

# The Beneficial Use of Melanin in Inhibiting and Treating HCC Through Preventing CBC, Liver Enzymes, Oxidative Stress, and Lipid Peroxidation Alterations

Mohamed Anwar K Abdelhalim (✉ [abdelhalimmak@yahoo.com](mailto:abdelhalimmak@yahoo.com))

King Saud University <https://orcid.org/0000-0002-3957-9078>

Yanallah H Al-Mohy

King Saud University

Mohammed S Al-Ayed

King Saud University

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## Short Communication

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# Abstract

Hepatocellular carcinoma (HCC) accounts for more than 90% of primary liver cancers and is a major global health problem. The present application relates to normalizing abnormal levels of hematological blood parameters, oxidative stress indicators, and liver enzyme levels in the rats using melanin, and particularly, to treating their abnormal levels, in addition to evaluate the role of melanin during and after the progression of HCC, which will be supplemented histologically. Hematological blood parameters such as white blood cells (WBCs), red blood cells (RBCs), hemoglobin (HGB), hematocrit (HCT), and platelets (PLTs); liver enzymes such as alanine transaminase (ALT) and aspartate transaminase (AST); oxidative stress such as glutathione peroxidase (GSH-PX) and superoxide dismutase (SOD); LP malondialdehyde (MDA) were evaluated in all groups of rats. Injection of Diethylnitrosamine (DEN) and 2-Acetyaminofluorine (2-AFF) induced HCC in the rats accompanied with a significant reduction in WBCs, RBCs, HGB, HCT, and PLTs, GSH-PX, and SOD; while a significant elevation was observed in ALT and AST, and MDA compared to the control. Melanin normalized all the above mentioned parameters during and after the progression of cancer towards their control values. These results demonstrate the beneficial use of melanin as a powerful antioxidant tool in inhibiting and treating HCC in the rats.

## Introduction

HCC is the most common cancer-related death worldwide. Contemporary methods of cancer research have been influenced by data that reveal the importance of inflammatory components in the microbial environment. HCC is affected by factors such as DEN, 2-AAF, phenobarbital (PB), alcohol, and viruses (B and C) [1–2]. Clinical observations indicated a possible relationship between the bloodstream and malignancy in cancer. Therefore, clarifying tissue perfusion of organs or cancerous tissue is necessary to predict the growth of cancer and malignant tumor in tumor-carrying animals or in human cancer patients.

Unlike other tumors caused by the liver and digestive system, the cause of HCC is still sophisticated and mysterious, making the disease more complex and challenging. Cancer is a disease that affects the metabolism of the entire body rather than targeting the organs.

Animal models are critical tools for studying HCC. Due to the physiological and genetic similarities between rodents and humans, short age, reproductive ability and a variety of manipulation methods, animal models are often used for cancer research [3].

Several experimental animal models of HCC have been described over the past decades. Studies on the induction of liver cancer in rats use chemical agents such as DEN and 2-AAF [4]. 2-AAF shows its carcinogenic effect through the formation of DNA adducts, on the production of reactive oxygen species (ROS) and oxidized DNA damage [5]. DEN is a nitrosamine compound that stimulates the formation of liver cancer. DEN increased lipid peroxidation in studies conducted. This may increase the tumor [6–7]. The most acceptable suggested by Farber et al. [8], combines chemical induction by DEN with partial

hepatic resection. Since then, DEN has been used to initiate liver cancer either alone or in conjunction with other cancer pathogens [9–11].

Advances in clinical and surgical treatments for cancer patients have increased overall survival rates. However, the growth of primary tumors and subsequent cancer tumors continue to cause deaths and prevent effective treatment [12, 13].

Clinical observations indicated a possible relationship between the bloodstream and malignancy in cancer [14, 15]. Therefore, clarifying tissue perfusion of organs or cancerous tissue is necessary to predict the growth of cancer and malignant tumor in tumor-carrying animals or in human cancer patients.

Unlike other tumors caused by the liver and digestive system, the cause of HCC is still sophisticated and mysterious, making the disease more complex and challenging [16]. Cancer is a disease that affects the metabolism of the entire body rather than targeting the organs [17].

Liver function, which indicates the degree of liver damage, partially reflects the condition of the tumor biology. In clinical practice, patients with degraded liver function often had an unsatisfactory diagnosis compared to patients with acceptable criteria.

Few studies have associated the temporal development of oxidative stress (OS) with GSH-Px and SOD, and LP, such as MDA, and damage of cells involved in HCC formation. Understanding the changes that occur from pre-tumor lesions to cancer lesions in OS, LP, and liver inflammation and fibrosis can be important to improve knowledge about the transmission of chronic liver disease to HCC [9–11].

