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The effect of "3, 6 diamino-5-cyano-4-(p-chloro phenyl) - Pyrazolo [3, 4- b] pyridine" on Ehrlich ascites carcinoma in mice

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ABSTRACT

Pyrazolopyridine derivatives are organic substance with novel properties. They have many beneficial uses as antibacterial and antifungal etc; so this study was designed to investigate the antitumor and hepatoprotective effect of "3, 6 diamino-5-cyano-4-(p-chloro phenyl) - Pyrazolo [3, 4- b] pyridine" in comparison to folfox (5-flouro uracil 50 mg/kg &6mg/kg oxaloplatine). Thirty five adult male mice (24-26 g) was divided into five groups (7 each).first one considered as a negative control, second as positive control, third and fourth treated intraperitoneal with 35 mg/kg B.WT. Of compound from the 1st day and after one week of EAC injected respectively. Serum analysis of liver enzymes showed a significant increase in ALT, AST and significant decrease in serum Albumin and total protein activity, also there were an elevation in inflammatory gene expression of cytokine TNF-alpha and insignificant elevation in gene expression of CDKn2D. and after treatment, the liver function showed normal level and significant decreases of gene expression of cytokine TNF-alpha. In addition, gene expression of CDKn2D showed significant elevation in it is level .

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INTRODUCTION

When abnormal cells divide in an uncontrolled way is known as cancer. Some cancers may eventually spread into other tissues and if the spread not

controlled, it will cause death. There are more than 200 different types of cancer [1]. Cancer starts when changes of gene make one cell or a few cells begin to grow and divided too much, this growth called a tumor [2].Ehrlich ascites carcinoma (EAC)

is one of the important tumors. EAC is referred to as an undifferentiated carcinoma and is originally hyperdiploid, has high transplantable capability, no-regression, fast proliferation, shorter life span, 100% malignancy. Also, does not have tumor-specific transplantation antigen "TSTA" [3]. EAC resembles human tumors which are the most sensitive to chemotherapy due to the fact that they are undifferentiated and that they have a rapid growth rate. The ideal drug being ineffective or minimally effective for normal cells have been focused on, and at this point, the usage of natural sources as an alternative cancer therapy is thought to have a great value for cancer control and programs destruction [4]. Synthesis of the pyridine ring system and its derivatives occupy an important place in the realm of synthetic organic chemistry, due to their therapeutic and pharmacological properties [5]. The pyridine ring is also an integral part of anticancer [6]. 1H-Pyrazolo [3, 4-b] pyridine used in treating cancer, [7]. Cyanopyridine is important intermediates in the pharmaceutical industry for the synthesis of nicotinamide, nicotinic acid and isonicotinic acid and anti- cancer [8]. Pyrazolopyridine derivative and cyanopyridine is an effective drug for liver mice bearing EAC, it has hepatoprotective effect and antitumor effect. It maintains liver enzymes in normal level Anupa et al., (2017) [9]. Our aim is to Investigate the effect of 3, 6 diamino-5-cyano-4-(p-chloro phenyl) - Pyrazolo [3, 4- b] pyridine & folfox as antitumor drugs.

MATERIAL AND METHODS

Chemicals

3, 6 diamino-5-cyano-4-(p-chloro phenyl) - Pyrazolo [3, 4- b] pyridine "was prepared in faculty of science of Zagazig university in Egypt by Amer et al., (2005) [10].

Animals:-

Forty nine adult male mice (24-26 g) obtained from the central animal house,

faculty of medicine, Zagazig University were used in this study. A commercial standard pellet diet and water were available to animals *ad libitum*. Animal maintained in a controlled environment (25 ± 2 C with (12:12h) light-dark cycles in an experimental room stimulating natural conditions [11]. Animals were acclimatized under control conditions at the laboratory for one week before use. Food and water were kept in special open containers fixed in the walls of the cages. Cleaning and changing water and food for all animals were performed twice a day.

