# Malvidin Abrogates Oxidative Stress and Inflammatory Mediators to Inhibit Solid and Ascitic Tumor Development in Mice

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**ABSTRACT:** The anticancer activity of malvidin was studied in Dalton's lymphoma ascites (DLA)–induced solid and ascitic tumor mice models. Malvidin is a natural compound belonging to the family of O-methylated anthocyanidin and plays a predominant role in regulating both short- and long-term cellular activities. Animals were injected with DLA cells ($1.5 \times 10^6$ cells/animal) to induce solid and ascitic tumors. The administration of malvidin (5 mg/kg bw and 10 mg/kg bw) was carried out for 10 consecutive days from the day of tumor induction for both solid and ascitic tumors. Cyclophosphamide, CTX (25 mg/kg bw), used as the standard drug, was also administered for 10 consecutive days. Treatment with malvidin showed a significant reduction in tumor volume and elevated white blood cell (WBC) count when compared to the DLA-bearing control animals. The treatment also maintained the body weight and hemoglobin level, and decreases in aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT) were also noted. This investigation also reported the decreased levels of cellular glutathione (GSH) in ascitic tumor groups. Malvidin reduced inflammatory mediator and cytokine levels, such as tumor necrosis factor level alpha (TNF-α) and interleukin-6 (IL-6), which serve as molecular targets for cancer prevention. A decrease in the level of reactive oxygen species (ROS), like nitric oxide (NO), was observed. Histopathological examination revealed altered morphological changes in tumor tissue and the alleviation of hepatic architecture due to DLA. Immunohistochemical analysis revealed the inhibition of iNOS. This study demonstrated that malvidin exhibited significant *in vivo* antitumor activity and that it was reasonably imputable to its increasing endogenous mechanism. We accent the pertinence of malvidin as a potential naturally derived drug target for tumor control.

**KEY WORDS:** Dalton's lymphoma ascites, malvidin, reactive oxygen species, tumor, cytokines, CTX

## I. INTRODUCTION

Cancer is the second leading death-causing disease globally, and 9.8 million deaths from cancer were reported in 2018 (as retrieved from the World Health Organization, 2020). Cancer causes a deregulation of normal cellular activities, and this leads to an uncontrolled proliferation of genetically altered cells. There are more than 200 types of cancer, and solid tumor is one of the commonest forms.[1] In general, solid tumors come to mind when patients hear the word *cancer*, and these cases essentially involve the growth of harmful cancer cells. Causative factors include anything that can lead to the development of abnormal cells, and some of the major categories are chemical or toxic substances (asbestos, cadmium, vinyl chloride), tobacco smoke (which contains 66 unknown carcinogenic chemicals and toxins), and genetics. Genetic changes that occur after conception are defined as *acquired* changes. Given the prevalence of cancer, there is an ongoing need to develop new therapeutic and diagnostic choices with less toxicity and minimal side effects. Chemotherapy is one of the most promising approaches, complementary to many other conventional therapies. However, chemotherapeutics can inflict damage on active cells, presenting risk factors that may be irreversible.[2] The limitations of anticancer drugs as cytotoxic compounds cannot be ignored in clinical practice. These agents exhibit a lack of selectivity and often kill normal cells during treatment, which poses a major drawback.[3] Hence, there is a need to

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evolve drugs that can target malignant cells without inducing any damage to normal cells.

Since time immemorial, plants and plant-derived products have been used as a primary source in the field of drug discovery from natural sources. As is well known, researchers have shown a keen interest in the area of exploring antioxidant-capacity-rich compounds to develop plant-derived anticancer products. Malvidin (O-methylated anthocyanidin) is a 3',5'-methoxy derivative of delphinidin anthocyanidin and is abundant in a variety of berries, including *Vaccinium corymbosum* (family: Ericaceae), *Vitis vinifera*, *Amelanchier alnifolia*, and *Anagallis monelli*.[4,5] The structure of malvidin is represented in Fig. 1. Anthocyanin dissociated from fruits and vegetables blocks the translocation of NF-κB in a nuclear factor erythroid 2–related factor (Nrf-2) independent manner, resulting in a reduction of the secretion and expression of pro-inflammatory cytokines.[6] Malvidin, the top anthocyanin in blueberries, has emerged as a promising agent that possess promising antioxidant and anti-proliferative properties.[7]

Malvidin holds an excellent antioxidant capacity, with high free-radical-scavenging properties *in vitro*.[8] Preliminary experimental studies imply that the antioxidant activity of malvidin is responsible for regulatory action; however, certain studies reported malvidin to activate apoptosis by inducing oxidative damage in malignant cells.[9] One of the highlightable properties of malvidin is that it activates Nrf-2/Keap (Kelch-like ECH-associated protein-1) signaling and upregulates cytoprotective enzymes for regulating oxidative stress.[10] The chemotherapeutic efficacy of blueberries in the Hamster Buccal Pouch Carcinoma (HBP) model, based on its ability to abrogate transforming growth factor beta (TGF-β) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathways and modulate specific microRNA.[11] Studies recently evidenced that malvidin could act as a STAT-3 inhibitor in a cellular context, using the oral cancer cell line SCC131.[12] In recent years, targeted therapy, including precision medicine, kinase inhibitors, antibody-drug conjugates (ADCs), monoclonal antibodies, small molecule-drug conjugates (SMDCs), and antisense technology approaches, has become a robust strategy for anticancer therapeutics.[13]

To date, malvidin's potential to modulate various cellular activities has been explored; however, its role as an antitumor agent has not been investigated. Therefore, in the present study, an attempt was made to investigate the *in vivo* antitumor activity of malvidin, in comparison with the standard drug cyclophosphamide (CTX). The selection of this standard drug was also based on previous studies.[16] The results of the present study were expected to provide better insight into the targeting mechanisms of malvidin in terms of inhibiting tumor proliferation, which would prove helpful in effective cancer therapeutics.