More recently, melanin has been extracted from various sources, and the immune role of melanin has been reported in plants and skin [18]. Many diseases are associated with an increase or decrease in melanin production. Melanin can be found in various organs, tissues and in the blood of living organisms. Melanin's ability to act as free-source feed has been demonstrated in vitro and in vivo [19].

The ability of melanin to act as an antioxidant has been proven to inhibit fat oxidation [20]. Melanin pigments are produced by melanocytes and are believed to act as antioxidants based on the belief that melanin can inhibit the electron-induced states and scavenge the free radicals [19, 20].

The present application relates to normalizing abnormal levels of hematological blood parameters, oxidative stress indicators, and liver enzyme levels in the patient using melanin, and particularly, to treating their abnormal levels caused by hepatocellular carcinoma.

It has become apparent that inflammation has a dual effect on the development of cancer. Thus, a method of normalizing hematological blood parameters and liver enzyme levels solving the aforementioned problems is desired. This study aims to generate HCC in rats, then to evaluate the total CBC, liver enzymes, OS, and LP as well as to evaluate the role of melanin during and after the development of HCC which will be confirmed histologically.

# Materials And Methods

## Animals

Forty Wistar-Kyoto male ( $120 \pm 10$  g, 4–6 weeks) Obtained from the Central Animal House at the college of Pharmacy, King Saud University, Riyadh. They were housed in plastic (polypropylene) cages in groups of five rats per cage and kept in a room maintained at  $25 \pm 2$  °C with a 12-h light/dark cycle. By following the guiding standard for animal care and handling by KSU Local Animal Care and Use Committee Consideration. All experimental protocols were approved by KSU and/or licensing committee.

## Chemicals and reagents

DEN and 2-AAF were purchased from Sigma Chemical Company, USA. All other chemicals and reagents were of the analytical grade were purchased from R&D Systems Company, USA and Thermo Fisher Scientific Company, USA.

## Experimental protocols

Rats were randomly divided into four groups of animals. The experimental design and treatment protocol were as follows: **Group one (G1, n = 10)** normal control animals were orally administered saline. **Group two (G2, n = 20)** injected i.p. with a single dose of 200 mg/kg body weight of diethylnitrosamine (DEN). After 1 week of recovery, the DEN-treated animals were divided into two groups. **Group three (G3, n = 10)** were given repeated doses (150 mg/kg body weight) of 2-acetyaminofluorine (2-AAF) combined with 1% carboxymethyl cellulose as a promoting agent every three days for one month in combination with a melanin dose of 150 mg/kg body weight orally every two days for one month. **Group four (G4, n = 10)** were only given repeated 2-AAF doses of 150 mg/kg body weight every three days for one month, After this period repeated melanin doses of 150 mg/kg body weight were given orally every two days for other one month.

## Preparation of tissues

At the end of experimental periods, rats were anesthetized by inhalation of the ether, and blood was collected from the dorsal aorta. The serum was separated by centrifugation at  $1200 \times g$  for 10 minutes and stored at  $-80$  °C prior to analysis. Their livers were immediately removed and divided into two parts, one in formalin 10% and the other under  $-80$  °C for analysis.

## Complete blood count (CBC)

Blood samples were collected in EDTA tubes, and hematological blood parameters such as white blood cells (WBCs), red blood cells (RBCs), hemoglobin (HGB), hematocrit (HCT), and platelets (PLTs) were evaluated using Full Blood Analysis CBC Genius KT-6400 Hematology Analyzer Device at King Khalid University Hospital, King Saud University, Riyadh.

## Biochemical estimation

Examination of liver enzymes such as alanine transaminase (ALT) and aspartate transaminase (AST) were prepared using ELISA Gen 5 microplate reader. Examination of OS such as GSH-PX and SOD, and liver tissue LP (MDA) by liver tissue was prepared using ELISA Gen 5 microplate reader with GSH-PX Elisa kit, SOD Elisa and MDA in college of Pharmacy, King Saud University, Riyadh.

## Histological examinations

For histological examination, hepatic sections of the liver were stained from different groups with hematoxylin and eosin (H & E). Briefly, at the end of the experiment, rats were anesthetized by the ether and transcidentally perfused with saline. The liver was quickly removed and fixed in formalin buffered (10%) for 24 hours. After fixation is complete, slices (3–4 mm) of these tissues were dehydrated and embedded in paraffin. At least four cross sections of each tissue were taken with a thickness of 5 microns and stained with H & E. After two washing with xylene (2 min each), the tissue sections were fitted with a DPX mountant. The slides were observed for pathological changes. Microscopic images were taken using the Olympus BX50 microscope system (Olympus, Japan) at King Khalid University Hospital, King Saud University, Saudi Arabia.

