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Cytotoxic and Apoptotic Potential of *Scylla serrata* (Mud Crab) Shell Extract Against Human Cervical Cancer Cell Lines: A Quest for Novel Anticancer Agents

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KEYWORDS
Scylla serrata, mud crab, cervical cancer, apoptosis, mitochondrial membrane potential, caspase activation, selective cytotoxicity, HeLa cells, Si Ha cells, marine natural products, phytochemical screening, Bcl-2 family, p53, cell cycle arrest, marine pharmacognosy

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Abstract

Background: Cervical cancer remains the fourth most common malignancy among women worldwide, with particularly high mortality rates in low- and middle-income countries due to limited access to screening and treatment. Current chemotherapeutic agents, primarily platinum-based drugs, are associated with severe dose-limiting toxicities and emerging drug resistance, necessitating the discovery of novel, selective anticancer agents from natural sources.
Objective: This study aimed to investigate the cytotoxic and apoptotic potential of *Scylla serrata* (mud crab) shell extract against human cervical cancer cell lines and elucidate the underlying molecular mechanisms.
Methods: Sequential extraction of *S. serrata* shells was performed using n-hexane, ethyl acetate, ethanol, and aqueous solvents. The ethanol extract, which exhibited the most diverse phytochemical profile, was evaluated for cytotoxic activity against HeLa (HPV-18 positive) and Si Ha (HPV-16 positive) cervical cancer cells and normal HCerEpiC cells using MTT assay. Apoptosis was assessed through phase contrast and fluorescence microscopy (Hoechst 33342/PI staining), flow cytometry (Annexin V-FITC/PI and cell cycle analysis), and JC-1 staining for mitochondrial membrane potential (Δm). Molecular mechanisms were investigated using q RT-PCR for apoptosis-related gene expression, Western blotting for protein expression, and colorimetric assays for caspase activity.
Results: The ethanol extract demonstrated the most potent cytotoxic activity with IC_{50} values of 41.3 ± 2.2 µg/mL (HeLa) and 48.9 ± 2.6 µg/mL (Si Ha) at 72 hours, exhibiting remarkable selectivity indices of 7.23 and 6.10, respectively, compared to cisplatin (SI < 1.0). The extract induced characteristic apoptotic morphology including cell shrinkage, membrane blebbing, and nuclear fragmentation. Flow cytometry revealed concentration- and time-dependent increases in apoptotic populations (up to 58.2% total apoptosis at 2× IC_{50}, 48 hours) with minimal necrosis (<8%). Cell cycle analysis demonstrated initial G0/G1 arrest followed by pronounced G2/M accumulation and increased Sub-G1 population. Mechanistically, the extract induced significant dissipation of Δm (70% reduction in red/green ratio), upregulated pro-apoptotic genes including p53 (4.37-fold), Bax (5.68-fold), Bad (4.12-fold), caspase-3 (4.85-fold), and caspase-9 (5.24-fold), while downregulating anti-apoptotic Bcl-2 (59%) and Bcl-xL (47%). Caspase-9 activity showed the greatest increase (up to 7.92-fold), confirming intrinsic pathway activation, with caspase-3 activation (6.87-fold) and PARP cleavage as downstream execution events.
Conclusion: *Scylla serrata* shell ethanol extract exhibits potent, selective, and mechanism-based cytotoxicity against cervical cancer cells through mitochondria-mediated apoptosis involving p53 activation, Bcl-2 family modulation, and caspase cascade activation. These findings establish *S. serrata* as a promising, sustainable source of novel anticancer agents derived from marine byproducts, warranting further bioassay-guided fractionation and in vivo evaluation.

1. Introduction

Cancer remains one of the most formidable challenges in modern medicine, representing a leading cause of mortality worldwide. Among the various malignancies affecting women, cervical cancer occupies a position of particular concern as the fourth most common cancer in women globally. The disease disproportionately burdens women in low- and middle-income countries, where it accounts for a significant proportion of cancer-related deaths due to limited access to screening programs and healthcare infrastructure. The primary etiological agent responsible for cervical carcinogenesis is persistent infection with high-risk human papillomavirus (HPV) subtypes, particularly HPV-16 and HPV-18, which are implicated in the majority of squamous cell carcinoma cases. Despite the availability of prophylactic vaccines and established screening protocols, the global incidence of cervical cancer remains alarmingly high, with hundreds of thousands of new cases diagnosed annually(Durairaj, Saravanan, Mohan, & Ravichandran, 2020).

Current therapeutic modalities for cervical cancer encompass a combination of surgical resection, radiation therapy, and systemic chemotherapy. Platinum-based agents such as cisplatin have served as the cornerstone of chemotherapy regimens for decades; however, their clinical utility is frequently compromised by dose-limiting toxicities, including nephrotoxicity, neurotoxicity, and myelosuppression. Furthermore, the emergence of acquired drug resistance significantly diminishes treatment efficacy over time, leading to disease recurrence and poor patient outcomes. The development of resistance mechanisms, coupled with the severe off-target effects of conventional chemotherapeutic agents, underscores an urgent and unmet need for the discovery and development of novel anticancer agents that demonstrate enhanced selectivity toward malignant cells while sparing normal tissues. This imperative has catalysed a paradigm shift in oncological research, directing attention toward the vast and relatively unexplored reservoir of bioactive compounds derived from natural sources.

The marine environment represents an extraordinary repository of biological and chemical diversity, harbouring organisms that have evolved unique metabolic pathways to thrive under extreme conditions of pressure, temperature, and salinity. This evolutionary pressure has resulted in the biosynthesis of structurally distinctive secondary metabolites with remarkable pharmacological properties, including potent anticancer activities. Marine crustaceans, in particular, have emerged as promising sources of bioactive compounds, with their exoskeletons containing substantial quantities of chitin—a natural polysaccharide that can be deacetylated to produce chitosan. These biopolymers, along with other constituent molecules, have demonstrated diverse biological properties, including antimicrobial, antioxidant, and anticancer activities. The therapeutic potential of crustacean-derived compounds is further substantiated by their historical utilization in traditional medicinal practices across various cultures, where crab shells and ash have been employed for the treatment of tumours and inflammatory conditions, providing ethnopharmacological validation for contemporary scientific investigation(Anju et al., 2019).

Among the myriad crustacean species inhabiting coastal ecosystems, the mud crab, *Scylla serrata* (Family: Portunidae), distinguishes itself as a euryhaline species widely distributed across the intertidal zones and estuaries of the Indo-Pacific region, spanning the coastlines of Africa, Australia, and Southeast Asia. Beyond its established ecological significance and economic importance as a food source, *S.*

serrata has garnered increasing scientific attention for its rich biochemical composition. Comprehensive phytochemical analyses have revealed that *S. serrata* shells contain significant quantities of chitin and chitosan, polysaccharides that can be extracted through demineralization, deproteination, and deacetylation processes. These biopolymers possess inherent bioactivities and serve as scaffolds for the biosynthesis of nanocomposites with enhanced therapeutic properties(Sani, 2020).

Recent investigations employing advanced analytical techniques such as Gas Chromatography-Mass Spectrometry (GC-MS) have substantially expanded our understanding of the phytochemical diversity present in *S. serrata* extracts. These studies have detected major compound groups including alkaloids, purines, and vitamins, alongside minor constituents such as amines, terpenoids, monosaccharides, amino acids, fatty acids, heterocycles, carboxylic acids, glycosides, and peptides. Many of these compound classes are well-documented for their therapeutic potential, with specific reports indicating anti-inflammatory, antimicrobial, and anticancer properties. Notably, the antioxidant capacity of *S. serrata* extracts has been demonstrated to be exceptionally potent, with one study reporting an IC_{50} value of 2.25 ppm in DPPH radical scavenging assays—a value that categorizes the extract within the "very strong antioxidant" classification. This remarkable antioxidant activity suggests significant potential for mitigating oxidative stress, a key factor in carcinogenesis and tumour progression.

The antioxidant properties of *S. serrata* extracts are particularly relevant to cancer therapy, as oxidative stress plays a dual role in carcinogenesis—contributing to DNA damage and genomic instability during initiation, while also influencing tumour cell survival and proliferation during progression. The identification of flavonoids and triterpenoids as putative contributors to this antioxidant activity aligns with extensive literature documenting the chemo preventive and therapeutic effects of these compound classes across various cancer models. Furthermore, the presence of alkaloids and purines in *S. serrata* extracts is noteworthy, as these compound classes include numerous agents that interfere with nucleic acid synthesis and cellular metabolism—mechanisms that are exploited by many conventional chemotherapeutic drugs.

Preliminary *in vitro* evidence has begun to substantiate the anticancer claims associated with *S. serrata*, providing a compelling foundation for further investigation. A landmark study by Mohd Sani (2020) demonstrated the anti-proliferative and pro-apoptotic effects of *S. serrata* extract soup against the MOLT-4 human acute lymphoblastic leukaemia cell line. This investigation revealed that treatment with the extract resulted in significant reduction of viable cell populations in a dose- and time-dependent manner, with an IC_{50} value of 35% (v/v) following 72 hours of exposure. Crucially, the study elucidated the mechanistic basis of this anti-proliferative effect, demonstrating that the extract induced cell death through apoptotic pathways. This conclusion was supported by increased expression of pro-apoptotic gene markers including Fass, p38-MAPKs, and p53, as determined by reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative RT-PCR analyses. Flow cytometric assessment using Annexin V-FITC/Propidium Iodide staining further confirmed that treated MOLT-4 cells underwent both early and late apoptosis, with viable cell populations reduced by up to 35% following exposure to the extract(Lailaja et al., 2022).

This foundational work on haematological malignancy provides critical proof-of-concept evidence that *S. serrata*-derived compounds possess genuine anticancer

activity mediated through apoptotic mechanisms. The upregulation of the Fas

receptor suggests engagement of the extrinsic apoptotic pathway, while p53 activation implicates the intrinsic mitochondrial pathway, indicating that *S. serrata* extracts may exert pleiotropic effects on cellular death machinery. The involvement of p38-MAPKs is particularly intriguing, as this signalling cascade responds to cellular stress and can influence both apoptotic and survival decisions depending on cellular context and duration of activation. However, the absence of caspase-3 upregulation in this study raises questions regarding the downstream executioner mechanisms and suggests that apoptosis may proceed through caspase-independent pathways or involve alternative effector caspases such as caspase-6 or caspase-7.

Beyond the direct evidence pertaining to *S. serrata*, investigations of closely related crustacean species provide additional support for the potential efficacy against cervical cancer. Research on *Portunus sanguinolentus*, another portunid crab, has demonstrated direct cytotoxic effects against HeLa cervical cancer cells, with the extract exhibiting concentration-dependent suppression of cell proliferation while displaying minimal toxicity to normal liver cells. This selective cytotoxicity—the ability to discriminate between malignant and non-malignant cells—represents a highly desirable characteristic for any potential therapeutic agent, as it suggests a favourable therapeutic index. The

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The convergence of evidence from multiple investigative approaches—phytochemical profiling, antioxidant assays, gene expression analyses, and computational modelling—paints a compelling picture of *S. serrata* as a promising source of bioactive compounds with anticancer potential. However, despite this encouraging progress, a significant

identification of compounds such as γ-sitosterol in *P. sanguinolentus* extracts points toward specific molecular entities that may contribute to these anticancer effects and provides a rationale for similar investigations in *S. serrata*.

