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GC-MS Profiling and Anticancer Evaluation of Ethanolic Extract of *Annona squamosa* Flower on Cervical Cancer Cells

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ABSTRACT

Background: Natural plant extracts are rich sources of bioactive compounds with potential therapeutic properties. *Annona squamosa*, traditionally known for its medicinal value, was investigated for its anticancer activity using ethanolic flower extract. **Objective:** To identify the phytochemical constituents of *Annona squamosa* flower extract using GC-MS and evaluate its cytotoxic, apoptotic, and anti-migratory effects on human cervical cancer (SiHa) cells. **Methods:** The ethanolic extract of *Annona squamosa* flower was subjected to GC-MS analysis to identify major phytochemicals. Cytotoxicity was assessed using MTT assay. Morphological changes were observed via phase-contrast microscopy. Apoptosis was confirmed through ethidium bromide staining and RT-PCR for apoptotic gene expression. A scratch wound assay was conducted to evaluate the anti-migratory effect of the extract. **Results:** GC-MS analysis revealed 20 bioactive compounds, including 4H-Pyran-4-one, Germacrene D, γ-Elemene, and Palmitic acid with known therapeutic properties. The extract showed dose-dependent cytotoxicity with significant morphological changes indicating apoptosis. Ethidium bromide staining confirmed nuclear condensation, and RT-PCR demonstrated upregulation of apoptosis-related genes. The wound healing assay indicated reduced cell migration in treated cells. **Conclusion:** The ethanolic extract of *Annona squamosa* flower possesses significant anticancer potential through its ability to induce apoptosis and inhibit cell migration. This extract could serve as a promising candidate for cervical cancer therapy after further validation.

INTRODUCTION:

Cervical cancer is a significant global health issue, primarily caused by persistent infection with high-risk human papillomavirus (HPV). It ranks as the fourth most common cancer among women and is a leading cause of cancer-related mortality, particularly in low- and middle-income countries where screening is inadequate(Clinical Outcomes of Women who attend t...", 2023) (Jain & Limaiem, 2021). Early detection through screening and vaccination can prevent the progression of pre-

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cancerous lesions to invasive cancer, yet awareness and access remain critical challenges(Kondhalkar & Anwar, 2024) (Sahasrabuddhe, 2024).High-risk HPV types, particularly 16 and 18, are the primary etiological agents(Jain & Limaiem, 2021).Smoking, early sexual activity, and multiple sexual partners increase risk(Kondhalkar & Anwar, 2024). Women in low-socioeconomic areas face higher incidence rates due to limited access to screening(Jain & Limaiem, 2021).HPV vaccines can significantly reduce the incidence of cervical cancer(Natelauri, 2023).**Screening Methods**: Transition from Pap smears to HPV-based testing enhances early detection(Sahasrabuddhe, 2024).Treatment Options **Surgical Interventions**: Hysterectomy and brachytherapy are effective for early-stage cancer, with over 98% five-year survival rates(Natelauri, 2023).**Multidisciplinary Approach**: Treatment may include chemotherapy, radiation, and palliative care for advanced cases(Natelauri, 2023).In recent years, there has been growing emphasis on the discovery of novel therapeutic agents derived from natural sources for cancer treatment. This study focuses on the potential therapeutic application of *Annona squamosa* Flower flower extract in the context of cervical cancer. The investigation aims to evaluate its anticancer efficacy through phytochemical profiling, in vitro cytotoxicity, and molecular interaction studies, contributing to the development of plant-based interventions in cervical cancer research.

MATERIAL AND METHODS:

Annona squamosa flower powder

Healthy flower buds were carefully plucked by hand during the early morning hours to ensure minimal contamination and maximum phytochemical retention. Flowers were selected based on maturity (usually unopened or semi-opened) and the absence of pests or diseases. The flowers were sorted to remove any unwanted parts such as twigs, dried petals, or insects. They were then gently rinsed with clean water to remove dust, dirt, or external pollutants. After rinsing, the flowers were air-dried or wiped with clean blotting paper to remove surface moisture. The cleaned flowers were shade-dried for 5–7 days to preserve phytochemicals, as direct sunlight could degrade the bioactive compounds. The dried flowers appeared crisp and brownish, as shown in the image. Once thoroughly dried, the flowers were ground using a mechanical grinder to form a fine brown powder. The powder was sieved to ensure uniform particle size and then stored in an airtight container away from moisture and light.