## Results

WBCs significantly ( $*p < 0.05$ ) reduced to  $11.03 \pm 0.18$  ( $10^3$ cells/ $\mu$ L) in **G2** rats compared to  $14.17 \pm 0.2$  ( $10^3$ cells/ $\mu$ L) in control rats (**G1**). While WBCs in **G3** and **G4** rats significantly increased to  $12.64 \pm 0.25$ ,  $12.92 \pm 0.71$  ( $10^3$ cells/ $\mu$ L) respectively compared to **G2** rats, but with no significant effect with **G1** rats (**Table 1**).

RBCs significantly ( $*p < 0.05$ ) reduced to  $7.12 \pm 0.17$  ( $10^6$ cells/ $\mu$ L) in **G2** rats compared to  $8.43 \pm 0.079$  ( $10^6$ cells/ $\mu$ L) in control rats **G1**. While RBCs in **G3** and **G4** rats significantly increase to  $8.17 \pm 0.12$ ,  $7.99 \pm 0.12$  ( $10^6$ cells/ $\mu$ L) respectively compared with the **G2** rats, but with no significant effect with **G1** rats (**Table 1**).

**Table.1** represents CBC in four groups of the rats

Complete blood count (CBC)	Mean ± SE n = 40			
White Blood Cells (WBC) (10 <sup>3</sup> cells/μL)	14.17 ± 0.2	11.03 ± 0.18*	12.64 ± 0.25* <sup>a</sup>	12.92 ± 0.71* <sup>a</sup>
Red Blood Cells (RBC) (10 <sup>6</sup> cells/μL)	8.43 ± 0.079	7.12 ± 0.17*	8.17 ± 0.12* <sup>a</sup>	7.99 ± 0.12* <sup>a</sup>
Hemoglobin (HGB) (g/dL)	154.2 ± 1.5	134 ± 2.50*	151.87 ± 1.16* <sup>a</sup>	149.03 ± 2.53* <sup>a</sup>
Hematocrit (HCT) (%)	46.44 ± 1.14	41.46 ± 0.65*	44 ± 0.82* <sup>a</sup>	45.69 ± 1.52* <sup>a</sup>
Platelet count (PLTs) (10 <sup>3</sup> cells/μL)	1127.33 ± 20.5	760 ± 26.39*	1079.33 ± 15.5* <sup>a</sup>	994.67 ± 20.34* <sup>a</sup>

HGB significantly (\*p<0.05) reduced to 134 ± 2.5 (g/dL) in **G2** rats compared to 154.2 ± 1.5 (g/dL) in control rats **G1**. While HGB in **G3** and **G4** rats significantly increase to 151.87 ± 1, 149.03 ± 2.53 (g/dL) respectively compared with the **G2** rats, but with no significant effect with **G1** rats (**Table 1**).

HCT (\*p<0.05) significantly (\*p<0.05) reduced to 41.46 ± 0.65 (%) in **G2** rats compared to 46.44 ± 1.14(%) in control rats **G1**. While HCT in **G3** and **G4** rats significantly increase to 44 ± 0.82, 45.69 ± 1.52 (%) respectively compared with the **G2** rats, but with no significant effect with **G1** rats (**Table 1**).

PLTs significantly (\*p<0.05) reduced to 760 ± 26.39 (10<sup>3</sup>cells/μL) in **G2** rats compared to 1127.33 ± 20.5 (10<sup>3</sup>cells/μL) in control rats **G1**. While PLTs in **G3** and **G4** rats significantly increase to 1079.33 ± 15.5, 994.67 ± 20.34 (10<sup>3</sup>cells/μL) respectively compared with the **G2** rats, but with no significant effect with **G1** rats (**Table 1**).

**Fig.1** ALT significantly (\*p<0.05) increased to 56.31±3.2 (U/mg) protein in **G2** rats compared to 25.23±1.8 (U/mg) protein in control rats **G1**. While ALT in **G3** and **G4** rats significantly reduced to 43.81±3.1, 50.02±3.05 (U/mg) protein respectively compared with the **G2** rats, but with no significant (\*ap<0.05)effect with **G1** rats.

**Fig.2** AST significantly (\*p<0.05) increased to 4.04±0.27 (U/mg) protein in **G2** rats compared to rats 1.52±0.2 (U/mg) protein in control rats **G1**. While AST in **G3** and **G4** rats significantly reduced to 1.93±0.23, 2.83±0.25 (U/mg) protein respectively compared with the **G2** rats, but with no significant (\*ap<0.05)effect with **G1** rats.

