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The effect of Bee venom as anti-inflammatory, antioxidant and antitumor agent in mice with hepatocellular carcinoma.

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ABSTRACT

Background: Bee venom is a medicine that is frequently used Because of the bioactive compounds and its pharmacological properties. Necrosis, cytotoxicity, impacts on proliferation, induction of apoptosis, and a decrease in the growth of different cancer cell types are only a few of the effects of B.V. that have recently been observed. **A*im:*** This research aims to verify the effectiveness of bee venom in the prevention and treatment of liver cancer. **M*ethod:*** Fifty male albino mice, weighing around 20–25 g, were residing in the animal house of the Faculty of medicin, Zagazig University. mice used in the experiment were separated into 5 groups, as follows: Group (1) control group: normal mice. Group (2) positive group: HCC induction was performed utilizing carbon tetra chloride (CCL4) 2ml/kg was given IP twice weekly for two months to develop HCC. Group (3**)** Treatment group: mice treated with Bee venom (0.1mg/kg/orally) for 45 day after HCC induction. Group (4) protective group: mice took Bee venom (0.1 mg/kg /orally) daily for 45day then took carbon tetra chloride for 2 months. Group (5) Cisplatin group: mice administered cisplatin (1.5 mg/kg /i.p) after induction of HCC. **Result:** After treatment was over, the animals were sacrificed with an injection of urethane (1g/kg body weight). All the animals in the various experimental groups received their blood and liver tissues. These activities were assessed by investigating the liver enzymes ALT, AST, Alb and immunohistochemical marker AFP. Increases in ALT, AST, ALP and immunohistochemical marker AFP were observed in mice who received CCl4 only, in contrast to mice that took Bee venom after CCl4 administration and protective group with statistically significant value $p<0.05$.

C*onclusion:* The current study indicate that the Bee venom may assist in the therapy and improving the recovery of the HCC. due to its anti-inflammatory, antioxidant and anticancer effects.

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INTRODUCTION

One of the most crucial components of in our body is the liver. It facilitates proper food digestion, creates elements required for life, and gets rid of toxins from the body. The liver also serves a protective purpose by removing toxins that are produced during metabolism or ingested from the outside. If the liver quits functioning, the entire body may experience severe issues [1].

Hepatocellular carcinoma (HCC) is one of the most prevalent and lethal cancers [2]. The majority of HCC tumors develop with cirrhosis, which is typically brought on by non-alcoholic fatty liver disease (NAFLD) or infection with HBV/HCV [3]. Due to delayed diagnosis and the ineffectiveness of current medications, the prognosis is still poor Despite current therapy breakthroughs, with a 5-year survival rate of only 15% [3,4]. There are few treatment options for advanced HCC [5].

HCC is a highly malignant tumor with little resistance to chemotherapeutic treatments, and after examining the seriousness of the side effects of radiotherapy and chemotherapy and how dangerous they are to the body [6]. To improve the health outcomes of people with the HCC, innovative pharmacologic treatments must be developed. Natural products are now being used as an alternative or a supplement to treatment by scientists. By triggering the antitumor, antiproliferative, anti-inflammatory, and antioxidant systems, natural compounds, such as the venom of some species, block the mechanisms that lead to the development of cancer and stimulate the mechanisms involved in disease prevention [7].

Recently, doctors and certified apitherapies have started using honeybee venom to treat patients with autoimmune and chronic disorders. Testing in the lab and clinical trials have demonstrated how effective honeybee venom is as a biotherapy [8]. Recently, the venom has also been tested for use in the treatment of several cancer types [9]. The literature suggested that, additional to its medicinal advantages, bee venom may lessen the side effects of other types of pharmaceuticals and traditional medication [8].

According to numerous publications, bee venom contains several of active compounds, including peptides and enzymes like phospholipase A2 and hyaluronidase, in addition to non-peptide substances like histamine, dopamine, and norepinephrine. Melittin is a crucial element of bee venom which comprises roughly 50% of dry venom and is made up of 26 amino acids [8].

Studies conducted in vivo and in vitro have investigated the pharmacology of bee venom. The bee venom has several different pharmacological effects, including those that are anti-arthritic, anti-metastatic, anti-radioprotective, anti-hepatotoxic, cytoprotective, anti-oxidant, anti-microbial, anti-viral, anti-inflammatory, and anti-tumor properties [10,11].

Bee venom is a mixed-up collection of peptides, enzymes, and other bioactive components [12]. Various human ailments have traditionally been treated with bee venom therapy [13].

In this study, we provide a viewpoint on the future of advanced HCC therapy by presenting a summary of therapeutic effect of bee venom that has shown effectiveness in mice trials.

MATERIAL AND METHODS

Bee venom:

The Carniolan bee venom specimen (Apis mellifera carnica) was purchased from the Agriculture Research Centre department of the Beekeeping Research of the Plant Protection

Research Institute of in Doki, Giza governorate, Egypt.

Honeybee venom is a clear liquid that has a 4.5–5.5 pH range, a 1.13 specific gravity, and a harsh flavor. The honeybee venom soon dries up and crystalline when it comes into touch with the air [14]. Due to the oxidation of some venom proteins, dried venom turns a light-yellow tint and some commercial preparations have a brown appearance. Alcohol and ammonium sulphate are insoluble; however, it is soluble in water. There are several highly volatile chemicals in bee venom that are easily lost during collection [15].

