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Research Article

In- vitro Pharmacological Evaluation of Flavonoid Rich Fractions of Betula Utilis and Annina Squamosa on Colon Cancer

Pragati Singh^{*1}, Amit Kumar Nigam², Akash Ved³, Ajay Kumar Singh Rawat⁴

¹Maharishi School of Pharmaceutical Sciences, Lucknow, Uttar Pradesh, India.

²Deen Dayal Upadhyay Gorakhpur University, Gorakhpur, Uttar Pradesh, India.

³Dr. A.P.J Abdul Kalam Technical University, Lucknow, Uttar Pradesh, India.

⁴Pharmacognosy and Ethnopharmacology Division, National Botanical Research Institute, Lucknow, Uttar Pradesh, India.

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ABSTRACT

The majority of colon cancers are one of the principal causes of cancer-related deaths all over the world. The search for good and safe medicinal therapy is just a recent interest; flavonoid-rich fractions from exclusive plants have received attention in connection with this goal. The present study revolves around pharmacological assessments in vitro of flavonoid-rich fractions derived from Betula and Annona squamosa for colon malignancies. The fractions were found to exert notable anti-inflammatory, antiproliferative, and apoptotic actions on colon cancer cells. These effects were mediated through the modulation of signaling pathways. Thus, this study supports the possible use of flavonoid-rich fractions of Betula utilis and Annona squamosa as therapeutics for the majority of colon malignancies.

INTRODUCTION

Cancer is the leading cause of morbidity and mortality globally. About 10 million deaths were reported in 2020. The new cases of cancer were Lung carcinomas (2.21 million), Breast cancer (2.26 million), Colorectal cancer (1.93 million), prostate cancer (1.41 million), non-melanoma skin cancer (1.20 million), and gastric cancer (1.09 million) are reported worldwide in the year 2020

[16,17]. Cancer is a chronic problem that is rapidly expanding in the 21st era and is estimated to influence more than 22 million individuals by 2030 [18,19]. Colon cancer is one of the major public health concerns throughout the world, with over 1.9 million new cases and 935,000 deaths attributable to it in 2020 alone [4]. Despite advances in surgery and chemotherapy, the prognosis for colon cancer patients remains poor;

***Corresponding Author:** Pragati Singh

Address: Maharishi School of Pharmaceutical Sciences, Lucknow, Uttar Pradesh, India.

Email ✉: pragathakur25@gmail.com

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thus, there is almost a yearning for ideal agents that are effective and safe. [1,2] Flavonoids, a class of compounds from plant sources, have been evident to have an anti-cancer action that entails activation against cell growth, induction of apoptosis, and anti-inflammatory properties [5]. Colon cancer or colorectal cancer takes place in the rectum or colon. [3,14,15]. The incidence of colon cancer varies widely from region to region in different countries, but the highest rates are found in North America, Europe, and Australia [5,6]. Colon cancer is a complex and multifactorial disease whose development converses processes involving geometrical, environmental, and life-style modulations. The pathogenesis includes the accumulation of genetic mutations in colon cells, resulting in the anti-adoma, the appearance of adenomatous polyps, and, ultimately, invasiveness by carcinomas [2,11,13]. *Betula utilis* and *Annona squamosa* are two plants that have been used in traditional medicine for centuries. *Betula utilis* has anti-inflammatory, antimicrobial, and antioxidant properties. Furthermore, *Annona squamosa* is said to possess antineoplastic, anti-inflammatory, and antimicrobial properties. *Betula utilis* has been reputed in the traditional medicine field for the treatment of different diseases ranging from skin diseases, fever, and rheumatism and *Annona squamosa* is reputed in the traditional medicine-field to treat a host of diseases like cancer, inflammation, and infectious diseases.[1] Sugar apple tree, scientifically called *Annona squamosa*, is a tropical tree native to the Americas [12]. It has been in use in traditional medicine for centuries to treat similar complaints, including cancer, inflammation, and infectious diseases. This review is intended as a rudimentary introduction to the traditional uses, phytochemistry, and pharmacological activity of *Annona squamosa*. *Annona squamosa* is a traditional medicinal plant with a wide range of pharmacological activities. Parts of the plants that presumably possess effects

on anti-cancer, anti-inflammatory, and antimicrobial activities are compounds occurring in it, such as the annonaceous acetogenins, flavonoids, and alkaloids [7]. Further research is necessary to explore the full therapeutic potential of *Annona squamosa* [10]. It is still grown as a backyard tree and the Philippines is considered as one of the largest producers in the world [1]. Current studies support various ranges of activities of *A. squamosa* including but not limited to anticancer, anti-oxidant, antidiabetic, antihypertensive, hepatoprotective, antiparasitic, antimalarial, insecticidal, microbicidal and molluscicidal activities [8].