Preparation of extraction:

The powdered flower was mixed with ethanol in a 1:3 w/v ratio. The mixture was placed in a conical flask with a stopper and left to macerate for 72 hours at room temperature. It was stirred occasionally (every 12 hours) to improve the extraction. The flask was kept away from light to prevent degradation of sensitive compounds. After maceration, the mixture was filtered using Whatman No. 1 filter paper or muslin cloth. The ethanolic extract was collected in a clean beaker. The solvent was evaporated using a rotary evaporator or water bath at 40–50°C. Alternatively, the extract was allowed to air dry in a shallow dish covered with foil in a dust-free environment. The dried extract was stored in a labeled, amber-colored bottle at 4°C for further use in biological assays or formulation.

Cell line maintenance:

Human cervical cancer cell lines (SiHa) were obtained from the NCCS, Pune. The cells were grown in T25 culture flasks containing DMEM and RPMI supplemented with 10% FBS and 1% antibiotics. Cells were maintained at 37◦C in a humidified atmosphere containing 5% CO2. Upon reaching confluency, the cells were trypsinized and passaged.

Cell viability (MTT) assay:

The cell viability of Sample-A treated cervical cancer cell line was assessed by MTT assay. The assay is based on the reduction of soluble yellow tetrazolium salt to insoluble purple formazan crystals by metabolically active cells. The cervical cancer cell line was plated in 96 well plates at a concentration of $5x10^3$ cells/well 24 hours after plating, cells were washed twice with 100µl of serum-free medium and starved by incubating the cells in serum-free medium for 3 hours at 37ºC. After starvation, cells were treated with different concentrations of Sample-A (10- 160µg/ml) for 24 hours. At the end of treatment, the medium from control and treated cells were discarded and 100µl of MTT containing DMEM (0.5 mg/ml) was added to each well. The cells were then incubated for 4h at 37ºC in the CO_2 incubator. The MTT containing medium was then discarded and the cells were washed with 1x PBS. Then the formazan crystals formed were dissolved in dimethyl sulfoxide (100µl) and incubated in dark for an hour. Then the intensity of the color developed was assayed using a Micro ELISA plate reader at 570 nm. The number

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of viable cells was expressed as a percentage of control cells cultured in serum-free medium. Cell viability in the control medium without any treatment was represented as 100%. The cell viability is calculated using the formula: % cell viability = [A570 nm of treated cells/A570 nm of control cells]×100.

Morphology study:
Based on MTT assay we selected the optimal doses (IC-50: 80µg/ml of Sample-A for cervical cancer cell line) for further studies. Analysis of cell morphology changes by a phase contrast microscope. 2×10^5 cells were seeded in 6 well plates and treated with PR extract for 24h. At the end of the incubation period, the medium was removed and cells were washed once with a phosphate buffer saline (PBS pH 7.4). The plates were observed under a phase contrast microscope.

Determination of mode of cell death by ethidium bromide (EtBr) staining:
The effects of Sample-A on cervical cancer cell death were also determined by EtBr staining as described previously. The cells were treated with Sample-A 80µg/ml for 24 h and then the cells were harvested, washed with ice-cold PBS. The pellets were resuspended in 5 µl of acridine orange (1 mg/mL) and 5 µl of EtBr (1 mg/mL). The apoptotic changes of the stained cells were then observed by using a fluorescence microscope.

Scratch wound Assay:
Human lung cancer cells (2×10^5 cells/well) were seeded onto six-well culture plates. The cell monolayer was scratched using 200µl tip to create wound, washed with PBS and photographed in inverted microscope. Sample-4 (40µg/ml) treated for 24 h and control cells were received with serum-free culture medium, after the treatment period, the wounded area was photographed using the same microscope. And the experiments were repeated in triplicate for each treatment group.

Real Time PCR:
The gene expression of apoptosis signaling molecules was analysed using real-time PCR. The total RNA was isolated by the standardized protocol using Trizol Reagent (Sigma). 2µg of RNA used for cDNA synthesis using reverse transcription using a PrimeScript, 1st strand cDNA synthesis kit (TakaRa, Japan). The targeted genes were amplified using specific primers. PCR reaction was performed with GoTaq® qPCR Master Mix (Promega), it contains SYBR green dye and all the PCR components. Real time-PCR was performed in a CFX96 PCR system (Biorad). The results were analyzed by comparative C_T method and $2^{-\Delta\Delta C_T}$ method was used for fold change calculation described by Schmittgen and Livak.