Chemicals and KITS:

Basal diets, analytical grade carbon tetrachloride (CCl_4), were sourced from Sigma Chem. Co., (St. Louis, U.S.A), whereas Cisplatin was purchased from (Sigma Aldrich). Chemical kits for ALT, AST and ALP were from Sigma Aldrich.

Animals:

Male albino mice weighing 20–25 g were kept in the faculty of medicine's animal house at Zagazig University. Mice were housed in a lab environment with a 20–25°C temperature range, a 60–65°F humidity range, and a 12-hour cycle of light and dark. Mice were fed a regular chow diet and had unlimited access to tap water.

Ethical approval:

The Ethical Committee of Zagazig University approved the experimental design and animal care (Approval number ZU-IAUUC/1/F/25/2020).

Experimental design:

The mice acclimatized for one week, then they were grouped into 5 groups (n = 10). control group: normal mice, positive group: We used carbon tetra chloride (CCL4) to induce HCC. I.P. dosage of 2ml/kg was given twice weekly. for three months to progress HCC [16], Treatment group: mice treated with Bee venom (0.1mg/kg/orally) for 45 day [17] after induction of HCC, protective group: mice took Bee venom (0.1 mg/kg /orally) daily for 45day then took carbon tetra chloride for 2 months and Cisplatin group mice administred cisplatin (1.5 mg/kg /i.p) after induction of HCC [18].

Samples collection:

After treatment was over, the animals were sacrificed using urethane injections. (1g/kg body weight). All the animals in the various experimental groups received their blood and liver tissues. Centrifuging the serum (at 2500 rpm for 10 minutes) separated it, and it was then stored at 80 °C for later investigation. For histological and biochemical investigation, a sample of the liver tissue was kept in 10% neutral buffered formalin for 24 hours.

Measurement of serum biochemical parameters

Liver function test

ELISA (Sigma kit) was used to measure liver function assays (alanine aminotransferase (ALT) Catalog Number MET-5123, aspartate aminotransferase (AST) Catalog Number MAK055, and alkaline phosphatase (ALP) Catalog Number MAK446.

Immunohistochemical assay

the immunohistochemical examination of Alpha-fetoprotein (AFP) immunostaining were performed. Five micron paraffin sections were mounted on positively charged glass slices. Sections of paraffin were overnighted in Xylene to achieve appropriate deparaffinization, and then the sections were then transferred to distilled water using ethanol concentration of 100%, 95%, 75% and 50%. For antigen retrieval: Slides were placed in a plastic bag that wasn't sealed and had enough antigen retrieval solution inside (Citrate buffer solution, pH 6). The

plastic container was placed in an open plastic tray to catch boil-over. In a microwave (Samsung 800 Watts with digital control), slides were heated at power 10 for 5 minutes. To keep slides from drying, the amount of fluid in the container was examined, and water was added as necessary. Microwave on power 10 for an extra five minutes. After being removed from the oven, the container was given 15 minutes to cool. The slides were then placed in phosphate buffer saline (PBS) for 5 minutes after being repeatedly rinsed in deionized water. Sections of tissue were incubated with an endogenous peroxidase blocking reagent containing hydrogen peroxide and sodium azide (DAKO peroxidase blocking reagent, Cat. No. S 2001). Slides were dried, with the exception of the tissue section, once extra buffer was removed. One to two drops of the supersensitive primary monoclonal antibody [against alpha fetoprotein (AFP) were then applied to the sections. Slides were incubated for 60 minutes horizontally in a humid environment at room temperature. Excess reagent was discarded and the slides were rinsed for 5 minutes in two PBS (phosphate buffer saline) jars placed side by side. After wiping away extra buffer with a cloth, 1-2 drops of the DAKO Envision + system were applied and left on for 20 minutes at room temperature. Sections were subsequently blotted and washed with PBS as before. Chromogen used was DAB (diaminobenzidine), 1-2 drops for 10-20 minute until the desired shade of brown was obtained. Next, buffer was used to wash the slides. The sections were placed in distilled water then nuclear counter staining was done using Mayer's hematoxylin (Hx):Sections were put in Hx solution for 3-5 min. based on the intensity of nuclear staining .Then, sections were washed in tap water and seperated in acid-alcohol, then washed again in water. Slides were mounted in Canada balsam after being allowed to dry in the air [19].

Morphometric analysis

Analysis of images Slides were photographed with a 40X objective, 1/2X picture adaptor, and Olympus LC20 digital camera mounted on an Olympus BX-50 microscope in Tokyo, Japan. The result images were analyzed on Intel® Core I3® based computer using Video Test Morphology 5.2 software (Russia) with a specific built-in routine for immunohistostaining analysis and stain quantification. The technique calculated the percentage of a region that expresses alpha-fetoprotein positively.

Images from five slices per tissue were taken 200 μm apart. Using image analysis software (JID801D), five visions were randomly picked from each slice to evaluate the presence of positive cells. The positive cells' average grayscale was automatically determined. Average grayscale was used to express the severity of the immune response. Values <160 was considered high, 160–170 medium and 170–180 low [20].

Statistical analysis:

Excel program was used to analyze the outcomes of the statistical study. The data were shown as a mean and SEM. The Two-Factor Without Replication ANOVA test was used to establish the statistical significance. P values less than 0.05 ($P \leq 0.05$)were considered statistically significant [21].

RESULTS

Effect of Bee venom on liver enzymes in all groups: Table (1):

The data obtained demonstrated that the positive control group had significantly higher liver enzymes (AST, ALT, and ALP) than the negative control group. The administration of Bee venom and cisplatin to mice with HCC resulted in lower levels of liver enzymes when compared to the positive control group.