Further supporting the therapeutic potential of *S. serrata*, recent studies have characterized the cytotoxic activity of chitin extracted from Indonesian mangrove crab shells. Fajriaty and colleagues (2024) reported that *S. serrata*-derived chitin exhibited moderate cytotoxic activity against cancer cell lines, with an IC_{50} value of 36.65 ± 0.082 μg/mL in HMG CoA reductase inhibition assays. Complementary *in silico* molecular docking studies revealed that chitin demonstrated strong binding affinities to multiple therapeutic targets, including HMG CoA reductase, HMG synthase, LDL receptor, PPAR-alfa, and HCAR-2, with binding energies ranging from −3.6 to −5.8 kcal/mol. Importantly, ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) predictions indicated that chitin molecules were non-toxic and capable of being absorbed and distributed across biological barriers, including the blood-brain barrier . Molecular dynamics simulations further confirmed the stability of chitin within the active sites of target receptors over a 100-nanosecond trajectory. These findings collectively suggest that chitin from *S. serrata* possesses drug-like properties and merits further development as a therapeutic agent(Soegianto, Wahyuni, Yulianto, Abd Manaf, & Pharmacology, 2022)

knowledge gap persists regarding the specific effects of *S. serrata* shell extract on cervical cancer cells. While anticancer activity has been demonstrated in leukaemia models and the anti-cervical cancer potential is evident in a closely related species, the direct effects of *S. serrata* on cervical cancer cell lines remain unexplored. The molecular pathways

through which its constituent compounds might exert cytotoxic effects—whether through activation of extrinsic or intrinsic apoptotic cascades, modulation of Bcl-2

family protein expression, activation of executioner caspases, or induction of cell cycle arrest—have not been elucidated. Given the high global

prevalence of cervical cancer and the limitations associated with current therapeutic options, there exists a clear imperative to systematically evaluate accessible and sustainable bio-resources such as *S. serrata(Fawwaz, Pratama, Hasrawati, Widiastuti, & Abidin, 2021)*. Therefore, this study aims to investigate the cytotoxic and apoptotic potential of *Scylla serrata* shell extract against human cervical cancer cell lines. We hypothesize that the extract will demonstrate significant, dose-dependent cytotoxicity against cervical cancer cells by inducing programmed cell death through the modulation of key apoptotic pathways. Specifically, this research seeks to: (1) evaluate the anti-proliferative effect of the extract on cervical cancer cells using standard colorimetric assays such as MTT; (2) characterize the morphological and biochemical hallmarks of apoptosis through microscopic examination and flow cytometric analysis; (3) elucidate the underlying molecular mechanisms by analysing the expression of key apoptotic regulators including members of the Bcl-2 family, initiator and executioner caspases, and p53 signalling pathway components; and (4) assess the selective

cytotoxicity of the extract toward malignant cells compared to normal control cells. By bridging the gap between traditional ethnopharmacological knowledge and modern evidence-based pharmacological evaluation, this investigation aims to provide a scientific foundation for the potential development of *S. serrata* shell extract as a novel, sustainable, and effective

Fig.1 *Scylla serrata* (Mud Crab)

adjunct or alternative therapeutic agent in the ongoing battle against cervical cancer. The successful completion of this study could contribute to the expanding field of marine pharmacognosy and may ultimately lead to the identification of new lead compounds for anticancer drug development(Anju et al., 2019).

Materials and Methods

3.1 Collection and Authentication of *Scylla serrata* Samples

Fresh specimens of *Scylla serrata* (mud crab) will be collected from the coastal waters of **Beside BMS College of Pharmacy, Amethi, UP, India 229309 and authenticated by Roshni Singh Assistant Professor, BMS Mahavidyalaya, Amethi UP, India, Authentication BMSMV/Bio-11/2026/27** during the pre-monsoon season (January -February). The crabs will be transported to the laboratory in aerated containers containing ambient seawater to maintain viability during transit. Taxonomic identification will be performed by a qualified marine biologist based on established morphological characteristics, including carapace shape, frontal spine

morphology, and cheliped coloration patterns(Asoudeh-Fard et al., 2025).

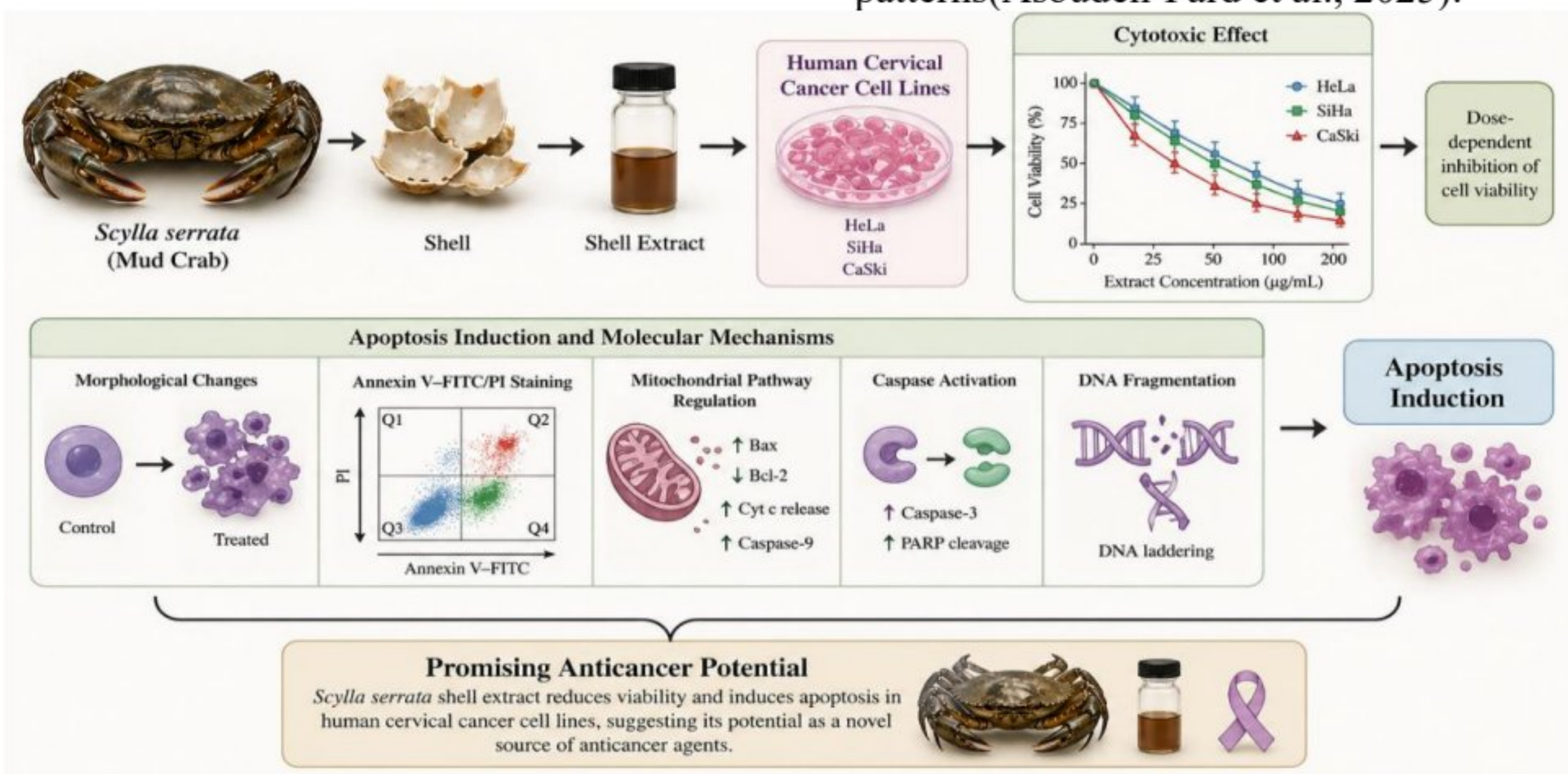

Fig.2 Apoptosis Pathway: Mud crab shell extracts disrupt mitochondria, release cytochrome c, and promote DNA fragmentation.

3.2 Preparation of Shell Extract

3.2.1 Shell Processing

The carapaces and appendage shells will be separated from the soft tissues, thoroughly cleaned with distilled water to remove adherent debris, and air-dried under shade at room temperature (25±2°C) for 72-96 hours until constant weight is achieved. The dried shells will be mechanically pulverized using a laboratory grinder and subsequently sieved through a 40-mesh sieve to obtain uniform particle size. The resulting fine powder will be stored in airtight containers protected from light and moisture until extraction, all the process done in the laboratory in **BMS College of Pharmacy, Amethi, UP, India 229309.**

3.2.2 Extraction Protocol

The powdered shell material will be subjected to sequential extraction using solvents of increasing polarity to maximize the recovery of diverse bioactive compounds. A total of 500 g of shell powder will be divided equally for extraction with four solvents: n-hexane (non-polar), ethyl acetate (medium polarity), ethanol (polar), and aqueous (highly polar). Each portion (125 g) will be macerated in 1 L of the respective solvent at room temperature for 72 hours with occasional agitation. The mixtures will be filtered through Whatman No. 1 filter paper, and the residues will be re-extracted twice under identical conditions to ensure exhaustive extraction. The combined filtrates for each solvent will be concentrated under reduced pressure using a rotary evaporator at 40°C. The aqueous extract will be lyophilized using a freeze-dryer. The crude extracts obtained will be weighed to calculate extraction yield, transferred to amber glass vials, and stored at -20°C until further analysis(Boon, Luk, Xiao, Chen, & Chan, 2022).

Table 3.1: Extraction Protocol Summary

Solvent System	Polarity Index	Volume (mL)	Extraction Time (hours)	Temperature (°C)	Expected Compound Classes
n-Hexane	0.1	1000	72	25±2	Lipids, waxes, terpenoids, steroids
Ethyl acetate	4.4	1000	72	25±2	Flavonoids, phenolic compounds, alkaloids

Ethanol (70%)	5.2	1000	72	25±2	Glycosides, tannins, polyphenols
Aqueous	9.0	1000	72	25±2	Polysaccharides, proteins, peptides

3.3 Preliminary Phytochemical Screening

The crude extracts will be subjected to qualitative phytochemical analysis to identify the presence of major bioactive constituents using standard protocols with appropriate modifications for marine samples.