MATERIAL AND METHODS

Collection and Authentication of *Betula utilis* leaves, *Annona squamosa* roots

Collection and authentication of the selected plant materials from the taxonomic division of Banaras Hindu University (BHU), Varanasi. A voucher specimen were deposited for future reference.

- *Betula utilis* leaves (Ref. No. *Betula*. 2023/1).
- *Annona squamosa* roots (Ref. No. *Annona*. 2023/1).

Chemicals and Reagents

The various chemicals and reagents were used: DPPH, methanol, Eagle's minimal essential medium (EMEM), fetal bovine serum (FBS) and 1 % antibiotic solution (10,000 U/ml penicillin and 10 mg/ml streptomycin), trypsin, DMSO, BUL and ASME, 2'-7'-dichlorodihydrofluorescein diacetates (DCFH-DA),

Pharmacological evaluation

In-vitro Antioxidant Assays

Determination of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity

The free radical scavenging activity of the leaf extract was carried out to evaluate its ability to scavenge the stable DPPH radical by preparing 0.1 mM of DPPH solution in methanol. This solution (1 ml) was added to 3 ml. of different extracts in methanol and made up to various concentrations



(10, 25, 50, 75, and 100 µg/ml). The different concentrations were made by the dilution method. The whole mixture was shaken vigorously and allowed to stand for 30 minutes at room temperature followed by measurement of absorbance which was calculated at 517 nm using a spectrophotometer [UV-VIS Shimadzu]. The standard compound used was ascorbic acid and the experiment was performed in triplicate. The percent DPPH scavenging effect was calculated using the following equation.

% Inhibition = [Control absorbance- Sample absorbance]/ Control absorbance × 100

The IC₅₀ value of the sample, which is the concentration of the sample required to inhibit 50% of the DPPH free radical, was calculated using the Log dose inhibition curve.

***In-vitro* Anticancer activity**

Cell line and culture

Human colon cancer cell lines (HCT-116) were procured from the cell repository- National Centre for Cell Sciences (NCCS), Pune, India. The cells were cultured in Eagle's minimal essential medium (EMEM) along with 10 % (v/v) fetal bovine serum (FBS) and 1 % antibiotic solution (10,000 U/ml penicillin and 10 mg/ml streptomycin) in a 5% CO₂ humidified air .at 37°C. Cells were cultured to a confluency of 80% and were subsequently digested with 0.25% trypsin and seeded into new plates at the required density.

Cell viability assay

The *in-vitro* cell viability of methanolic extract of both plants BUL and ASME was investigated using an MTT assay. The viable cells were seeded at a density of 1×10^4 (100 µl/well) in 96-well plates in 100 µl EMEM media for one day and incubated in a humidified atm of 5% CO₂ and 95% air at 37°C for 24h to generate cell monolayer. Treatment with various concentrations viz. 25, 50, 100, 200, and 300 µg/ml of methanolic BUL and ASME was carried out for 24 h incubation of HCT-116 cells. After 21 h exposure, 10 µl of MTT

solution (5 mg/ml stock solution) was added to each and then re-incubated for 3 h at 37°C until formazan blue crystals developed. Culture media was discarded from each well and 100 µl of DMSO was added to dissolve formazan crystals for 10 min at 37°C. The absorbance was recorded at 540 nm by a microplate reader (BIORAD-680) and relative percentage cell viability was evaluated.

% Cell viability = [HCT-116 [Treated Cells]/ HCT-116 [Control Cells] ×100

Determination of IC₅₀

A plot of percentage viability versus sample concentration was used to calculate the inhibitory concentration to 50% of the cells (IC₅₀). Cellular morphology studies were also carried out under an inverted phase-contrast microscope.