The effect on immunohistochemistry analysis: (Table 2)

Investigation of immune-stained hepatic tissue of different experimental groups pointed out the percentage of expression of cytoplasmic brownish stainability to the used fibro-carcinogenic marker alpha fetoprotein (AFP). The estimation of the positive cells was carried out in three microscopic fields at a high-power focus using ImageJ software. Average calculated percentage of positive cells for control negative, control positive, treatment, protective and chemotherapy treatment groups were 1.22, 86.25, 42.23, 41.01 and 18.48 respectively. **(Figures 1,2,3,4,5)**

Morphometric analysis

Used to measure the area percentage of Alpha-fetoprotein positive expression. **(Figure 6)**

DISCUSSION

Hepatocellular carcinoma is the most prevalent type of liver cancer and the main reason for cancer deaths globally. Liver cancer death rates have risen by about 3% annually, in contrast to other major malignancies' diminishing patterns. Hepatitis B or C virus-related chronic liver infection (HBV or HCV, respectively) and alcoholism are the most common causes of HCC [22].

Due to their lack of or little side effects, the use of medicinal herbs as chemotherapeutic agents in the treatment of cancer is gaining interest [23]. In the meanwhile, venoms like bee venom have shown a variety of biological actions that additional research may help identify newer and safer HCC treatment alternatives. Chemoresistance has grown to be a significant obstacle in the treatment of cancer. However, chemo sensitizers made from natural sources have caught the interest of researchers. Following extensive clinical and preclinical research, natural products for the treatment of cancer have shown to be very promising. These natural products' bioactive components have been examined for their potential to fight cancer. These substances function either by themselves or in conjunction with traditional chemotherapeutic treatments to lessen their side effects while also defending nearby healthy cells from damage [24].

In our study, we improved the anti-cancer properties of bee venom against HCC brought on by CCL_4 in a mice model. We have compared a component of bee venom with cisplatin to support our hypothesis. Cisplatin is a frequently employed metal coordination substance that has been given clinical approval for the treatment of several malignancies. Its primary molecular mode of action is the creation of DNA adducts that cause the start of programmed cell death by activating important signaling pathways [25,26].

It was discovered that exposure to CCl_4 causes substantial hepatotoxicity, characterized by fibrosis, cirrhosis, and increased bile duct proliferation. Although the exact mechanism of CCl_4-induced liver cancer is unknown, mounting evidence suggests that CCl_4-induced cytotoxicity is reliant on triggering the generation of reactive oxygen species (ROS), which in turn cause oxidative stress [26].

The research was done to determine whether bee venom can prevent HCC from being produced by CCl_4 in an experimental animal model. Bee

venom demonstrated a variety of pharmacological properties, including anti-inflammation and antioxidant, and it had a crucial role in preventing the progression of HCC, as this study shown.

Our findings demonstrated that CCl_4 treatment caused a number of changes, including a notable increase in the blood levels of liver enzymes Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and Alkaline phosphatase (ALP). Significant increases in liver enzyme levels are typically used by doctors as additional markers to help with the diagnosis of various disorders. Finding the concentration of this enzyme is a more accurate technique to check for liver issues since it primarily distinguishes the necrosis of the hepatocellular layer, which is the most prevalent indicator of hepatotoxicity when its level is high in serum [27].

Mice were administered the liver-toxicant CCL_4 before and after receiving bee venom, which helped bring the levels of ALT, AST, and ALP back to roughly normal levels.

In the current study, after bee venom administration, increased levels of the liver function test ALT, AST, and ALP with CCL_4 treatment were decreased in treated and protective groups.

Bee venom administration returned the evaluated biochemical values to normal levels. This research established the bee venom's defensive properties.

For the detection and monitoring of HCC, the tumor marker alpha fetoprotein (AFP) has been employed [28]. AFP is a glycoprotein which is similar to albumin. The serum AFP level is more specific, and immunohistochemistry is more sensitive [29]. Investigation of immuno-stained hepatic tissue of different experimental groups pointed out the percentage of expression of cytoplasmic brownish stainability to the used fibro-carcinogenic marker alpha fetoprotein (AFP). In the current study, the percentage of positive cells decreased in treated and protective groups compared to positive group.

Morphometric analysis calculated the percentage of the area of expressed Alpha-fetoprotein. The positive cells' average grayscale was automatically determined. Immunoreactive intensity were expressed by average grayscale. Compared to the treated group, the area percent of positive cells containing AFP was high.

CONCLUSION

In the current research, we proposed that the antineoplastic activity of bee venom may be attributed to its antioxidant and anti-inflammatory qualities. significantly, the bee venom has shown anti-tumor properties against different cancer cell lines.

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Table (1): Effect of Bee venom on liver function test in all groups for observation of therapeutic activity.

