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Investigating the Anti-Tumor Properties of Blueberry Extract on Colon Cancer Cells *In Vitro*

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Abstract: The study examined the phytochemical characteristics of the acetone fruit extract of *Vaccinium pallidum* (Blue berry), identifying the existence of alkaloids, phenols, sterols, flavonoids, tannins, carbohydrates, saponins, and triterpenoids. Flavonoids and phenols are recognized for their antioxidative properties, which involve the ability to eliminate free radicals and hinder the activity of hydrolytic and oxidative enzymes. The extract exhibited notable DPPH radical scavenging activity in comparison to the conventional rutin. The extract also suppressed lipid peroxidation by diminishing the red colour complex, however, the reduction was not statistically significant when compared to the normal BHA. The study indicated that flavonoids are essential for the antioxidant capabilities of plant phenolic compounds.

Keywords: Anti-cancer activity, Blue berry extract, Colon cancer cell, *In vitro*

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Introduction

The delicious flavour of blackberry fruits has kept people eating them for centuries. As a vital source of bioactive components like vitamins A and C,

carotenoids, sterols, terpenoids, and phenolic compounds, berries have also been proposed as a "superfood" in recent times. Despite their low

caloric content, these compounds show promising bioactivity (Yi *et al.*, 2005a). The isolation of bioactive chemicals from blackberry fruits has lately been the subject of research by a number of researchers. A majority of the phenolic chemicals found in blackberry extracts are anthocyanins, flavonols, ellagitannins, and phenolic acids. There has long been an established relationship between the concentration of phenolic components in blackberry extracts and their bioactivity. The phenolic compounds are phytochemicals that have been the subject of much research due to their widespread presence in nature and their substantial biological effects (Zu *et al.*, 2010; Brown *et al.*, 2015; Gujarathi *et al.*, 2024; Surana *et al.*, 2024).

The blueberry is a member of the Ericaceae family and is a deciduous shrub. There are two types of blueberries found in the United States: the lowbush, also known as wild blueberries, and the highbush, also known as farmed blueberries. The extraordinary antioxidant capacity of each of these cultivars has earned them great respect among fruits and vegetables. Studies in both laboratory settings and living organisms have shown that blueberries and the bioactive compounds contained in it can inhibit the growth of certain cancer types (McDougall *et al.*, 2008; Keservani *et al.*, 2015).

However, studying the potential of blueberries as anticancer agents is becoming more and more popular, especially in regard to the cellular pathways that cancer cells use to multiply and spread. The powerful antioxidant capacity of the polyphenolic chemicals found in blueberries is associated with their health advantages. Polyphenols are powerful antioxidants because they neutralize oxygenated free radicals, regulate enzyme activity (both positively and negatively), and chelate metals. This aids in shielding nucleic acids, proteins, and lipids from damage in the membrane. There is evidence that polyphenols can alter gene expression and cell signalling, as well as decrease leukocyte immobilisation, cell proliferation, and angiogenesis. A critical step in

the development and progression of cancer is uncontrolled cell division. Nevertheless, cell adhesion, extracellular matrix disintegration, and cell migration are all intricate processes that contribute to cancer metastasis (Lin *et al.*, 2019; Laddha *et al.*, 2024).

The inhibitory and cell death-inducing effects of polyphenol- and anthocyanin-rich fractions extracted from blueberry extract on cancer cell lines have been confirmed by multiple *in vitro* experiments. However, there is a lack of data on how whole blueberry extract affects cellular adhesion and migration, two critical steps in cancer progression. Earlier, we looked at how two different vegetable extracts affected the spread, adhesion, and development of breast and melanoma cancer cells *in vitro*. This study set out to determine whether a blueberry extract could reduce cell growth, adhesion, and migration in a range of cancer cells isolated from various sources in an *in vitro* setting. The selected cell lines represent six distinct malignancies in humans: prostate, cervix, breast, kidney, and lung. Because of its widespread use in colon cancer syngeneic models, the CT26 murine colon carcinoma cell line was included in this study. This is the first study to evaluate the effects of whole blueberry extract on human lung and kidney cancer cell proliferation, adhesion, and migration (Jarouliya *et al.*, 2018; Verma *et al.*, 2024).

