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Research Article

Isolation of Alpha-linolenic acid (ALA) from tasar, *Antheraea mylitta* pupal oil and its anticancer activity

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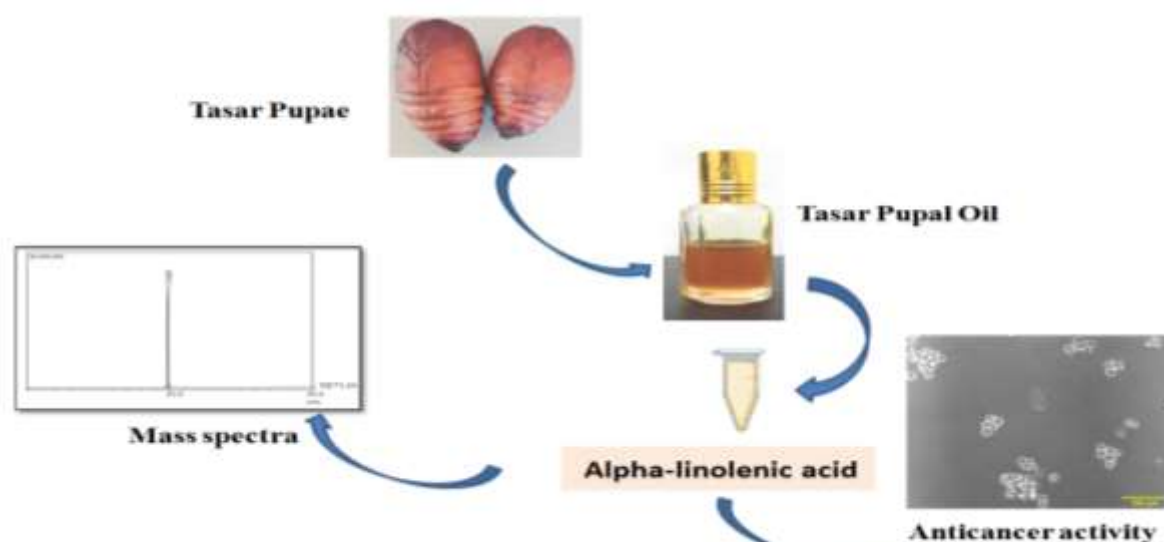
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Abstract

Previously, we reported that tasar silkworm pupal oil (TPO) is a rich source of alpha-linolenic acid (ALA) (37%). In this study, we have used three chromatographic techniques, namely preparative HPLC, gas chromatography-mass spectroscopy (GC-MS), and gas chromatography flame ionization detection (GC-FID), to purify, determine, and quantify alpha-linolenic acid (ALA), respectively, from TPO. Additionally, to determine the potential of the purified ALA as an anticancer drug, the sulforhodamine B (SRB) assay was utilised for in vitro investigation. Results revealed that ALA was successfully purified in a fraction of TPO by preparatory HPLC. It was discovered that only ALA was present in the purified fraction of ALA when it underwent GC-MS analysis, further confirming its purity. In addition, the quantification analysis using GC-FID showed that the concentration of ALA was 11760 ppm. Furthermore, anticancer analysis revealed purified ALA significantly inhibited the growth of a colon cancer cell line, (COLO-205) with a GI 50 $\leq$ 20. This is the initial investigation into isolating and purifying ALA from Tasar pupal oil.

Keywords: Alpha-linolenic acid; GC-FID; HPLC; Omega-3Preparative, Tasar pupal oil.



Graphical Abstract

INTRODUCTION

Alpha-linolenic acid (ALA) (Fig. 1) (18:3n-3) is a plant-based (legumes, green leafy vegetables, walnuts, canola, flaxseed, soybeans, etc.) essential omega-3 fatty acid consisting of 18 carbon atoms and three cis double bonds. It belongs to the well-known n-3 polyunsaturated fatty acid (PUFA) family, which is the precursor of other longer-chain n-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) ^{1,2}. Various studies have reported that ALA has multiple pharmacological actions such as anti-diabetic, cardioprotective, anti-inflammatory, antioxidant, neuroprotective, anti-cancer, etc. (Fig. 2) ³.