**Fig.3** GSH-PX significantly (\*p<0.05) reduced to 5.2±0.55 (U/mg) protein in **G2** rats compared to 8.54±0.49 (U/mg) protein in control rats **G1**. While GSH-PX in **G3** and **G4** rats significantly increase to 7.75±0.48, 8.2±0.54 (U/mg) protein respectively compared with the **G2** rats, but with no significant (\*ap<0.05) effect with **G1** rats.

**Fig.4** SOD significantly ( $*p \leq 0.05$ ) reduced to  $2.63 \pm 0.082$  (U/mg) protein in **G2** rats compared to  $3.49 \pm 0.054$  (U/mg protein) in control rats **G1**. While SOD in **G3** and **G4** rats significantly increase to  $3.32 \pm 0.105$ ,  $3.23 \pm 0.075$  (U/mg) protein respectively compared with the **G2** rats, but with no significant ( $*p \leq 0.05$ ) effect with **G1** rats.

**Fig.5** MDA significantly ( $*p \leq 0.05$ ) increased to  $0.639 \pm 0.021$  (U/mg) protein in **G2** rats compared to  $0.19 \pm 0.015$  (U/mg) protein) in control rats **G1**. While MDA in **G3** and **G4** rats significantly reduced to  $0.543 \pm 0.032$ ,  $0.442 \pm 0.041$  (U/mg) protein respectively compared with the **G2** rats, but with no significant ( $*p \leq 0.05$ ) effect with **G1** rats.

**Fig.6** shows the change in the liver sections stained by H&E. (A) section of the liver obtained from the control group. Central vein with surrounding normal hepatocytes was observed (H/E stain X 400). (B) Section of liver obtained from group 2. Note the emergence of a new population of atypical hepatocytes showing large nuclei (arrowhead) and coarse chromatin: H/E stain 600. (C) Section of liver obtained from group 3. Note the presence of some residual atypical hepatocytes indicative of partial improvement. Arrowhead is pointing towards a residual enlarged hepatocyte with abnormal nucleus (H/E stain X 600). (D) Section of liver obtained from **G4**. High power microscopic view showing many normal hepatocytes showing post mortem changes with one residual atypical hepatocytes showing a mitotic figure indicative of regeneration (arrowhead). The features are consistent with partial improvement with some compensatory regeneration (H/E stain X 600).

## Discussions

In this study, injection of DEN and 2-AFF induced HCC in the rats. In these treated DEN + 2-AFF rats, a significant reduction in hematological parameters such as WBCs, RBCs, HGB, HCT, and PLTs was observed.

The number of WBCs has been reported to be more frequent in cancer patients with malignant tumors [21]. WBCs are produced by bone marrow and high WBCs indicate trauma, allergies, and leukemia or inflammation [22]. Other blood factors such as RBCs, HGB, HCT need further tests and studies to learn more about their relationship to cancer in general and liver cancer specifically.

PLTs are the primary mediators of pathological thrombosis [23]. Previous empirical evidence has shown that PLTs support tumor malignancy [24]. Platelet activation and thrombocytopenia have been shown to play a crucial role in the development of cancer. PLTs contribute to the survival of malignant tumor cells, suggesting that it may provide a new therapeutic way to treat metastatic cancer [25].

To evaluate importance of liver enzymes on the progression of cancer, we measured ALT and AST. Our study indicated significant elevation in ALT and AST compared to the control. Studies have shown that expressions of abnormal liver enzymes often lead to a bad diagnosis in many cancers. ALT and AST have been reported to influence the diagnosis in some cancers [26, 27].

There has been a significant increase in liver enzyme activity in the blood, especially when liver cell damage leads to liver function abnormalities. These enzymes (AST, ALT, and ALP) have been shown to exhibit higher activity in the abnormally functioning liver, demonstrating their presence as an indicator of liver function recovery in patients with liver transplantation [28]. A significant increase in AST, ALT and ALP serum levels was also reported after liver tumors were induced by CCl<sub>4</sub> in mice [29]. While DEN has cancer, it is also a potent analgesic and shows toxicity.

Oxidative stress is a major factor during carcinogenesis [30]. Fat peroxide is one of the freest biologically studied free radicals series. It attacks the radical fatty acids or asyl fatty side chain of any chemical type which has sufficient interactivity to extract hydrogen atom from methylene carbon side chain. It may lead produce many toxic products such as MDA and 4-hydroxynonenal, which can attack DNA, stimulating cancer-causing mutations [30].

In our study, the hepatic GSH-PX and SOD levels were significantly reduced indicating an elevation in OS, while the MDA levels were significantly increased. Our data were in consistent with those reported [31]. Glutathione effectively wipe up all kinds of poisons and free radicals. Earlier results have elucidated the regulatory influence in GSH level in animals injected with natural antioxidants post ZnO-NPs administration. MDA is the main produce of fatty acid peroxidation, it has been confirmed to function as carcinogen, and it causes DNA damage by producing alterations in neoxyguanosine, deoxyadenosine and deoxycytidine, and leading to apoptosis [32].