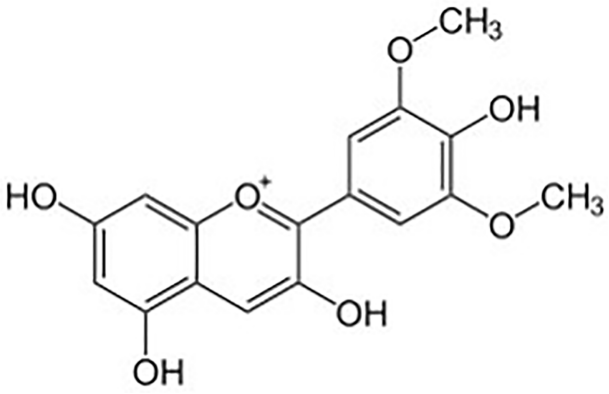


**FIG. 1:** Structure of malvidin

## II. MATERIALS AND METHODS

### A. Drugs and Chemicals

Malvidin was purchased from Sigma-Aldrich (Bangalore, India); Drabkin's solution was purchased from Agappe Diagnostics Ltd. (Coimbatore, India); α-napthyl-ethylene diamine dihydrochloride, ethylene diamine tetraacetic acid (EDTA), and dithionitrobenzene were purchased from Hi-Media (Mumbai, India); and cyclophosphamide (CTX) was purchased from Zydus Onco Sciences (Goa, India).

### B. Animals

Male Swiss albino male mice (20–25 g) were procured from the animal facility at the PSG Institute of

Medical Science and Research (PSGIMSR), in Coimbatore. The animals were maintained in the animal house facility under controlled conditions (temperature 25 ± 2°C, 12 hour dark/light cycle, and humidity 55%). They were fed a standard pellet diet and water *ad libitum*. The experiments involving animals were performed after obtaining approval from the Institutional Animal Ethics Committee (IAEC) Government of India (Approval Number: 415/2018/IAEC).

## C. Induction of Tumor

Dalton's lymphoma ascites (DLA) cell lines were used for the present study. The DLA cells were provided by Amala Cancer Research Centre, Trissur, Kerala. The cells were maintained in mice (*in vivo*) by intraperitoneal (IP) transplantation. The tumor cells aspirated in 0.05% phosphate-buffered saline (PBS) from the peritoneal cavity, and $1.5 \times 10^6$ cells were injected in the mice to develop solid and ascitic tumor models, with the exception of those in the normal (untreated) group. For *in vivo* experiments, malvidin was suspended in phosphate-buffered saline (PBS), and the nontoxic dosage level used in the present study (i.e., 5 mg/kg bw and 10 mg/kg bw) was selected based on the toxicity studies from the literature cited previously.[12,14,15]

## D. DLA-Induced Ascitic Tumor Model

The mice were divided into five groups, with n = 6 animals in each group. Group I mice served as the untreated normal group; group II mice served as the tumor-bearing control and received PBS vehicle only; and group III mice were treated with the standard drug cyclophosphamide, CTX (25 mg/kg bw). Groups IV and V were treated with malvidin at a dosage of concentration 5 mg/kg bw and 10 mg/kg bw, respectively. All treatments were administered as IP (100 µl injections), starting 24 hours after the DLA tumor challenge. All groups were inoculated with $1.5 \times 10^6$ of DLA cells, except for Group I (normal; untreated). Groups II–V were treated for 10 consecutive days with malvidin (0.1 ml) and standard drug CTX through IP injection. The blood was collected from each group by using the tail vein method at 3-day intervals, until the 15th day, to assess hematological parameters, such as white blood cell (WBC) count and hemoglobin. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), and nitric oxide (NO) were measured on the 15th day alone (i.e., at the end of the experiment period). In addition to this, ascitic fluid was aspirated on 15th day, and the cellular glutathione (GSH) level was also measured. To determine the mean survival time (MST) and percentage increase of life span (%ILS), separate sets of mice were used and treated with DLA cells, along with other treatment groups, as outlined previously. The animals were monitored for 50 days, as per our earlier protocols.[16]

## E. DLA-Induced Solid Tumor Model

The mice were divided into five groups, n = 6 animals in each group. Group I mice served as an untreated normal; group II mice served as a tumor-bearing control and received PBS vehicle only; and group III mice were treated with the standard drug cyclophosphamide, CTX (25 mg/kg bw). Groups IV and V were treated with malvidin at a dosage of concentration 5 mg/kg bw and 10 mg/kg bw, respectively. All treatments were administered as IP (100 µl injections), starting 24 hours after the DLA tumor challenge. Induction of solid tumor was carried out by injecting DLA cell lines ($1.5 \times 10^6$ cells) through intramuscular injection of the right hind limb of the mice. Then, the mice in all groups, except for Groups I and II, were treated for 10 consecutive days with malvidin (0.1 ml) and standard drug CTX, respectively, through IP injection. Blood was collected from each group to analyze the hematological profile (WBC, hemoglobin, on an interval of every 5 days). Serum markers (ALT, AST) and cytokine (tumor necrosis factor level alpha [TNF-α] and interleukin-6 [IL-6]) levels were measured on the 25th day alone. At the end of the experiment, blood samples were collected by cardiac puncture from all the groups; the animals were euthanized by using ketamine xylazine overdose anesthesia. Tumor volume was calculated per the following formula: $V = 4/3\pi r_1^2 \times r_2$. Body weights (bw) of all mice were measured at 5-day intervals from day 0 to day 25, as per the previously noted protocols.[16]

### F. DNA Fragmentation Assay

The DLA cells aspirated from Group II–V were subjected to isolation of genomic DNA using a standard DNA isolation kit (Pure Link DNA Mini-kit, Thermo Scientific, India). The isolated DNA was subjected to agarose gel (1%) electrophoresis, as per standard protocol, followed by ethidium bromide (Et-br) staining, and the DNA fragmentation pattern was observed.

### G. Histopathological Analysis

A small portion of tumor tissue from the solid tumor model and liver from the ascitic model was recovered from each group and fixed in 5% formalin after washing with 0.5% saline. After several treatments for alcohol dehydration, sections (~ 4 μm thickness) were cut and stained with hematoxylin and eosin, and monitored under a light microscope at a magnification of 40×.

### H. Immunohistochemical Analysis

Tumor tissues were collected from DLA-induced, CTX-treated, and malvidin-treated groups. After performing several steps, sections (~ 4–5 μm thickness) were mounted and treated with buffered blocking solution (3% BSA in PBS) and then co-incubated with primary antibody against iNOS (polyclonal murine anti-iNOS antibody; ThermoScientific, IL, US) and secondary antibody SS polymer horseradish peroxidase-HRP (Abcam, US) separately at room temperature for about 1 hour, respectively. The sections were then co-incubated with 3,3'-diamino-benzidine (DAB) solution in the dark, and stained with hematoxylin and monitored under a light microscope (magnification 40×) to check for brown-colored staining. The detailed protocol was described in our earlier study.[16]

### I. Statistical Analysis

All the experiments were done in triplicates. The data were represented as mean ± SD, using a one-way analysis of variance (ANOVA), followed by Dunnet's test using GraphPad Instat version 5.0 (GraphPad Software, San Diego, CA, USA). p values significantly differed from the DLA tumor (non-extract-treated) control (*$p < 0.05$, **$p < 0.01$).