Intracellular Reactive oxygen species (ROS) generation analysis

The intracellular ROS production in Human colon cancer cell lines (HCT-116) cells was studied with the help of fluorescence microscopic imaging after the exposure to numerous concentrations of BUL and ASME at 12h, using the fluorescence probes 2'-7'-dichlorodihydrofluorescein diacetate (DCFH-DA) as previously reported. The intracellular fluorescence of HCT-116 cells was seen through a fluorescence inverted microscope (Nikon ECLIPSE Ti-S, Japan) and images were captured. In quantitative analysis of ROS, 1×10^4 HCT-116 cells per well were seeded in 96 well black bottom cell culture plate and treated with 50 µg/ml and 100 µg/ml, concentrations of methanolic BUL and ASME. After the treatment period, cells were incubated with DCFH-DA (10 mM) and the fluorescence intensity of cells was observed by the multiwall microplate fluorimeter (Synergy Hybrid Multi-Mode Microplate Reader, BioTek) (excitation: 485 nm; emission: 528 nm). The percentage of fluorescence intensity was shown as the relative fluorescence percentage of the treated and control group.

Cell morphology analysis



The effect of BUL and ASME methanolic extract was analyzed for morphological changes in the cultured cells. The cells were seeded at a density of 1×10^4 cells/well in a 96-well culture plate. After overnight incubation, the cells were treated with different concentrations of BUL and ASME for 24 h. The cellular morphology was observed under an inverted phase-contrast microscope.

Nuclear apoptosis analysis/ DAPI for fluorescence imaging

The nuclear and chromatin structural alterations in HCT-116 cells after BUL and ASME exposure was examined by using the fluorescent nuclear dye DAPI (4',6-diamidino-2-phenylindole) as per the previous protocol. Apoptotic cells were visualized and quantified under an inverted fluorescent microscope (Nikon ECLIPSE Ti-S, Japan).

Statistical Analysis

The values were expressed as the mean $\pm$ standard deviation (SD) of results obtained from the independent experiments. Levels of statistical significance were calculated by one-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison Test using Graph Pad Prism (Version 5.01) software. Results with the $p < 0.05$ level, were considered statistically significant.

RESULTS

Pharmacological activity

In-vitro anticancer activity of MeOH extract of *Betula utilis*

HCT-116 Cell viability assay/ Cell morphology analysis

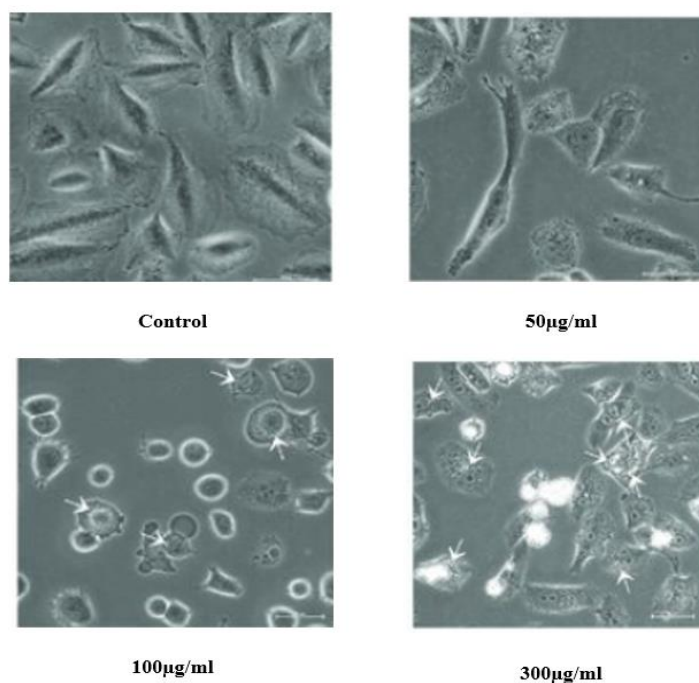


Figure 1. Effect of MeOH extract of *Betula utilis* doses on cell viability HCT-116 cells.

(A) Cellular Morphological alterations of viable and dead HCT-116 cells after the treatment of 50 $\mu\text{g/ml}$ to 300 $\mu\text{g/ml}$ MeOH extract of *Betula utilis* concentrations. (B) Percentage cell viability was measured by MTT assay after 24 h exposure of

various concentration of MeOH extract of *Betula utilis* on HCT-116 cells. At least three independent experiments were performed and the values are expressed as mean $\pm$ SEM, $**p < 0.01$ and $***p < 0.001$ as compared to the control.