The used melanin plays an effective hepatoprotective role against CBC count, AST, ALT, GSH-PX, SOD, and MDA alterations caused by DEN and 2-AAF toxicity. Co-administration of melanin to treated DEN and 2-AAF rats successfully and significantly returned the above mentioned to near their control values.

Our results were in consistent with those reported previously, in which melanin has many attractive properties, such as being an oxidizing agent in addition to its activities in removing free radicals [18, 33]. Previous studies of melanin have revealed that melanin combines with oxygen-containing substances such as oxygen [34], radical hydroxyl, and negatively charged superoxide ion [33]. Melanin pigments can scavenge the free radicals [19, 20].

To completely confirm and insure the HCC, additional experiments related to DNA damage and ROS might be performed.

## **Conclusions**

To generate cancer in the rats, the rats were injected with a single dose of DEN followed by repeated doses of 2-AAF for one month. According to an embodiment, melanin can be administered to a patient to normalize abnormal levels of hematological blood parameters, oxidative stress indicators, and liver enzyme levels in the patient. In an embodiment, the patient can be suffering from hepatocellular carcinoma (HCC). Hematological blood parameters can include white blood cells (WBCs), red blood cells (RBCs), hemoglobin (HGB), hematocrit (HCT), and platelets (PLTs). Liver enzymes can include alanine

transaminase (ALT), aspartate transaminase (AST), liver glutathione peroxidase (GSH-PX), and liver superoxide dismutase (SOD). Oxidative stress parameters can include reactive oxygen species (ROS), and LP malondialdehyde (MDA). As described herein, HCC can cause a significant reduction in WBCs, RBCs, HGB, HCT, and PLTs, GSH-PX and SOD, while a significant elevation in ALT, AST, and MDA levels. The administration of melanin during and after the progression of HCC can return all of these parameters towards their normal control values.

## **Declarations**

### **Ethical Approval and Consent to participate**

All procedures concerning animal care as required for animal research of KSU were strictly followed.

### **Availability of supporting data**

Not Applicable

### **Competing interests**

The authors assert that they have no known conflict of interests.

### **Patent Number**

### **Docket No. 33032.24**

Re: Office Action in Patent Application for METHOD OF NORMALIZING BLOOD PARAMETERS, OXIDATIVE STRESS INDICATORS, AND LIVER ENZYME LEVELS USING MELANIN Serial No. 16/734,100

### **Authors' contributions**

MAKA has participated in designing the study. MAKA and YHA carried out the practical work. MAKA and YHA have analyzed the data. MAKA, YHA, and MSA have prepared the manuscript draft. Final draft of manuscript has been modified and revised by MAKA. The final draft of the manuscript has been approved by all the authors.

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### **Conflict of Interest:**

The authors of this manuscript declare that they have no conflict of interest.

### **Statement of Significance:**

These results demonstrate the beneficial use of melanin as a powerful tool in inhibiting and treating the HCC through preventing CBC, liver enzymes, oxidative stress, and lipid peroxidation alterations.