ALA is mostly synthesized from plant-based sources. However, both mulberry and non-mulberry silkworm pupal oils have been identified as new sources of ALA in recent days ^{4,5,6}. Among the silkworm pupal oils, *Antheraea mylitta* (Tasar silkworm) (Lepidoptera: Saturniidae) pupal oil has emerged as a rich source of ALA, as it revealed 37% of ALA in it ⁷.

Therefore, plants and silkworms are significant sources of ALA, and attaining the highest purity of ALA from such sources while extracting is also crucial. Hence, finding suitable chromatographic techniques is also a need of the hour when it comes to their further applications in pharmacology and natural product research ⁸. Chromatography techniques are important biophysical techniques for separating and purifying the components of a mixture for qualitative and quantitative studies ⁹. Due to their precision and accuracy, one can achieve better results from chromatography in the separation and purification of natural compounds, including oils, when compared to existing separation methods such as molecular

distillation, low-temperature crystallization, and urea adduct formation ^{10,11,12}. Preparative HPLC is one of the significant chromatographic techniques where we can separate and purify omega-3 fatty acids, particularly ALA, EPA, and DHA ^{13,14}. Gas chromatographies such as GC-MS and GC-FID are considered the most precise and sensitive tools for the identification and quantification of the majority of fatty acids, including ALA ^{15,16}.

Cancer is the leading cause of death worldwide. Malignancy is now the third leading cause of death in many developing countries, after infectious and cardiovascular disorders ¹⁷. Many biological activities of ALA have been discovered, including anti-cancer properties ^{18,19}. As a result, ALA appears to be a viable chemotherapeutic drug with desired properties ²⁰.

In this investigation, we used three chromatographic techniques to extract, purify, and quantify alpha-linolenic acid (ALA) from the oil of tasar silkworm (*Antheraea mylitta*) pupae. Further, purified ALA was tested in vitro to determine its potential as an anticancer drug.

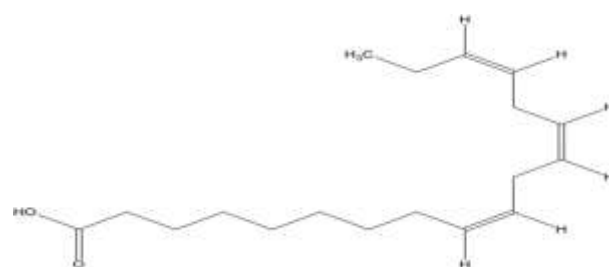


Figure 1: Alpha-linolenic acid

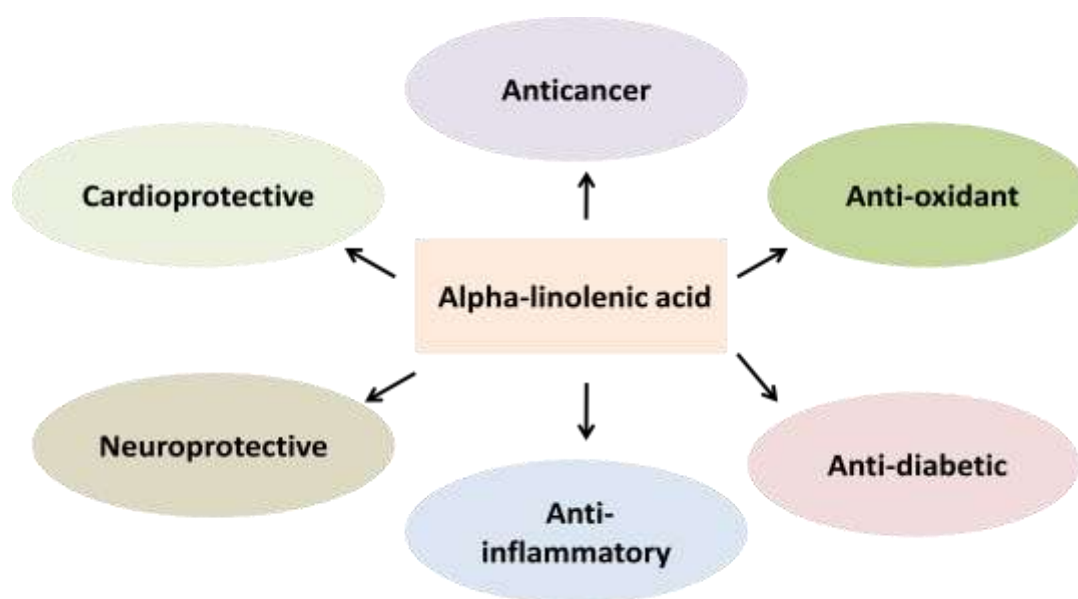


Figure 2: Various therapeutic activities of alpha-linolenic acid

MATERIALS AND METHODS

Materials

To extract oil, tasar pupae were purchased from the local farmers through State Silk Board, Kanpur, Uttar Pradesh, India.

