

Preliminary Phytochemical screening for the qualitative and quantitative confirmation of constituents present in the plant extracts and fractions of *Betula utilis* leaves and *Annona squamosa* roots

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ABSTRACT

The abundance of bioactive compounds in medicinal plants plays a crucial role in the development of new pharmaceuticals. This ongoing study focuses on the preliminary phytochemical screening and qualitative and quantitative analyses of phytoconstituents present in extracts and fractions from *Betula utilis* leaves and *Annona squamosa* roots. Plant materials were collected, dried, ground into powder, and extracted using solvents, including methanol, ethanol, and water. A preliminary phytochemical screening was conducted on crude extracts and fractions to identify the main classes of secondary metabolites. Standard chemical methods were employed to perform qualitative phytochemical analysis, identifying alkaloids, flavonoids, tannins, saponins, glycosides, phenolic compounds, terpenoids, steroids, and carbohydrates in plant extracts. Although the fractions varied in quantity, the analysis showed that both plant extracts contained a diverse array of bioactive compounds. The leaf extracts of *Betula utilis* were found to be rich in flavonoids, phenolics, and tannins, which are known for their antioxidant properties. Additionally, alkaloids, glycosides, and saponins were detected in the root extracts of *Annona squamosa*, which may have potential pharmacological applications such as antibacterial and anticancer effects. Quantitative analysis of certain phytochemicals, including total phenolic content, total flavonoid content, and alkaloid content, was also performed to determine the concentration of major compounds.

The findings indicated that solvent polarity significantly influenced the efficiency of phytochemical extraction, with some solvent fractions containing higher concentrations of active phytoconstituents. Overall, the study confirms that *Betula utilis* leaves and *Annona squamosa* roots are valuable sources of important phytochemicals and hold promise for further pharmacological and therapeutic research. The preliminary phytochemical screening of plant extracts provides a scientific foundation for future studies on the isolation, characterization, and evaluation of bioactive compounds responsible for various medicinal properties.

Keywords: *Betula utilis*, *Annona squamosa*, qualitative analysis, quantitative analysis, alkaloids, flavonoids.

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INTRODUCTION

Medicinal herbs have been proven to be safe and efficient natural treatments. Folk medicine utilizes a variety of plant species to treat a wide range of illnesses. In many underdeveloped nations, plant-based chemicals are still used extensively as medicinal treatments in primary healthcare. Secondary metabolites with intriguing biological functions are abundant in plants. These secondary metabolites, known for their varied structural forms and properties, often serve as a major source.[1] Notable examples of these compounds include flavonoids, phenols, phenolic glycosides, saponins, and cyanogenic glycosides. [2,3] Traditionally, antibiotics have been sourced from natural products originating from

microorganisms. However, with the increasing acceptance of herbal medicine as an alternative treatment, it has become essential to examine medicinal plants for active compounds, as they may serve as sources for antibiotic prototypes. [4,5] In vitro screening methods have been shown to provide the initial insights needed to select crude plant extracts with potentially beneficial properties for further pharmacological and chemical studies. 6 Since ancient times, humans have explored plants to discover new medications, leading to the use of numerous medicinal plants to treat a wide range of ailments. Eighty percent of people in developing nations only use traditional medicine for their basic medical needs, with the

majority of these treatments being the use of plant extracts, according to the World Health Organization (WHO, 2008). [7] Materials that are naturally occurring and supplied by the Earth are referred to as natural resources because they cannot be produced by humans. Alternatively, they can be altered and utilized in ways that are advantageous to people. Numerous plant species that produce chemically unique natural goods, such as biotic (wood, silk), biobased (bioplastics, cornflour), bodily fluids (plant exudates), and other natural materials (soil, coal), are examples of nature's limitless natural resources. Natural goods continue to offer a substitute for contemporary treatment in drug discovery all around the world. [8] Since the beginning of time, plant-based products have been acknowledged as an essential part of the medical industry. Phytochemicals found in the various portions of plants with medicinal qualities are what cure illnesses. The chemical compounds found in plant materials help heal a variety of ailments in both humans and animals by eliciting or altering certain physiological responses in the afflicted individual. [9, 10] The diversity and abundance of medicinal plant resources in traditional medicine are influenced not only by the richness of the local flora but also by the accessibility and ease of using these therapeutic plant resources, as well as the wisdom that goes along with using them as herbal remedies. Human society has amassed information about this traditional medicine over time, which has been influenced by regional culture and environment and transmitted from one generation to the next. Traditional medicine continues to play an important part in basic healthcare even in this day and age, and it has been shown to be a promising source of new medication candidates.