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## Figures

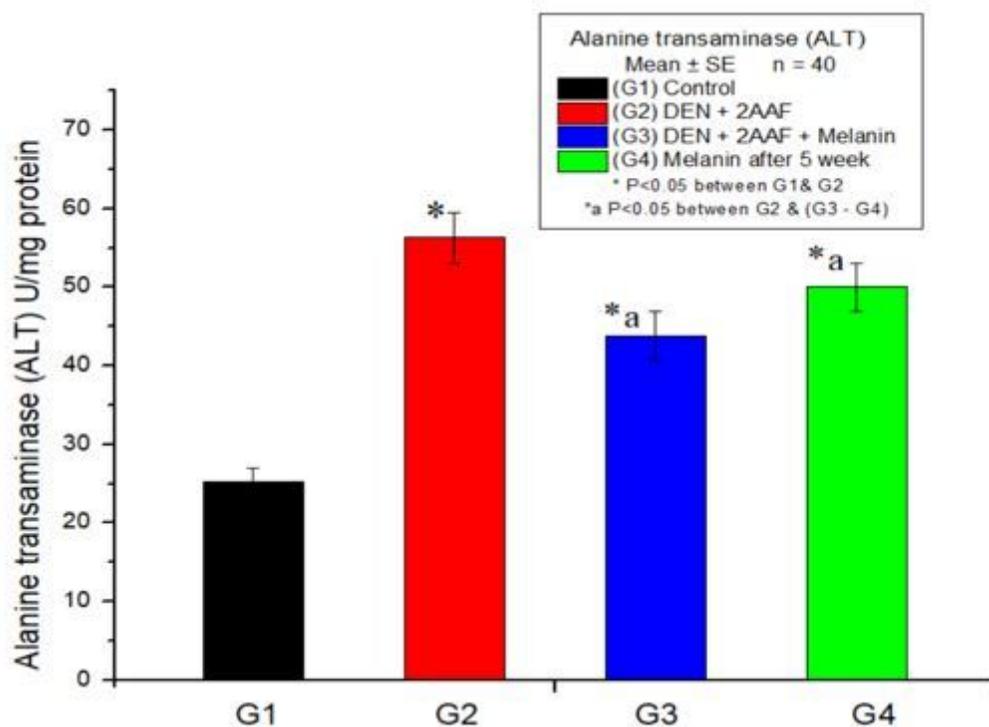


Figure 1

Change in Alanine transaminase (ALT) in four groups of the rats

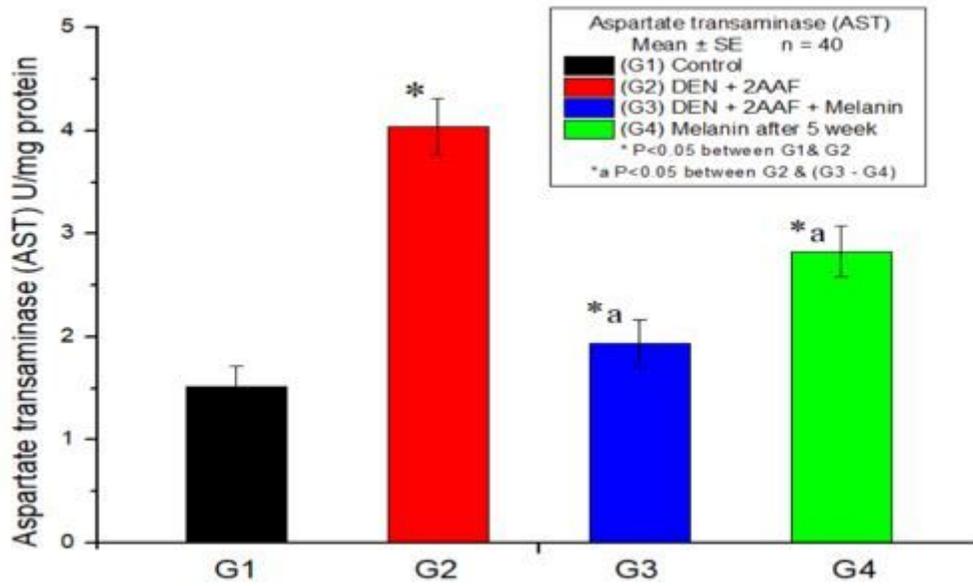


Figure 2

Change in Aspartate transaminase (AST) in four groups of the rats