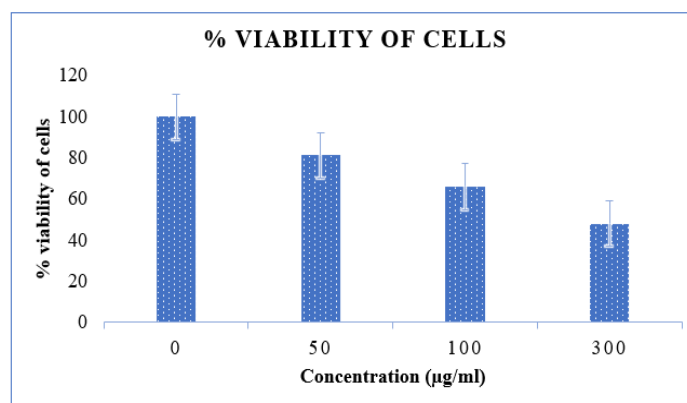


Figure 2. Effect of various concentrations of MeOH extract of *Betula utilis* on HCT-116 cells viability.

Nuclear fragmentation Assay

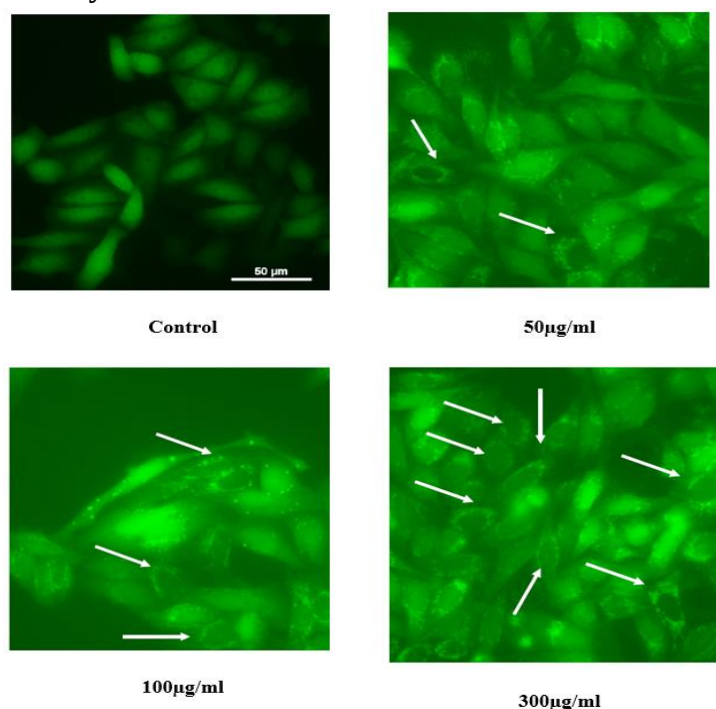


Figure 3. Nuclear fragmentation on effective concentrations of MeOH extract of *Betula utilis* on HCT-116 cells. (A)

Photomicrographs exhibits the characteristic fragmented and condensed nuclei (indicated by arrow) in MeOH extract of *Betula utilis* HCT-116 cells. **(B)** Statistical data expressed as percent of apoptotic cells as compared to the control.

Minimum three independent experiments were performed and the values are expressed as means $\pm$ SEM, $**p < 0.01$ and $***p < 0.001$ as compared to the control.

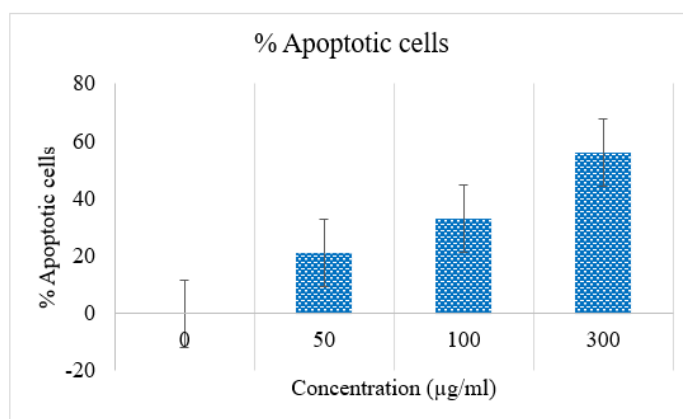


Figure 4. MeOH extract of *Betula utilis* induces Nuclear condensation in a dose dependent manner on HCT-116 cells