## III. RESULTS

### A. Effect of Malvidin on Body Weight and Tumor Volume in DLA-Bearing Ascitic and Solid Tumor Models

The body weight and solid tumor volume were measured after tumor inoculation from the beginning, and then the monitoring was repeated every 5th day (solid tumor model) and 3rd day (ascitic tumor model) until the end of the experiment duration by using vernier caliper. Average increase in the body weight was also determined. Body weight was measured every 5 days (solid tumor) and 3 days (ascitic tumor). At the end of the study, there was a significant increase in the body weight of the tumor-alone group, and the malvidin-treated group showed a decrease in body weight, as indicated in Figs. 2(A) and 3(B), in both the solid and ascites tumor models. Similarly, the standard CTX-treated animals also showed similar results at the end, and the observed results were statistically significant from the tumor control (**$p < 0.01$). Regarding tumor volume, the malvidin-treated group showed a reduction in tumor volume ($4.94 \pm 1.67$ mm$^3$, $4.90 \pm 0.94$ mm$^3$) when compared to DLA ($9.99 \pm 0.99$ mm$^3$) treated groups on 25th day. Malvidin- and CTX-treated groups ($4.30 \pm 0.21$ mm$^3$) both showed significant reduction in the tumor development compared to that seen in the normal group, as shown in Fig. 3(A).

### B. Effect of Malvidin on Hematological Parameters in DLA-Bearing Ascitic and Solid Tumor Models

Hematological parameters measurement helps assess health and disease conditions; the reference ranges are particularly very useful for measuring the condition of tumor progression. The hematological parameters were significantly altered after day 15 in the solid tumor models and day 25 in the ascitic tumor models, when compared with the DLA-bearing tumor group. The total WBC count also increased in the DLA control group ($6.20 \pm 0.2 \times 10^3$ cells/

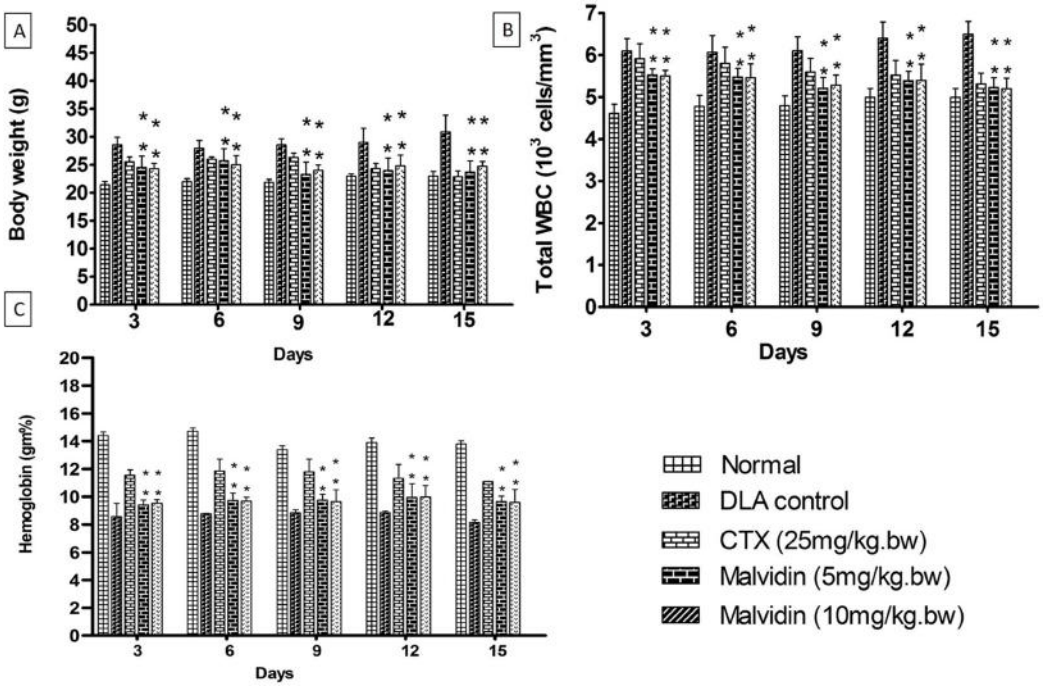


**FIG. 2:** Effect of malvidin on morphological and hematological parameters of a DLA-bearing ascitic tumor mice model: (A) body weight, (B) WBC, and (C) hemoglobin. Values are expressed as mean ± SD for six animals in each group. p values significantly differ from the DLA tumor (non-extract-treated) control (*$p < 0.05$, **$p < 0.01$).

$mm^3$ and $6.50 \pm 0.302 \times 10^3$ cells/$mm^3$), whereas hemoglobin decreased in the DLA control group, which was found to be 9.64 ± 0.20 gm %. After treatment with malvidin (dosage of 5 mg/kg bw and 10 mg/kg bw), the hematological parameters were normalized on day 15 and 25, respectively, which was found to be in the near normal level observed in Group I (untreated). The WBC count was significantly decreased in malvidin-treated groups ($5.10 \pm 0.12 \times 10^3$ cells/$mm^3$, $5.00 \pm 0.1 \times 10^3$ cells/$mm^3$); in the ascitic tumor model group, as shown in Fig. 2(B); and in the solid tumor model group ($5.23 \pm 0.23 \times 10^3$ cells/$mm^3$, $5.20 \pm 0.25 \times 10^3$ cells/$mm^3$), as shown in Fig. 3(C). Similarly, the hemoglobin level was significantly mitigated to a near normal level of 11.96 ± 0.38 gm %, 11.88 ± 0.28 gm %, in the ascitic tumor model group, as shown in Fig. 2(C), and was 9.66 ± 0.38 gm %, 9.59 ± 0.728 gm %, in the solid tumor model group, as shown in Fig. 3(D), in both malvidin-treated groups after day 15 in solid tumor models and day 25 in ascitic tumor models. The observed results suggests that the compound malvidin is effective at both the doses of 5 mg/kg bw and 10 mg/kg bw.