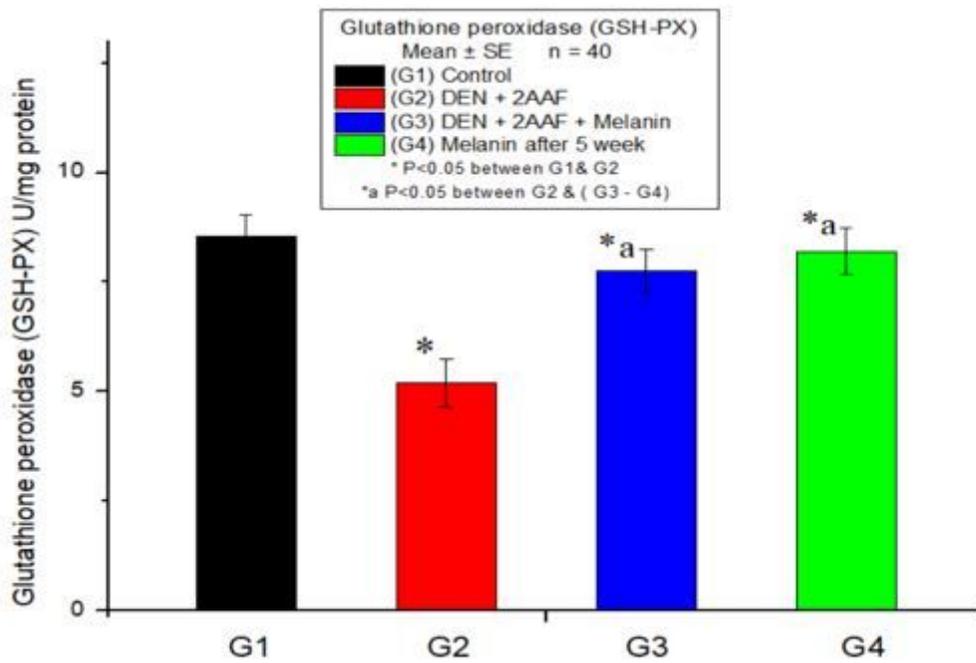


Figure 3

Change in glutathione peroxidase (GSH-PX) in four groups of the rats

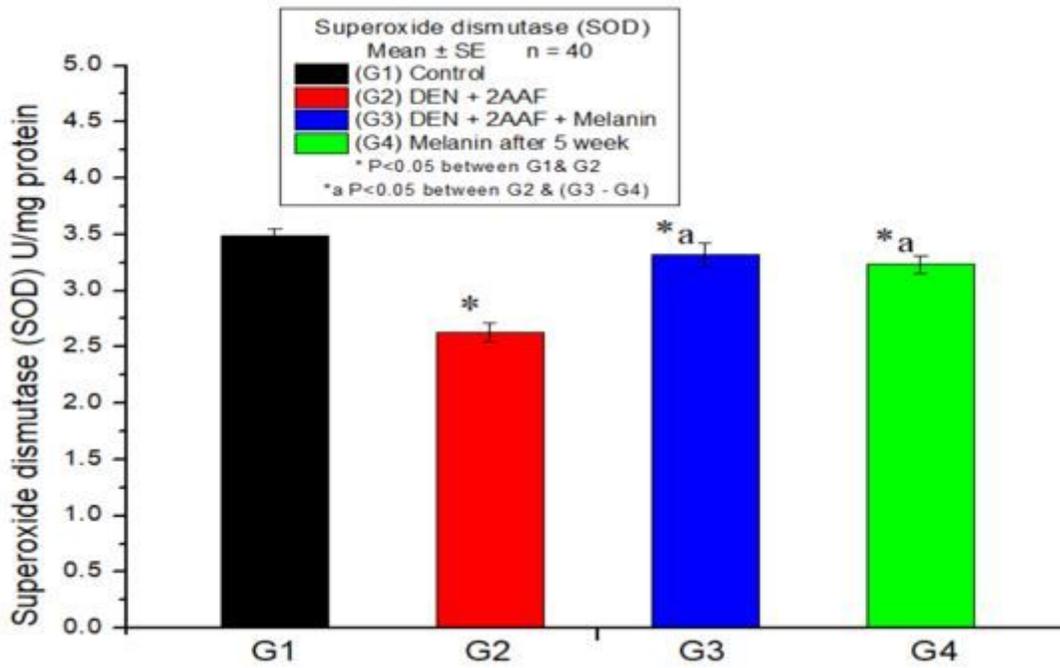


Figure 4

Change in Superoxide dismutase (SOD) in four groups of the rats

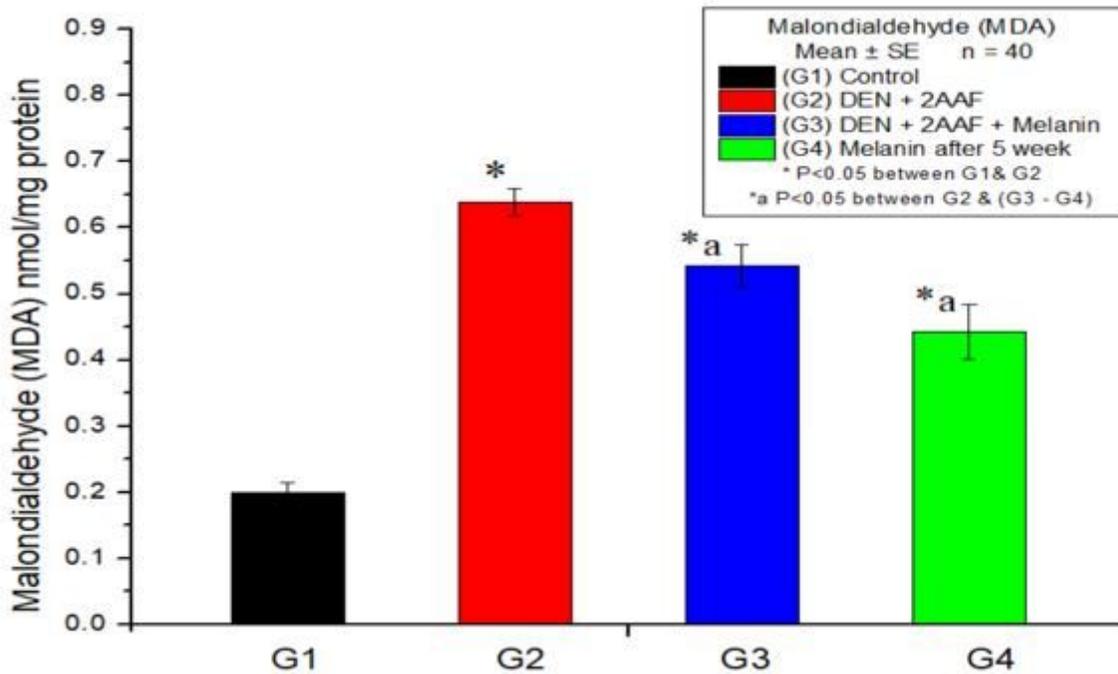