Herbal medicine plays a crucial role in shaping modern healthcare systems worldwide, with a pronounced impact in developing countries. These herbal remedies meet the essential healthcare needs of nearly four billion people, representing 80% of the population in these regions. Plant-derived chemical compounds have shown significant efficacy in this area. Bioactive phytochemicals are particularly valued for their ability to selectively target cancer cells while sparing normal cells. The process of carcinogenesis is complex, involving a multitude of signaling pathways. Phytochemicals, with their diverse functional capabilities, interact with these pathways in various ways, making them excellent candidates for anticancer drug development. [12,13,14,15,16]. Although herbal treatments

are becoming increasingly popular worldwide, we all know very little concerning the active ingredients and molecular mechanisms in lots of these remedial plants [17,18]. Phytochemicals are suitable candidates for anticancer drug development due to their pleiotropic actions on target events with multiple manners [18]. India has distinctive plant diversities, which have been intensively studied for anticancer elements [19].

Betula utilis is a multipurpose, broad-leaved deciduous tree native to the Himalayan region. It is one of the dominant tree species at the Himalayan tree line. One of the most significant tree species in the Himalayan highlands, *Betula utilis*, can reach heights of 4,500 m (14,800 ft). Its family is Betulaceae. Medium-sized *Betula utilis* trees can grow to a height of 20 m. The tree has several branches and a bole that is typically uneven. Its immature leaves, bracts, and stalks are covered in short, silky hairs. The leaves are oval and irregularly serrated and are alternately oriented. They are deciduous. The bark is white or reddish-white. The exterior bark is composed of several smooth layers that peel off in horizontal flakes. The inner cortex is reddish. [20] Many native groves of *B. utilis* (locally known as bhojpatra in the Indian Himalaya) have lost their habitat due to deforestation resulting from the exploitation of the tree. In 1993, the first high-altitude bhojpatra nursery was founded in Chirbasa, which is located close to Gangotri, the site of a popular Hindu pilgrimage to the Ganges River's source. Himalayan mountaineer Dr. Harshvanti Bisht founded the first nursery and is still working to increase bhojpatra reforestation in the Gangotri region and within Gangotri National Park. By 2000, the region had approximately 12,500 bhojpatra seedlings planted. Attempts to outlaw the gathering of bhojpatra trees in the Gangotri region have been made in recent years. [21-25]

In certain regions, *B. utilis* serves as a foundation for numerous biochemical compounds that exhibit anticancer, anti-HIV, antioxidant, antimicrobial, and ant fertility properties. Additionally, *B. utilis* is a source of essential oil, which includes geranic acid, seleneol, linalool, sesquiphellendrene, champacol, and 1, 8-cineol, all of which demonstrate potent antimicrobial effects against the fungus *Candida albicans* and both Gram-positive and Gram-negative human pathogenic bacteria. This tree species also has a suppressive influence on microbial communities within the rhizosphere [25-28]. In Ayurvedic medicine, the bark is utilized to treat a variety of ailments, such as wound healing, leprosy, skin infections, bronchitis, convulsions, and diseases affecting

the blood and ear. Various biochemical compositions found in different parts of the tree are employed for diverse pharmacological activities[28-32]. The primary biochemical compounds are pentacyclic triterpenes, identified as betulin, lupeol, acetyloheanollic acid, betulic acid, lupenone, β -sitosterol, methyl betulonate, and methyl betulate, while the minor compounds reported include oleanolic acid, ursolic acid, and betulinic aldehyde [32-37].