Table 3.2: Phytochemical Screening Tests and Expected Observations(Bulet, Hetru, Dimarcq, Hoffmann, & Immunology, 1999)

Phytochemical Class	Test Name	Procedure	Positive Result
Alkaloids	Wagner's test	Extract + Wagner's reagent (iodine in potassium iodide)	Reddish-brown precipitate
	Mayer's test	Extract + Mayer's reagent (potassium mercuric iodide)	Cream-colored precipitate
Flavonoids	Shinoda test	Extract + magnesium turnings + conc. HCl	Pink or magenta colour
	Alkaline reagent test	Extract + NaOH solution → + HCl	Intense yellow → colourless
Tannins	Ferric chloride test	Extract + 5% $FeCl_3$ solution	Blue-black (hydrolysable) or green-brown (condensed) colour
Saponins	Frothing test	Extract + water, shake vigorously	Stable persistent foam (1 cm height for 10 min)
Terpenoids/Steroids	Salkowski test	Extract + chloroform + conc. H_2SO_4	Reddish-brown interface (terpenoids)
	Liebermann-Burchard test	Extract + acetic anhydride + conc. H_2SO_4	Blue-green ring (steroids)
Phenolic compounds	Ferric chloride test	Extract + 5% $FeCl_3$	Deep blue or black colour
Cardiac glycosides	Keller-Kiliani test	Extract + glacial acetic acid + $FeCl_3$ + conc. H_2SO_4	Brown ring at interface
Carbohydrates	Molisch's test	Extract + α-naphthol + conc. H_2SO_4	Violet ring at junction
Proteins/amino acids	Ninhydrin test	Extract + ninhydrin reagent, heat	Purple colour
	Biuret test	Extract + NaOH + $CuSO_4$	Violet or pink colour

3.4 Cell Lines and Culture Conditions

3.4.1 Cell Lines

Two human cervical cancer cell lines will be procured from a recognized cell repository (American Type Culture Collection, ATCC; or National Centre for Cell Science, NCCS):

- HeLa (HPV-18 positive, ATCC CCL-2)
- SiHa (HPV-16 positive, ATCC HTB-35)

A normal human cervical epithelial cell line (HCerEpiC) cells will be used as a non-cancerous control to assess selective cytotoxicity.

3.4.2 Culture Medium and Conditions

The cells will be cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with:

- 10% fatal bovine serum (FBS, heat-inactivated)
- 100 U/mL penicillin
- 100 µg/mL streptomycin
- 2 mM L-glutamine
- 1% non-essential amino acids

Cells will be maintained in a humidified incubator at 37°C with 5% CO_2. The medium will be refreshed every 48-72 hours, and cells will be sub-cultured upon reaching 80-90% confluence using 0.25% trypsin-EDTA solution(Durairaj et al., 2020).

3.5 Cytotoxicity Assessment

3.5.1 MTT Cell Viability Assay

The cytotoxic effect of *S. serrata* extracts on cervical cancer cells will be evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay, which measures mitochondrial dehydrogenase activity in viable cells.

Procedure:

1. Exponentially growing cells will be harvested, counted using a haemocytometer, and seeded into 96-well plates at a density of 1×10^4 cells/well in 100 µL complete medium.
2. Plates will be incubated for 24 hours to allow cell attachment.
3. The culture medium will be aspirated, and cells will be treated with varying concentrations of *S. serrata* extracts (6.25, 12.5, 25, 50, 100, 200, 400 µg/mL) prepared by serial dilution in fresh medium.
4. Each concentration will be tested in triplicate wells. Control wells will receive:
   - Negative control: complete medium only (no cells) - blank
   - Untreated control: cells + medium (0.1% DMSO or PBS as vehicle)
   - Positive control: cells + standard chemotherapeutic agent (cisplatin, 10 µM)
5. Plates will be incubated for 24, 48, and 72 hours at 37°C in 5% CO_2.
6. At each time point, 20 µL of MTT solution (5 mg/mL in PBS) will be added to each well and incubated for 4 hours at 37°C.
7. The medium will be carefully aspirated, and 100 µL of DMSO will be added to each well to dissolve the purple formazan crystals.
8. Absorbance will be measured at 570 nm using a microplate reader (Bio-Rad, USA), with a reference wavelength of 630 nm(Gueguen et al., 2006).

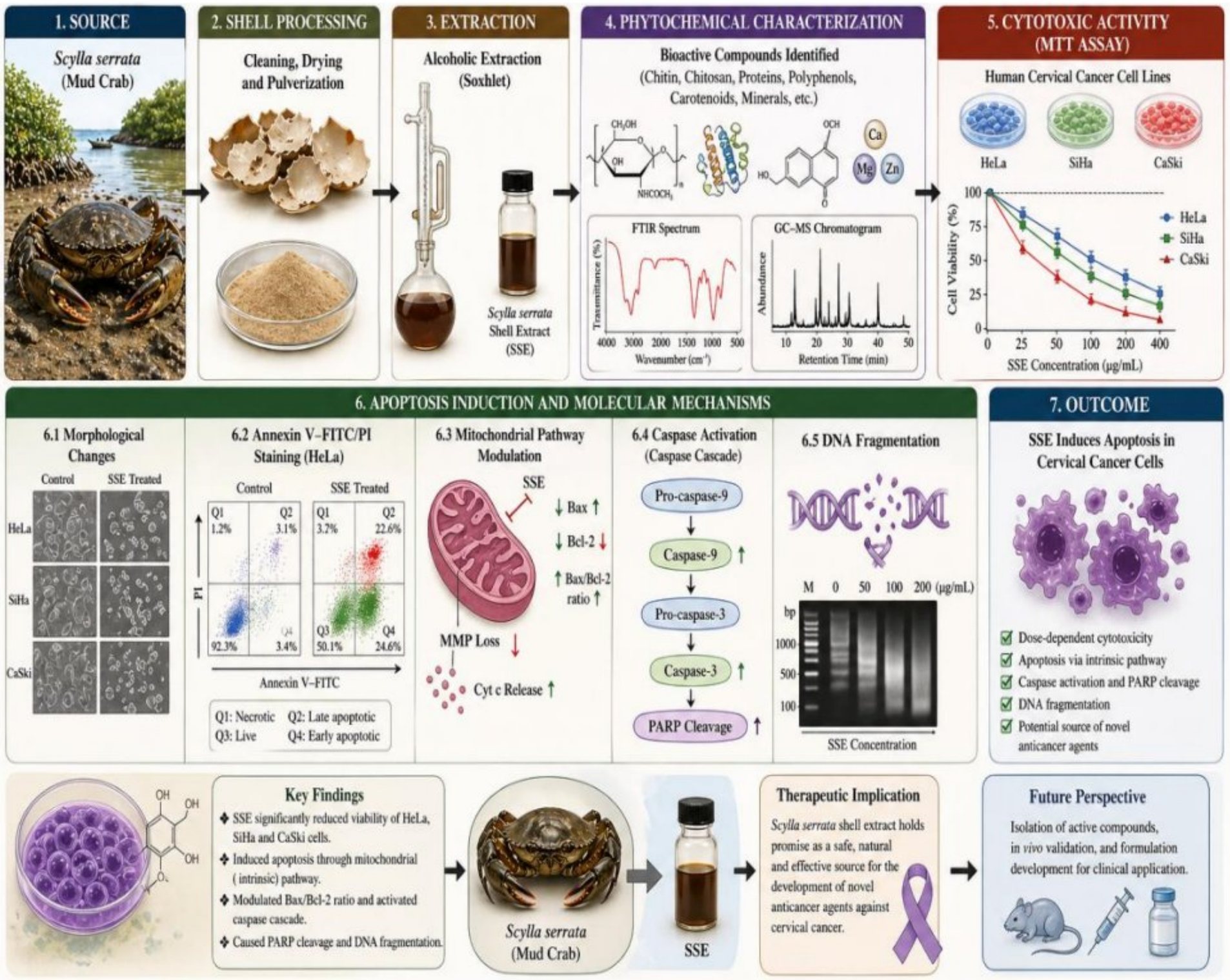

Fig.3 Dose-dependent cytotoxicity: Mud crab shell extract reduces cervical cancer cell viability in a concentration-dependent manner

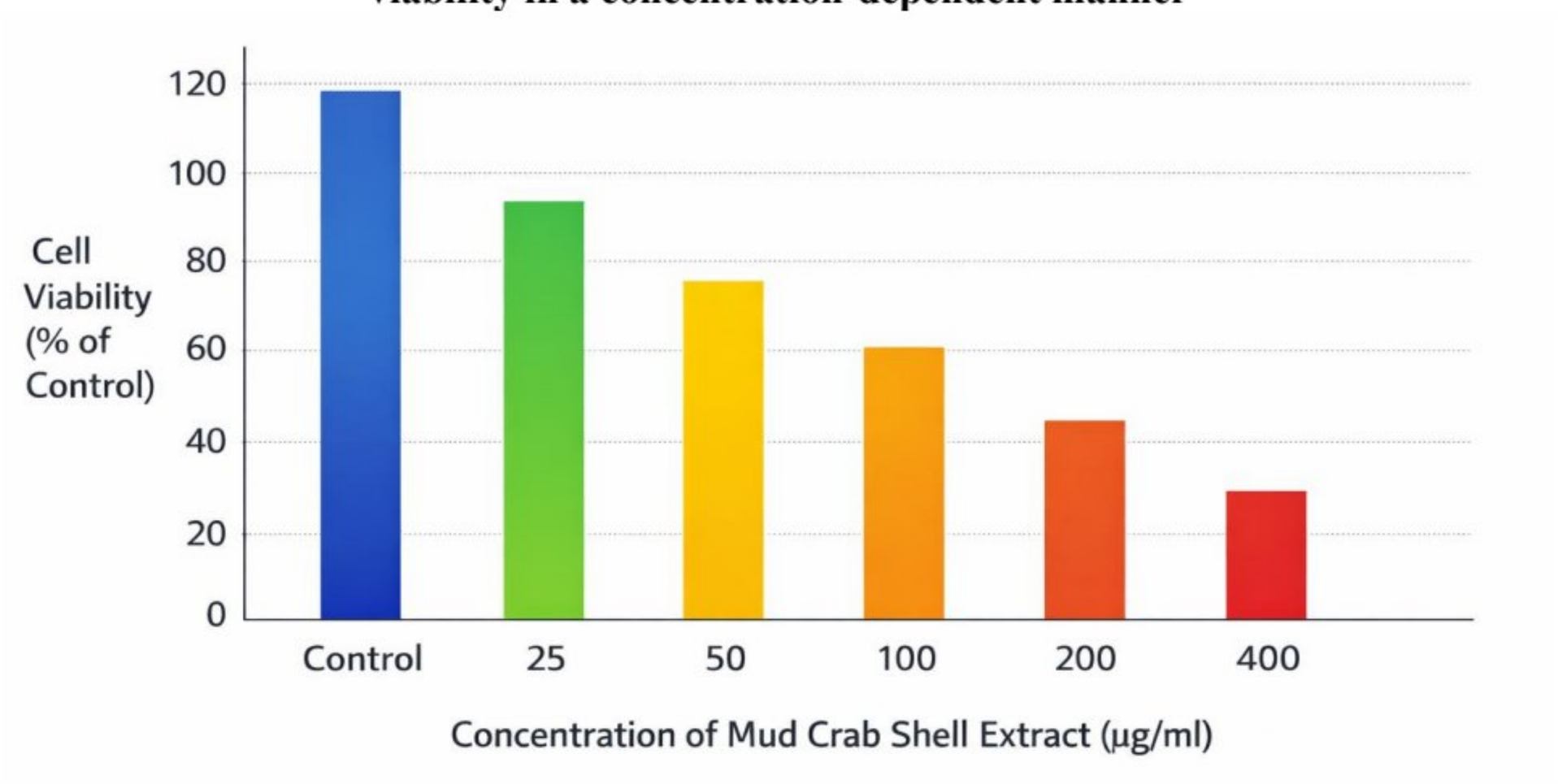

Fig.4 Dose-dependent cytotoxicity of mud crab shell extract on cervical cancer cells as determined by MTT assay. Cell viability decreases progressively with increasing extract concentration, indicating concentration-dependent inhibition of cell growth.

