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Therapeutic potential of alpha-linolenic acid from Sacha Inchi oil in cervical cancer: an *in vitro* study on HeLa cells

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ABSTRACT

This study investigated the potential of alpha-linolenic acid (ALA) from Sacha Inchi oil as a therapeutic agent for cervical cancer through an *in vitro* study on HeLa cells. Cervical cancer is one of the most common types of cancer in women, which is often caused by human papillomavirus (HPV) infection. Although chemotherapy therapy is one of the main methods in cancer treatment, this approach often causes side effects and drug resistance. ALA, which is one of the main components of Sacha Inchi oil, is known to have antioxidant and anti-cancer activities. In this study, Sacha Inchi oil was analyzed using liquid chromatography-high resolution mass spectrometry (LC-HRMS) for identification of its active components. Cytotoxic assays were performed using the MTT method on HeLa cells, which showed that ALA significantly inhibited cancer cell viability at low concentrations, with low IC_{50} values compared to the positive control compound cisplatin. These results suggest that ALA has potential as an effective anti-cancer agent against cervical cancer cells. This study concludes that ALA from Sacha Inchi oil can be a strong candidate in the development of safer and more effective cervical cancer therapy.

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1. INTRODUCTION

Cervical cancer remains one of the most prevalent malignancies affecting women globally, with over 604,000 new cases and 311,000 deaths reported annually [1], [2]. There are 311,365 cervical cancer deaths and 569,847 new cases per year. The primary risk factor is the human papillomavirus (HPV), and prolonged high-risk HPV infection has been linked to cervical cancer. Sexual contact can spread HPV, which has been implicated in 99% of cases of cervical squamous cell cancer [3], [4]. HPV infection, especially high-risk subtypes such as HPV 16 and 18, is required to cause cervical neoplasia, although HPV infection alone is not sufficient to cause cancer [5]. There are up to 225 different varieties of HPVs, a diverse collection of double-stranded DNA viruses, and new types are constantly being discovered [6]. HPV infection is strongly associated with the development of cervical cancer, especially high-risk HPV types such as 16, 18, 31, 33, and 45, which are found in approximately 97% of cervical cancer patients [1]. The pentose phosphate pathway (PPP) can be abnormally activated by HPV infection; PPP plays an important role in the development and progression of cervical cancer [7].

Given these limitations, there is a growing need for alternative therapeutic agents that are safer, more effective, and derived from natural sources. One promising candidate is Sacha Inchi, also known as *Plukenetia volubilis L.*, an Amazonian plant known for its high content of polyunsaturated fatty acids,

particularly alpha-linolenic acid (ALA) [8], [9]. Linolenic acid (LA) has been shown to exert anti-cancer properties through various mechanisms, including the induction of apoptosis, inhibition of cancer cell proliferation, and enhancement of the cytotoxic effects of chemotherapeutic agents [10], [11].

Several *in vivo* and *in vitro* studies have indicated that ALA may serve as a potential adjuvant or primary agent in cancer treatment [12], [13]. Sacha Inchi is usually used as a source of edible oil and protein. Oil from Sacha Inchi seeds is known to be rich in unsaturated fatty acids and antioxidants [10]. Sacha Inchi is known for its oil, which is highly valued for its high nutritional content. The oil contains 52% LA and 34% LA, a total of 85% essential fatty acids [14]. This oil is obtained through cold pressing and is known for its moisturizing effect on the skin. It is light and good for dry skin, comparable to olive oil [15].

Sacha Inchi has phytochemicals that may function as an anti-cancer. The oil from Sacha Inchi seeds contains phenolic compounds and antioxidants that may help prevent and treat cancer. In addition, the phenolic compounds and phytosterols in Sacha Inchi seeds have also been evaluated for their antioxidant activity, which is important for fighting oxidative stress often associated with cancer [16]. ALA, with the chemical formula C18H30O2, has several benefits in cancer treatment. It is known that ALA reduces tumor cell viability and proliferation and increases apoptosis in various types of cancer cells, including neuroblastoma and breast cancer. In addition, when ALA is combined with chemotherapy or radiotherapy (RT) agents, it exhibits a synergistic effect, which enhances the cytotoxic effect of the treatment. ALA also functions as a radiosensitizer, increasing cell sensitivity [16]. ALA is reported to have cardiovascular protection, neuron protection, anti-cancer, anti-osteoporosis, anti-inflammatory, and antioxidant effects. ALA has the ability to control the no signal release and lipid peroxidation in tumor therapy. In addition, ALA can activate the mitogen-activated protein kinase (MAPK) signaling pathway, which causes mitochondrial damage in cancer cells [17].