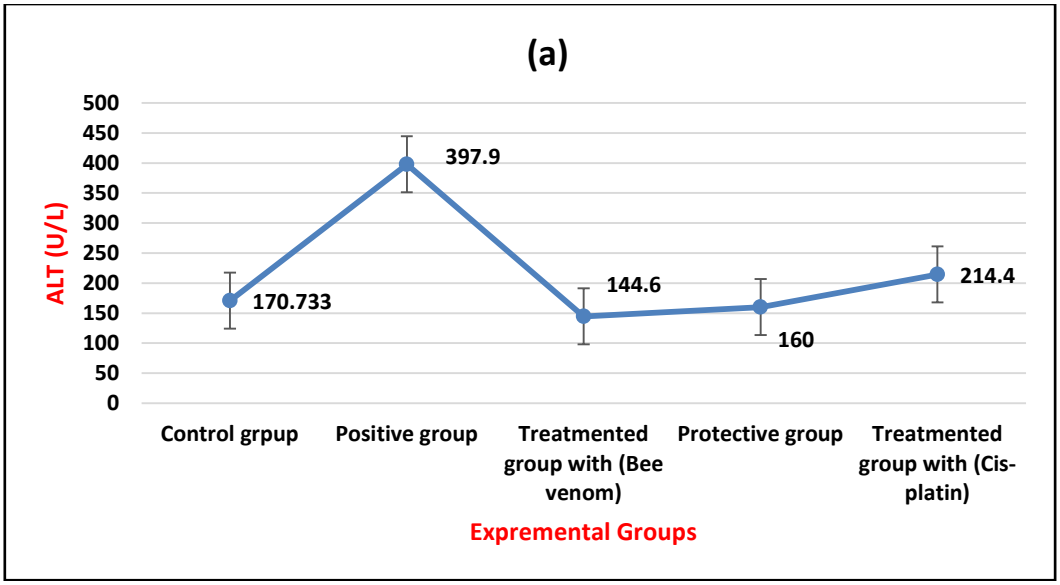
Groups	ALT	AST	ALP
Control	170.733±4.12^{*a}	107.7±64.18^{*a}	93.5±6.062^{*a}
Positive	397.9±47.67	366.3±28.81	275.7±14.72
Treatment (BV)	144.6±4.273^{*b}	222±20.03^{*b}	205±6.351^{*b}
Protective	160±34.37^{*b}	50.62±41.94^{*b}	113.3±12.41*^{b}
Treatment (CP)	214.4±11.69^{*b}	313.3±8.603^{*b}	138±6.928^{*b}

Values are expressed as mean ± SEM., n = 10. Comparisons between the four groups represented statistical significance as a P-value of ≤0.05 indicating significant difference. (*a) indicate a significant difference between negative group and positive group. (*b) indicate a significant difference between treatment groups and positive group.

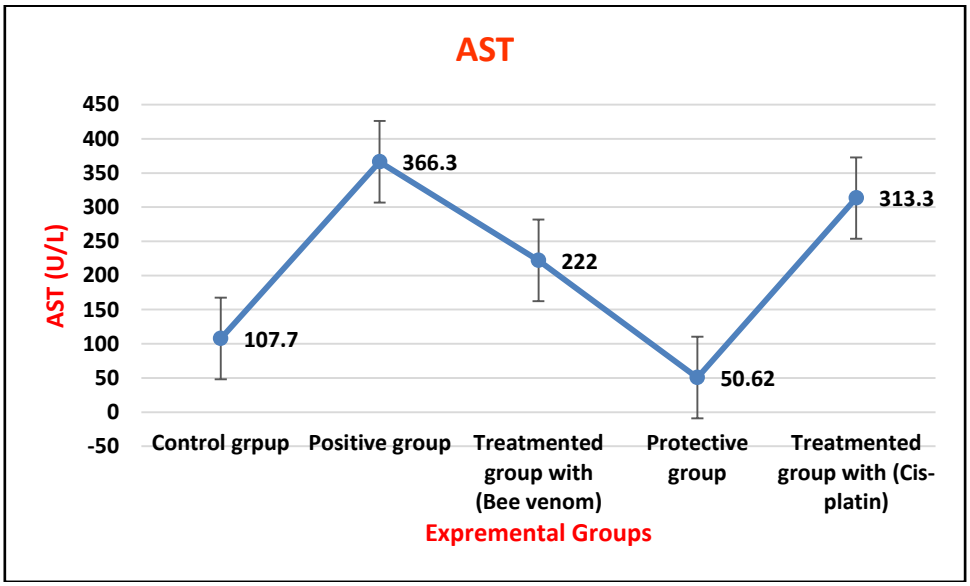
Table (2): Effect of Bee venom on immunohistochemistry AFP in all groups for observation of therapeutic activity.

Groups	AFP (immunohistochemistry)
Control	**1.227*^{a}**
Positive	**86.26**
Treatment (BV)	**42.23*^{b}**
Protective	**41.01*^{b}**
Treatment (CP)	**18.48*^{b}**