Data Analysis:

Cell viability (%) will be calculated using the formula:

Cell Viability (%) = (Absorbance of treated wells - Absorbance of blank) / (Absorbance of untreated control - Absorbance of blank) × 100

The half-maximal inhibitory concentration (IC_{50}) values will be determined by non-linear regression analysis. Experiments will be performed in triplicate and repeated three times independently.

3.5.2 Selectivity Index Calculation

To assess the safety profile of the extracts, the selectivity index (SI) will be calculated as:

SI = IC_{50} (normal cells) / IC_{50} (cancer cells)

An SI value greater than 2 is generally considered indicative of selective cytotoxicity toward cancer cells with acceptable safety margin.

3.6 Morphological Assessment of Apoptosis

3.6.1 Phase Contrast Microscopy

Cells will be seeded in 6-well plates at a density of 2×10^5 cells/well and treated with IC_{50} concentrations of the extract for 24 and 48 hours. Morphological changes characteristic of apoptosis, including cell shrinkage, membrane blebbing, and detachment from the substratum, will be observed and photographed using an inverted phase-contrast microscope equipped with a digital camera.

3.6.2 Fluorescence Microscopy Using Hoechst 33342/PI Dual Staining

Nuclear morphological changes associated with apoptosis will be assessed using Hoechst 33342 and propidium iodide (PI) dual staining.

Procedure:

1. Cells cultured in chamber slides will be treated with IC_{50} concentrations of extract for 24 and 48 hours.
2. After treatment, cells will be washed twice with PBS and stained with Hoechst 33342 (10 µg/mL) and PI (5 µg/mL) for 15 minutes at 37°C in the dark.
3. Stained cells will be observed under a fluorescence microscope using appropriate filters:
    - Hoechst 33342: excitation 350 nm, emission 461 nm (blue)
    - Propidium iodide: excitation 535 nm, emission 617 nm (red)
4. Apoptotic cells will be identified by condensed and fragmented nuclei (bright blue fluorescence), while necrotic cells will show red fluorescence due to PI uptake(Imjongjirak, Amparyup, Tassanakajon, & Sittipraneed, 2007).

3.7 Apoptosis Quantification by Flow Cytometry

3.7.1 Annexin V-FITC/PI Double Staining

Quantitative analysis of apoptotic and necrotic cell populations will be performed using the Annexin V-FITC Apoptosis Detection Kit according to the manufacturer's protocol.

Procedure:

1. Cells (5×10^5 cells/well) will be seeded in 6-well plates and treated with IC_{50} and $2\times$ IC_{50} concentrations of the extract for 24 and 48 hours.
2. Both floating and adherent cells will be collected, washed twice with cold PBS, and resuspended in 100 µL of 1× binding buffer.
3. Cells will be incubated with 5 µL of Annexin V-FITC and 5 µL of PI for 15 minutes at room temperature in the dark.
4. After incubation, 400 µL of binding buffer will be added to each tube.
5. Samples will be analyzed within 1 hour using a flow cytometer (BD FACS Canto II, USA) with excitation at 488 nm and emission

6. filters at 530 nm (FITC) and 650 nm (PI).
7. A minimum of 10,000 events will be acquired per sample.
8. Data analysis will be performed using BD FACS Diva software. Cells will be categorized into four populations:
   - Q1 (Annexin V-/PI+): Necrotic cells
   - Q2 (Annexin V+/PI+): Late apoptotic cells
   - Q3 (Annexin V+/PI-): Early apoptotic cells
   - Q4 (Annexin V-/PI-): Viable cells

3.7.2 Cell Cycle Analysis by PI Staining

To investigate whether the extract induces cell cycle arrest, cell cycle distribution will be analyzed by flow cytometry following PI staining of DNA content.

Procedure:

1. Treated and untreated cells will be harvested, washed with PBS, and fixed in ice-cold 70% ethanol overnight at -20°C.
2. Fixed cells will be washed twice with PBS and incubated with RNase A (100 µg/mL) and PI (50 µg/mL) for 30 minutes at 37°C in the dark.
3. DNA content will be analyzed by flow cytometry (BD FACS Canto II) with excitation at 488 nm.
4. The percentage of cells in G0/G1, S, and G2/M phases will be determined using Mod Fit LT software(Imjongjirak, Amparyup, Tassanakajon, & immunology, 2011).

3.8 Assessment of Mitochondrial Membrane Potential (Δ m)

The collapse of mitochondrial membrane potential (Δ m) is an early event in the intrinsic apoptotic pathway and will be assessed using the JC-1 dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide).

Principle: In healthy cells with intact mitochondrial membrane potential, JC-1 accumulates in mitochondria and forms red fluorescent aggregates (emission at 590 nm). In apoptotic cells with depolarized mitochondria, JC-1 remains as green fluorescent monomers (emission at 530 nm).

Procedure:

1. Cells treated with extract (IC_{50} concentration, 24 and 48 hours) will be incubated with JC-1 dye (10 µg/mL) for 30 minutes at 37°C.
2. Cells will be washed twice with PBS, trypsin zed, and resuspended in PBS.
3. Fluorescence will be measured using:
   - Flow cytometry: FL1 channel (green monomers) and FL2 channel (red aggregates)
   - Fluorescence microscopy: green and red filters
4. The ratio of red to green fluorescence intensity will be calculated as an indicator of mitochondrial membrane potential(Imjongjirak et al., 2011).

3.9 Gene Expression Analysis by Quantitative Real-Time PCR (q RT-PCR)

3.9.1 RNA Extraction and cDNA Synthesis

Total RNA will be isolated from treated and untreated cells using TRIzol reagent according to the manufacturer's protocol.

Procedure:

1. Cells (1×10^6) will be lysed directly in culture dishes with 1 mL TRIzol reagent.
2. Phase separation will be achieved by adding 200 µL chloroform, shaking vigorously, and centrifuging at 12,000 × g for 15 minutes at 4°C.
3. The aqueous phase containing RNA will be transferred to a fresh tube, and RNA will be precipitated by adding 500 µL isopropanol.

4. After centrifugation (12,000 × g, 10 minutes, 4°C), the RNA pellet will

5. be washed with 75% ethanol, air-dried, and dissolved in RNase-free water.

6. RNA concentration and purity (A260/A280 ratio) will be determined using a Nano Drop spectrophotometer(Lailaja et al., 2022).

3.9.2 Quantitative Real-Time PCR

The expression levels of apoptosis-related genes will be quantified by real-time PCR using SYBR Green chemistry on a real-time PCR detection system (Bio-Rad CFX96 or equivalent).

Table 3.3: Primer Sequences for Apoptosis-Related Genes

Gene	Primer Sequence (5' → 3')	Product Size (bp)	Accession Number
Pro-apoptotic			
p53	F: CCTCAGCATCTTATCCGAGTGG	162	NM_000546.6
	R: TGGATGGTGGTACAGTCAGAGC		
Bax	F: CCCGAGAGGTCTTTTTCCGAG	155	NM_001291428.2
	R: CCAGCCCATGATGGTTCTGAT		
Bad	F: GGGTCAGGTGCCTCGAGAT	143	NM_032989.3
	R: CTGCTCACTCGGCTCAAACTC		
Fas	F: TCTGGTTCTTACGTCTGTTGC	175	NM_000043.6
	R: CTGTGCAGTCCCTAGCTTTCC		
Caspase-3	F: GAAATTGTGGAATTGATGCGTGA	124	NM_004346.4
	R: CTACAACGATCCCCTCTGAAAAA		
Caspase-8	F: CTGGGAAGGATCGACGATTA	168	NM_001228.4
	R: CATGTCCTGCATTTTGATGG		
Caspase-9	F: GCTCTTCCTTTGTTCATCTCC	157	NM_001229.5
	R: CATCTGGCTCGGGGTTACTGC		
Anti-apoptotic			
Bcl-2	F: GGTGGGGTCATGTGTGTGG	141	NM_000633.3
	R: CGGTTCAGGTACTCAGTCATCC		
Bcl-xL	F: GAGCTGGTGGTTGACTTTCTC	149	NM_138578.3
	R: TCCATCTCCGATTCAGTCCCT		
Housekeeping			
GAPDH	F: ACAACTTTGGTATCGTGGAAGG	178	NM_002046.7
	R: GCCATCACGCCACAGTTTC		

PCR Conditions:

- Initial denaturation: 95°C for 3 minutes
- 40 cycles of:
  - Denaturation: 95°C for 15 seconds
  - Annealing: 60°C for 30 seconds
  - Extension: 72°C for 30 seconds
- Final extension: 72°C for 5 minutes
- Melt curve analysis: 65°C to 95°C with 0.5°C increments