In research on cervical cancer, HeLa cells are used. In this study, HeLa cells were used to study the potential mechanism of 5, 6, 7, and 4'-tetramethoxyflavone against cancer cells; this study specifically concentrated on its inhibitory effects through various signaling pathways [18]. When exposed to nonsense-mediated mRNA decay (NMD) and mouse double minute 2 homolog (MDM2) inhibitors, HeLa cells showed significant synergistic effects. HeLa cells died, and the cell cycle stopped with this treatment [19]. Sacha Inchi seed oil has anti-cancer activity, according to research. Ex *vivo*, a Sacha Inchi oil-based diet (1 g/kg body weight daily for four weeks) reduced tumor mass and tumor cell proliferation in tumor-bearing Walker 256 mice. In addition, plasma levels of glycemia, triglycerides, and inflammatory cytokines decreased, and lipoperoxidation increased in Walker 256 tumor tissues. Additional research used 1, 2-dimethylhydrazine to induce colon cancer in Sprague-Dawley and Wistar rats. The results showed that the group of rats given Sacha Inchi seed oil at a dose of 150 µL/(kg-day) showed a 12.5% increase in the number of individuals protected from tumor induction [20], [21]. This study was conducted using the MTT assay method. MTT assay is a method used to assess the cytotoxicity of free drugs and drug combinations against HeLa cells [22]. MTT is converted to a colorful, insoluble formazan inside the cells. Through the use of an enzyme-linked immunosorbent assay (ELISA) reader to measure the optical density of the formazan product at 495 nm, this procedure enables the quantification of live cells in multi-well microplates [23], [24].

This study tests pure ALA isolated from Sacha Inchi oil against HeLa cervical-cancer cells. Unlike prior work that used crude extracts, we evaluate the purified fatty acid itself, finding that its IC_{50} is lower than cisplatin's, marking it as the more potent agent. We also compare ALA from two oil sources, conventional plants vs. those grown with shrimp-waste fertilizer, an angle not yet reported. The results provide an early foundation for safer, sustainable natural adjuvants in cervical cancer therapy.

2. RESEARCH METHOD

The MTT assay using HeLa cells includes a number of important steps. It begins with cell analysis and separation and ends with an assay using the MTT method to evaluate viability. To determine the metabolic activity of the cells, the procedure involves the separation and embedding of cells in a 96-well plate, incubation with MTT reagent, and measurement of absorbance results.

2.1. Materials

This study used two Sacha Inchi oil samples. "Sachi" oil was pressed from nuts grown in Batang, Central Java: 100 g of dried, shelled beans were mechanically pressed at 60 °C, allowed to settle for 2-3 days, and yielded about 100 mL of crude oil. "Ali" oil, supplied by CV. Inovasi Anak Negeri in West Java served as the source for isolating ALA.

2.2. Procedures

2.2.1. Liquid chromatography-high resolution mass spectrometry assay

The analysis method uses liquid chromatography-high resolution mass spectrometry (LC-HRMS) to identify and characterize ALA in Sacha Inchi oil with a high degree of accuracy, allowing detection of