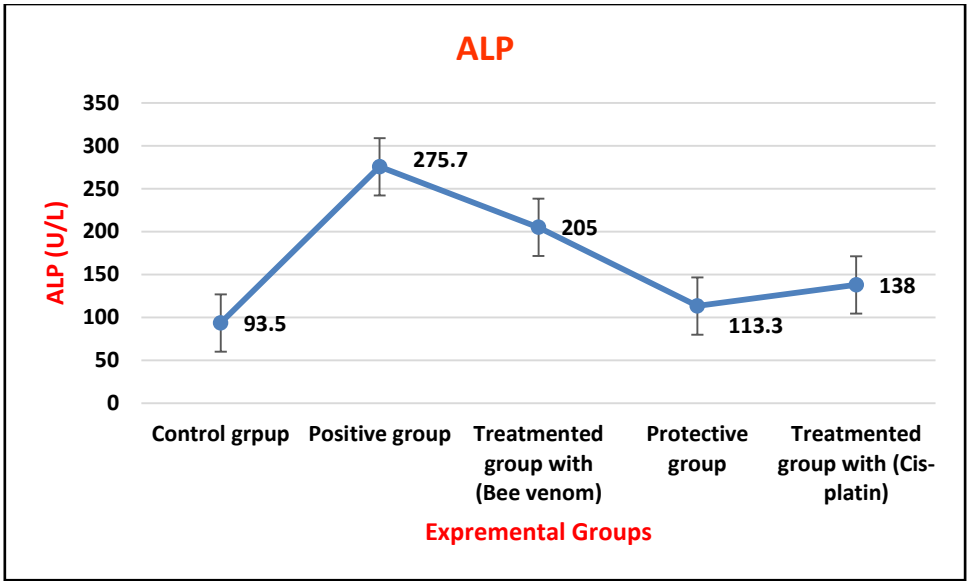
Values are expressed as mean ± SEM., n = 10. Comparisons between the four groups represented statistical significance as a P-value of ≤0.05 indicating significant difference. (*a) indicate a significant difference between negative group and positive group. (*b) indicate a significant difference between treatment groups and positive group.



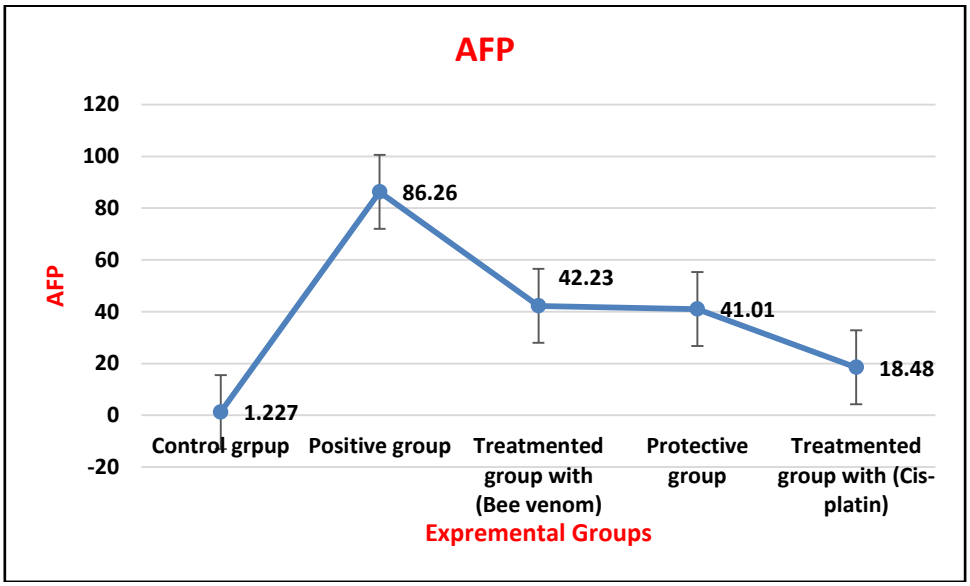
Graph (1): Activities of biochemical variables (ALT) in the groups.



Graph (2): Activities of biochemical variables (AST) in the groups



Graph (3): Activities of biochemical variables (ALP) in the groups.



Graph (4): Activities of Alpha fetoprotein (AFP) in the groups.