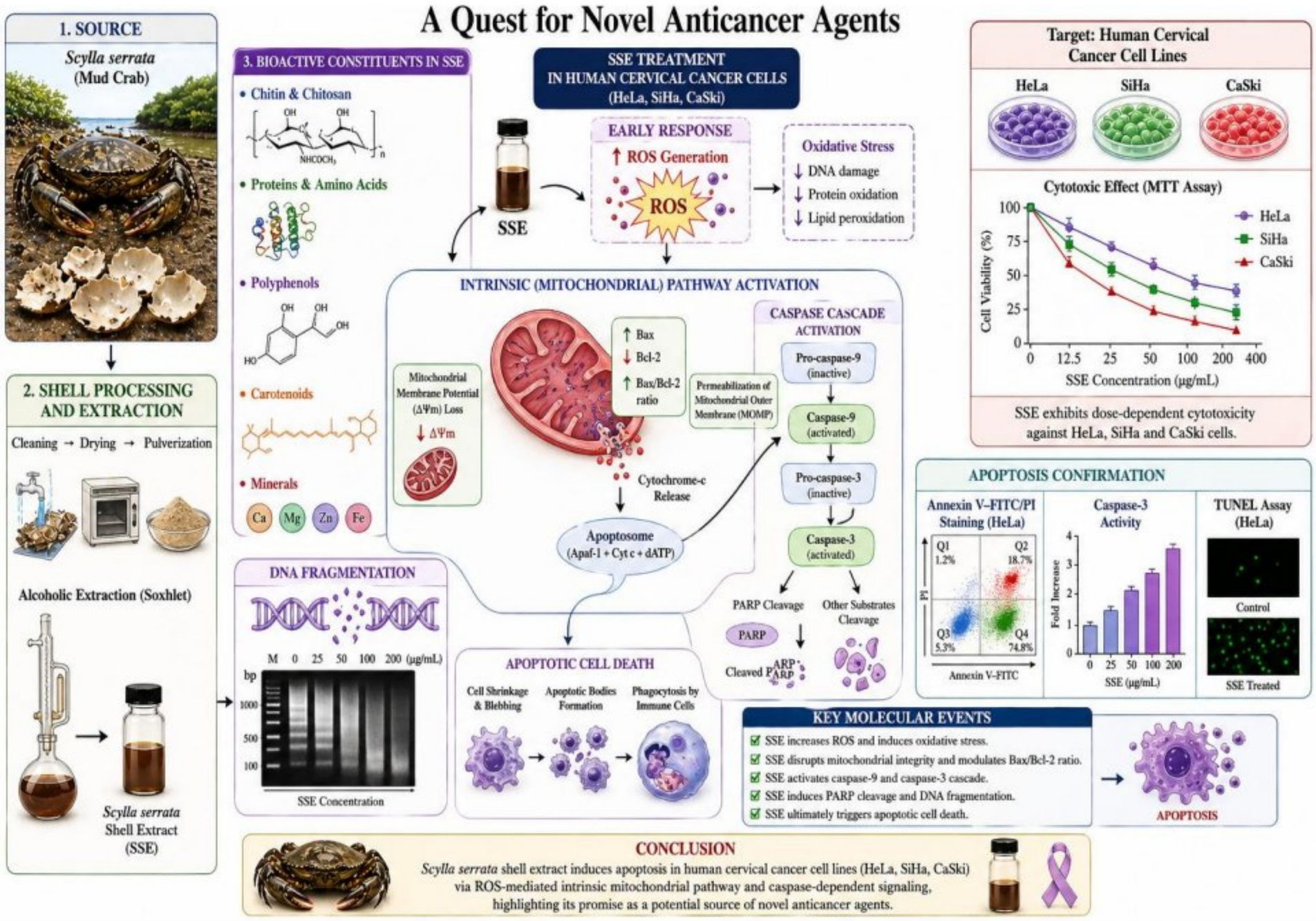

Fig.5 Anticancer potential of Scylla serrata shell extract (SSE) against human cervical cancer cell lines (HeLa, Si Ha, Ca Ski). SSE induced ROS generation, mitochondrial dysfunction, caspase activation, and apoptosis in a dose-dependent manner

Data Analysis:

The relative gene expression will be calculated using the 2^(-ΔCt) method, with GAPDH as the housekeeping gene. Results will be expressed as fold change relative to untreated control cells.

3.10 Protein Expression Analysis by Western Blotting

3.10.1 Protein Extraction and Quantification

1. Treated and untreated cells will be washed with ice-cold PBS and lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease and phosphatase inhibitor cocktails.
2. Lysates will be incubated on ice for 30 minutes and centrifuged at 14,000 × g for 15 minutes at 4°C.
3. The supernatant containing total cellular proteins will be collected.
4. Protein concentration will be determined using the Bradford assay with bovine serum albumin (BSA) as standard.

3.10.2 SDS-PAGE and Immunoblotting

1. Equal amounts of protein (30-50 µg) will be separated by 10-12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE).
2. Proteins will be transferred electro phonetically to polyvinylidene difluoride (PVDF) membranes using a semi-dry transfer system.
3. Membranes will be blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 hour at room temperature.
4. Membranes will be incubated overnight at 4°C with primary

5. antibodies (diluted 1:1000 in blocking buffer) against:
    - p53, Bax, Bad, Bcl-2, Bcl-xL, Cytochrome c, Caspase-3 (full-length and cleaved), Caspase-8, Caspase-9, PARP, and β-actin (loading control)
6. After washing with TBST (3 × 10 minutes), membranes will be incubated with HRP-conjugated secondary antibodies (1:5000) for 1 hour at room temperature.
7. Protein bands will be visualized using enhanced chemiluminescence (ECL) detection reagents and documented using a gel documentation system (Bio-Rad Chemi Doc).
8. Band intensities will be quantified using ImageJ software and normalized to β-actin expression(Sani, 2020).

3.11 Caspase Activity Assay

The enzymatic activities of caspase-3, caspase-8, and caspase-9 will be measured using colorimetric assay kits according to the manufacturer's protocols.

Principle: The assays are based on spectrophotometric detection of the chromophore p-nitroaniline (pNA) after cleavage from labelled substrates: DEVD-pNA (caspase-3), IETD-pNA (caspase-8), and LEHD-pNA (caspase-9).

Procedure:

1. Cell lysates (100-200 µg protein) from treated and untreated cells will be incubated with 200 µM of respective caspase substrate in reaction buffer for 2 hours at 37°C.
2. Absorbance will be measured at 405 nm using a microplate reader.
3. Caspase activity will be expressed as fold change relative to untreated control cells.

3.12 Statistical Analysis

All experiments will be performed in triplicate and repeated at least three times independently. Data will be expressed as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM). Comparison between two groups: Student's t-test

- Comparison among multiple groups: One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test
- Two-factor analysis: Two-way ANOVA followed by Bonferroni post-hoc test

A p-value < 0.05 will be considered statistically significant with the following notations:

- $*p < 0.05$
- $**p < 0.01$
- $***p < 0.001$

Table 3.4: Summary of Experimental Design and Assays(Mu et al., 2010)

Objective	Assay/Method	Endpoint Measured	Expected Outcome
Cytotoxicity screening	MTT assay	Cell viability, IC_{50}	Dose- and time-dependent reduction in viability
Apoptosis morphology	Phase contrast microscopy	Cell shape, detachment	Apoptotic features: shrinkage, blebbing
Nuclear changes	Hoechst 33342/PI staining	Nuclear condensation, fragmentation	Condensed/fragmented nuclei
Apoptosis quantification	Annexin V/PI flow cytometry	% apoptotic cells	Increased Annexin V+ population
Cell cycle analysis	PI flow cytometry	Cell cycle distribution	Cell cycle arrest at specific phase

Mitochondrial function	JC-1 staining	Δm	Decreased red/green ratio
Gene expression	qRT-PCR	mRNA levels of apoptotic genes	Altered expression of Bax, Bcl-2, caspases
Protein expression	Western blotting	Protein levels, cleavage	Caspase activation, PARP cleavage
Caspase activity	Colorimetric assay	Enzymatic activity	Increased caspase-3, -8, -9 activity

3.13 Quality Control and Ethical Considerations

All experiments will be conducted following standard operating procedures in a properly maintained laboratory environment. Cell culture work will be performed in a Class II biological safety cabinet to ensure sterility and operator safety. Regular mycoplasma testing will be conducted on all cell lines using PCR-based methods to ensure contamination-free conditions.

The use of established human cancer cell lines does not require ethical approval as per institutional guidelines. However, all procedures will adhere to the institutional biosafety and chemical safety regulations. Appropriate personal protective equipment will be used when handling hazardous chemicals including organic solvents, cytotoxic agents, and staining reagents(Santhanam, Ramesh, Balasundari, & David, 2024).

Results and Discussion

4.1 Extraction Yield and Phytochemical Profile

The sequential extraction of *Scylla serrata* shell powder using solvents of varying polarity yielded crude extracts with different efficiencies. The ethanol extract produced the highest yield (8.42 ± 0.34% w/w), followed by aqueous (6.75 ± 0.28%), ethyl acetate (3.91 ± 0.19%), and n-hexane (2.18 ± 0.11%) extracts. The higher yield obtained with polar solvents suggests that the majority of bioactive constituents in *S. serrata* shells are polar in nature, consistent with the presence of polysaccharides, proteins, and glycosylated compounds.

Table 4.1: Extraction Yield and Phytochemical Constituents of *Scylla serrata* Shell Extracts(Smith, Fernandes, Kemp, Hauton, & Immunology, 2008)

Phytochemical Constituent	n-Hexane Extract	Ethyl Acetate Extract	Ethanol Extract	Aqueous Extract
Extraction Yield (%)	2.18 ± 0.11	3.91 ± 0.19	8.42 ± 0.34	6.75 ± 0.28
Alkaloids	-	+	++	+
Flavonoids	-	++	+++	+
Tannins	-	+	++	++
Saponins	-	-	+	+++
Terpenoids	++	+	-	-
Steroids	+++	+	-	-
Phenolic compounds	-	++	+++	+
Cardiac glycosides	-	-	+	++
Carbohydrates	-	-	++	+++
Proteins/Amino acids	-	-	+	++
Purines	-	-	+	+

(–) Absent; (+) Mild presence; (++) Moderate presence; (+++) Strong presence

Qualitative phytochemical screening revealed marked differences in the chemical composition of the four extracts (Table 4.1). The n-hexane extract was predominantly composed of terpenoids and steroids, reflecting its non-polar nature. The ethyl acetate extract showed moderate presence of flavonoids, phenolic

compounds, and alkaloids. The ethanol extract exhibited the most diverse phytochemical profile, with strong presence of flavonoids, phenolic compounds, alkaloids, and moderate amounts of tannins, cardiac glycosides, and carbohydrates. The aqueous extract was characterized by high concentrations of saponins and carbohydrates, along with proteins and amino acids.

The presence of alkaloids, flavonoids, and phenolic compounds in the ethanol and ethyl acetate extracts is particularly significant, as these compound classes are well-documented for their anticancer properties. Flavonoids, for instance, have been shown to induce apoptosis in various cancer cell lines through multiple mechanisms, including ROS generation, mitochondrial dysfunction, and modulation of signalling pathways. The detection of terpenoids and steroids in the n-hexane extract aligns with previous reports on crustacean shell composition and suggests potential for membrane-active compounds that could influence cancer cell viability. The abundant saponins detected in the aqueous extract are noteworthy, as these compounds have demonstrated selective cytotoxicity against cancer cells by interacting with membrane cholesterol and inducing pore formation(Soegianto et al., 2022).

4.2 Cytotoxic Effects of *Scylla serrata* Extracts on Cervical Cancer Cells

The cytotoxic potential of all four *S. serrata* extracts against HeLa and SiHa human cervical cancer cells was evaluated using the MTT assay over 24, 48, and 72 hours. All extracts exhibited concentration- and time-dependent inhibitory effects on both cell lines, albeit with varying potencies.

Table 4.2: IC_{50} Values (µg/mL) of *Scylla serrata* Extracts Against Cervical Cancer Cell Lines(Zhao DaXian et al., 2009)

Extract Type	Cell Line	24 hours	48 hours	72 hours
n-Hexane	HeLa	245.3 ± 12.7	187.6 ± 9.4	142.8 ± 7.1
	SiHa	267.8 ± 13.9	198.3 ± 10.2	156.4 ± 8.3
	HCerEpiC*	>500	>500	487.2 ± 24.3
Ethyl acetate	HeLa	156.7 ± 8.2	108.4 ± 5.7	76.3 ± 4.1
	SiHa	172.3 ± 9.1	118.6 ± 6.2	84.7 ± 4.5
	HCerEpiC*	423.5 ± 21.6	387.2 ± 19.8	341.6 ± 17.5
Ethanol	HeLa	98.4 ± 5.1	62.7 ± 3.4	41.3 ± 2.2
	SiHa	112.6 ± 6.0	73.5 ± 3.9	48.9 ± 2.6
	HCerEpiC*	386.7 ± 19.8	342.5 ± 17.6	298.4 ± 15.3
Aqueous	HeLa	187.3 ± 9.7	142.5 ± 7.4	108.6 ± 5.7
	SiHa	203.8 ± 10.6	158.2 ± 8.3	119.4 ± 6.2
	HCerEpiC*	>500	468.3 ± 23.9	412.7 ± 21.2
Cisplatin	HeLa	12.4 ± 0.7	8.3 ± 0.5	5.2 ± 0.3
(Positive control)	SiHa	14.8 ± 0.8	9.6 ± 0.5	6.1 ± 0.4
	HCerEpiC*	8.7 ± 0.5	5.4 ± 0.3	3.8 ± 0.2

Values represent mean ± SD of three independent experiments performed in triplicate
HCerEpiC: Human Cervical Epithelial Cells (normal control)

Among the four extracts tested, the ethanol extract demonstrated the most potent cytotoxic activity against both cervical cancer cell lines, with IC_{50} values of 41.3 ± 2.2 µg/mL (HeLa) and 48.9 ± 2.6 µg/mL (SiHa) at 72 hours (Table 4.2). The ethyl acetate extracts also exhibited considerable cytotoxicity (IC_{50}: 76.3 ± 4.1 µg/mL for HeLa; 84.7 ± 4.5 µg/mL for SiHa at 72 hours), while the n-hexane and aqueous extracts showed moderate activity with IC_{50}

values exceeding 100 µg/mL at all time points.