compounds in low concentrations as well as differentiation between structurally similar isomers. LC-HRMS, which uses Thermo Scientific's Vanquish™ UHPLC Binary Pump and Thermo Scientific's Q Exactive™ Hybrid Quadrupole-Orbitrap™ High Resolution Mass Spectrometer, with high-performance liquid chromatography (HPLC) using Thermo Scientific's Accucore™ Phenyl-Hexyl column. The mobile phases were mass spectrometry (MS)-grade water containing 0.1% formic acid (A) and MS-grade methanol containing 0.1% formic acid (B), applied under a gradient elution program at a flow rate of 0.3 mL/min. The gradient was initiated at 5% B, increased linearly to 90% within 16 min, held for 4 min, and then returned to the initial condition (5% B) by 25 min. The injection volume was 3 µL. Mass spectrometric detection was performed using electrospray ionization (ESI) in positive ion mode, with a capillary voltage of 3.30 kV, a capillary temperature of 320 °C, and a scan range of 66.7–1000 m/z. This combination of ultra-high-performance liquid chromatography and high-resolution Orbitrap mass spectrometry enabled high sensitivity, accurate mass measurement, and comprehensive characterization of both target compounds and complex metabolites.

2.2.2. Isolation of alpha-linolenic acid

The study uses flash chromatography to isolate alpha-linolenate from a large sample of 1.2 g, analyzing it in an 8:2 ratio. The sample is dissolved in chloroform for 15 minutes, and purification is performed using flash chromatography C-850. The flash chromatography column is characterized by a station and equilibration. Sacha Inchi oil samples were extracted using the cold-pressing method, then purified by liquid-liquid extraction using n-hexane solvent. LC-HRMS analysis was performed using a C18 column (100 mm×2.1 mm, 1.7 µm) with a temperature of 40 °C, a flow rate of 0.3 mL/min, and a mobile phase of 0.1% formic acid in water: methanol (30:70 v/v). Detection was performed at a wavelength of 215 nm, with positive ion electrospray ionization (ESI+) mode to identify the specific m/z of ALA.

2.2.3. Culture HeLa cells

The MTT assay involves using HeLa cells that reach 70-80% confluence in a 75 cm^2 t-flask. The cells are washed, incubated in a 37 °C, 5% CO_2 incubator, and then transferred to a centrifugation tube. Complete media is added, and the cells are separated and subcultured in 96-well plates. The remaining cells are then subcultured in 75 cm^2 t-flasks. HeLa cells were seeded in 96-well plates using 100 µL of cells, a 5 times dilution, and 2,000,000 cells per well. One plate was used to test three compounds and a positive control, cisplatin, and cells were incubated overnight in a 37 °C, 5% CO_2 incubator.

2.2.4. MTT assay cytotoxicity test

The seeded cells were observed for confluency, and if 90% confluency was reached, they were ready for the MTT assay. Three test compounds (Sachi, Ali, and ALA) were varied in concentration using stratified dilution in Figure 1. Ali and Sachi were used as a control, while ALA was used as a positive control.

The 96-well plate was incubated with MTT solution dissolved in basal media for 4 hours, followed by the addition of test samples. Formazan crystals were observed after 4 hours, and dissolved with 20% sodium dodecyl sulfate (SDS) stop reaction solution. After 24, 48, and 72 hours of treatment with ALA, 10 µL of MTT solution (5 mg/mL in phosphate-buffered saline (PBS)) was added to each well and incubated for 4 hours at 37 °C. The medium was removed, and 100 µL dimethyl sulfoxide (DMSO) was added to dissolve the formazan formed. Absorbance was measured using an ELISA reader at a wavelength of 570 nm with a reference filter of 630 nm.

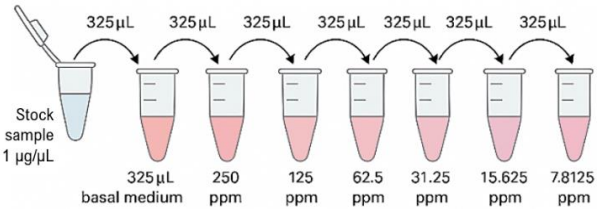


Figure 1. Dilution process of test compound for MTT assay

3. RESULTS AND DISCUSSION

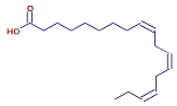
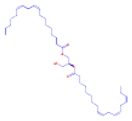
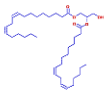
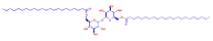
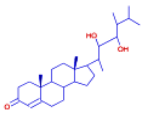
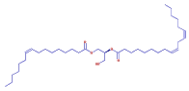
This study utilized ALA isolated from Sacha Inchi oil B, where the Sacha Inchi plants were fertilized using shrimp waste. In contrast, Sacha Inchi oil A was obtained from plants cultivated using conventional fertilization methods. The comparison between these two cultivation systems aimed to evaluate

the potential effect of organic shrimp waste fertilizer on oil quality. This distinction provides valuable insight into sustainable agricultural practices and their impact on bioactive compound production.