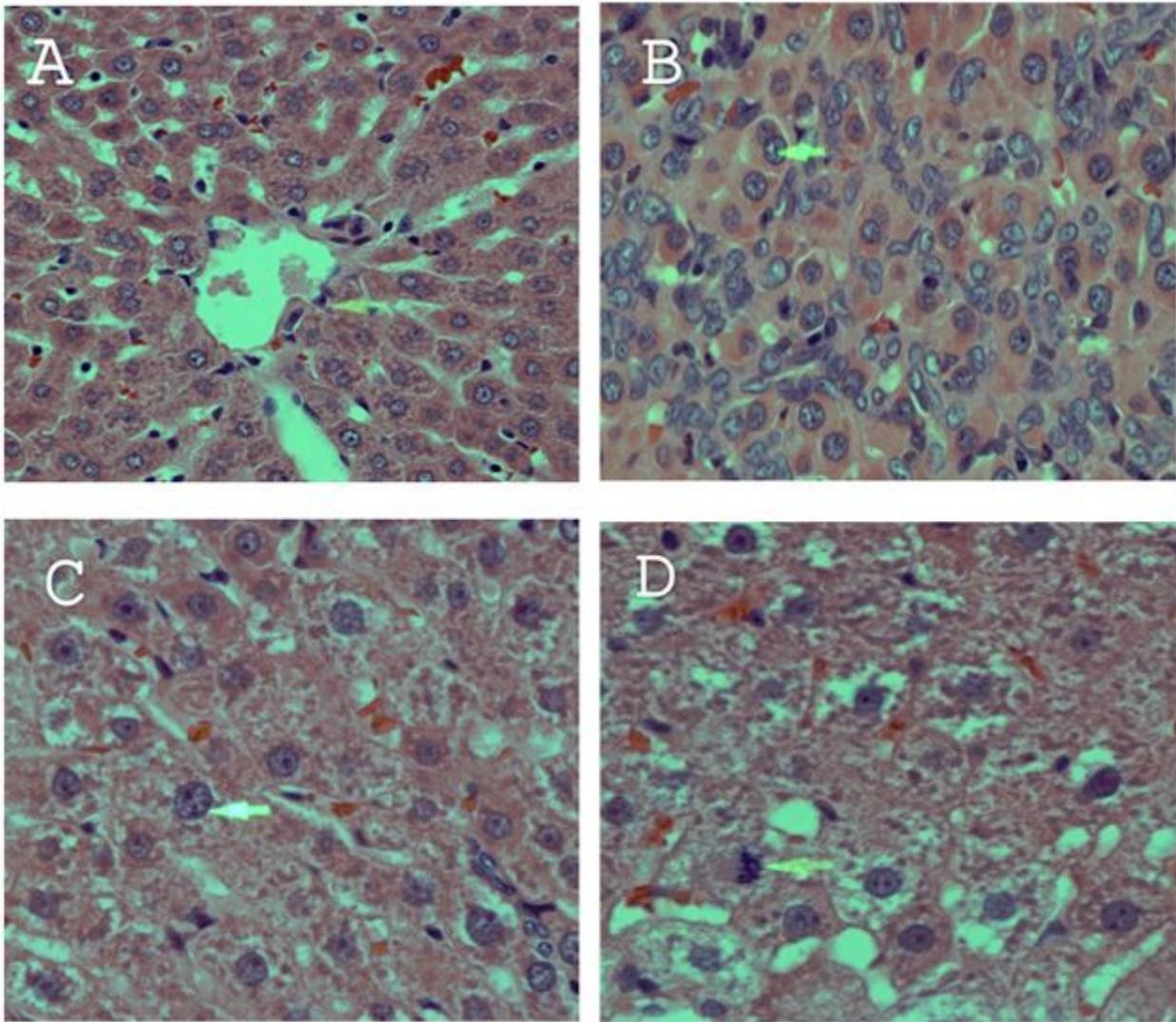


Figure 5

Change in malondialdehyde (MDA) in four groups of the rats



**Figure 6**

Photomicrographs of 400×. (A) G1: control rats. (B) G2: DEN+2AAF rats. (C) G3: DEN + 2AAF + Melanin rats. (D) G4: melanin after 5 weeks.