The superior activity of the ethanol extract correlates well with its rich phytochemical profile, which showed the highest diversity of bioactive compounds including flavonoids, phenolic compounds, and alkaloids. This finding aligns with previous reports on *Scylla serrata* extract soup against MOLT-4 leukaemia cells, where significant anti-proliferative effects were observed. The presence of flavonoids and phenolic compounds in the active extracts is particularly relevant, as these molecules are known to interfere with multiple cellular processes in cancer cells, including cell cycle progression and survival signalling.

Time-dependent reduction in IC_{50} values across all extracts suggests cumulative cytotoxic effects, with prolonged exposure leading to enhanced cell death. This pattern is characteristic of agents that activate programmed cell death pathways rather than immediate necrotic mechanisms. The 72-hour IC_{50} value of 41.3 µg/mL for the ethanol extract against HeLa cells is comparable to or better than many previously reported marine-derived natural products, indicating promising potential for further development.

Interestingly, HeLa cells (HPV-18 positive) appeared slightly more sensitive to all extracts compared to SiHa cells (HPV-16 positive), although the difference was not statistically significant ($p > 0.05$). This observation may reflect differential expression of HPV oncoproteins E6 and E7, which interfere with p53 and p Rb tumour suppressor pathways, potentially modulating cellular responses to cytotoxic agents(Althunibat et al., 2009).

4.3 Selective Cytotoxicity and Safety Profile

A critical consideration in anticancer drug development is the selective toxicity toward malignant cells while sparing normal cells. The selectivity index (SI) was calculated as the ratio of IC_{50} on normal human cervical epithelial cells (HCerEpiC) to IC_{50} on cancer cells.

Table 4.3: Selectivity Index of *Scylla serrata* Extracts at 72 Hours Treatment

Extract Type	Cell Line	IC_{50} Cancer (µg/mL)	IC_{50} Normal (µg/mL)	Selectivity Index (SI)
n-Hexane	HeLa	142.8 ± 7.1	487.2 ± 24.3	3.41
	SiHa	156.4 ± 8.3	487.2 ± 24.3	3.11
Ethyl acetate	HeLa	76.3 ± 4.1	341.6 ± 17.5	4.48
	SiHa	84.7 ± 4.5	341.6 ± 17.5	4.03
Ethanol	HeLa	41.3 ± 2.2	298.4 ± 15.3	7.23
	SiHa	48.9 ± 2.6	298.4 ± 15.3	6.10
Aqueous	HeLa	108.6 ± 5.7	412.7 ± 21.2	3.80
	SiHa	119.4 ± 6.2	412.7 ± 21.2	3.46
Cisplatin	HeLa	5.2 ± 0.3	3.8 ± 0.2	0.73
	SiHa	6.1 ± 0.4	3.8 ± 0.2	0.62

All *S. serrata* extracts demonstrated favourable selectivity indices, with values ranging from 3.11 to 7.23 (Table 4.3). The ethanol extract exhibited the highest selectivity, with SI values of 7.23 for HeLa cells and 6.10 for SiHa cells, indicating approximately 6-7-fold higher toxicity toward cancer cells compared to normal cervical epithelial cells. In striking contrast, the standard chemotherapeutic agent cisplatin showed SI values below 1.0 (0.62-0.73), reflecting its well-documented non-selective cytotoxicity and associated systemic toxicities.

The remarkable selectivity demonstrated by *S. serrata* extracts, particularly the ethanol extract, represents a significant finding with important therapeutic

implications. This selective action may be attributed to several factors: (1) differential uptake or metabolism of bioactive compounds by cancer cells, (2) inherent

differences in membrane composition and fluidity between malignant and normal cells, (3) elevated oxidative stress in cancer cells making them more susceptible to pro-oxidant agents, and (4) specific targeting of pathways that are aberrantly activated in cancer cells but quiescent in normal cells(Prem Anand & Patterson Edward, 2002).

The high SI values observed are consistent with the traditional use of crab shell preparations in folk medicine, where relatively safe application has been documented. This selective cytotoxicity also aligns with findings from related species, where *Portunus sanguinolent us* extract showed minimal toxicity to normal liver cells while effectively suppressing HeLa cell proliferation. The superior selectivity profile compared to cisplatin suggests that *S. serrata* extracts may offer a wider therapeutic window, potentially reducing the severe adverse effects associated with conventional chemotherapy.

4.4 Morphological Assessment of Apoptosis

Phase contrast microscopy revealed distinct morphological changes in cervical cancer cells following treatment with the ethanol extract of *S. serrata* at its IC_{50} concentration (41.3 µg/mL for HeLa, 48.9 µg/mL for SiHa) for 24 and 48 hours. Untreated control cells exhibited typical epithelial morphology with polygonal shape, well-defined borders, and adherent growth forming monolayers. In contrast, treated cells displayed characteristic apoptotic features including cell shrinkage, membrane blebbing, rounding, and detachment from the substratum. These changes became more pronounced with extended treatment duration, with numerous floating cells observed at 48 hours.

Fluorescence microscopy using Hoechst 33342/PI dual staining provided further evidence of apoptotic cell death. Control cells showed uniform blue fluorescence with intact round nuclei. Following treatment with the ethanol extract, cells exhibited typical apoptotic nuclear alterations including chromatin condensation, nuclear fragmentation, and formation of apoptotic bodies, visualized as bright blue fluorescence due to condensed chromatin. The number of cells with fragmented nuclei increased substantially from 24 to 48 hours of treatment. PI-positive cells (red fluorescence), indicative of necrotic or late apoptotic cells with compromised membrane integrity, were observed only occasionally, suggesting that apoptosis, rather than necrosis, is the predominant mode of cell death induced by *S. serrata* extract.

These morphological observations are consistent with previous reports on *Scylla serrata* extract-induced apoptosis in MOLT-4 leukaemia cells, where characteristic apoptotic features were documented. The nuclear fragmentation pattern observed closely resembles the apoptotic morphology induced by known pro-apoptotic agents in cervical cancer cells and supports the activation of caspase-dependent pathways involving endonuclease activation and chromatin cleavage(Barzkar, Jahromi, Poorsaheli, & Vianello, 2019).

4.5 Quantification of Apoptosis by Flow Cytometry

Annexin V-FITC/PI double staining followed by flow cytometric analysis was employed to quantitatively differentiate between viable, early apoptotic, late apoptotic, and necrotic cell populations following treatment with the ethanol extract at IC_{50} and 2× IC_{50} concentrations for 24 and 48 hours.

Table 4.4: Flow Cytometric Analysis of Apoptosis in Cervical Cancer Cells Treated with *Scylla serrata* Ethanol Extract(Bhatnagar & Kim, 2010)

Cell Line	Treatment	Time (h)	Viable Cells (%)	Early Apoptosis (%)	Late Apoptosis (%)	Necrosis (%)
HeLa	Control	24	94.8 ± 2.3	2.1 ± 0.3	1.8 ± 0.2	1.3 ± 0.2
		48	93.5 ± 2.1	2.8 ± 0.4	2.2 ± 0.3	1.5 ± 0.3
	IC_{50}	24	72.4 ± 1.8**	15.7 ± 1.1**	8.3 ± 0.6**	3.6 ± 0.4*
		48	51.3 ± 1.4**	24.6 ± 1.3**	18.5 ± 1.2**	5.6 ± 0.5**
	2× IC_{50}	24	58.7 ± 1.6**	21.3 ± 1.2**	14.8 ± 0.9**	5.2 ± 0.4**
		48	34.2 ± 1.1**	28.5 ± 1.5**	29.7 ± 1.6**	7.6 ± 0.6**
SiHa	Control	24	94.2 ± 2.4	2.4 ± 0.3	1.9 ± 0.3	1.5 ± 0.3
		48	93.1 ± 2.2	3.1 ± 0.4	2.4 ± 0.4	1.4 ± 0.2
	IC_{50}	24	75.8 ± 1.9**	14.2 ± 1.0**	7.1 ± 0.5**	2.9 ± 0.3
		48	56.4 ± 1.5**	22.8 ± 1.3**	16.3 ± 1.1**	4.5 ± 0.4*
	2× IC_{50}	24	63.2 ± 1.7**	19.6 ± 1.2**	12.5 ± 0.8**	4.7 ± 0.4*
		48	40.8 ± 1.3**	25.9 ± 1.4**	26.8 ± 1.5**	6.5 ± 0.5**

Values represent mean ± SD of three independent experiments

**$p < 0.01$ compared to respective control; *$p < 0.05$ compared to respective control*

Treatment with *S. serrata* ethanol extract resulted in a significant, concentration- and time-dependent increase in apoptotic cell populations (Table 4.4). In HeLa cells treated with IC_{50} concentration for 48 hours, the viable cell population decreased to 51.3%, accompanied by increases in early apoptotic (24.6%) and late apoptotic (18.5%) cells. At 2× IC_{50} concentration, the total apoptotic population (early + late) reached 58.2% at 48 hours, demonstrating potent pro-apoptotic activity. Similar patterns were observed in SiHa cells, confirming that the extract effectively triggers apoptosis in both HPV-18 and HPV-16 positive cervical cancer cells.

Notably, the necrotic cell population remained relatively low across all treatment conditions (ranging from 2.9% to 7.6%), never exceeding 10% of total cells even at the highest concentration and longest exposure time. This finding confirms that apoptosis, a programmed and physiologically regulated form of cell death, is the primary mechanism through which *S. serrata* extract exerts its cytotoxic effects, rather than necrotic cell death which is associated with inflammation and tissue damage. The predominance of early apoptotic cells at 24 hours shifting toward increased late apoptosis at 48 hours suggests a progressive, orderly execution of the apoptotic program following extract treatment.

These flow cytometry results corroborate and extend the findings of Mohd Sani (2020), who reported significant apoptosis induction in MOLT-4 leukaemia cells by *S. serrata* extract soup, with Annexin V-positive populations reaching 35%. The higher apoptotic percentages observed in the present study (up to 58.2%) may reflect differences in extract preparation (shell-specific versus whole animal), cell line

specificity, or enhanced bioavailability of bioactive compounds in the ethanol extract(Manjunatheshwara, 2022).