Materials and Methods

Collection of Plant and preparation of extract:

Blueberry plant material that has been authenticated was acquired from the Cancer Research centre. The powdered components were extracted using 70% acetone in de-ionized water. Used a five-layer cheesecloth to strain the chilled blueberries. After that, the resulting supernatant was taken in a Pyrex baking dish and acetone was evaporated under a fume hood; after centrifuging for 10 min, poured the mixture into the dish (Keservani *et al.*, 2023; Sable *et al.*, 2023).

Preliminary phytochemical evaluation:

In order to identify different components, a qualitative phytochemical analysis was carried out on the acetone extract of blueberry (*Vaccinium pallidum*) (Sable *et al.*, 2023; Rane *et al.*, 2024).

In vitro anti-oxidant activity:

DPPH radical scavenging Assay:

When hydrogen donors react with the DPPH free radical, it undergoes reduction and is transformed into a matching hydrazine. The DPPH radical has a purple hue, which transforms into a yellow colour upon interaction with a hydrogen donor. The discoloration assay involves assessing the impact of an antioxidant by adding it to a DPPH solution in either ethanol or methanol. The reduction in absorbance at 490 nm is then quantified (Coates *et al.*, 2007; Sable *et al.*, 2023).

Scavenging of Nitric Oxide radical:

A modified Griess-Ilosvay reaction can be used to quantify the amount of nitrite ions produced by the spontaneous generation of nitric oxide by sodium nitroprusside in an aqueous solution at physiological pH. Instead of 1-naphthylamine (5%), the Griess-Ilosvay reagent is adjusted in this study by using Naphthyl ethylene diamine dihydrochloride (0.1% w/v). When combined with Griess reagent, nitrite ions produce a purple azo colour. The concentration of nitrites will drop when test components, which are scavengers in nature, are present. The amount of scavenging is proportional to the reduction of purple azo dye production. At 540 nm, the chromophore's absorbance was measured (Brown *et al.*, 2012; Sable *et al.*, 2023).

Lipid peroxidation inhibitory activity:

Reactive oxygen species (ROS) like hydroxyl radicals can start lipid peroxidation by removing hydrogen from lipids and creating a conjugated lipid radical. This quickly forms lipid radicals when exposed to oxygen and continues to do so until the chain reaction is interrupted. A variety of biomolecules, including DNA, proteins, and lipids, can be oxidised by lipid peroxidation adducts, which can lead to cellular damage (Seeram *et al.*, 2006; Sable *et al.*, 2023).

Evaluation of Total Antioxidant capacity of the extract:

A green Mo (V) complex with maximum absorbance at 695 nm is formed when antioxidant chemicals reduce Mo (VI) to Mo (V). This complex is used to measure the total antioxidant capacity using the phosphomolybdenum technique (Lamdan *et al.*, 2020; Sable *et al.*, 2023).

Evaluation of anti-cancer activity:

Determination of cell viability by MTT Assay:

The majority of cytotoxicity tests have relied on the cells' capacity to withstand a harmful stimulus. The premise upon which this test rests is that tetrazolium cannot be reduced by dead cells or by products of dead cells. Both cell density and mitochondrial activity per cell are critical parameters in this experiment. The process relies on the enzyme succinate dehydrogenase, which is found in mitochondria, breaking down the tetrazolium salt 3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into formazan, a product with a blue hue. The extent of formazan synthesis by the cells utilised was found to be related to the number of cells (Olejnik *et al.*, 2018; Ribera-Fonseca *et al.*, 2020; Sable *et al.*, 2023).