Annona squamosa Linn. is a small, semi-deciduous tree that reaches a height of 3 to 7 meters. It features a wide, open crown with branches that spread unevenly. The inner bark is light yellow and slightly bitter, whereas the twigs become brown with light-brown spots known as lenticels. *A. squamosa* is primarily a desert fruit found throughout tropical regions[38-42]. Although its original habitat is uncertain, *A. squamosa* is indigenous to tropical America and the West Indies. The leaves and bark of the custard apple contain alkaloids, whereas the fruit is rich in iron, calcium, fiber, amino acids, vitamins, carotene, thiamine, riboflavin, niacin, and ascorbic acid. [25,26] *A. squamosa* is the most extensively cultivated species of *Annona*, originating in the New World tropics, likely in the Caribbean[43-45]. This plant is also referred to as sugar apple or sweetsop and has various regional names, including custard apple (India), anon (Portuguese), and noi-na (Thailand). Sugar apple is a popular fruit in Cuba and is also prevalent in the West Indies. Sweetsop is widely grown in Taiwan as well. It continues to be cultivated as a backyard tree, with the Philippines being one of the largest producers globally [45-47]. Phytochemical analysis of *Annona squamosa* extracts has identified numerous phytochemicals, such as proteins, carbohydrates, saponins, alkaloids, flavonoids, phenolics, and glycosides. *Annona squamosa* is a lowland tropical shrub with significant pharmaceutical potential for treating cardiac ailments, thyroid-related disorders, diabetes, and cancer. Various research studies on *A. squamosa* have demonstrated its anticancer, antioxidant, antidiabetic, antihypertensive, hepatoprotective, antiparasitic, antimalarial, insecticidal, microbicidal, and molluscicide properties [48].

MATERIAL AND METHODS

Collection and Authentication

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

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

REAGENTS AND CHEMICALS

Materials: 1-amino-2-naphthol-4-sulfuric acid, acetic acid, acetic anhydride, acetone, acetyl acetone, albumin, alkaline phosphatase (ALP), ammonium molybdate, ascorbate, aspartate transaminase (AST), carboxymethyl cellulose, catechol, chloroform, dipotassium-monohydrogen phosphate, DPPH, ethanol, ethyl acetate, formalin, gamma glutamyl transferase, hydrogen peroxide, isopropanol, and methanol.

Calculation of Total Phenol and Flavonoids content in MeOH extract of *Betula utilis* leaves (BUL) and *Annona squamosa* roots (ASME)

Total phenolic content

The total phenolic content of the ethanolic extract was determined using a method involving the Folin Ciocalteu reagent (FCR) as the oxidizing agent and gallic acid as the standard.

Principle

The Folin Ciocalteu reagent was used to assess the total phenolic content in various plant parts. This reagent essentially evaluates the reducing capacity of a sample. Although the precise chemical composition of the FCR is not fully understood, it is thought to include heteropolymetaphosphates and molybdates. (29-31).

Reagent and Apparatus

- Folin-Ciocalteu Reagent (10 times diluted)
- Sodium carbonate solution (Na_2CO_3) 7.5 %
- Methanol
- Gallic acid (Reagent grade)
- Micropipette (10-100 μl)
- Pipette (1-10ml)
- UV-spectrophotometer

Preparation of Reagents (3)

- **Folin-Ciocalteu Reagent (FCR)**

2 ml of FCR reagent was taken in beaker and specific water was added for 10 times dilution.

- **Preparation of 7.5 % Na_2CO_3 Solution**

Na_2CO_3 (7.5 g) was added to a 100 mL volumetric flask, and a small amount of distilled water was added, and the mixture was shaken to dissolve Na_2CO_3 . The volume was then made up to the mark with distilled water.

- **Preparation of Standard Gallic acid solution**

A stock solution was prepared by dissolving 1 mg of gallic acid in 1 mL of distilled water,

resulting in a concentration of 1 mg/mL or 1000 µg/mL. This solution was then used to prepare serial dilutions to obtain various concentrations.

- **Preparation of Blank Solution**

The blank solution contained 5 mL FCR, 1 mL methanol, and 4 mL sodium carbonate solution.

Experiment Procedure

A 1 mL aliquot of the plant extract or standard solution of varying concentrations was placed in a test tube. To this, 5 ml of FCR reagent solution, diluted tenfold with water, was added. Subsequently, 5 mL of a 7.5% sodium carbonate solution was added to the test tube. The mixture was then incubated at 25°C for 20 min to allow the reaction to proceed. Following incubation, the absorbance of the solution was recorded at 760 nm using a UV spectrophotometer, with a blank serving as the reference. The blank solution contained all the reagents except for the plant extract or standard solution. Test drug (0.25 g) was added to 2.5 mL of ethanol, followed by centrifugation at 20 °C for 10 min. The supernatant solution was collected. The test solution was re-separated with 80% ethanol. The pooled cleared supernatant fractions were concentrated. At that point, 3 ml of water was added in to dried supernatant. Finally added FCR reagent and 20% sodium carbonate. The chemical reaction was set in a water bath for 60 s, and the UV absorbance was estimated at 650 nm using a UV-visible spectrophotometer. Gallic acid (mg/g) was used as the standard.