3.1. Compound identification in Sacha Inchi oil

The LC-HRMS analysis of Sacha Inchi oil sample B (cultivated with shrimp waste fertilizer) identified 26 active compounds, including ALA, diglycerides, mandenol, and other bioactive lipids. ALA, with a retention time of 14.494 minutes and a molecular formula of $C_{18}H_{30}O_2$, was confirmed as the dominant anti-cancer compound in Table 1. These findings are consistent with prior studies showing Sacha Inchi's rich unsaturated fatty acid profile and antioxidant potential. This compound showed a retention time, which is the time required to pass through the chromatography column, of 14.492. These results confirmed that ALA ($C_{18}H_{30}O_2$) was successfully identified in the Sacha Inchi oil sample.

Table 1. Analysis results of anti-cancer compounds in Sacha Inchi oil

Structure	Name	Formula	Retention time (min)	Percentage (%)
	ALA	$C_{18}H_{30}O_2$	14.494	4.452
	DG (18:2(9Z, 12Z)/18:3 (9Z.12Z, 15Z)/0:0)	$C_{39}H_{66}O_5$	19.657	20.09
	1, 2-Dilinoleoyl-sn-glycerol	$C_{39}H_{68}O_5$	20.202	15.48
	Mandenol	$C_{20}H_{36}O_2$	14.977	11.8
	Trehalose-6, 6-dibehenate	$C_{56}H_{106}O_{13}$	21.15	6.533
	3-[(15Z, 19Z)-11, 12-dihydroxy-15, 19-doctriacontadien-1-y1]-5-methyl-2(5H)-furanone	$C_{37}H_{66}O_4$	20.416	5.792
	1-oleoyl-2-linoleoyl-sn-glycerol	$C_{39}H_{70}O_5$	20.726	5.701
	(22R, 23R, 24S)-22, 23-dihydroxyergost-4-en-3-one	$C_{28}H_{46}O_3$	18.5	3.787
	2-amino-1, 3, 4-octadecanetriol	$C_{18}H_{39}NO_3$	9.933	3.548
	1-(9Z-hexadecenoyl)-2-(9Z, 12Z-octadecadienoyl)-sn-glycerol	$C_{37}H_{66}O_5$	19.97	1.255

3.2. Isolation of alpha-linolenic acid

To isolate ALA, thin-layer chromatography (TLC) was first conducted to visualize compound separation. Figure 2 shows distinct bands indicating the presence of multiple compounds, including ALA, based on polarity differences. The separation was further refined using flash chromatography, allowing efficient recovery of ALA. These methods ensured high purity of the isolated compound before bioassay testing. The

optimized eluent (80% n-hexane:20% ethyl acetate) proved effective in selectively separating ALA, as shown in Figure 2. Figure 2(a) shows the profiling with TLC and Figure 2(b) shows the profiling with TLC results.

TLC separates mixture components by polarity. A drop of the sample is spotted on a silica-gel plate, the plate is placed in solvent, and capillary action carries the solvent upward. Non-polar compounds travel far, polar ones lag near the baseline. Applied to Sacha Inchi oil, the plate shows distinct spots whose matching retention factor (Rf) values reveal the oil's individual constituents and confirm compositional similarity between samples [24], [25]. The TLC analysis reveals multiple components in Sacha Inchi oil, including ALA (Figure 3(a)). The separation profile of the compound was confirmed using TLC and flash chromatography, as presented in Figure 3. The TLC result (Figure 3(a)) shows distinct spots indicating the presence of the target compound, while the flash chromatography profile (Figure 3(b)) demonstrates successful isolation with a clear fraction pattern. Prior to purification, the oil sample undergoes 15 minutes sonication to enhance separation efficiency. The purified fractions are then validated by TLC, confirming the successful isolation of target compounds. ALA was the compound found in the flash chromatography and TLC results, indicating that the separation and analysis process performed successfully identified the main component of the sample. The similar results for both methods indicate that the separation method used was well optimized and sensitive and selective enough to find ALA.