## C. Effect of Malvidin on Liver and Serum Markers in DLA-Bearing Ascitic and Solid Tumor Models

Tumor markers or biomacromolecules are normal endogenous products that are synthesized at a greater rate by cancer cells. Tumor progression can influence these biomarkers—mainly liver marker enzymes. After DLA inoculation in mice, on day 15, levels of GGT, ALP, AST, and ALT were found to be significantly increased. The observed results indicated that liver marker enzymes were elevated in DLA-bearing ascitic groups (27.01 ± 0.32, 174.23 ± 12.36, 86.63 ± 1.51, 60.95 ± 1.67 U/L), as shown in Fig. 4(A–E). Malvidin-treated groups showed reduced levels of these serum enzymes, and it was observed that levels were normalized by treatment with malvidin at the dosages of 5 mg/kg bw and 10 mg/kg bw in the ascitic tumor model. Similarly,

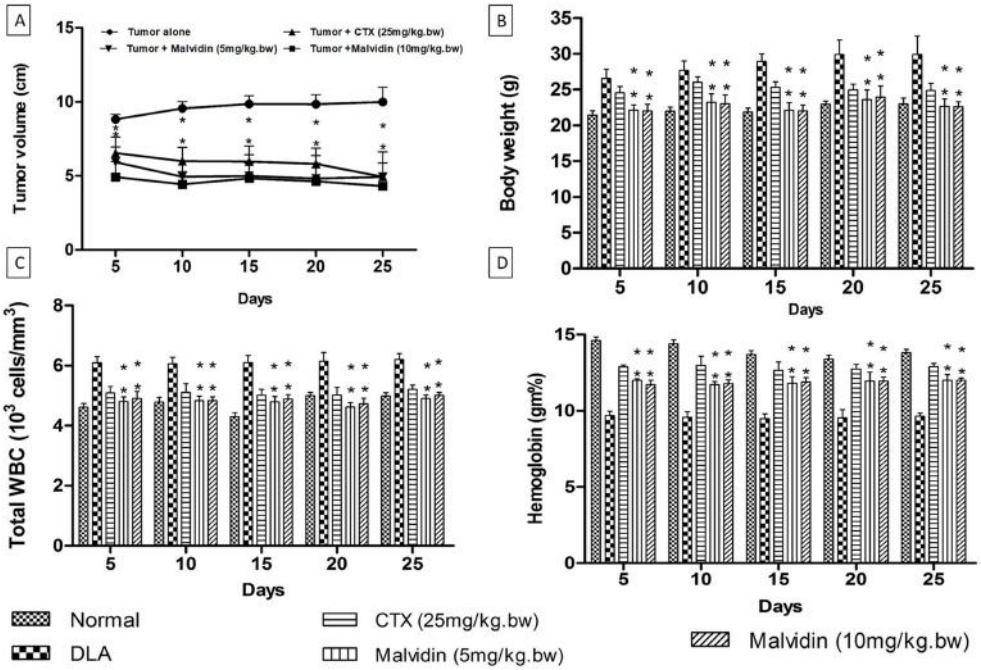


**FIG. 3:** Effect of malvidin on morphological and hematological parameters of a DLA-bearing solid tumor mice model: (A) tumor volume, (B) body weight, (C) WBC, and (D) hemoglobin. Values are expressed as mean ± SD for six animals in each group. p values significantly differ from the DLA tumor (non-extract-treated) control (*$p < 0.05$, **$p < 0.01$).

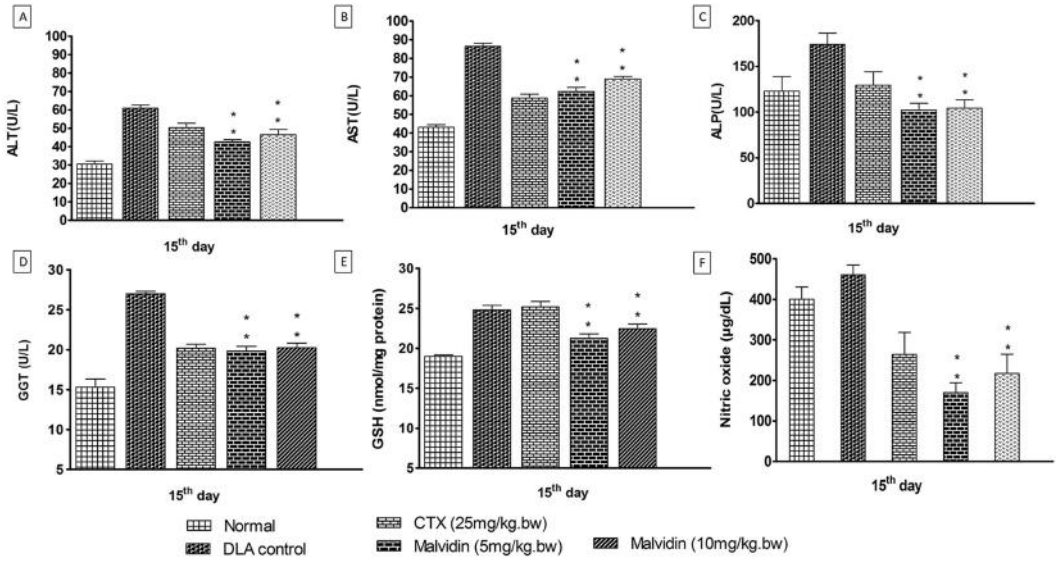


**FIG. 4:** Effect of malvidin on liver marker enzymes and oxidative stress markers in a DLA-bearing ascitic tumor mice model: (A) ALT, (B) AST, (C) ALP, (D) GGT, (E) cellular GSH, and (F) NO. Values are expressed as mean ± SD for six animals in each group. p values significantly differ from the DLA tumor (non-extract-treated) control (*$p < 0.05$, **$p < 0.01$).

in the solid tumor model, a significant reduction (**p < 0.01) in AST (67.25 ± 2.302, 60.06 ± 1.35 U/L) and ALT (51.62 ± 1.23, 42.51 ± 2.75 U/L) levels was observed on day 25 with malvidin dosages of 5 mg/kg bw and 10 mg/kg bw, respectively, as shown in Fig. 5(A–B). The observed results were comparable with those of the standard CTX-treated group, which were similar. After administration of malvidin, the level of serum markers was normalized compared to the tumor-alone group.