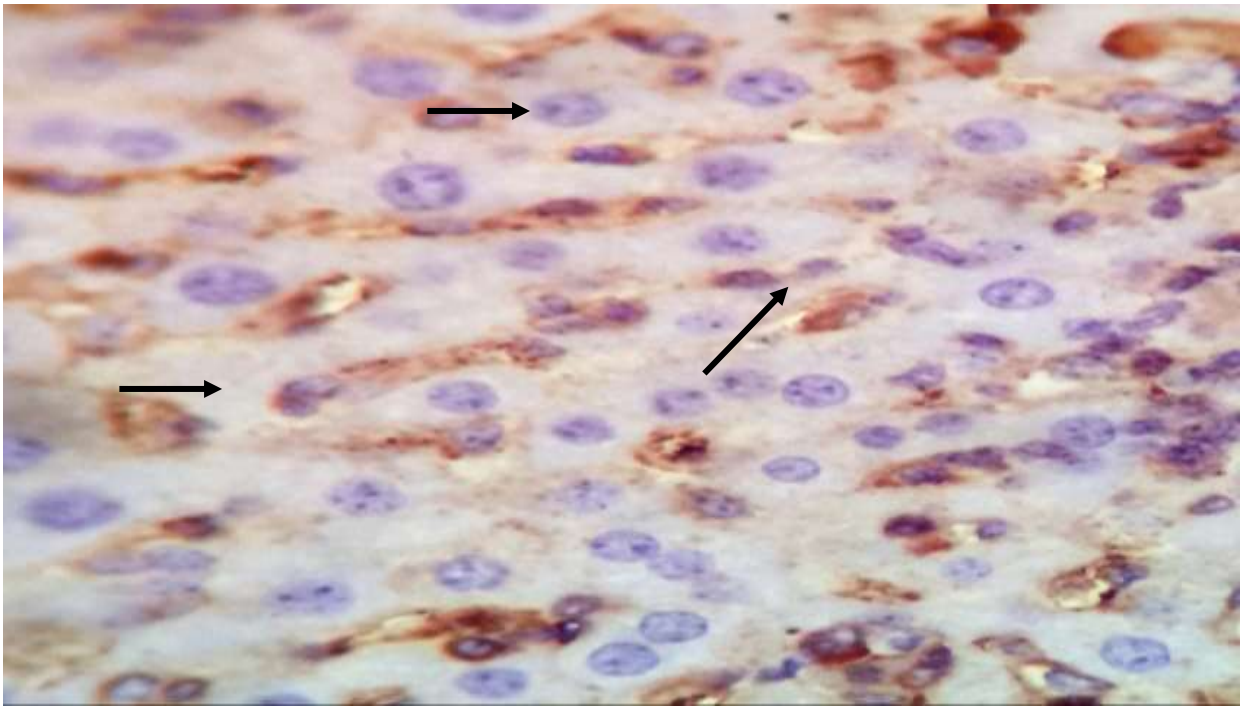


Figure (1): Photomicrograph of G1 showing the positively expressed hepatocytes and occasionally other cells. Positive cells show brownish cytoplasmic stainabilities to AFP at a Poor density. (AFP X 400)

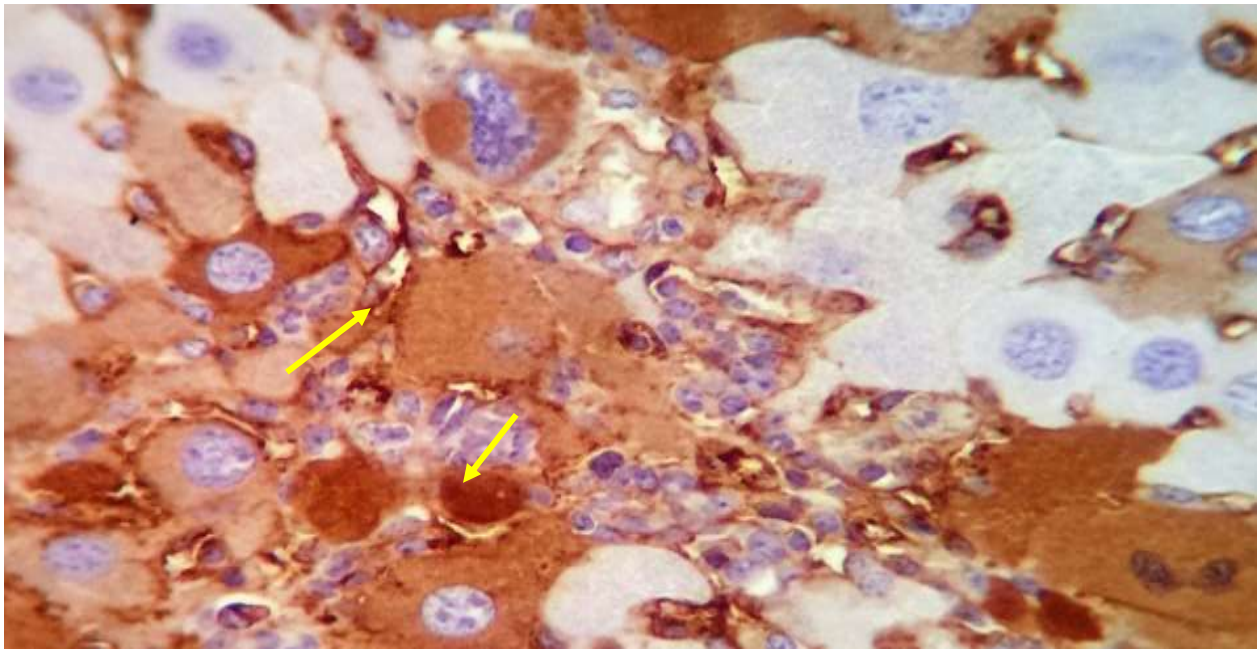


Figure (2): Photomicrograph of G2 showing the positively expressed hepatocytes and occasionally other cells. Positive cells show brownish cytoplasmic stainabilities to AFP at a very strong density. (AFP X 400).