Chemicals and solvents

Standard ALA was purchased from Sigma Aldrich, Acetonitrile, n-hexane, methanol, ethanol, and sulfuric acid were of

analytical grades. The in vitro anticancer activities were tested at ACTREC in Mumbai, India.

Extraction of tasar silkworm pupal oil

Tasar silkworm pupae were dried in a hot air oven at a temperature of 70°C until all moisture was eliminated. The oil was subsequently extracted from the pupae using the Soxhlet process with n-hexane as the solvent. A vacuum evaporator was used to extract the solvent, and the oil was then placed in storage for examination ⁶.

Preparation of Fatty Acid Ethyl Esters (FAEEs)

Prior to the purification of ALA by prep-HPLC, the extracted oil was converted to fatty acid ethyl esters (FAEEs) as suggested by Bligh ²¹. A 1:10:0.4 (w/w) mixture of pupal oil, ethanol, and sulfuric acid was used in the reaction, which took place at 70 °C for two hours. After the reaction was finished, the reactants were moved to a separating funnel, where water was added to separate them into two phases. Two layers were created when the water was introduced; the lower layer was removed, and the upper layer (the FAEEs layer) received 500 ml of hot water. This process was repeated until the lower layer was completely removed. The remaining upper layer was then dried in a vacuum.

Purification of alpha-linolenic acid (ALA)

ALA purified from free fatty acid ethyl ester was analyzed by preparative high-performance liquid chromatography (model: LC -20AP, make: SHIMADZU, Japan) equipped with a C18 column (4.6 × 250 mm; 5 µM). Solvent acetonitrile: methanol: n-hexane in the ratio 90:8:2. Flow rate 2ml/min at 210 nm. The run time was 30 min, and only the B pump was used ¹². First, the standard ALA was loaded and the retention time was observed. After the retention time of the standard ALA was determined, the FAEEs derived from oil were loaded (15 µl) and the fraction of ALA was collected at the same retention time as the standard ALA.

Determination of extracted ALA

The extracted ALA was loaded into GC/MS (Shimadzu, Japan) for analysis. The column oven temperature and injection temperatures were 60 °C and 260 °C, respectively, at a pressure of 73.3 kPa. The total flow rate remained at 16.3 ml/min, while the column flow rate remained at 1.21 ml/min.

Quantitative analysis of extracted ALA

Quantitative analysis of purified ALA was performed using GC-FID as suggested by Oh et al. (2020) 0.5 µl each of the standard solution ALA and the purified solution ALA were injected separately into the instrument GC-FID (Agilent 8890 GC, 7697 A HS with GC/TQ, 7010B, USA) and the data were recorded and analysed.

Anticancer activity of ALA

The sulforhodamine B (SRB) assay was used to test the anticancer effect of ALA against two different cancer cells MIA-PA-CA -2 (pancreas), and Colo-205 (colon) as suggested by ^{22, 23}. The percentage of growth inhibition was determined as follows:

$$[Ti/C] \times 100\%$$

RESULTS AND DISCUSSION

Extraction of alpha-linolenic acid (ALA)

In this study, preparative HPLC was used to extract the free fatty acid ALA from Tasar pupal oil. ALA in the free fatty acid sample was identified by comparing the retention time with the standard ALA. The peak of sample ALA from tasar pupal oil was found to be symmetrical. The retention time of standard ALA and sample ALA was 15 minutes (Figs. 3 and 4). In this study, we achieved significant purity of ALA from tasar pupal oil by preparative HPLC. Similarly, Oh et al. ¹⁰ purified DHA from the oleaginous microalgae *Shizochytrium sp.* SH103 using a preparative column containing approximately 34% DHA. Our study is in close agreement with this study.