Total flavonoids

The plant extract was mixed with a hydroalcoholic solution at two distinct ratios: 9:1 and 1:1. The resulting homogenate was separated, and these two ratios were combined and evaporated to dryness until most of the ethanol was removed. The concentrated liquid was extracted in a separating funnel using non-polar solvents. The solvent-extracted aqueous fractions were purified, and the concentrate was pipetted into a test tube. Then, 4 mL of 1% vanillin sulfuric acid was added, and the mixture was heated in a boiling water bath for up to 15 min. Absorbance was measured at 360 nm. A reference standard solution was prepared using quercetin (mg/g). For the standard, 11 mg of catechol was dissolved in distilled water (100 mL), where 1 mL corresponds to 110 µg/mL of catechol.

Statistical analysis

Data are presented as the mean±standard deviation (SD) derived from independent experiments. Statistical significance was assessed using one-way analysis of variance (ANOVA), followed by Dunnett's multiple

comparison test, with the aid of GraphPad Prism (version 5.01) software. A p-value of less than 0.05 was deemed statistically significant.

RESULTS

Estimation of TPC & TFC

Estimation of total phenolic content of *Betula utilis* leaves extracts

Phenolic compounds are considered potent antioxidants that interrupt chain reactions. Phenols play a crucial role in plants because of their ability to scavenge free radicals, thanks to their hydroxyl groups. Table 1 presents the TPC Figure 1 and TFC Figure 2 values of different extracts of *Betula utilis* leaf extracts Table 1.

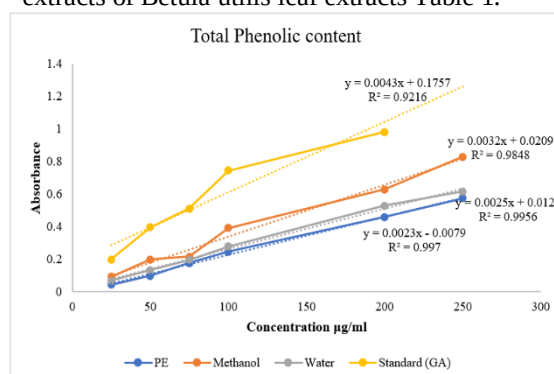


Figure 1 Absorbance at (760 nm) of various concentrations (µg/mL) of standard (gallic Acid) and *Betula utilis* leaves in total phenolics content

Estimation of Total flavonoid content *Betula utilis* leaves extracts

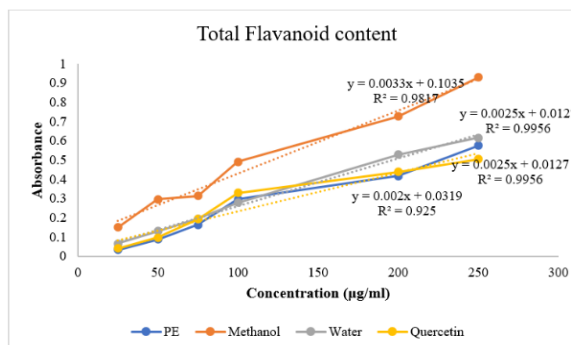


Figure 2 Absorbance at (760 nm) of various concentrations (µg/mL) with reference to Quercetin Equivalent

Plants can produce optional metabolites called flavonoids that have antioxidant effects. Our results showed that the PE extract was not as rich in phenols and flavonoids as the ethanolic and aqueous solutions of *Betula utilis* leaves (7.78 0.063 and 4.23 0.042, respectively). The most positive effects of flavonoids are attributed to their chelating and antioxidant properties. Table 1 summarizes the total flavonoid content of various leaf concentrations of *Betula utilis* leaves.