Statistical analysis:
All data obtained were analyzed by One way ANOVA flowed by Students-t-test using SPSS, represented as mean ± SD for triplicates. The level of statistical significance was set at $p<0.05$.

RESULTS:

Gc-Ms Annalysis Of Ethanolic Extract Of Annona Squamosa Flower

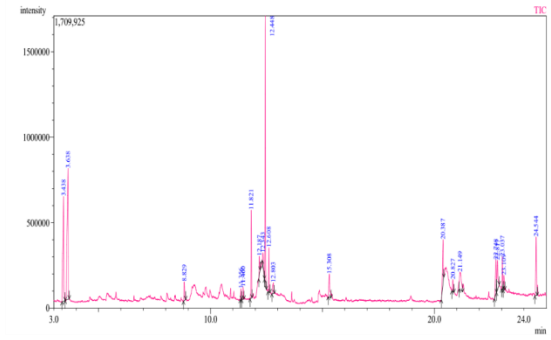

Fig-2 Chromatogram

Identification of componentsInterpretation of mass spectrum GC-MS was conducted using the database of National Institute Standard and Technique (NIST08s), WILEY8 and FAME having more patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST08s, WILEY8 and FAME library. The Name, Molecular weight, Molecular formula and Structure of the component of the test material was ascertained.

Table- 2 Bioactive compounds

1 & 2	Ethylene glycol monoisobutyl ether	3.43 & 3.63	Solvent-like compound, may aid in extraction
3	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	8.83	Antioxidant, flavoring agent
4	Ylangene	11.36	Anti-inflammatory, antimicrobial
5	Aromandendrene	11.46	Antibacterial, insecticidal
6	Bicyclo[7.2.0]undec-4-ene derivative	11.82	Potential anti-inflammatory role
7	Humulene	12.18	Well-documented anti-inflammatory agent
8	1,8-Nonadien-3-ol	12.34	Fragrance component, may aid in dermal applications
9	Germacrene D	12.44	Major compound, known anti-inflammatory and antibacterial properties

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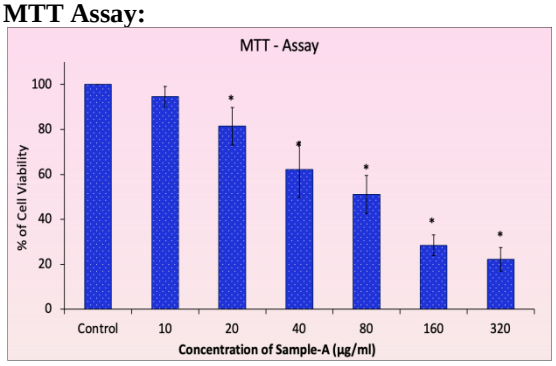
10	γ-Elemene	12.60	Antitumor, anti-inflammatory
11	Hexahydro-naphthalene derivative	12.80	Potential antioxidant activity
12	Isopropyl-dimethylenecyclodecene derivative	15.30	Structural analog of terpene, possible anti-inflammatory
13	n-Hexadecanoic acid (Palmitic acid)	20.39	Antioxidant, anti-inflammatory
14	Hexadecanoic acid, ethyl ester	20.82	Antimicrobial and emollient properties
15	Glucose	21.14	Hydrating and skin-b

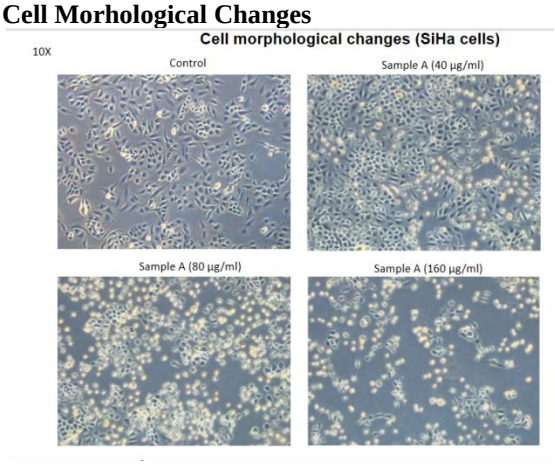
Twenty compounds were identified in the ethanol fraction of Annona squamosa flower extract by GC-MS analysis.The active principle, area of the peak, Concentration (%) and Retention Time (RT) are mentioned in above table

