was found to be lower than the above-mentioned pupal oils. However, the SV of TSPO showed a similar range to that of edible mustard oil, which had a SV of 158.55 mg KOH/g.[31]

The edibility and rancidity of oils are measured by their acid value [32]; this is beneficial in determining hydrolysis or the enzymes responsible for oil degradation.[33] Alajtal et al.[34] stated that the maximum acid value for olive oil is 17 mg KOH/g and for other edible oils is 0.6 mg KOH/g. Similarly, according to Food and Agriculture Organization (FAO) the maximum acceptable range of virgin palm oils is 10.0 mg KOH/g.[35] The AV value of TSPO falls in the middle range between olive oil and virgin palm oils it as contains a significant amount of PUFA. However, recently, it has been reported that the encapsulation technique improved the PV in vegetable oils.[36] Therefore, in the future, encapsulated TSPO can be tried and tested for the same.

The PV of TSPO was higher than the reference range; however, this can be acceptable as extra virgin olive oil also shows high PV (up to 20 meq/kg), which is significant according to the standards of the International Olive Oil Council and the Codex Alimentarius Commission.[37]

According to Parthasarathy et al.[38], iodine levels in excellent fat should be 25–50, ideally 30-45. IV of TSPO comes within permissible range.

The measurement of colour is an important part of oil quality. Colour measurement is one of the many tests that must be performed on edible oils and fats during the refining process. Colour measurements are used not just to assess visual quality but also to improve bleaching, deodorising, and other manufacturing processes. The majority, if not all, refined oils are sold by colour, and each type of oil will have its own unique "sell by colour" guidelines.[39] The most commonly used technique for determining the colour of commercial oils is Lovibond.[40,41] In the Lovibond method, colour is expressed as red and yellow components. In general, fully refined oil may be 0.8 R (red) and 8.0 Y (yellow). Frying oils are often discarded when their Lovibond red colour increases from 1.5–3.5 to 20–30.[42]

As per our knowledge, this was the first time we studied the *in vitro* anti-diabetic activity of pupal oil. We reported that TSPO exhibited α-amylase inhibition activity in a dose-dependent manner. However, when compared with standard acarbose, TSPO was found to be less effective.

CONCLUSION

In this study, we have extracted oil from the pupa of the tasar silkworm, which is a wild species of non-mulberry. Interestingly point that we reported that the edible properties of tasar pupal oil fall within the permissible range compared to other edible oils currently available in the market. As a result, tasar pupal oil is not only an alternative source of edible oil, but it is also an excellent source for therapeutic applications. Since it was the first screening for the anti-diabetic activity of TSPO, other in vitro anti-diabetic activities, such as β-glucosidase inhibition assay, might also be analysed before starting an *in vivo* trial. Furthermore, animal studies are required to analyze the biocompatibility and bioavailability of tasar pupal oil in an animal system before human trials and commercialization.

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authorship contribution

Devika Srivastava: Study design, sample collection, analysis, interpretation, manuscript writing.

Vandana Singh: Manuscript writing, manuscript editing.

Venkatesh Kumar R: Study design, manuscript editing.

Declaration of competing interest: None of the authors have any competing interest to declare.

Declaration of conflict of interest

None of the authors have any conflict of interest to declare

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