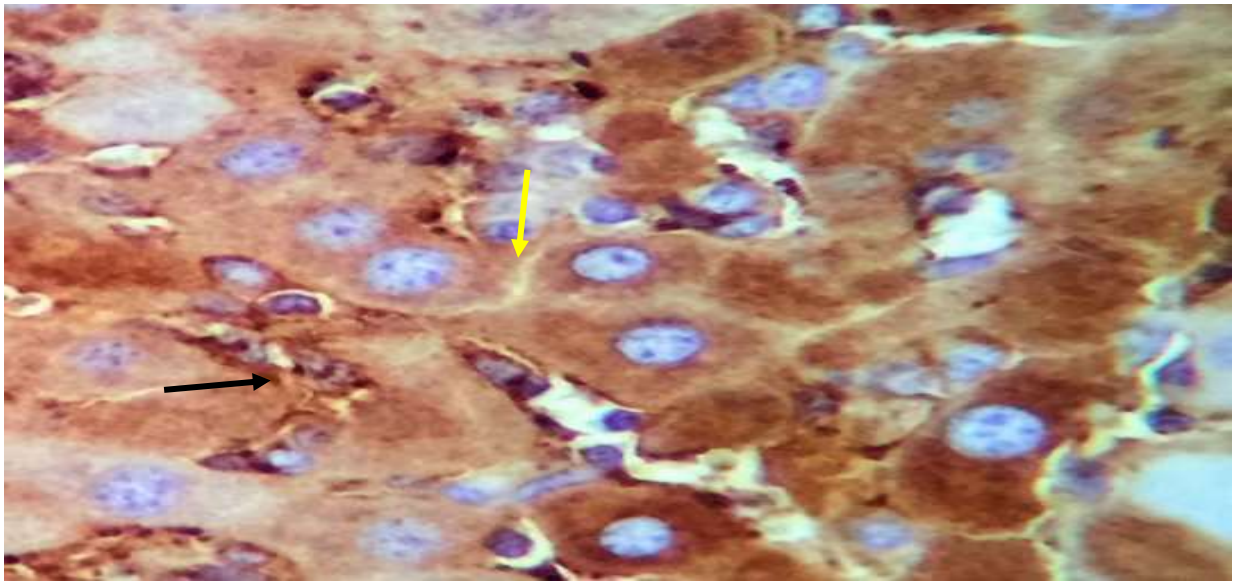


Figure (3): Photomicrograph of G3 showing the positively expressed hepatocytes and occasionally other cells. Positive cells show brownish cytoplasmic stainabilities to AFP at a moderate density. (AFP X 400).

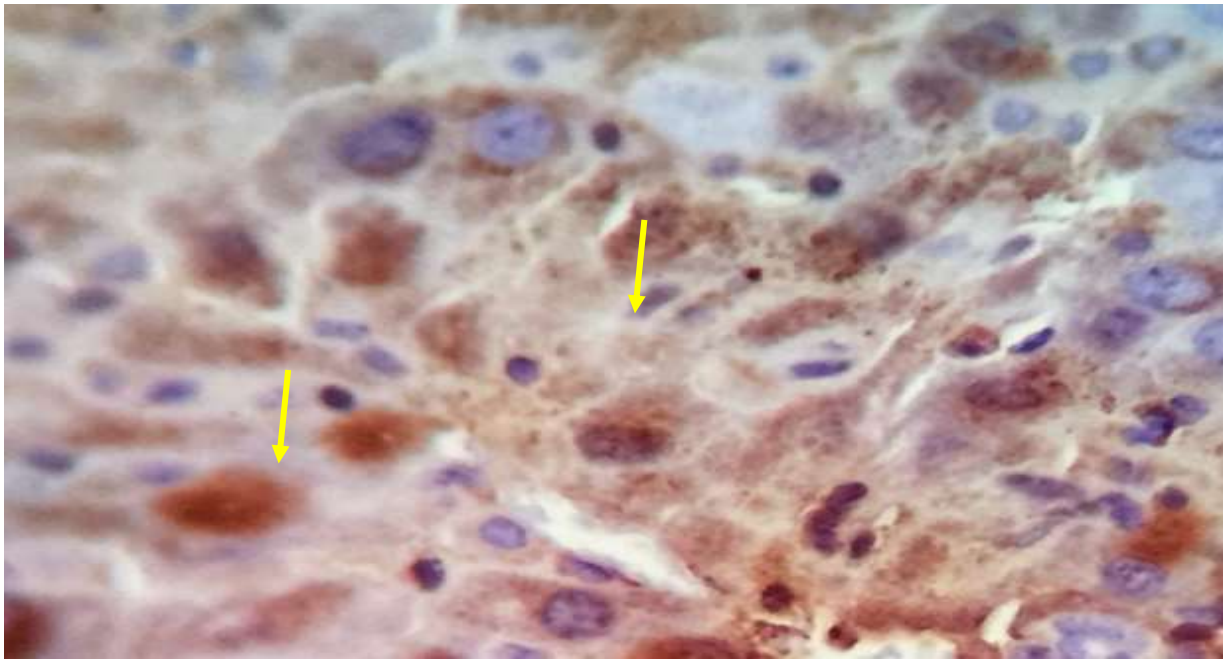


Figure (4): Photomicrograph of G4 showing the positively expressed hepatocytes and occasionally other cells. Positive cells show brownish cytoplasmic stainabilities to AFP at a mild density.
(AFP X 400).