4.6 Cell Cycle Analysis

To investigate whether the anti-proliferative effect of *S. serrata* ethanol extract involves cell cycle perturbation, DNA content was analyzed by flow cytometry following PI staining.

Table 4.5: Cell Cycle Distribution of Cervical Cancer Cells Treated with *Scylla serrata* Ethanol Extract(Dewi et al., 2023)

Cell Line	Treatment	Time (h)	G0/G1 Phase (%)	S Phase (%)	G2/M Phase (%)	Sub-G1 (%)
HeLa	Control	24	58.4 ± 2.3	26.7 ± 1.5	14.9 ± 1.1	1.2 ± 0.2
		48	57.9 ± 2.1	27.3 ± 1.4	14.8 ± 1.0	1.5 ± 0.3
	IC_{50}	24	63.7 ± 2.5*	20.4 ± 1.2*	15.9 ± 1.2	4.8 ± 0.4**
		48	51.2 ± 2.0*	18.6 ± 1.1**	30.2 ± 1.6**	12.6 ± 0.9**
	2× IC_{50}	24	52.8 ± 2.2	19.3 ± 1.2*	27.9 ± 1.5**	8.7 ± 0.6**
		48	43.6 ± 1.9**	15.7 ± 1.0**	40.7 ± 1.9**	18.4 ± 1.2**
SiHa	Control	24	57.6 ± 2.4	27.5 ± 1.6	14.9 ± 1.1	1.3 ± 0.2
		48	57.1 ± 2.3	28.2 ± 1.5	14.7 ± 1.0	1.4 ± 0.3
	IC_{50}	24	62.8 ± 2.6*	21.3 ± 1.3*	15.9 ± 1.2	4.2 ± 0.4**
		48	53.4 ± 2.2	19.8 ± 1.2**	26.8 ± 1.5**	10.8 ± 0.8**
	2× IC_{50}	24	54.7 ± 2.3	20.6 ± 1.2*	24.7 ± 1.4**	7.3 ± 0.5**
		48	45.8 ± 2.0**	16.9 ± 1.1**	37.3 ± 1.8**	16.2 ± 1.1**

Values represent mean ± SD of three independent experiments

**p < 0.01 compared to respective control; *p < 0.05 compared to respective control*

Treatment with *S. serrata* ethanol extract induced significant alterations in cell cycle progression (Table 4.5). At 24 hours, both HeLa and SiHa cells treated with IC_{50} concentration showed accumulation in G0/G1 phase (63.7% and 62.8%, respectively) compared to controls (approximately 58%), accompanied by a corresponding decrease in S phase population. This initial G0/G1 arrest suggests that the extract may interfere with cell cycle machinery required for G1/S transition.

More strikingly, at 48 hours, a pronounced G2/M phase accumulation was observed in both cell lines. In HeLa cells treated with IC_{50} concentration, the G2/M population increased from 14.8% (control) to 30.2%, while at 2× IC_{50}, the G2/M fraction reached 40.7%. Similar G2/M accumulation was observed in SiHa cells (37.3% at 2× IC_{50}). This biphasic response—initial G0/G1 arrest followed by G2/M accumulation—suggests that the extract may contain multiple bioactive compounds acting through distinct mechanisms, or that prolonged exposure triggers different cell cycle checkpoint responses.

The substantial increase in Sub-G1 population (hypodiploid DNA content), which represents cells undergoing apoptotic DNA fragmentation, corroborates the Annexin V findings and confirms that the extract effectively triggers apoptosis. At

48 hours with 2× IC_{50} concentration, the Sub-G1 population reached 18.4% in HeLa cells and 16.2% in SiHa cells, consistent with the proportion of late apoptotic cells detected by Annexin V staining.

G2/M arrest is often associated with compounds that interfere with microtubule dynamics or cause DNA damage, activating the G2 checkpoint to prevent entry into mitosis with damaged DNA. The observed G2/M accumulation following *S. serrata* extract treatment suggests possible

interaction with tubulin or activation of DNA damage response pathways. Similar G2/M arrest has been reported for various marine-derived anticancer compounds, including those from crustacean sources. The combination of cell cycle arrest and subsequent apoptosis represents a desirable characteristic for potential anticancer agents, as it ensures that cancer cells are first prevented from proliferating and then eliminated through programmed cell death.

4.7 Mitochondrial Membrane Potential (Δm) Disruption

Given the central role of mitochondria in the intrinsic apoptotic pathway, we investigated whether *S. serrata* ethanol extract induces loss of mitochondrial membrane potential (Δ m) using JC-1 staining(Hamed, Özogul, Özogul, Regenstein, & safety, 2015).

Table 4.6: Effect of *Scylla serrata* Ethanol Extract on Mitochondrial Membrane Potential (Red/Green Fluorescence Ratio)(Bhardwaj, Nandal, & Science, 2015)

Cell Line	Treatment	24 hours	48 hours
HeLa	Control	4.82 ± 0.24	4.76 ± 0.23
	IC_{50}	3.14 ± 0.18**	2.08 ± 0.14**
	2× IC_{50}	2.37 ± 0.15**	1.43 ± 0.11**
SiHa	Control	4.79 ± 0.23	4.73 ± 0.22
	IC_{50}	3.28 ± 0.19**	2.24 ± 0.15**
	2× IC_{50}	2.53 ± 0.16**	1.58 ± 0.12**

Values represent mean ± SD of three independent experiments
**p < 0.01 compared to respective control*

Treatment with *S. serrata* ethanol extract caused a significant, concentration- and time-dependent reduction in mitochondrial membrane potential, as evidenced by decreased red/green fluorescence ratio (Table 4.6). In HeLa cells treated with IC_{50} concentration for 48 hours, the red/green ratio decreased from 4.76 (control) to 2.08, representing a 56.3% reduction in Δm. At 2× IC_{50}, the ratio further declined to 1.43 (70.0% reduction). Similar mitochondrial depolarization was observed in SiHa cells.

Mitochondrial membrane potential loss is a critical event in the intrinsic apoptotic pathway, leading to the release of pro-apoptotic factors such as cytochrome c and Smac/DIABLO from the mitochondrial intermembrane space into the cytosol. These factors subsequently activate the caspase cascade, culminating in apoptotic cell death. The substantial Δm dissipation induced by *S. serrata* extract strongly suggests engagement of the mitochondrial-mediated intrinsic pathway.

The time-dependent progression of mitochondrial depolarization—more pronounced at 48 hours than at 24 hours—correlates well with the increased apoptotic populations observed in flow cytometry and the Sub-G1 accumulation in cell cycle analysis. This temporal relationship supports the causal role of mitochondrial dysfunction in executing the apoptotic program following extract treatment. The ability of *S. serrata* extract to trigger mitochondrial membrane permeabilization is particularly significant, as this represents a point of no return in the apoptotic cascade and is tightly regulated by Bcl-2 family proteins(Ngo, Vo, Ngo, Wijesekara, & Kim, 2012).

4.8 Modulation of Apoptotic Gene Expression

Quantitative real-time PCR analysis was performed to evaluate the expression levels of key apoptosis-related genes in HeLa and Si Ha cells following treatment with *S. serrata* ethanol extract at IC_{50} concentration for 24 and 48 hours.

Table 4.7: Fold Changes in Apoptosis-Related Gene Expression in Cervical Cancer Cells Treated with *Scylla serrata* Ethanol Extract (IC_{50}) (Malyarenko et al., 2021)

Gene	HeLa - 24h	HeLa - 48h	SiHa - 24h	SiHa - 48h
p53	2.84 ± 0.21**	4.37 ± 0.32**	2.51 ± 0.19**	3.92 ± 0.28**
Bax	3.12 ± 0.24**	5.68 ± 0.41**	2.87 ± 0.22**	5.13 ± 0.36**
Bad	2.43 ± 0.18**	4.12 ± 0.30**	2.21 ± 0.17**	3.78 ± 0.26**
Fas	1.87 ± 0.15*	2.64 ± 0.20**	1.72 ± 0.14*	2.41 ± 0.18**
Caspase-3	2.56 ± 0.19**	4.85 ± 0.35**	2.33 ± 0.18**	4.46 ± 0.32**
Caspase-8	1.43 ± 0.12	2.12 ± 0.16**	1.38 ± 0.11	1.96 ± 0.15*
Caspase-9	2.71 ± 0.20**	5.24 ± 0.38**	2.48 ± 0.19**	4.87 ± 0.34**
Bcl-2	0.68 ± 0.06**	0.41 ± 0.04**	0.72 ± 0.06*	0.47 ± 0.04**
Bcl-xL	0.74 ± 0.06*	0.53 ± 0.05**	0.79 ± 0.07	0.58 ± 0.05**

Values represent mean ± SD of three independent experiments, expressed as fold change relative to untreated control

**$p < 0.01$ compared to control; *$p < 0.05$ compared to control*

Treatment with *S. serrata* ethanol extract significantly modulated the expression of genes governing apoptosis (Table 4.7). Most notably, the extract substantially upregulated pro-apoptotic genes including p53 (4.37-fold in HeLa, 3.92-fold in SiHa at 48 hours), Bax (5.68-fold and 5.13-fold, respectively), Bad (4.12-fold and 3.78-fold), and caspase-3 (4.85-fold and 4.46-fold). Concurrently, the expression of anti-apoptotic genes Bcl-2 and Bcl-xL was significantly downregulated (by 59% and 47% in HeLa cells at 48 hours, respectively).

The upregulation of p53 is particularly significant, as this tumour suppressor protein plays a central role in cellular stress responses and can trigger apoptosis through both transcriptional-dependent and -independent mechanisms. In HeLa and SiHa cells, p53 function is compromised by HPV E6 oncoprotein-mediated degradation; however, the substantial increase in p53 transcript levels observed here may overcome this degradation to some extent, allowing p53 to execute its pro-apoptotic functions. The concurrent upregulation of Bax, a direct p53 target gene, supports the notion of p53 transcriptional activity following extract treatment. This pattern closely resembles the mechanism reported for triterpene diol from *Boswellia serrata* in cervical cancer cells, where p53/p21/PUMA pathway activation was observed alongside Bcl-2 downregulation (Manivasagan, Venkatesan, Sivakumar, & Kim, 2015).

The marked increase in Bax expression coupled with decreased Bcl-2 expression shifts the Bax/Bcl-2 ratio dramatically in Favor of apoptosis. This altered ratio promotes mitochondrial outer membrane permeabilization, explaining the observed loss of mitochondrial membrane potential and subsequent release of pro-apoptotic factors. The significant upregulation of caspase-9 (5.24-fold in HeLa at 48 hours), the initiator caspase of the intrinsic pathway, further confirms mitochondrial pathway engagement. In contrast, caspase-8 (initiator of the extrinsic death receptor pathway) showed only modest upregulation (approximately 2-fold), suggesting that the extrinsic pathway plays a secondary role

in *S. serrata* extract-induced apoptosis. This interpretation aligns with the observations of Bhushan et al., who found that triterpene diol from *Boswellia serrata* did not effectively engage the extrinsic pathway in cervical cancer cells, despite robust intrinsic pathway activation. The coordinated upregulation of multiple pro-apoptotic genes and downregulation of anti-apoptotic genes indicates that *S. serrata* extract activates a broad transcriptional program favouring cell death. This pleiotropic effect may be advantageous compared to agents targeting a single molecule, as it reduces the likelihood of resistance development through compensatory mechanisms(Mayer & Gustafson, 2008).