Experimental design

The experimental mice were divided into five groups each group comprising of seven mice. **Group 1(negative control group):** without EAC. **Group 2 (positive control group):** With Ehrlich cell **Group 3:** Mice received intra peritoneal injection from the first day of Ehrlich injection of a dose (35 mg/kg) of drug one (3, 6 diamino-5-cyano-4-(p-chloro phenyl) - Pyrazolo [3, 4- b] pyridine) as protection. **Group 4:** Mice receive intaperitoneal injection of a dose (35mg/kg) of this drug one after one week of Ehrlich injection. **Group 5:-** Mice receive intaperitoneal injection of folfox drug (Oxaloplatin 6mg /kg & 5-fluoro uracil 50mg/kg).

Blood collection

Serum sample: blood sample were collected from mice, kept for a time was centrifuged at 3000 r.p.m. for 15 minutes, [12] the resulting supernatant were collected and used for estimation of serum ALT, AST activity, total protein and albumin activities using kits supplied by SPINREACT KIT (Ctra.santa Coloma, SPAIN).

Molecular determinations of gene expression of (CDKn2D & TNF Alpha)::

Determination of TNF α , CDKn2D gene expression using a semi- quantitative RT-PCR according to Meadus [13]. The gene expression of TNF α , CDKn2D genes

were determined using RT-PCR technique. Total RNA was extracted from separated tissues using RNeasy Mini Kit (Qiagen, Cat. No.74104).First strand cDNA was synthesized using Revert Aid TM H Minus (Fermentas, life science, Pittsburgh, PA, USA). The PCR reaction was started by using SYBR® Green PCR Master Mix Catalog Number 2501130 (Master Mix) supplied by applied bio systems in a rotor gene apparatus (Biometra- Germany). The housekeeping gene GAPDH was used as a constitutive control for normalization. Primers were designed as previous published [14].Primers was provided by Sigma Aldrich (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and was listed in table (1) The quantitative fold's changes in mRNA expression were determined relative to the housekeeping controls (GAPDH mRNA) levels in each corresponding group and calculated using the 2- $\Delta\Delta$ CT method.(2- $\Delta\Delta$ CTthe relative Quantification level of target genes calculation.

Statistical analysis

The obtained data were analyzed and graphically represented using the statistical package for social science (SPSS, 15.0software) [15] for obtaining means and standard error. The data were analyzed using one way ANOVA to determine the statistical significance of differences among groups. Duncan's test was used for making a multiple comparisons among the groups for testing the inter-grouping homogeneity.

RESULTS

Result were presented as means \pm SE of seven mice in each group. Values of $p < 0.05$ were regarded as statistically significant and the data are represented as mean \pm SE for the absolute values or percent of controls as indicated in the vertical axis legend of figures. The statistical significance of differential

findings between the experimental groups and control were determined and that are represented by symbols (a,b,c,d,e,f).

Table (2) demonstrated the ALT & AST & T.P & Alb activities in all studied groups. The mean activity of ALT&AST in control positive group (injected with EAC cells) showed significant increase when compared to control negative without EAC injection. Otherwise, the mean activity of total protein &albumin in control positive (injected with EAC) showed significant decrease when compared to control negative group. On the other hand the activity of ALT &AST were significantly to normal level in groups treated with (3, 6 diamino-5-cyano-4-(p-chloro phenyl) - Pyrazolo [3, 4- b] pyridine) at dose of (35mg/Kg)[16] in the first day and after one week. Also the ALT &AST activity were significantly decreased in groups treated with folfox drug when compared to control positive group. Other wise, the mean activity of total protein &albumin showed significant increase in groups treated with (3, 6 diamino-5-cyano-4-(p-chloro phenyl) - Pyrazolo [3, 4- b] pyridine) at dose of (35mg/Kg) in the first day and after one week when compared to control positive group. Additionally, group treated with fofox drug showed significant increase in T.P &Alb when compared to control positive group.