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Table 1 TPC and TFC of *Betula utilis* leaves extracts

S. No.	Extracts	Total phenol content (mg/g of gallic acid)*	Total flavonoids content (mg/g Quercetin)*
1	Pet. Ether extract of <i>Betula utilis</i> leaves	1.342±0.054	0.627±0.46
2	Methanol extract of <i>Betula utilis</i>	7.78±0.063	3.155±0.12
3	Aqueous extract of <i>Betula utilis</i>	4.23±0.042	2.208±0.26

TPC and TFC of *Annona squamosa* roots extracts

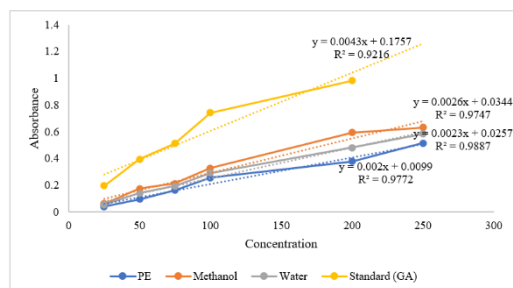


Figure 3 Absorbance at (760 nm) of various concentrations (µg/mL) Total Polyphenolic Content of Gallic Acid Std

Estimation of total phenolic content of *Annona squamosa* roots extracts

Phenolic compounds are considered potent antioxidants that interrupt chain reactions. Phenols play a crucial role in plants because of their ability to scavenge free radicals, thanks to their hydroxyl groups. Table 2 presents the TPC and TFC values of different extracts from the roots of *A. squamosa*.

Estimation of total Flavonoid content of *Annona squamosa* roots extracts

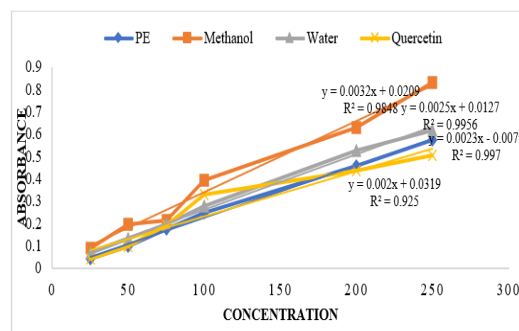


Figure 4 Absorbance at (760 nm) of various concentrations (µg/mL) Total flavonoid Content of Quercetin Std

Table 2 lists the total phenolic content of various concentrates of *Annona squamosa* roots extracts

S. No.	Extracts	Total phenol content (mg/g of gallic acid)*	Total flavonoids content (mg/g Quercetin)*
1	Pet. Ether extract of <i>Annona squamosa</i> roots	1.868±0.034	0.921±0.51
2	Methanol extract of <i>Annona squamosa</i> roots	9.25±0.0813	4.325±0.24
3	Aqueous extract of <i>Annona squamosa</i> roots	3.84±0.091	1.858±0.62

DPPH Scavenging activity

The results represent the mean ±SD of three separate studies. Each randomized test employed three duplicates for each therapy group. substantially different from that of the control group ($p < 0.001$). The cell inhibitory outcomes of three separate tests are presented as the mean ±SD. The Graph Pad Prism application was used to perform a one-way ANOVA and overall Dunnett's Multiple Comparisons Analysis with a level of significance of $p \leq 0.05$ Figure 5,6 & Table 3,4.

Impact of *Betula utilis* leaf extracts in DPPH assay

Table 3 Percentage inhibition of extract of *Betula utilis* leaves extracts by DPPH Assay

	Pet Ether	Methanol	Aqueous
Control	0.4213	0.4312	0.2314
10	35.2	40.58	32.16
25	37.95	47.85	39.25
50	42.25	62.26	50.86
100	54.18	84.52	72.32
IC50	6.28	5.53	3.826

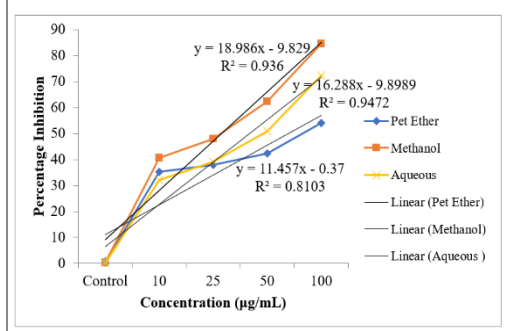


Figure 5 Percentage of inhibition of extracts of *Betula utilis* leaves by DPPH assay

Impact of *Annona squamosa* root extract on DPPH assay.