Intracellular ROS production

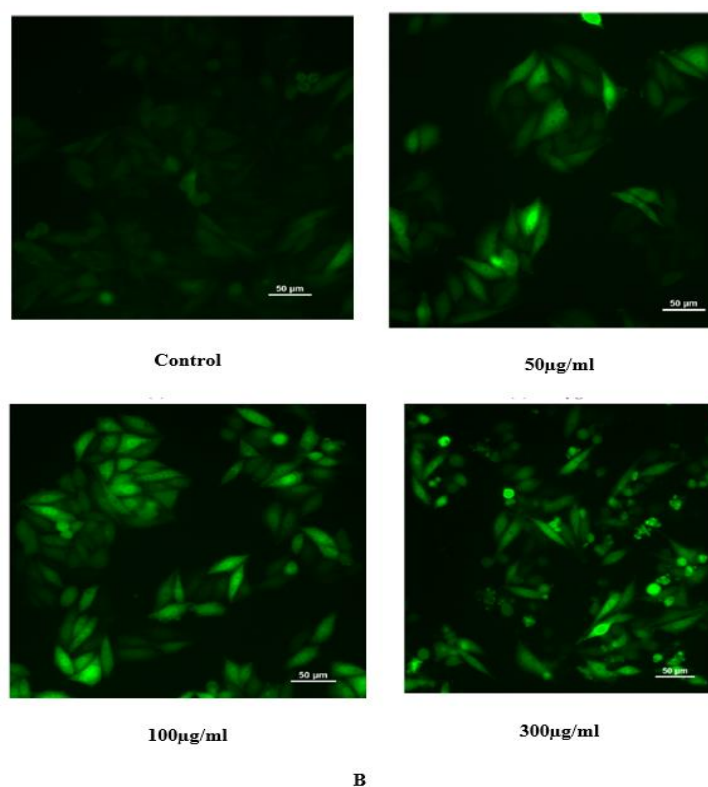


Figure 5. Intracellular ROS production on effective concentrations of MeOH extract of *Betula utilis* on HCT-116 cells (A)

The photomicrographs show induction in intracellular ROS on HCT-116 cells at 50 µg/ml, and 100 µg/ml concentrations of MeOH extract of *Betula utilis*. (B) Quantitative data represents the percentage of fluorescence intensity on HCT-116 cells with respect to the control.

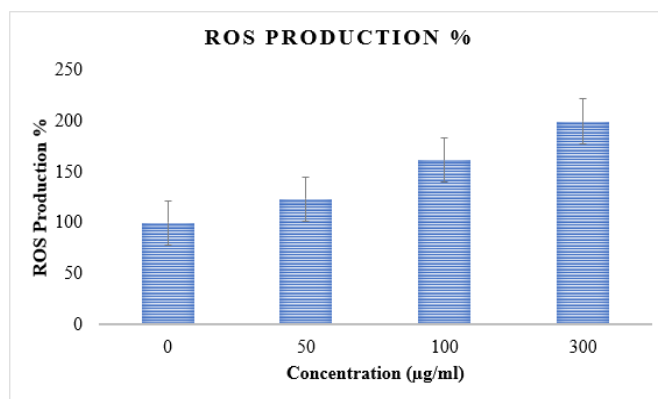


Figure 6. Intracellular ROS production at effective concentrations of MeOH extract of *Betula utilis* on HCT-116 cells