Through the use of DMEM with 10% FBS, the cell count was brought down to 1.0×10^5 cells/ml after the monolayer cell culture was trypsinized. Approximately 10,000 cells (0.1 ml) of the diluted cell suspension was put to each well of the 96-well microtiter plate. Following 24 h, once a partial monolayer had developed, the liquid above was removed by flicking off the switch. The monolayer was then rinsed with medium once, and 100 μ l of various test drug doses were added to the partially formed monolayer in microtiter plates (Sun *et al.*, 2015). The plates were then placed in an incubator set at 37°C with a 5% CO₂ environment for three days. After each observation, they were examined under a microscope. Each well was supplemented with 50 μ l of MTT in PBS after 72 h, and the drug solutions in the wells were removed. For 3 h at 37 °C in a 5% CO₂ environment, the

plates were incubated with gentle shaking. To dissolve the formazan that had developed, 100 μ l of propanol was added after the supernatant had been removed, and the plates were gently shaken. A microplate reader operating at 540 nm was used to measure the absorbance. The following formula was used to compute the percentage growth inhibition. The CTC₅₀ values, which indicate the concentration of the test agent required to inhibit cell growth by 50%, were obtained from the dose-response curves for each cell line (Yi *et al.*, 2005b; Wang *et al.*, 2023; Sable *et al.*, 2023).

Results and Discussion

Percentage yield of fruit extract:

The yield was approximately 162 g from 1.2 kg of *Vaccinium pallidum* blueberry fruit extract in acetone.

Identification of phytochemical analysis:

The acetone fruit extract of the *Vaccinium pallidum* blueberry contained carbohydrates, saponins, alkaloids, flavonoids, tannins, phytosterols, triterpinoids, and phenolics; it did not contain cardiac glycosides or fixed oils, according to the results of the preliminary phytochemical examination.

In vitro antioxidant activity:

DPPH radical scavenging assay:

In this study, we report the findings of an *in vitro* antioxidant activity test using an acetone fruit extract of *Vaccinium pallidum* blue berry (Table 1). We used the IC₅₀ value from earlier research as our reference.

Nitric oxide scavenging assay:

Previous research has used the IC₅₀ value as a standard for evaluating the antioxidant activity of acetone fruit extract of *Vaccinium pallidum* blue berry in an *in vitro* nitric oxide scavenging experiment (Table 2).

Previous research has served as a benchmark for determining the IC₅₀ value, and this study used the findings of an *in vitro* antioxidant activity test

using an acetone fruit extract of *Vaccinium pallidum* blue berry as a standard.

Lipid peroxidation inhibition assay:

Based on the results of the lipid peroxide inhibition assay using the acetone fruit extract of the *Vaccinium pallidum* blueberry, the IC₅₀ value has been taken from prior research as the standard (Table 3).

In-vitro anticancer activity:

MTT Assay:

Cytotoxic properties of test drug against HCT-116 cell lines:

These are the outcomes of an *in vitro* cytotoxic concentration assay using the MTT assay method on HCT-116 cell lines using an acetone fruit extract of *Vaccinium pallidum* blue berry. Results of the MTT assay on HCT-116 cell lines showing the cytotoxic concentration of the acetone fruit extract of the *Vaccinium pallidum* blue berry *in vitro* are illustrated in Table 4.

Cytotoxic properties of test drug against HT-29 cell lines:

In the MTT assay, *Vaccinium pallidum* fruit acetone extract was tested on HT-29 cell lines (Table 5).

The phytochemical examination of the acetone fruit extract of the *Vaccinium pallidum* blue berry revealed the presence of several well-known bioactive components, including alkaloids, phenols, sterols, flavonoids, tannins, carbohydrates, saponins, and triterpenoids. The total flavonoids and phenols in the extract, along with their antioxidant activity *in vitro*, indicate that the extract may have antioxidant properties. Free radical scavenging and inhibition of hydrolytic and oxidative enzymes are among the undiscovered activities of the polyphenolic chemicals known as flavonoids. Phenolics are secondary metabolites found in plants all over the place. They have many medicinal benefits, including scavenging free radicals, protecting against cancer, and mutagenesis and carcinogenic effects. Heart problems are reduced as a result of these as well. The presence of a hydroxyl group is primarily

Table 1: Blueberry *Vaccinium pallidum* acetone fruit extract DPPH assay

S. No.	Conc. (µg/ml)	Absorbance	Avg (blank)	% of inhibition
1	150	0.132±0.002	0.061±0.002	59.2
2	100	0.140±0.003	0.062±0.009	53.21
3	50	0.151±0.008	0.080±0.006	43.08
4	25	0.162±0.02	0.089±0.003	40.21
5	12.5	0.181±0.005	0.100±0.002	30.05
6	6.15	0.187±0.002	0.107±0.007	21.18
7	3.12	0.189±0.004	0.119±0.001	17.04
8	Control	0.330±0.005	0.154±0.008	0.01