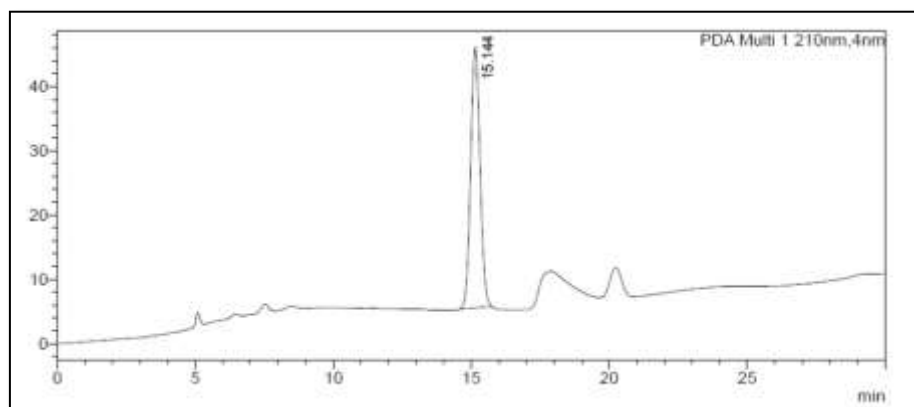


Figure 3: Chromatogram of standard ALA

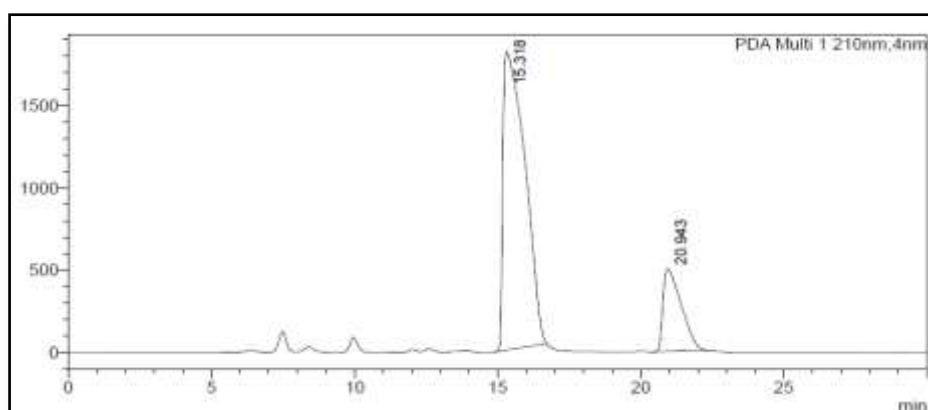


Figure 4: Chromatogram of ALA extracted from TPO

Determination of extracted ALA

To confirm that only ALA was extracted from the FAEs using Prep-HPLC, GC-MS analysis of the extracted ALA was performed. This confirmation is shown in Figure 4, which shows a single peak of ALA with a retention time of 19.883 and an area of 100% (Fig. 5) (Table 1) on the chromatograph. No other peaks were detected.

We determined the purity of ALA using GC-MS and reported that the collected fractions contained only ALA. Gas chromatography (GC) is a highly sensitive instrument for detecting and quantifying the fatty acid content of lipids in a variety of samples, providing results with great precision and consistency²⁴. GC instruments are also particularly effective in confirming or denying the purity of substances, and thus can often detect minute amounts of impurities²⁵.