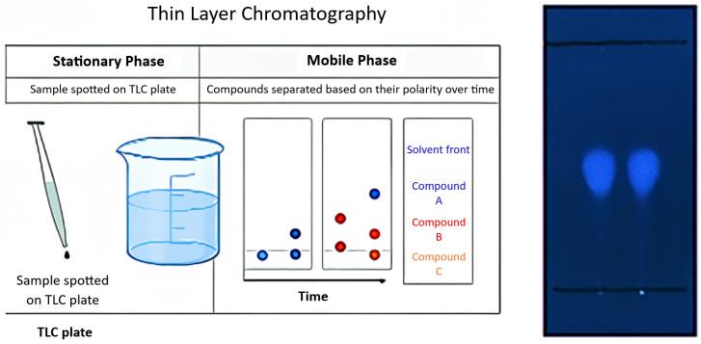


Figure 2. Profiling with (a) TLC and (b) TLC results

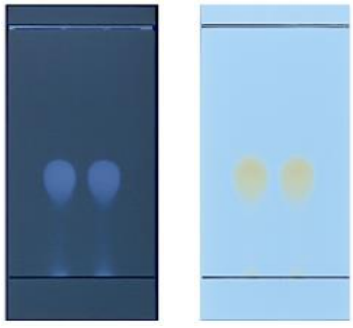


Figure 3. Result of (a) TLC and (b) flash chromatography

3.3. MTT assay using HeLa cells

The MTT cytotoxicity test on HeLa cells revealed pronounced differences in potency among the samples. Cisplatin, the positive control, sharply reduced cell viability above 31 ppm, yielding an IC_{50} of 16.7 ppm. In contrast, Sacha Inchi oil maintained high viability across the tested doses, with an IC_{50} of 225.9 ppm, indicating weak cytotoxicity. Ali oil, however, lowered viability even at low concentrations and produced an IC_{50} of 16.1 ppm, nearly matching cisplatin's effect. The most powerful response came from purified ALA: tested over a much narrower concentration range, it produced the greatest inhibition, with an exceptionally low IC_{50} (~9×10^{-26} ppm), underscoring its superior potential as an anti-cancer agent.

These results collectively support the potential of ALA as a strong anti-cancer agent with higher effectiveness at lower concentrations compared to both crude oil samples and standard chemotherapy agents. Figure 4 shows the visual differences in cell viability trends affirm the relative cytotoxic strength of each

tested compound. Figure 4(a) shows the comparative cytotoxicity of cisplatin, Figure 4(b) shows the Sachi, Figure 4(c) shows the Ali, and Figure 4(d) shows the ALA on HeLa cells. The MTT assay eluates the cytotoxicity of ALA, Sacha Inchi oil sample A (Sachi), and sample B (Ali) on HeLa cells. The results showed cisplatin had the lowest IC_{50} (16,73 ppm), indicating strong cytotoxicity. Sample Sachi had the highest IC_{50} (225.9 ppm), showing the weakest cytotoxicity effect, while sample Ali (IC_{50}: 16.10 ppm) and ALA (IC_{50}: 9.557e+02) showed stronger inhibition at lower concentrations, with concentrations, with ALA being the most potent. These findings suggest ALA is a promising anti-cancer compound due to its high efficacy at minimal concentrations.

Dose-dependent cytotoxicity shows in bar charts in Figure 4(a) cisplatin (control)–IC_{50}=16.73 ppm, Figure 4(b) Sachi–IC_{50}=225.9 ppm, Figure 4(c) Ali–IC_{50}=16.10 ppm, and Figure 4(d) linolenic acid–IC_{50}=9.557e+02 ppm. Data are presented as mean standard deviation (SD), with decreasing cell viability as concentration increases. The IC_{50} values indicate the concentration required to inhibit 50% of cell viability. ALA has a large inhibitory effect despite its smaller concentration range, as shown by the IC_{50} value in Figure 4, which is very small. This value indicates that even at low concentrations, ALA is very effective in inhibiting cancer cell growth. The results of the cell viability test and IC_{50} indicate that ALA has the potential to be a very strong anti-cancer agent.