MTT Assay:



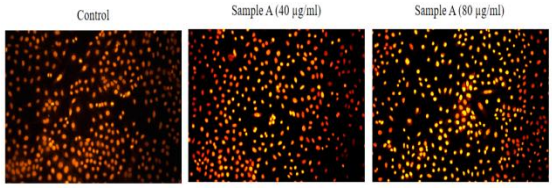
Effect of sample A on the viability of cervical cancer cells. The cells were treated with the indicated concentrations of sample A (10 to 320 µg/ml) for 24 h. Cell viability was assessed using the MTT assay, and the results are expressed as the percentage of surviving cells over control cells. Each value is presented as the mean ± SD and is representative of the results obtained from three independent experiments. The significance was determined by the Student's t-test (*p<0.05, compared with control group).

Cell Morhological Changes



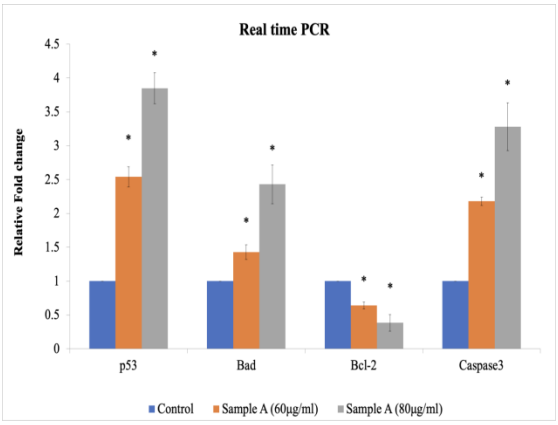
Effect of sample A on cell morphology of human cervical cancer cells (SiHa). Cells were treated with sample A (40, 0 and 160 µg/ml) for 24 h and cells were observed under an inverted phase contrast microscope. The number of cells decreased after sample4 treatment and the cells exhibited cell shrinkage and cytoplasmic membrane blebbing.

Ethidium Bromide Staining:

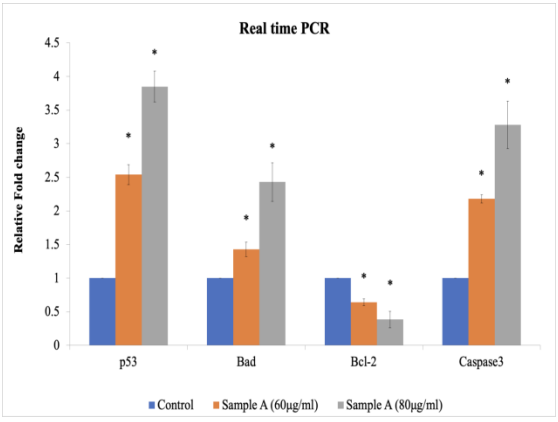


Human cervical cancer cells were treated with Sample A (40 and 80 µg/ml) for 24 h along with the control group. After the treatment, the cells were incubated with EtBr staining. Images were obtained using an Inverted Fluorescence Phase contrast microscope.

Real Time PCR



Effect of sample A in apoptosis gene expression in cervical cancer cells. Total RNA was prepared for reverse transcriptase PCR (RT-PCR) analysis of apoptosis signalling molecules gene expression in lung cancer cells. The experiment was repeated three times. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as internal controls for the RT-PCR analyses.



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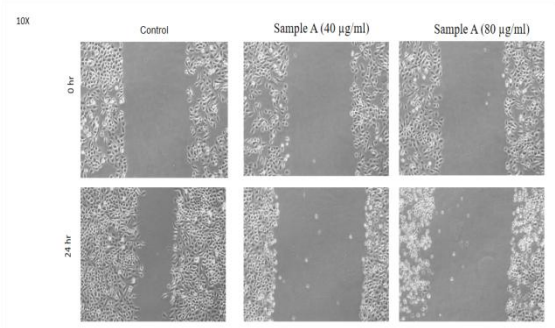
Scratch Wound Assay



Scratch wound assay to determine cell migration potential of sample A treated cervical cancer cells. Wound-healing assays were performed at 0 and 24h in SiHa cells in IC-50 dose of sample A (40 and 80 µg/ml) treated cells and untreated cells were used as controls. Representative phase-contrast microscope images showing the area covered by the cells at 0, and 24 after wounding. Original magnification 10×.