In-vitro anticancer activity of MeOH extract of *Annona squamosa*

HCT-116 Cell viability assay/ Cell morphology analysis

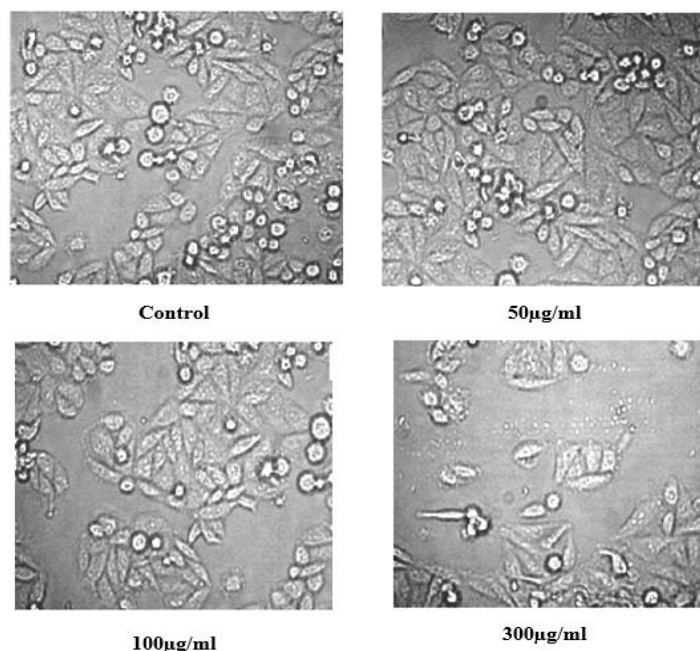


Figure 7. Effect of MeOH extract of *Annona squamosa* doses on cell viability HCT-116 cells.

(A) Cellular Morphological alterations of viable and dead HCT-116 cells after the treatment of 50 µg/ml to 300 µg/ml MeOH extract of *Annona squamosa* concentrations. (B) Percentage cell viability was measured by MTT assay after 24 h exposure of various concentration of MeOH

extract of *Annona squamosa* on HCT-116 cells. At least three independent experiments were performed and the values are expressed as mean±SEM, ** $p < 0.01$ and *** $p < 0.001$ as compared to the control.

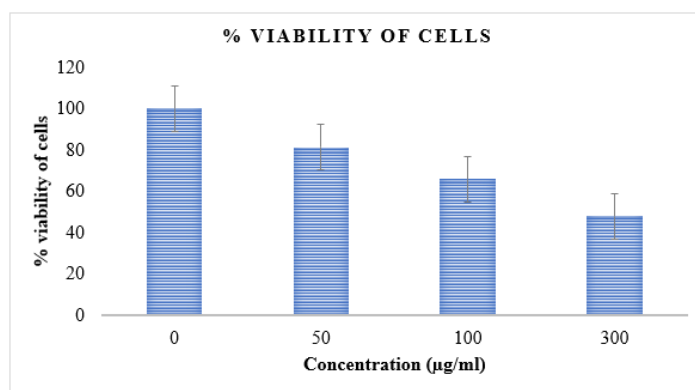


Figure 8. Effect of various concentrations of MeOH extract of *Annona squamosa* on HCT-116 cells viability

Nuclear fragmentation Assay

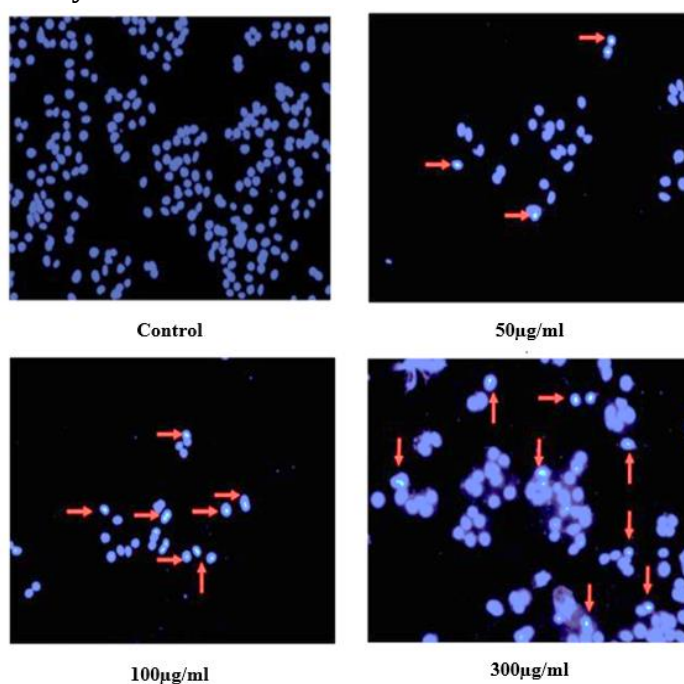


Figure 9. Nuclear fragmentation on effective concentrations of MeOH extract of *Annona squamosa* on HCT-116 cells. (A)

Photomicrographs exhibits the characteristic fragmented and condensed nuclei (indicated by arrow) in MeOH extract of *Annona squamosa* HCT-116 cells. (B) Statistical data expressed as percent of apoptotic cells as compared to the

control. Minimum three independent experiments were performed and the values are expressed as means $\pm$ SEM, $**p < 0.01$ and $***p < 0.001$ as compared to the control.