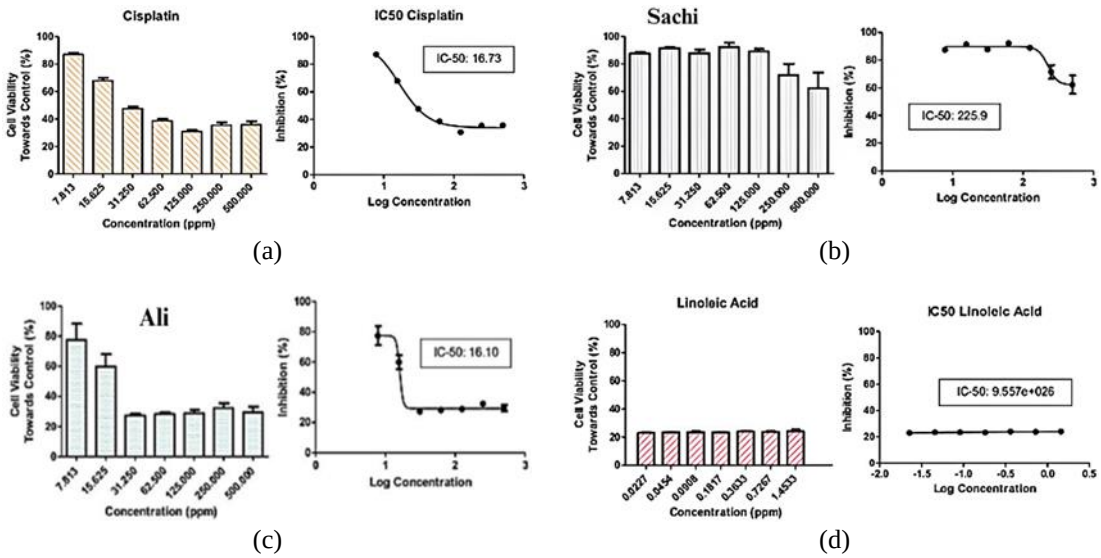


Figure 4. Effect of different compounds on cell viability of (a) comparative cytotoxicity of, (b) Sachi, (c) Ali, and (d) ALA on HeLa cells

Prior to the MTT assay, cell culture of helical cells was carried out to ensure that the cells could grow and develop optimally in an appropriate culture environment, as shown in Figure 5. In the condition of helical cells before seeding, as shown in Figure 5(a). The cells are numerous and scattered all over the surface with a round or oval shape typical of HeLa cells, indicating that they are healthy and have not experienced full attachment to the culture medium. Then, the seeding process is carried out on HeLa cells in Figure 5(b), and it can be seen that the cells begin to adapt and adhere to the culture medium. After culture, HeLa cells were tested by adding test parameters or samples. Figure 5(a) is a normal HeLa cell without any treatment.

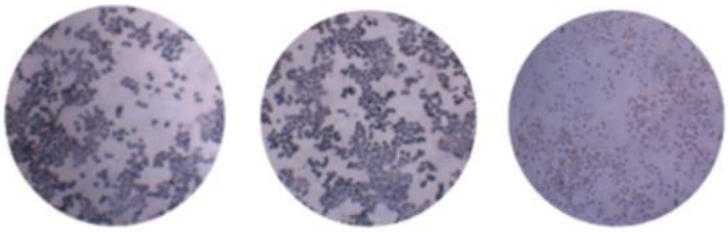
Figure 6 shows the HeLa cells with addition of Sachi and Ali. Figure 6(a) is an image of HeLa cells to which the Sachi sample was added, showing that the cell distribution and morphology remained the same as the untreated HeLa cells. There were no significant changes in shape or signs of cell damage, such as abnormal rounding or clustering, which are indicative of toxic effects. The Sachi can be considered safe for HeLa cells under the conditions tested because no significant cytotoxicity or adverse effects on cell viability, proliferation, or morphology were observed during the experimental period, as shown in Figure 6. Figure 6(a) shows that Sachi A oil has no toxic effect on HeLa cells, as the cells remain viable and maintain their structure well. HeLa cells show signs of cell death or damage after treatment with Sachi, as shown in Figure 6(b). The absence of needle crystal formation suggests cytotoxicity, causing a decrease in cell density and empty areas around damaged cells, affecting cell development and survival.