DISCUSSION

The present investigation demonstrates the therapeutic potential of the ethanolic flower extract of *Annona squamosa* through comprehensive GC-MS profiling and its anticancer effects on cervical cancer (SiHa) cells. A total of 20 phytochemicals were identified, many of which have been previously reported for their pharmacological activities.

Among the identified compounds, **Germacrene D**, a sesquiterpene, is well known for its **anti-inflammatory and antibacterial properties**, and has shown potential in various cancer models by modulating oxidative stress and inflammation (Wang et al., 2020). **γ-Elemene**, another abundant compound, is a documented **antitumor agent** that induces apoptosis and cell cycle arrest in cancer cells through mitochondrial pathways and inhibition of PI3K/Akt signaling (Liu et al., 2013). The presence of **n-Hexadecanoic acid (palmitic acid)** and its ethyl ester also contributes to the observed anticancer activity. Palmitic acid has been reported to exhibit **pro-apoptotic and anti-inflammatory effects**, partly by inducing mitochondrial dysfunction in tumor cells (Sanchez-Martinez et al., 2021). Additionally, **4H-Pyran-4-one derivatives** have antioxidant and cytoprotective roles which may enhance the extract's efficacy by reducing oxidative stress in cancer cells (Choudhury et al., 2021).

Biological assays conducted in this study revealed a **dose-dependent cytotoxic effect** of the extract on SiHa cervical cancer cells, as measured by MTT assay. This aligns with prior studies showing that *Annona squamosa* leaf and seed extracts possess cytotoxic effects against various cancer cell lines, including breast and lung cancer (Gajalakshmi et al., 2011). The morphological changes observed—such as cell shrinkage and blebbing—are characteristic of apoptosis, which was further confirmed by **ethidium bromide staining** and **RT-PCR expression of apoptotic genes**.

Furthermore, the **scratch wound assay** indicated that the extract significantly inhibited the **migration of cancer cells**, implying its **anti-metastatic potential**. Similar observations were made by Pandey et al. (2018), who reported anti-migratory effects of plant-derived bioactives in cervical cancer models.

Collectively, these findings support the hypothesis that the cytotoxicity and apoptosis observed in SiHa cells are largely attributable to the synergistic activity of the phytoconstituents present in the extract. The combination of antioxidant, anti-inflammatory, and apoptotic mechanisms may contribute to its overall anticancer efficacy.

SUMMARY:

The current study explored the phytochemical constituents and anticancer potential of ethanolic flower extract of *Annona squamosa* using GC-MS and various biological assays. GC-MS profiling revealed twenty bioactive compounds with known therapeutic properties, including antioxidant, antimicrobial, anti-inflammatory, and antitumor activities. Notable compounds such as Germacrene D, γ-Elemene, and Palmitic acid were identified.

Biological evaluations on human cervical cancer (SiHa) cells demonstrated that the extract induced a dose-dependent reduction in cell viability as shown by MTT assay. Morphological changes including cell shrinkage and membrane blebbing indicated the onset of apoptosis. Ethidium bromide staining further confirmed apoptotic changes. RT-PCR analysis revealed the upregulation of apoptosis-related genes, supporting the extract's pro-apoptotic effect. Moreover, the scratch wound assay showed impaired cell migration in treated groups, suggesting potential anti-metastatic activity.

CONCLUSION:

The ethanolic flower extract of *Annona squamosa* exhibits potent anticancer effects against SiHa cervical cancer cells by promoting apoptosis and inhibiting cell migration. The presence of multiple bioactive compounds identified through GC-MS supports its pharmacological potential. These findings suggest that *Annona squamosa* flower extract can be further explored as a natural therapeutic agent for cervical cancer treatment. However, additional studies including in vivo validation and mechanistic investigations are

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necessary to fully establish its clinical applicability.

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