## D. Effect of Malvidin on Oxidative Stress Marker Enzymes Cellular GSH and NO in DLA-Bearing Ascitic and Solid Tumor Models

Oxidative stress plays a role in the initiation and progression of any tumor. Altered redox status is a common biochemical aspect that has been observed in all cancer conditions. The cellular GSH and NO levels in the serum were determined, and it was observed that the cellular GSH (24.24 ± 0.76 nmol/mg protein) and NO (460.45 ± 24014 μm) levels in the ascites group were increased in the DLA-alone group on day 15. In malvidin- and CTX-treated groups, these levels were significantly reduced on day 15, as shown in Fig. 4(E–F). Similarly, in the solid tumor group, the NO level was found to be increased in the DLA-alone group (460.45 ± 24.14 μm), and this was found to be significantly (**p < 0.01) decreased in the malvidin-treated (170.08 ± 24.90, 217.08 ± 47.44 μm) groups at both concentrations, as shown in Fig. 5(C). Similarly, administration of standard CTX produced comparable results. This attenuation of GSH and NO levels validates the potent antitumor effect of malvidin at both doses.

## E. Effect of Malvidin on Cytokines in DLA-Bearing Solid Tumor Models

The serum levels of TNF-α and IL-6 were significantly increased (97.76 ± 8.90, 176.50 ± 17.30 pg/ml) in the state of tumor-alone mice in comparison with the normal group (20.22 ± 2.40, 34.67 ± 3.40 pg/ml). The malvidin-treated mice were shown to possess a significant (**p < 0.01) decrease in the level of TNF-α at 5 mg/kg bw and 10 mg/kg bw (72.34 ± 7.30, 69.84 ± 6.60 pg/ml) and IL-6 (83.92 ± 12.30, 79.042 ± 8.20 pg/ml), as shown in Fig. 5(D–E). Treatment with standard drug CTX at a dose of 25 mg/kg bw was found to be reversed in enhanced levels of TNF-α and IL-6 (58.92 ± 6.30, 69.32 ± 7.80 pg/ml) when compared to a DLA-alone treated group.

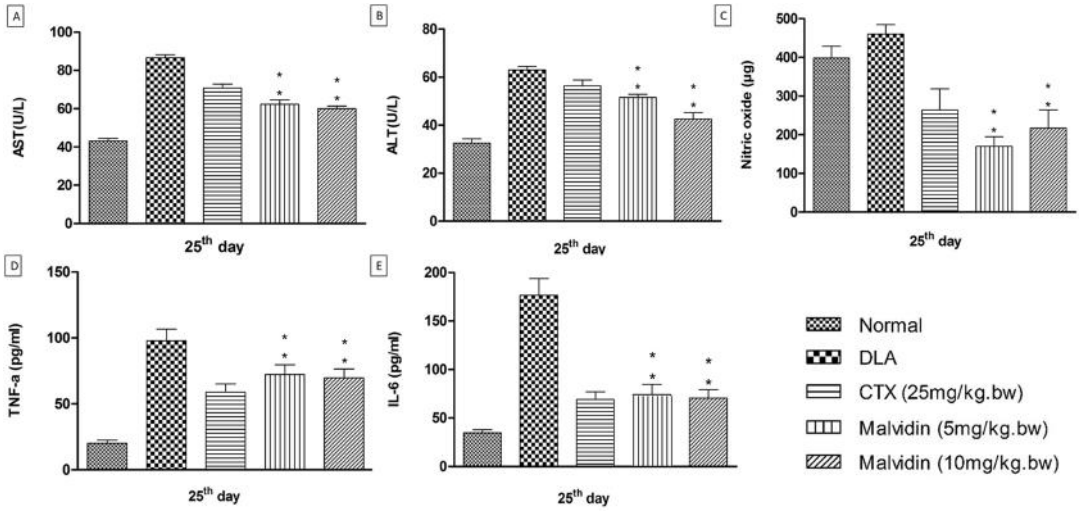


**FIG. 5:** Effect of malvidin on serum marker enzymes, NO, and cytokine levels in a DLA-bearing solid tumor mice model: (A) AST, (B) ALT, (C) NO, (D) TNF-α, and (E) IL-6. Values are expressed as mean ± SD for six animals in each group. p values significantly differ from the DLA tumor (non-extract-treated) control (*p < 0.05, **p < 0.01).

### F. Effect of Malvidin on Histopathology of Tumor Tissue in Solid Tumor Models

Experimental DLA-bearing mice in the intramuscular region, which formed solid tumor, revealed substantial pathological alterations distributed throughout the muscular region, as represented in Fig. 6. Examination of tumor sections obtained from the muscular region of mice treated with the standard CTX drug and malvidin revealed mild ameliorations, including pleomorphic cells, infiltration of cells, and moderate eosinophilic cytoplasm. Multinucleated tumor giant cells and necrosis were noted in the DLA solid tumor section in Group II.

### G. Effect of Malvidin on Histopathology of Liver Tissue in Ascitic Tumor Models

Histopathological observations were made via the liver specimens collected from Groups I–V at the end of the investigational period (i.e., day 15). The normal group showed sections with normal lobular architecture. In the DLA tumor control group, the liver sections showed altered lobular architecture. Hepatocytes were found to have polymorphic nucleoli. The central vein exhibited both congestion and dilatation, and the sinusoid showed dilatation. The CTX-treated group (25 mg/kg bw) showed slightly altered lobular architecture, individual hepatocyte parenchymal necrosis, cytoplasmic vacuolation, binucleation, and mild peripheral inflammation. The central vein and sinusoid exhibited dilatation and congestion. Malvidin-treated mice (both 5 mg/kg bw and 10 mg/kg bw) showed slightly altered lobular architecture polymorphic vesicular nuclei. Multiloculated giant cells are also noted. Individual hepatocytes show parenchymal necrosis. Focal centrilobular necrosis is also seen. The central vein was congested and dilated. The sinusoid also exhibited dilatation, as shown in Fig. 7.