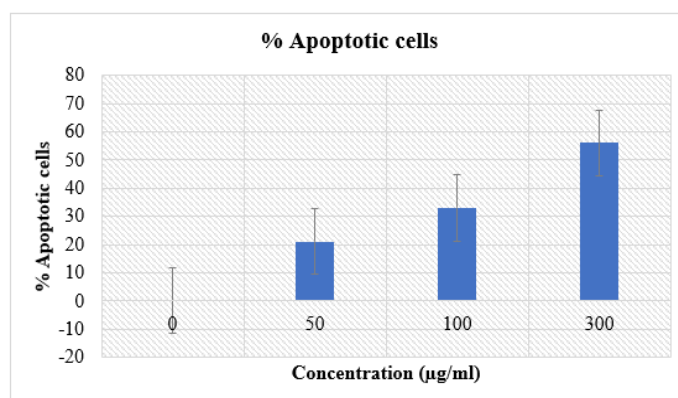


Figure 10. MeOH extract of *Annona squamosa* induces Nuclear condensation in a dose dependent manner on HCT-116 cells

Intracellular ROS production

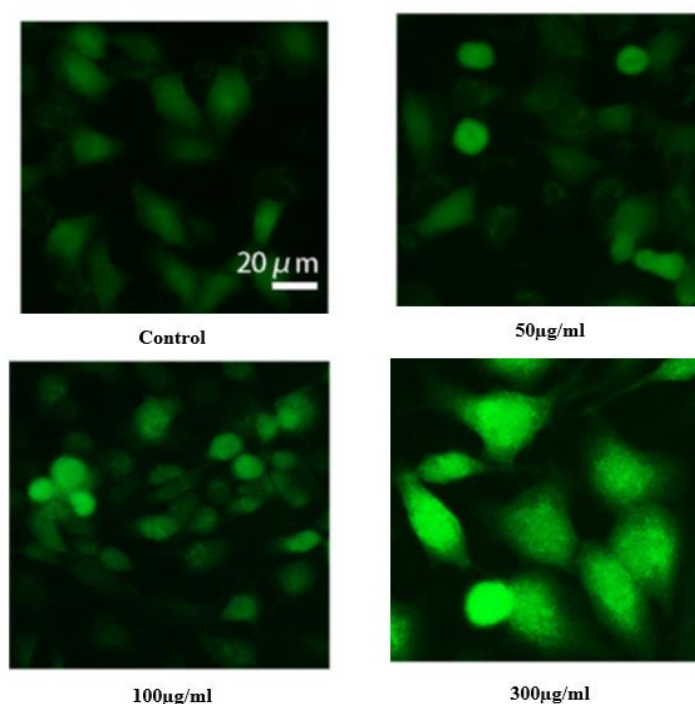


Figure 11. Intracellular ROS production on effective concentrations of MeOH extract of *Annona squamosa* on HCT-116 cells (A)

The photomicrographs show induction in intracellular ROS on HCT-116 cells at 50 µg/ml, and 100 µg/ml concentrations of MeOH extract of

Annona squamosa. (B) Quantitative data represents the percentage of fluorescence intensity on HCT-116 cells with respect to the control.

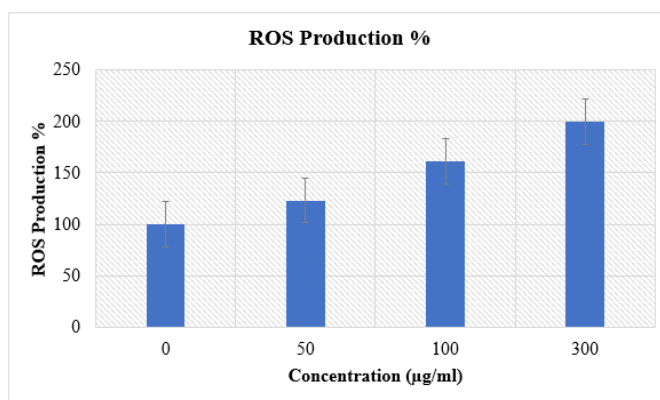


Figure 12. MeOH extract of *Annona squamosa* induces Nuclear condensation in a dose dependent manner on HCT-116 cells