Figure 7 shows the MTT assay results reveal gradations of color for cisplatin, Sachi, Ali, and ALA on HeLa cells, with cisplatin showing the palest color (indicating high cell death), while ALA exhibits the

most intense purple (suggesting lower cytotoxicity). Previous studies indicate that ALA inhibits HeLa cell growth by up to 54.3% through apoptosis and cell cycle arrest, mediated by increased pro-apoptotic protein expression (bax, caspase-3) and suppression of the PI3K/Akt/mTOR pathway. These findings suggest ALA's potential as a natural anti-cancer agent, either alone or as a chemotherapy adjuvant, though further *in vivo* studies are needed to confirm efficacy and safety. Nanoemulsion formulations or combination therapies could enhance its bioavailability and therapeutic potential.

(a) (b)

Figure 5. HeLa cells (a) before seeding process and (b) after seeding process



(a) (b)

Figure 6. HeLa cells with addition of (a) Sachi and (b) Ali

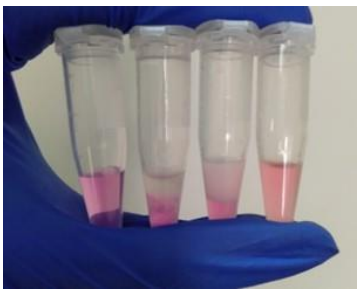


Figure 7. MTT assay results

4. CONCLUSION

This study highlights ALA from Sacha Inchi oil as a promising therapeutic agent for cervical cancer by inducing apoptosis and inhibiting HeLa cell proliferation. MTT assay confirmed its strong cytotoxic effect, while LC-HRMS validated its anti-cancer potential, showing lower IC_{50} values than cisplatin, suggesting higher efficacy at lower doses. These findings support ALA's role as a safer, nature-based therapy that can be used as an adjuvant in chemotherapy to enhance treatment effectiveness while reducing side effects and drug resistance. Future research should focus on *in vivo* studies, nanoemulsion formulation for better bioavailability, and combination therapy with chemotherapeutic agents to optimize its clinical potential for cervical cancer treatment.

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AUTHOR CONTRIBUTIONS STATEMENT

This journal uses the Contributor Roles Taxonomy (CRediT) to recognize individual author contributions, reduce authorship disputes, and facilitate collaboration.

Name of Author	C	M	So	Va	Fo	I	R	D	O	E	Vi	Su	P	Fu
Adi Permadi	✓	✓	✓	✓	✓	✓		✓	✓	✓			✓	
Mutiara Wilson Putri		✓				✓		✓	✓	✓	✓	✓		
Muhammad Ali Akbar	✓		✓	✓			✓			✓	✓		✓	✓

C : **C**onceptualization
M : **M**ethodology
So : **So**ftware
Va : **Va**lidation
Fo : **Fo**rmal analysis
I : **I**nvestigation
R : **R**esources
D : **D**ata Curation
O : Writing - **O**riginal Draft
E : Writing - Review & **E**diting
Vi : **Vi**sualization
Su : **Su**pervision
P : **P**roject administration
Fu : **Fu**nding acquisition

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no financial or personal relationships that could inappropriately influence this research. None of the authors has any ownership interests, patents, or financial benefits related to the subject matter discussed in this article. The collaboration with CV. Inovasi Anak Negeri was limited to providing research materials and did not influence the study design, data collection, analysis, or interpretation. All authors have conducted this research independently and objectively to ensure scientific integrity. Therefore, the authors state that there is no conflict of interest associated with this publication.

DATA AVAILABILITY

The data supporting the findings of this study are available within the article. Additional raw data are available from the corresponding author, [AP], upon reasonable request.

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