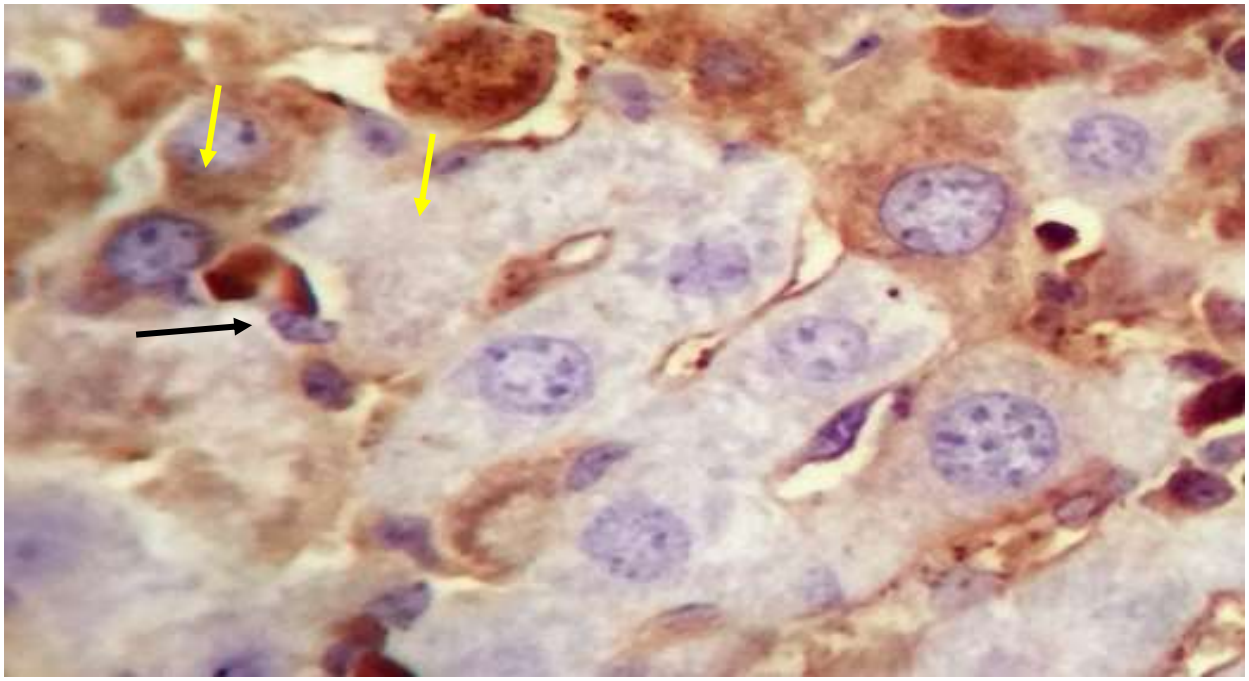


Figure (5): Photomicrograph of G5 showing the positively expressed hepatocytes and occasionally other cells. Positive cells show brownish cytoplasmic stainabilities to AFP at mild density.
(AFP X 400).

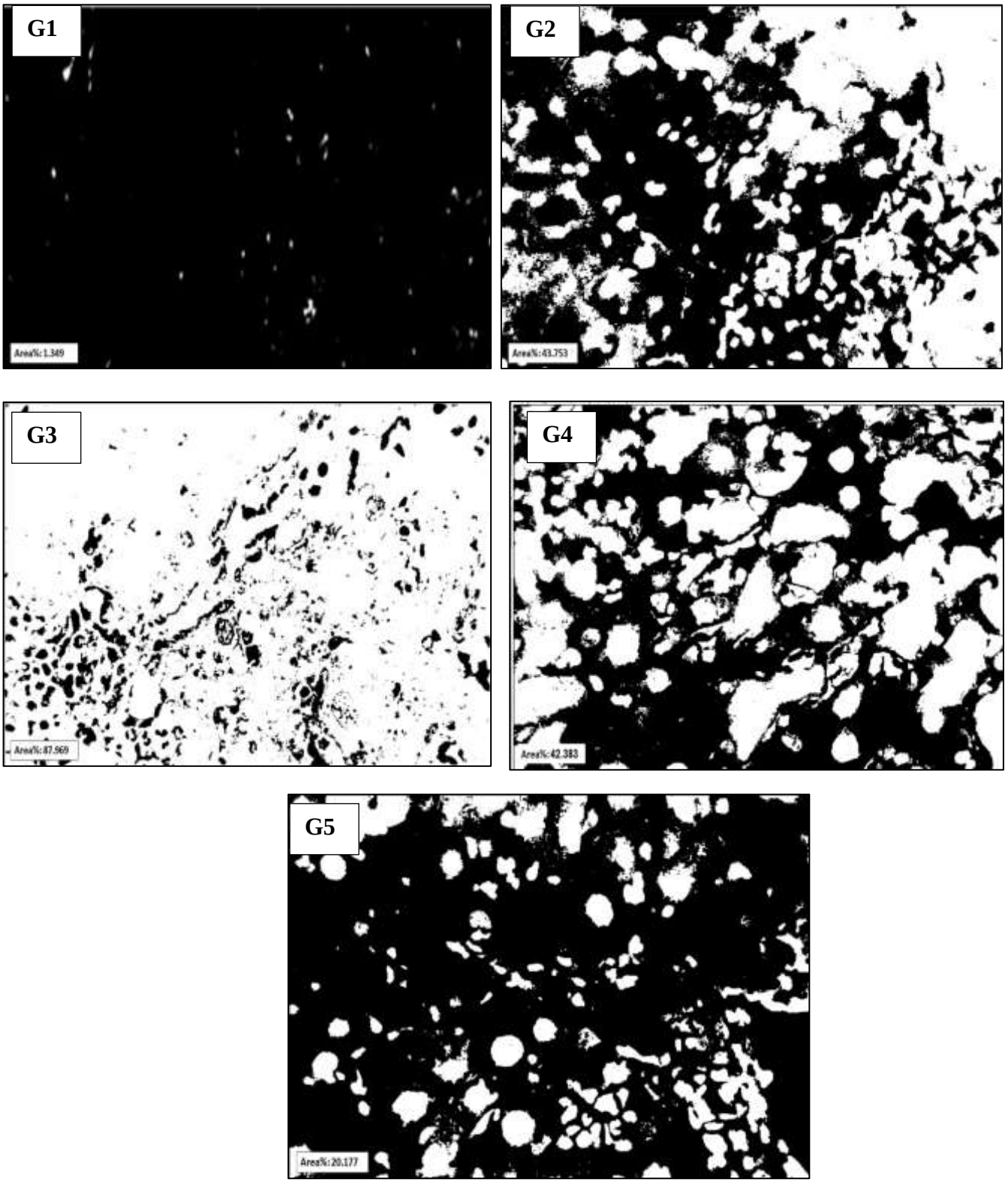


Figure (6): morphometric analysis of different experimental groups, showing the percentage areas of positivists (area %) to AFP.