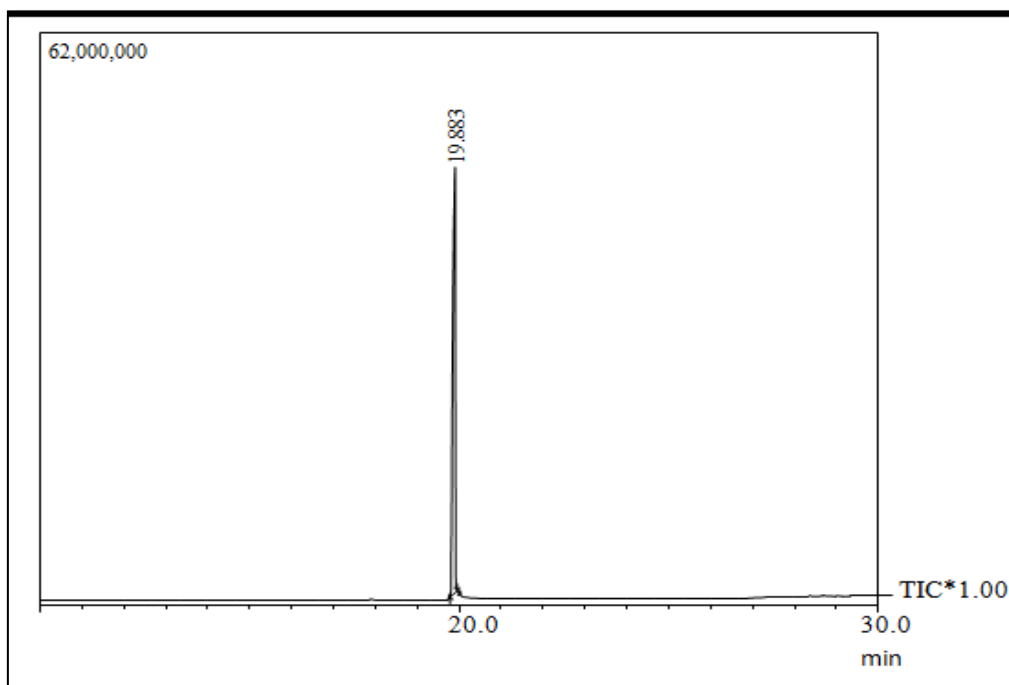


Figure 5: GC-Chromatogram of ALA extracted from TPO

Table 1 showing peak: total Ion Chromatogram (TIC)

Peak	R. Time	Area	Area%	Name of compound
1	19.883	186499460	100.00	9,12,15-Octadecatrienoic acid (ALA)

Quantitative analysis of extracted ALA

Although several analytical methods have been used in the last decade, GC-FID is still the most effective, complete, and widely used method to identify and quantify fatty acids in foods and essential oils²⁶. In this study, we determined the concentration of purified ALA by GC-FID and compared it with the standard ALA. We reported that the concentration of ALA

extracted from TPO was 11760 ppm (2.5 mg/ml) (Fig. 6). Further, the concentration of Standard ALA was found to be 1000 ppm (1mg/ml) (Fig. 7).

We have determined the concentration of purified ALA using GC-FID and obtained accurate and meaningful results. Previous studies have reported that GC-FID is a remarkable tool for the quantification of fatty acids^{27, 28}.

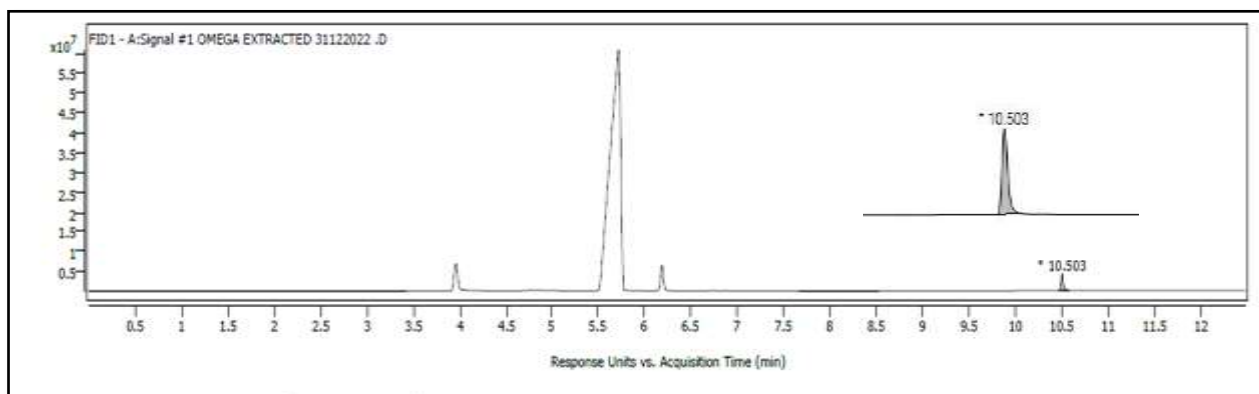


Figure 6: Chromatogram of extracted ALA (Retention time 10.503)