Figure 1&2 demonstrated that the transcriptional levels of mRNA of (CDKn2D) gene and TNF-alpha gene in all studied groups. The mean transcriptional level of mRNA of (CDKn2D) was insignificantly increased in control positive group. While the groups treated with (3, 6 diamino-5-cyano-4-(p-chloro phenyl) - Pyrazolo [3, 4- b] pyridine) showed increased significantly in groups treated from the first day and after one week of injection. Otherwise, the mean transcriptional level of mRNA of (TNF-alpha) was significantly increased in control positive group. While the groups treated with (3, 6 diamino-5-cyano-4-(p-chloro phenyl) - Pyrazolo [3, 4- b]

pyridine) was significantly decrease in groups treated from the first day and after one week of injection. On the other hand, group treated with folfox showed significant increase in the level of mRNA of CDKn2D and significant decrease in TNF-alpha when compared to control positive group.

Histopathological investigation

In the histopathological study, the liver sections in the control negative group (G1) without any treatment; Figure 3 (1) showing normal, intact hepatic parenchyma (arrow) with normal central vein .in Sections of the liver of the control positive group (G2) injected with Ehrlich's ascites carcinoma (EAC) Figure3 (2) showing necrosis in the hepatic parenchyma. In Sections of the liver of the group 3 (G3) treated with 3, 6 diamino-5-cyano-4-(p-chloro phenyl) - Pyrazolo [3, 4- b] pyridine with Ehrlich at the same time Figure 3(3): showing mild necrosis in the hepatic parenchyma. In Sections of the liver of the G4 treated with 3, 6 diamino-5-cyano-4-(p-chloro phenyl) - Pyrazolo [3, 4- b] pyridine after one week from treating with EAC Figure3 (4): showing moderate necrosis in the hepatic parenchyma. In Sections of the liver of the G5 treated with folfox after one week from injected with EAC Figure3 (5): showing moderate necrosis in the hepatic parenchyma (arrow) and fibrosis (arrow head).

DISCUSSION

EAC tumor bearing, a regular rapid increase in ascitic tumor volume was observed. Ascitic fluid is the direct nutritional source for tumor cells and a rapid increase in ascitic fluid with tumor growth. Treatment with various synthesized compounds such as pyrazolopyridine inhibited the tumor volume, viable tumor cell count and increased the life span of the tumor bearing mice

Liver enzymes are substances produced by the liver that can be measured with a blood test. Any elevation in an enzyme level may be a sign of a liver problem. Alanine aminotransferase (ALT) are two of the enzymes central to such an investigation and aspartate aminotransferase (AST). AST and ALT were used to identify liver toxicity, liver disease, or liver damage. Moreover Liver function tests reflective the general status of the patient, have been reported to be important factors affecting the prognosis in many types of cancers [17]

The obtained result revealed significant elevation of ALT and AST in control positive group injected by Ehrlich cell (G2) compared with those in negative control group without any treatment (G1). Meanwhile, the statistical analysis showed that the groups treated by "3, 6 diamino-5-cyano-4-(p-chloro phenyl) - Pyrazolo [3, 4- b] pyridine" (G3 and G4) exhibited significant reduction of ALT and AST levels comparing with positive control groups. On the other aspect, group treated by folfox drug (G5) exhibited significantly decreasing ALT and AST enzyme level comparing with control positive group. The obtained results coincided with those recorded by Kapoor et al., (2014) [18] which indicated significant elevations of ALT and AST enzymes in mice groups injected by EAC cells comparing with those in control group. Moreover Noureldeen et al., (2017) recorded obviously higher AST and ALT levels in mice groups treated with EAC cells than those in control group [19]