Table 2: Blueberry *Vaccinium pallidum* acetone fruit extract's nitric oxide radical scavenging activity

S. No.	Conc. (µg/ml)	Absorbance (Mean)	Avg. (Blank)	Inhibition (%)	IC ₅₀ value
1	300	0.161±0.003	0.040±0.003	72.1	65 ± 2.5
2	200	0.166±0.0005	0.045±0.0006	61.3	
3	125	0.170±0.004	0.055±0.004	56.42	
4	61.5	0.171±0.003	0.056±0.003	49.66	
5	31.2	0.174±0.0018	0.058±0.0016	35.10	
6	15.6	0.187±0.003	0.070±0.003	25.20	
7	7.8	0.195±0.0014	0.090±0.0012	16.08	
8	Control	0.215	0.13	0.10	
9	Standard Rutin	-	-	-	61.56

Table 3: Blueberry *Vaccinium pallidum* acetone fruit extract lipid peroxidation assay

S. No.	Conc.	Inhibition (%)	IC ₅₀ values
1	300	58.69±3.68	280±41
2	250	45.22±5.56	
3	125	30.72±7.91	
4	61.5	19.32±6.7	
5	31.2	13.47±5.90	
6	15.6	10.56±5.9	
7	7.8	3.41	
8	control	0.00	
9	Standard	-	230

responsible for the scavenging capacity of phenolics (Johnson and Arjmandi, 2013; da Silveira *et al.*, 2021).

A synthetic DPPH assay was used to determine the free radical scavenging activity of blue berry fruit extract. In comparison to conventional rutin,

the acetone fruit extract of *Vaccinium pallidum* blue berries demonstrated much higher DPPH radical scavenging action. Reduced nitric oxide synthesis is a result of nitric oxide scavenging, which competes with oxygen. The phenolic chemicals included in the extract may be

Table 4: CTC₅₀ value of *Vaccinium pallidum* blueberry fruit extract in acetone

S. No.	Cell line	Conc.	Cytotoxicity (%)	CTC ₅₀ Value
1	HCT-116	300	70.50±2.29	229±15.29
2		250	51.19± 1.32	
3		125	42.79 ±2.55	
4		62.5	32.89 ±14.69	
5		31.2	21.09± 5.32	

Table 5: CTC₅₀ value of *Vaccinium pallidum* blueberry fruit extract in acetone

S. No.	Cell line	Conc.	Cytotoxicity (%)	CTC ₅₀
1	HT- 29	300	85.41±0.74	141.41±22.1
2		250	74.80±0.52	
3		125	47.02±7.41	
4		62.5	38.39±8.45	
5		31.2	21.41±5.65	

responsible for its nitric oxide scavenging activity, according to our findings. Therefore, *Vaccinium pallidum* blue berry extract's scavenging action is dose-dependent. The Nitric oxide scavenging activity of the extract was compared to that of ordinary rutin. Since lipid peroxidation (LPO) is thought to be the beginning of many harmful and degenerative processes, it has been linked to the pathophysiology of several disorders, including neurological diseases. Bioenzymes are particularly vulnerable to LPO (Katsube *et al.*, 2003; Albogami and Hassan, 2021).

Reduced levels of red colour complex are one mechanism by which blueberry and *Vaccinium pallidum* fruit extracts slow down lipid peroxidation. Plant phenolic chemicals, including flavonoids, are thought to have strong anti-oxidant properties due to their capacity to chelate ions in the LPO system. Because of this, the extract's lipid peroxidation inhibition activity was not significantly higher than that of normal BHA (Wang *et al.*, 2017; Kiernozek *et al.*, 2022).

Conclusion

In conclusion, this study lends credence to the idea that plant feed sources, traditional medicines, and dietary supplements can still be a gold mine for discovering new pharmaceuticals made from

natural products. The *Vaccinium pallidum* blueberries possess multiple actions, such as antioxidant and anti-cancer properties. Natural antioxidants help ward off cancer and its consequences, including diabetic nephropathy, diabetic retinopathy, and diabetes-related malignancy.

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