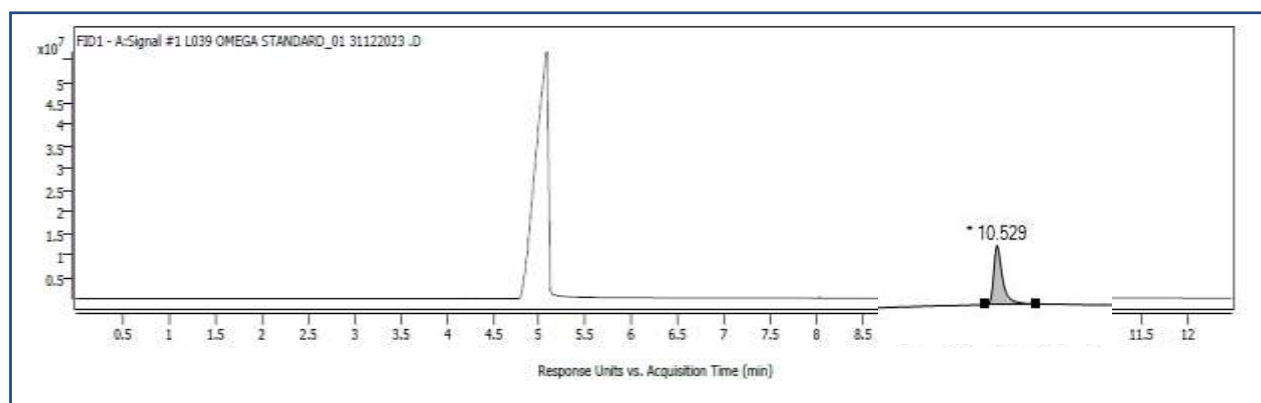


Figure 7: Chromatograph of standard ALA (Retention time 10.529)

Anticancer activity of ALA

In terms of GI50 (drug concentration resulting in 50% cell inhibition), the in vitro anticancer activity of ALA against human cancer cell lines including pancreatic cancer (MIA-PA-CA -2) and Colo-205 was investigated. ALA with a GI50 value of ≤ 20 $\mu\text{g/ml}$ (13.7) had drastically inhibited the growth of human colon cancer cell line (COLO-205) (Fig. 8 and Table 2), with morphological changes such as cell shrinkage and

decreased cell adhesiveness (Fig.10) (Table 2). In human pancreatic cell line, ALA at the highest concentration showed a mild anti-cancer effect (Figs. 9 and 10) Table 2.

We reported that purified ALA exhibited anticancer activity against colon and pancreatic cancer cell lines. Previous studies have shown that ALA has tremendous anticancer properties against various tested cancers ^{29,30,31}.