Table 4 Percentage inhibition of extract of *Annona squamosa* roots extracts by DPPH Assay

	Pet. Ether	Methanol	Water
Control	0.4231	0.4321	0.3214
10	15.52	30.35	22.48
25	30.85	46.25	35.16
50	40.21	50.23	43.56
75	45.36	54.67	48.35
100	51.47	61.25	55.98
IC50	5.27	4.248	4.956

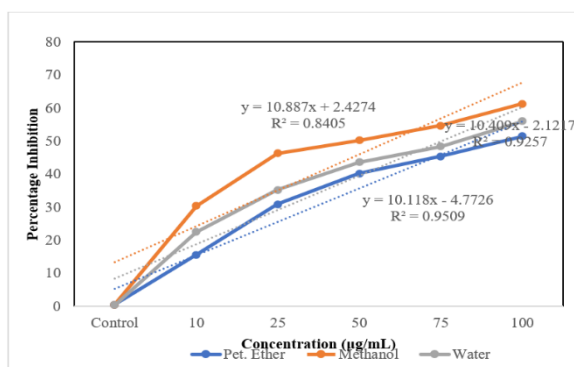


Figure 6 Percentage inhibition of extract of *Annona squamosa* roots extracts by DPPH Assay

DISCUSSION

The rising incidence 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 investigated 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. An exhaustive literature survey was conducted to identify gaps in current knowledge and to justify the selection of *Betula utilis* and *Annona squamosa*. Traditional texts have long mentioned these plants for their medicinal properties, including potential anticancer effects. Despite their documented uses, there is a lack of comprehensive studies examining their phytochemical and pharmacological profiles, particularly concerning colon cancer. This gap in the literature provided a strong rationale for our research. The authenticity of the plant samples was a critical step to ensure the reliability of the study. Plant specimens were collected from verified sources and authenticated using both botanical and molecular techniques. This step was crucial to eliminate any variability that might arise from using misidentified plant materials, thus ensuring the integrity of subsequent analyses. The establishment of preliminary pharmacognostic standards for *Betula utilis* and *Annona squamosa* involved detailed macroscopic and microscopic examinations. These standards serve as a baseline for identifying and verifying plant materials in future studies. Phytochemical screening revealed the presence of various bioactive compounds, including alkaloids, flavonoids,

saponins, and tannins. Quantitative assays further confirmed high levels of total phenolic and flavonoid content, particularly in the flavonoid-rich fractions. These compounds are known for their potent antioxidant properties, which could contribute to the anticancer activities of the plants.

The fractionation of methanolic extracts was aimed at isolating and identifying specific bioactive compounds responsible for the observed pharmacological effects. Using solvent-solvent partitioning and chromatographic techniques, we were able to obtain fractions enriched in phenolics and flavonoids. These fractions were further analyzed to determine their antioxidant capacities and potential cytotoxic effects on colon cancer cells. The DPPH free radical-scavenging assay demonstrated significant antioxidant activity in the extracts and fractions of both plants. The high antioxidant capacity is indicative of the presence of compounds capable of neutralizing free radicals, thereby reducing oxidative stress. Oxidative stress is a known contributor to cancer progression, and compounds that can mitigate this stress may help inhibit cancer cell growth. The ROS assay further quantified the ability of these extracts and fractions to reduce intracellular reactive oxygen species levels. A decrease in ROS levels suggests a protective effect against oxidative damage, which is crucial in preventing the initiation and progression of cancer.

The results of both antioxidant assays support the hypothesis that *Betula utilis* and *Annona squamosa* contain potent antioxidants that may play a role in cancer prevention and treatment. Future research should focus on isolating and characterizing the specific bioactive compounds responsible for these effects and on 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.

In conclusion, this research supports the traditional use of *B. utilis* and *A. 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, thereby contributing significantly to the global fight against cancer.

Authors Contributions:

My contribution was to design the study, conduct the data collection, and perform the statistical analysis. Dr. A.K.S. Rawat Sir also guided the study design and helped draft the manuscript. All authors read and approved the final manuscript.

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

Abbreviations:

ROS: Reactive oxygen species

TPC and TFC: Total phenolic content and Total flavonoid content

CRC: Colorectal cancer

FCR: Folin-Ciocalteu Reagent

DPPH: 2,2-diphenyl-1-picrylhydrazyl

HPTLC: High- performance thin layer chromatography

T.L.C: Thin- layer chromatography

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