### H. Effect of Malvidin on DNA Fragmentation Assay in Ascitic Tumor Models

The ascitic fluid was removed from the peritoneal cavity on day 15, and it was subjected to sonication

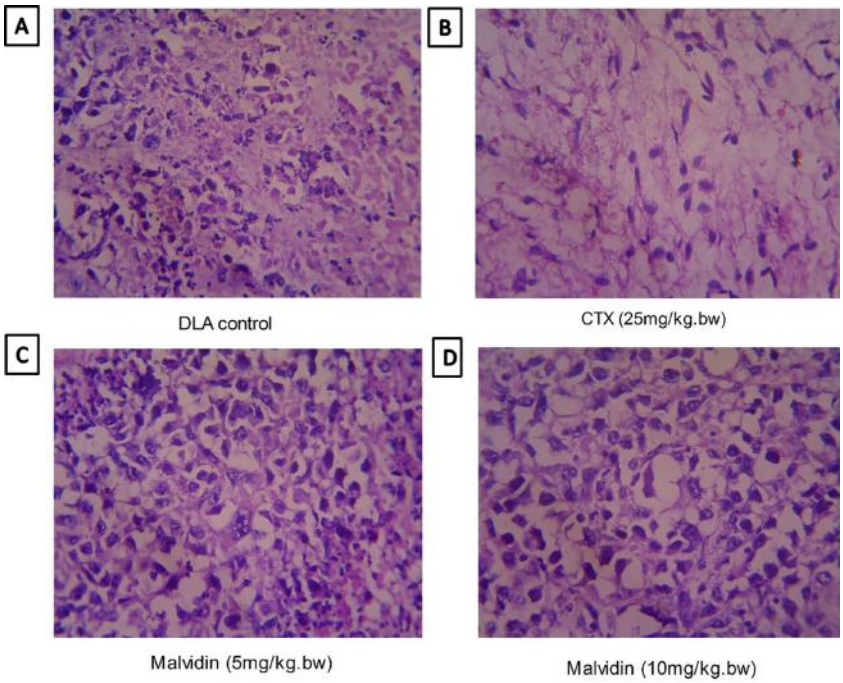


**FIG. 6:** Histopathology changes in DLA-induced solid tumor tissues treated with malvidin (magnification 40×), showing (A) positive control, (B) CTX (25 mg/kg bw), (C) malvidin (5 mg/kg bw), and (D) malvidin (10 mg/kg bw)

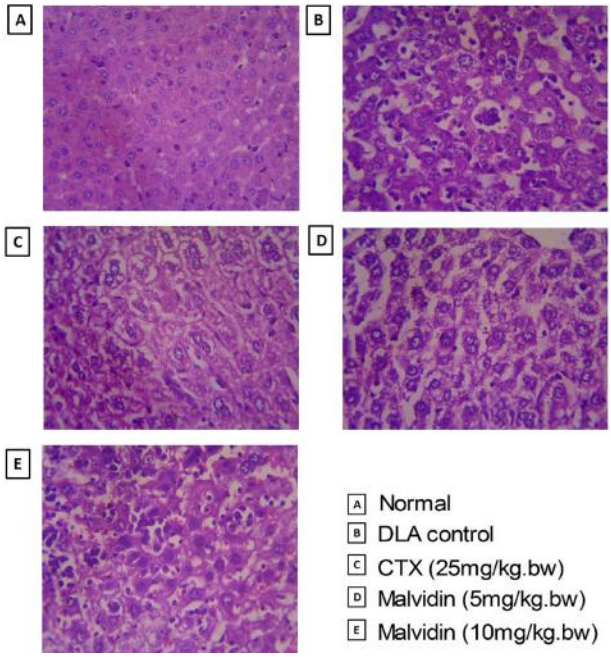


**FIG. 7:** Histopathology changes in liver tissues of a DLA-induced ascitic tumor model treated with malvidin (magnification 40×), showing (A) normal (untreated), (B) DLA control, (C) CTX (25 mg/kg bw), (D) malvidin (5 mg/kg bw), and (E) malvidin (5 mg/kg bw)

and used for detecting DNA fragmentation. The observed bands are shown in Fig. 8. In lane 1, DLA control was loaded, which shows slight shearing with intact bands. In lanes 2 and 3, malvidin-treated samples with concentrations 5 mg/kg bw and 10 mg/kg bw were loaded, which was observed with sheared DNA, indicating that malvidin is a potent inducer of apoptosis in DLA cells. In lane 4, the DNA marker was loaded with the size of 1 kb.

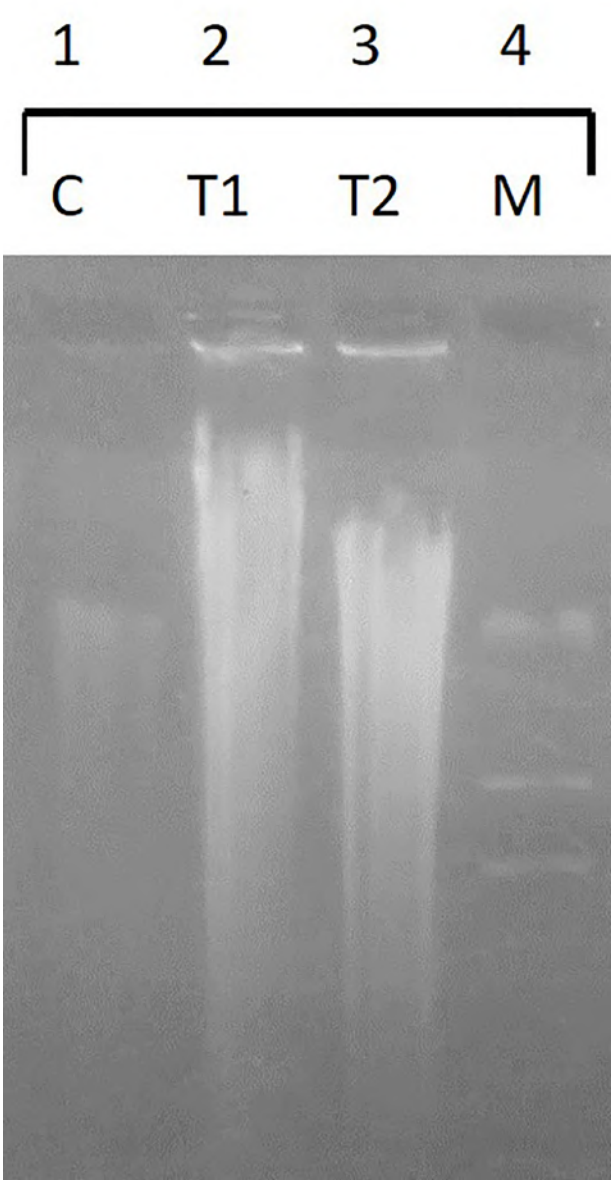


**FIG. 8:** DNA fragmentation assay using agarose gel electrophoresis. Lane 1: Positive control (C); Lane 2: T1-malvidin (5 mg/kg bw); Lane 3: T2-malvidin (10 mg/kg bw); Lane 4: DNA marker (M).