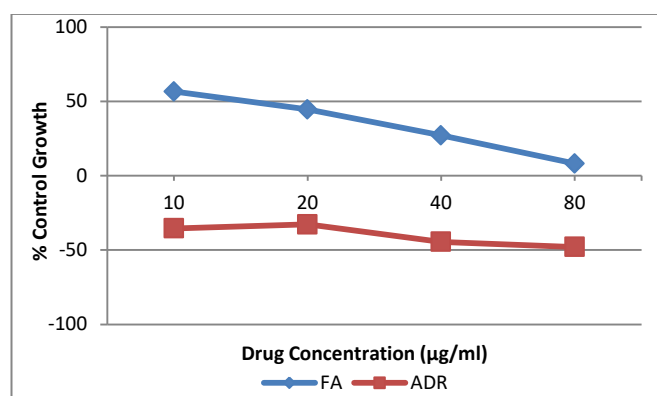


Figure 8: Growth curve of Human colon cancer cell line COLO-205

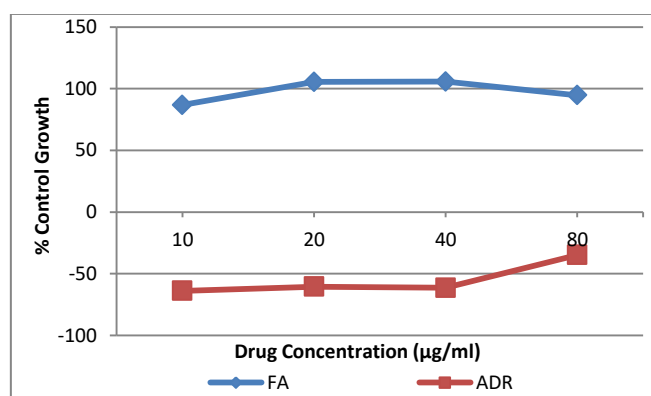
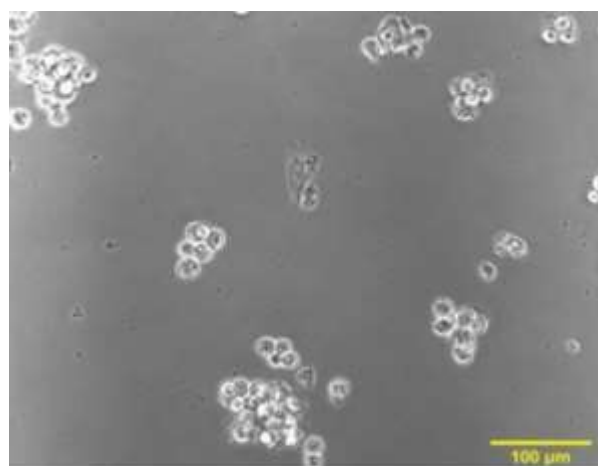
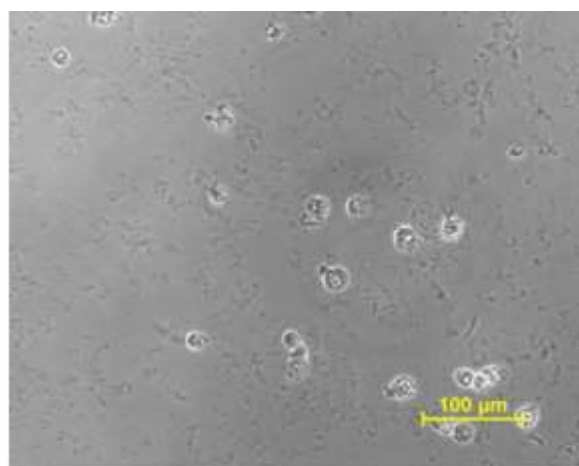


Figure 9: Growth curve: Human pancreatic cancer cell line Mia-PA-CA-2



COLO-205



Mia-PA-CA-2

Figure 10: Images showing effect of ALA on human colon and pancreatic cell lines

Table 2 showing % control growth vs. drug (ALA) concentration ($\mu\text{g/ml}$) against human cancer cell lines

Cell lines	Drug	% Control Growth															
		Drug Concentrations ($\mu\text{g/ml}$)															
		Experiment 1				Experiment 2				Experiment 3				Average values			
		10	20	40	80	10	20	40	80	10	20	40	80	10	20	40	80
COLO-2-5	ALA	62.3	40.8	43.9	25.9	69.4	49.1	22.7	2.7	38.3	43.7	14.7	-4.1	56.6	44.5	27.1	8.2
Mia-PA-CA-2		84.5	108.2	104.3	91.7	89.0	108.8	106.2	100.1	86.8	99.6	106.8	92.1	86.8	105.5	105.8	94.7

CONCLUSION

In the current study, we have purified ALA from tasar pupal oil, which has been shown to be a good source of ALA. We obtained a good yield of purified ALA, which was further validated by sophisticated instrumentation techniques, namely GC-MS and GC-FID. Preparative HPLC is a cornerstone for drug isolation and purification as it provides near 100% purity, which is a requirement for any drug before it is evaluated. Previous studies have shown that ALA is a pharmacologically active constituent that exhibits various therapeutic properties. In this we have also reported that purified ALA significantly reduced the growth of cancer cell lines which indicates that purification process did not alter the therapeutic activity of ALA. The recovery of purified ALA can be used to the fullest evaluate its efficacy against other dreadful diseases such diabetes, cardiovascular diseases etc. near future.

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Author Contributions

DS: Investigation, Visualization, Writing—original draft. VS: editing. M.K: Investigation.

V.K: Conceptualization, Supervision, Manuscript review and editing.

Competing interests/Conflict of interest

The authors have no competing/conflict of interests to disclose.

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