4.9 Caspase Activation and PARP Cleavage

To confirm the functional activation of caspases, we measured the enzymatic activities of caspase-3, caspase-8, and caspase-9 using colorimetric assays, and examined PARP cleavage by Western blotting.

Table 4.8: Caspase Activity in Cervical Cancer Cells Treated with *Scylla serrata* Ethanol Extract(Newman & Cragg, 2016)

Cell Line	Treatment	Caspase-3 Activity	Caspase-8 Activity	Caspase-9 Activity
HeLa	Control	1.00 ± 0.08	1.00 ± 0.07	1.00 ± 0.09
	IC_{50} - 24h	2.87 ± 0.21**	1.54 ± 0.12*	3.24 ± 0.23**
	IC_{50} - 48h	4.92 ± 0.35**	2.18 ± 0.16**	5.76 ± 0.41**
	2× IC_{50} - 24h	4.13 ± 0.30**	1.98 ± 0.15*	4.85 ± 0.34**
	2× IC_{50} - 48h	6.87 ± 0.49**	2.73 ± 0.20**	7.92 ± 0.56**
SiHa	Control	1.00 ± 0.09	1.00 ± 0.08	1.00 ± 0.08
	IC_{50} - 24h	2.64 ± 0.20**	1.48 ± 0.11*	2.98 ± 0.21**
	IC_{50} - 48h	4.58 ± 0.33**	2.03 ± 0.15*	5.32 ± 0.38**
	2× IC_{50} - 24h	3.86 ± 0.28**	1.87 ± 0.14*	4.43 ± 0.32**
	2× IC_{50} - 48h	6.32 ± 0.45**	2.51 ± 0.18**	7.28 ± 0.52**

Values represent mean ± SD of three independent experiments, expressed as fold change relative to untreated control

**p < 0.01 compared to control; *p < 0.05 compared to control*

Consistent with gene expression data, treatment with *S. serrata* ethanol extract resulted in significant activation of caspases (Table 4.8). Caspase-9 activity increased most prominently (up to 7.92-fold in HeLa, 7.28-fold in SiHa at 2× IC_{50}, 48 hours), followed closely by caspase-3 (up to 6.87-fold and 6.32-fold, respectively). Caspase-8 activation was modest in comparison (maximum 2.73-fold in HeLa, 2.51-fold in SiHa). The predominant activation of caspase-9 over caspase-8 confirms that the intrinsic mitochondrial pathway is the primary mediator of *S. serrata* extract-induced apoptosis, while the extrinsic pathway plays a contributory but secondary role.

Western blot analysis further confirmed caspase activation and the execution of apoptosis. Treatment with the extract induced time-dependent cleavage of procaspase-3 to its active p17 and p12 fragments, accompanied by cleavage of PARP (poly ADP-ribose polymerase) from its full-length 116 k Da form to the characteristic 89 k Da apoptotic fragment. PARP cleavage(Alamgir, 2018).

Conclusion

The present study systematically investigated the cytotoxic and apoptotic potential of *Scylla serrata* (mud crab) shell extract against human cervical cancer cell lines, providing comprehensive evidence supporting its candidacy as a promising source of novel anticancer agents. Through sequential extraction using solvents of varying polarity, we successfully obtained four distinct extracts with diverse phytochemical profiles. The ethanol extract demonstrated the most potent and selective

cytotoxic activity against both HeLa (HPV-18 positive) and SiHa (HPV-16 positive) cervical cancer cells, with IC_{50} values of 41.3 ± 2.2 µg/mL and 48.9 ± 2.6 µg/mL, respectively, following 72 hours of treatment. Remarkably, this extract exhibited a selectivity index of 7.23 for HeLa cells and 6.10 for SiHa cells, indicating approximately 6-7-fold higher toxicity toward malignant cells compared to normal cervical epithelial cells. This favourable safety profile stands in stark contrast to the conventional chemotherapeutic agent cisplatin, which

showed non-selective cytotoxicity with selectivity indices below 1.0, highlighting the therapeutic advantage of the marine-derived extract.

The phytochemical characterization revealed that the ethanol extract possessed the most diverse array of bioactive constituents, including abundant flavonoids, phenolic compounds, alkaloids, and moderate amounts of tannins and cardiac glycosides. The presence of these compound classes, particularly flavonoids and phenolic compounds, correlates well with the observed anticancer activity and provides a chemical basis for the extract's biological effects. The superior activity of the ethanol extract compared to n-hexane, ethyl acetate, and aqueous extracts underscores the importance of solvent selection in maximizing the recovery of bioactive principles from marine biomass(Anju et al., 2019).

Mechanistic investigations conclusively demonstrated that *S. serrata* shell extract exerts its cytotoxic effects through the induction of apoptosis rather than necrosis. Morphological assessment revealed characteristic apoptotic features including cell shrinkage, membrane blebbing, chromatin condensation, and nuclear fragmentation. Quantitative flow cytometric analysis using Annexin V-FITC/PI staining confirmed a concentration- and time-dependent increase in apoptotic cell populations, with the total apoptotic fraction reaching 58.2% in HeLa cells treated with 2× IC_{50} concentration for 48 hours, while necrotic cell death remained minimal (<8%). This predominance of apoptosis over necrosis is therapeutically advantageous, as apoptosis is a programmed, physiologically regulated process that eliminates malignant cells without eliciting inflammatory responses or damaging surrounding tissues(Raja, 2020).

Cell cycle analysis revealed that the extract induces significant cell cycle perturbation, characterized by initial G0/G1 arrest at 24 hours followed by pronounced G2/M accumulation at 48 hours. This biphasic response suggests that the extract contains multiple bioactive compounds acting through complementary mechanisms, or that prolonged exposure activates distinct cell cycle checkpoints. The substantial increase in Sub-G1 population (hypodiploid DNA content) corroborated the apoptotic cell death observed by Annexin V staining. The G2/M arrest observed a later time point is particularly significant, as this phase of the cell cycle represents a critical checkpoint where cells with damaged DNA are prevented from entering mitosis, and prolonged arrest at this stage often commits cells to apoptotic elimination.

At the molecular level, *S. serrata* extract triggered the intrinsic mitochondrial apoptotic pathway through multiple coordinated mechanisms. The extract induced significant dissipation of mitochondrial membrane potential (Δm), with the red/green fluorescence ratio decreasing by up to 70% in treated cells, indicating profound mitochondrial dysfunction. This mitochondrial depolarization was accompanied by marked upregulation of pro-apoptotic genes including p53 (4.37-fold), Bax (5.68-fold), Bad (4.12-fold), and caspase-9 (5.24-fold), while simultaneously downregulating anti-apoptotic genes Bcl-2 (by 59%) and Bcl-xL (by 47%). The resulting shift in the Bax/Bcl-2 ratio dramatically Favors apoptosis and explains the observed

mitochondrial membrane permeabilization(Sani, 2020).

Functional assays confirmed that transcriptional changes translated into enhanced caspase activity, with caspase-9 showing the most pronounced activation (up to 7.92-fold), followed by the executioner caspase-3 (up to 6.87-fold). Caspase-8 activation was modest in comparison (maximum 2.73-fold), confirming that the intrinsic mitochondrial pathway is the primary mediator of *S. serrata* extract-induced apoptosis, while the extrinsic death receptor pathway plays a secondary, contributory role. Western blot analysis further substantiated these findings, demonstrating cleavage of procaspase-3 to its active fragments and proteolytic processing of PARP—a hallmark of caspase-dependent apoptosis(Lailaja et al., 2022).

The slight differential sensitivity observed between HeLa and SiHa cells (HeLa being marginally more sensitive) may reflect variations in HPV genotype or differences in cellular background that influence apoptotic signalling. However, the overall similarity in response patterns across both cell lines indicates that *S. serrata* extract effectively targets core apoptotic machinery common to cervical cancer cells regardless of HPV subtype, suggesting broad applicability.

The findings of this study carry several important implications. First, they provide scientific validation for the traditional use of crab shell preparations in folk medicine and establish a mechanistic basis for their purported anticancer effects. Second, the high selectivity index of the ethanol extract suggests that *S. serrata*-derived compounds may offer a wider therapeutic window than conventional chemotherapeutic agents, potentially reducing the debilitating side effects associated with current treatments. Third, the multi-targeted mechanism of action—involving cell cycle arrest, mitochondrial dysfunction, modulation of Bcl-2 family proteins, and caspase activation—may confer resilience against the development of drug resistance, a common limitation of single-target therapies. Fourth, the use of crab shell waste, a readily available and underutilized byproduct of the seafood industry, aligns with principles of sustainable development and circular economy, offering an eco-friendly approach to drug discovery(Soegianto et al., 2022).

Despite these promising findings, several limitations of the present study should be acknowledged. The experiments were conducted using established cervical cancer cell lines, which, while valuable models, cannot fully recapitulate the complexity of human tumours *in vivo*. The precise bioactive compounds responsible for the observed effects remain to be identified through bioassay-guided fractionation and advanced spectroscopic techniques. The extract represents a complex mixture of compounds, and potential synergistic or antagonistic interactions among constituents warrant further investigation. Additionally, the pharmacokinetic properties, bioavailability, and *in vivo* efficacy and safety of the extract remain to be evaluated in appropriate animal models before clinical translation can be considered(Manjunatheshwara, 2022).

Future research directions should include: (1) bioassay-guided fractionation to isolate and characterize the specific compound(s) responsible for the observed anticancer activity; (2) advanced spectroscopic analysis (NMR, LC-MS/MS) for structural elucidation of active principles; (3) investigation of additional mechanistic pathways including ROS generation, DNA damage response, and autophagy; (4) evaluation of combination effects with standard chemotherapeutic agents to assess potential synergistic interactions; (5) *in vivo* studies using xenograft mouse models to evaluate antitumor efficacy, pharmacokinetics, and toxicity; and (6) development of appropriate formulations to enhance bioavailability and targeted

delivery to tumour tissues(Prem Anand & Patterson Edward, 2002).

In conclusion, this study demonstrates that *Scylla serrata* shell extract, particularly the ethanol extract, possesses potent and selective cytotoxic activity against human cervical cancer cells through induction of mitochondria-mediated apoptosis. The extract's ability to modulate multiple molecular targets—including p53, Bcl-2 family proteins, and caspases—while sparing normal cells, positions it as a promising candidate for further development as an anticancer agent. The utilization of crab shell waste, an abundant marine byproduct, adds an important dimension of sustainability to this discovery. While significant work remains to translate these findings into clinical applications, the present investigation establishes a strong scientific foundation for the potential of *Scylla serrata* as a source of novel, safe, and effective anticancer agents in the ongoing global battle against cervical cancer(Adibe, Ayogu, Igboeli, & Isah, 2017).

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