DISCUSSION

Flavonoid-rich extracts from *Betula utilis* and *Annona squamosa* appear to have great potential as adjunctive therapy in colon cancer treatments. Each fraction displayed a wide range of potency against colon cancer cells, encompassing anti-proliferative, pro-apoptotic, and anti-inflammatory activities, due to the presence of flavonoids like quercetin, kaempferol, and isorhamnetin. The rising burden of cancer worldwide necessitates the exploration of alternative treatment strategies beyond conventional Western medicine. Herbal medicine, with its long history of use in various cultures, presents a promising avenue for the discovery of novel anticancer agents. This study focused on investigating the in-vitro antioxidant and anticancer potential of *Betula utilis* leaves and *Annona squamosa* roots, two plants with documented traditional use but limited scientific validation in the context of colon cancer. The MTT assay was employed to evaluate the in-vitro anticancer activity of the extracts and fractions against colon cancer cell lines. The results indicated a dose-dependent decrease in cell viability, with certain fractions exhibiting pronounced cytotoxic effects. This cytotoxicity can be attributed to the presence of bioactive compounds that interfere with cancer cell proliferation and survival pathways. The observed

anticancer effects can be linked to several mechanisms. First, the antioxidant properties of the extracts may reduce oxidative stress, thereby inhibiting cancer cell growth. Second, specific bioactive compounds in the fractions may directly target and disrupt cellular processes essential for cancer cell survival. Further studies are needed to elucidate the exact signaling pathways involved and to identify the most potent compounds. A significant correlation was established between the phytochemical profiles and the pharmacological activities of the extracts and fractions. Fractions with higher phenolic and flavonoid content exhibited stronger antioxidant and anticancer activities. This correlation underscores the importance of these bioactive compounds in mediating the therapeutic effects of *Betula utilis* and *Annona squamosa*. This study provides scientific validation for the traditional use of *Betula utilis* and *Annona squamosa* in cancer treatment. The comprehensive analysis of their phytochemical and pharmacological profiles revealed potent antioxidant and anticancer properties, particularly against colon cancer cells. These findings contribute to the growing body of evidence supporting the use of herbal medicine as a viable alternative to conventional cancer therapies. Future research should focus on isolating and characterizing the specific bioactive compounds responsible for these effects, as well as

conducting in-vivo studies to confirm their efficacy and safety. The development of standardized extracts and formulations could pave the way for new, plant-based anticancer drugs, offering hope for more effective and less toxic treatment options for cancer patients worldwide.

CONCLUSIONS

Although colon cancer is one of the four most common cancers worldwide, with more than 1.9 million new cases and 935,000 deaths annually, the main ways to beware and control death from colon cancer are by means of detection and prevention, such as screening programs and lifestyle changes. In this regard, further research directed toward the development of more effective treatment and prevention strategies for colon cancer is in high demand. *Betula utilis* and *Annona squamosa* are medicinal plants that have been utilized since time immemorial. The chemical composition and medicinal actions of these plants leave an open door to the further research into these available natural therapeutic agents that may assist in curing a multitude of diseases. More research needs to be conducted to curtail the therapeutic perspectives of these medicinal plants. In conclusion, the flavonoid-rich fractions from *Betula utilis* and *Annona squamosa* potential therapeutic agents against colon cancer. Further studies are needed to evaluate the in-vivo efficacy and safety of these fractions, as well as to elucidate the underlying mechanisms of their anti-cancer activities. The MTT assay showed a dose-dependent cytotoxic effect on colon cancer cell lines, indicating the potential of these extracts to inhibit cancer cell growth and viability. A critical finding of this study is the correlation between the phytochemical content and pharmacological activities. Fractions rich in phenolics and flavonoids exhibited the strongest antioxidant and anticancer effects, underscoring the importance of these compounds in the therapeutic efficacy of *Betula utilis* and

Annona squamosa. In conclusion, this research supports the traditional use of *Betula utilis* and *Annona squamosa* in cancer treatment and highlights their potential as sources of new anticancer agents. These findings pave the way for further studies to isolate and characterize the specific bioactive compounds responsible for these effects. Future research should also include in-vivo studies to confirm the efficacy and safety of these compounds. The development of standardized plant-based formulations could offer more effective and less toxic treatment options for colon cancer, contributing significantly to the global fight against cancer.

Authors Contribution

My contribution was to design the study and carry out the data collection and statistical analysis. Dr. A.K.S. Rawat Sir and Dr. Amit Kumar Nigam Sir also guided the study design and helped draft the manuscript.

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Declaration Of Conflict of Interest

The author(s) declare(s) that there is no conflict of interest.

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