## I. Effect of Malvidin on MST and %ILS in Solid Tumor Models

The antitumor effect of malvidin was confirmed by examining the mortality of the animals due to tumor burden using MST and %ILS. It was calculated using the following equation: MST = (Day of first death + Day of last death)/2; %ILS = [(T – C)/C] × 100, where, T = no. of days the treated animals survived; C = no. of days the control animals survived. Administration of malvidin significantly prolonged the life spans of DLA-bearing tumor mice. The MST of treated mice was found to be 34 ± 1.46 and 35 ± 1.17 at both concentrations, compared to that of untreated DLA tumor groups at 23 ± 2.89, with an average increase in life span (of 30.57%), as shown in Table 1.

**TABLE 1:** Effect of malvidin on MST and %ILS during DLA-induced ascitic tumor progression

| S. No. | Treatment design | MST (in days) | %ILS |
|---|---|---|---|
| 1 | Normal | > 52 | — |
| 2 | DLA control | 23 ± 2.89 | — |
| 3 | CTX (25 mg/kg bw) | 36 ± 1.01 | 56.52 |
| 4 | Malvidin (5 mg/kg bw) | 34 ± 1.46** | 30.98 |
| 5 | Malvidin (10 mg/kg bw) | 35 ± 1.17** | 38.98 |

Values are expressed as mean ± SD (n = 6/group). p values significantly differ from the tumor control (i.e., non-drug-treated) group (**$p < 0.01$).

### J. Effect of Malvidin on Immunohistochemical Analysis of Tumor Tissue in Solid Tumor Models

The immunohistochemical staining of iNOS protein expression in the tumor tissue obtained from the muscular region of tumor-bearing mice showed positive expression, which was indicated via brown-color-stained tissues, along with infiltration of the neoplastic cells. However, treatment with malvidin showed mitigated expression and mild infiltration of the neoplastic cells with less brown-staining immunoreactive cells at both dosage levels of 5 mg/kg bw and 10 mg/kg bw. Correspondingly, the administration of CTX also revealed diminished levels of iNOS expression, as represented in Fig. 9.

## IV. DISCUSSION

Though we now have a wide number of anticancer agents, there is still no promising agent with enhanced efficacy to combat the dreadful disease. Hence there is an ongoing demand to develop novel active anticancer agents. Several anticancer agents derived from plants and plant-based products play an imperative role in protection against cancer, with good antioxidant potential and less or minimal side effects for host cells.[17] Although chemotherapy is an effective method to treat cancer, it is associated with a wide gamut of adverse effects, including cytotoxicity to normal cells (especially in the bone marrow cells), epithelial tissues, and the reticuloendothelial system.[18] Anthocyanin has been reported to possess effective antioxidant potential,[19,20] and it also prevents mutation by DNA damage.[21] Malvidin is a widely abundant anthocyanidin that holds strong anti-proliferative properties and can assist with carcinogenesis-mediating biological functions related to the inhibition of tumor progression. Malvidin showed potent cytotoxic effects in the DLA-induced solid tumor model and also showed promising effects in all parameters investigated in this present study. The administration of malvidin at concentrations of 5 mg/kg bw and 10 mg/kg bw showed an increase in average survival time and reduction in the percentage of body weight gained due to ascitic cells in both solid and ascitic tumor models. Similarly, hematological parameters were also monitored. The hematological parameters were significantly altered after treatment compared with the DLA control group. The total WBC count was increased in the DLA control; this condition may be due to severe infection or leukemia, which is secondary to malignancies. The decreased hemoglobin levels in the DLA control indicates the most common form of anemic condition. At the end of the study, the body weight and hematological parameters of the DLA groups were normalized, close to the values obtained for the normal group (untreated).

The present study attempted to verify if malvidin-mediated anticancer efficacy is locally (i.e., cytotoxic to DLA tumor cells) or systemically (i.e., where the solid tumor experimental model is employed) effective. The results showed an effective reduction in solid tumor levels induced by DLA cells in the malvidin-treated group at two different doses; there were significant reductions in tumor volume and body weight, which nearly resembled the standard group and contrasted with the tumor-bearing groups. This indicates that the effect of malvidin is systemic. The inhibitory effects of malvidin on

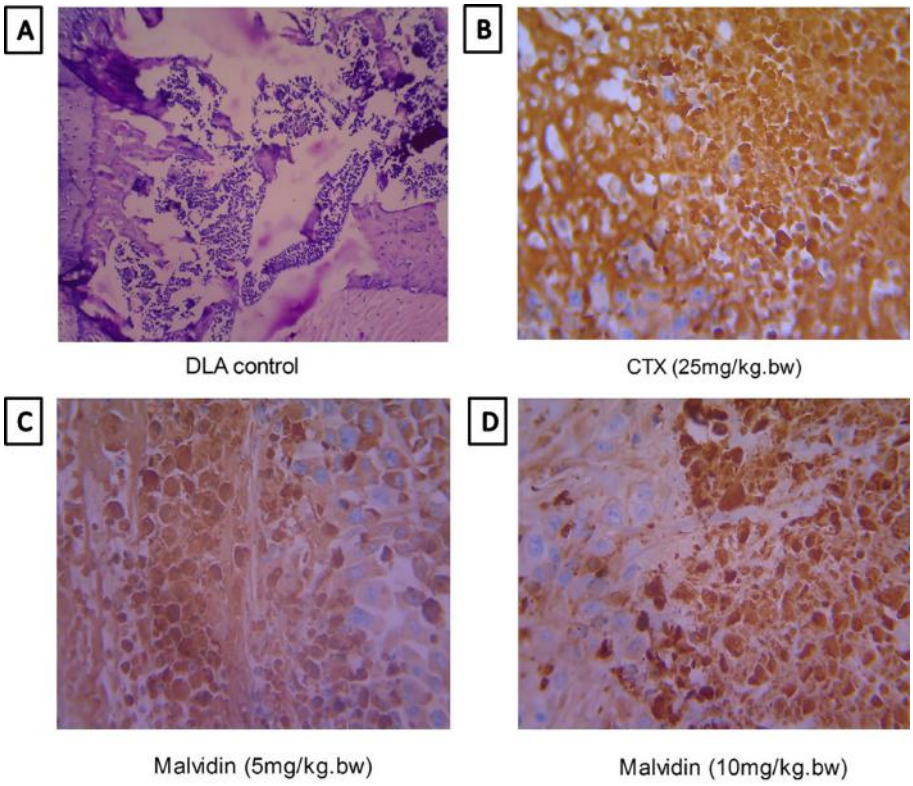


**FIG. 9:** Immunohistochemical analysis of DLA-induced solid tumor tissues treated with malvidin (magnification 40×), showing (A) DLA control (25 mg/kg bw), (B) CTX (25 mg/kg bw), (C) malvidin (5 mg/kg bw), and (D) malvidin (5 mg/kg bw)

tumor volume, body weight, and the hematopoietic system were similar to the results observed for the standard drug CTX. Similar results were also reported in earlier studies.[16,22]

DLA inoculation in mice significantly increased levels of serum AST and serum ALT. Abnormal levels of transaminases (AST and ALT) and increased levels of ALP, in a condition called *hyperphosphatasemia*, have also been reported with infiltrative liver disease.[16] The results of the present study suggested that DLA-induced tumors are characterized by many alterations and elevation of ALT and AST of mice blood sera; this may be due to the destruction of tissues and tumor growth response with infectious conditions. This condition usually represents hepatocellular injury. Abnormal levels of ALP in the blood also indicate a necrosis condition related to the liver. Increased levels of ALT, AST, and ALP are symptomatic of decreased liver function due to tumor progression and loss of integrity in cell membranes.[23] The previously mentioned effect was observed in the DLA-induced ascitic as well as solid tumor models. The administration standard drug CTX and malvidin separately diminished these elevated levels and reversed them to almost normal.

GGT, which has a direct role in GSH metabolism, is pointedly increased under tumor-bearing conditions, and it plays a major role in the proliferation of cells. The observed results clearly showed that malvidin treatment reduced increased GGT levels triggered by the DLA cells. This finding has been correlated with the role of malvidin in decreasing the hepatotoxic effect and mitigating the elevated level of GGT during an oxidative condition induced by DLA cells.[24]

Mice induced with DLA exhibited marked DNA fragmentation. The compound malvidin had

an inhibitory effect on DNA synthesis in aspirated DLA cells. Highly reactive molecules such as generated free radicals could damage the DNA through the formation of carcinogenic nitrosamines and the inhibition of the DNA damage repair system. Base repair in DNA damage is an inevitable phenomenon, but this process of extensive DNA damage involves complex molecular regulation in mammalian cells.[25] These findings were in accordance with the results we observed in the fragmentation assay.

NO synthases are a family of enzymes, among which iNOS is the main enzyme that catalyzes the synthesis of important cellular signaling molecule NO from L-arginine. NO seems to play a part in various stages of carcinogenesis, from initiation to progression, which is synthesized by iNOS. An increase in iNOS-mediated NO release in the tumor environment exerts harmful effects during DLA solid tumor progression. Our investigation reported that the NO level was increased in DLA-alone induced groups and that it was significantly decreased in malvidin-treated groups at both concentrations. Overall, the effect of NO transport depends on the expression level of iNOS.[26] These dynamics of iNOS expression were confirmed by immunohistochemical analysis. Likewise, histopathological alterations were also observed in DLA treated groups, which were reversed after the administration of standard CTX and malvidin.

Prolonged chronic inflammation leads to different forms of cancer. The inflammation related to cancer is mediated mainly by the pro-cytokines TNF-α and IL-6, which play a vital role in tumor development. IL-6 functions as a key component of the NF-κB/STAT-3 signaling pathway involved in tumor development.[27] Both cytokine levels were elevated during tumor progression in the DLA-alone induced tumor group. TNF and IL-6 fall under the well-established category of pro-tumorigenic cytokines, due to their activation of multiple signaling pathways. Specifically in cancer, these cytokines are involved in the activation of oncogenic transcription factors, including AP-1 (TNF), NF-κB, and STAT3 (IL-6), in epithelial cells.[28–30] It is well known that the stimulation of IL-6 causes tumor cells to proliferate and promotes their survival by activating the Ras/Raf/MEK/MAPK, PI3K/AKT, and JAK/STAT pathways via gp130 tyrosine phosphorylation.[31] Modulating the effects of CTX on host antioxidant and selective cytokine levels highlights the ability of malvidin to help alleviate/reverse the host-induced toxicity and immunotoxicity induced by CTX.[16] The enhanced level of both cytokines holds suppressive activity of tumor growth. The concomitant effect of malvidin in the present study, which was shown to significantly decrease the levels of pro-inflammatory cytokines TNF-α and IL-6 when compared to the tumor-alone-bearing animals, supports the aforementioned conclusion. Thus, both cytokines could serve as a molecular target for cancer prevention. Another possible mechanism in the treatment of cancer is the TNF-α signaling pathway (complex 2) that activates an ROS which induces necrotic cell death.[27] Decreased levels of TNF-α and ROS (like NO) at the end of the study reveal the reason for the decrease in tumor growth.

According to researchers, malvidin, an active constituent of blueberries, has the ability to target STAT-3, which ultimately inhibits the JAK/STAT-3 pathway and thereby modulates downstream targets that influence cell proliferation and apoptosis. Earlier reports show that blueberry and malvidin act as STAT-3 inhibitors in SCC131 in specific oral cancer cell lines.[12] The cytotoxic effect of malvidin has also been reported in human monocytic leukemia cells and HT-29 colon cancer cells, and they act by arresting the G(2)/M phase of cell cycle and apoptosis induction.[32,33]

## V. CONCLUSION

In conclusion, this study reveals that treatment with malvidin exhibited strong inhibitory effects on tumor progression in DLA ascitic mice models. The possible mechanism by which malvidin significantly inhibited tumor progression may be via the regulation of oxidative stress, with antioxidants balancing and suppressing inflammatory mediators. Therefore, the drug candidate malvidin could act as a potential antitumor agent and can be used in the treatment of cancer. The detailed mechanism of action is currently under investigation. Further studies will concentrate on using malvidin to investigate the expression and activation of multiple genes associated

with P13K/Akt, ERK, JNK, and MAPK pathways, which are key mechanisms of cancer progression.

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