The obtained result showed significant decrease of total protein and albumin in control positive group bearing EAC tumor (G2) comparing with control negative group without EAC injection (G1), while total protein and albumin has significant increased in group treated with "3, 6 diamino-5-cyano-4-(p-chloro phenyl) - Pyrazolo [3, 4- b] pyridine" from the 1st day to 28th day and from 8th

day to 28th day (G3 and G4) when compared with control positive group (G2). Furthermore, the levels of total protein and albumin in group treated with "3, 6 diamino-5-cyano-4-(p-chloro phenyl) - Pyrazolo [3, 4- b] pyridine" from 1st to 28th day are significantly higher than those treated with the same drug from 8th to 28th day. On the other hand, group treated with folfox drug from 8th day to 28th day (G7) showed significant increase of total protein when compared with control positive group (G2). The obtained results coincided with **Patel et al., (2016)** who showed that mice bearing EAC tumor resulted in a significant decrease in total protein and albumin levels [20]. Moreover, the total protein and albumin were found significantly decreased in the EAC control group when compared with the normal group (**Mushir et al., 2015**) [21]

In molecular findings we showed that insignificant increase of the level of gene expression of cyclin dependant kinas inhibitor (CDKn2D) and significant increase of TNF-alpha in control positive group injected ehrlich cell (G2) comparing with those in negative control group without any treatment (G1). Meanwhile, the groups treated with "3, 6 diamino-5-cyano-4-(p-chloro phenyl) - Pyrazolo [3, 4- b] pyridine" (G3 and G4) exhibited a significant increase of gene expression level of CDKn2D and significant decrease of TNF-alpha comparing with both positive and negative control groups. Group treated by drug 1 from 8th to 28th days (G3) had slightly increase in gene expression of CDKn2D than those in treated by the same drug from 1st to 28th days (G3). On the other hand, Group treated by folfox drug (G5) exhibited a significant increase of gene expression of CDKn2D and significant decrease of TNF-alpha when compared with the most examined groups. The obtained results coincided with those recorded by **Cassier et al., (2014)** who examined CDK deregulated in solid tumor; he showed that CDK is a target for cancer treatment in a

broad spectrum of solid tumors [22]. In addition, **Paiva et al., (2015)** obtained that CDK inhibitors are imperative for the development of effective cancer therapies [23]. **Gu et al., (2010)** who reported that in rat osteosarcoma model [24], TNF- α caused necrosis. In addition, **Kitaura et al., (2013)** showed that TNF- α could directly induce tumor cell proliferation in vitro and in vivo and can also induce cell death. [25] Also **Wu et al., (2014)** declared that TNF- α can also promote tumor expansion [26].

The histopathological investigation was carried out to judge and evaluate the anti-tumor activity of "3, 6 diamino-5-cyano-4-(p-chloro phenyl) - Pyrazolo [3, 4- b] pyridine" in comparison with folfox as anti-cancer drug against Ehrlich's ascites carcinoma (EAC). In this study the histopathological examination of the liver in the control negative group (G1) without lesions. The liver parenchyma of these groups was observed very homogenous, intact and consisting of numerous hepatic lobules that were difficult demarcated from each other's by a very thin connective tissue trabeculae in between. **On** the other hand, liver of the control positive group (G2) injected with Ehrlich's ascites carcinoma (EAC) revealed the presence of sever necrosis, degeneration and massive damage of hepatic tissues with loss of the hepatic architectures. liver section of EAC bearing mice showed various histopathological alternations including increased number of necrotic hepatocytes with deeply pyknotic nuclei, congestion associated with brown pigment deposition and thickening of wall on the central vein by increased collagen content, also reported that presence of tumors in the human body or in experimental animals is known to affect many functions of the vital organs especially the liver, even when the site of the tumor doesn't interfere directly with organ functions [27]. on the other hand femal mice bearing EAC tumor characterized with abundant basophilic

and dark stained cytoplasm as well as moderate sized nuclei [28].in addition, liver tissue from EAC bearing mice; indicated increase number of Kupffer cells, congested central vein with hemorrhage and dilated congested blood sinusoids Experimental [19]. Furthermore EAC control mice shows hydropic degeneration of the hepatocytes, loss of cell boundaries and ballooning degeneration, some other hepatocytes showed nuclear pyknosis and karyolysis [29]. Also Ehrlich ascites bearing mice revealed massive pathological alterations distributed throughout the hepatic tissue. And the liver showed enlarged and congested central vein, numerous focal lesions of leukocyte infiltration and Cytoplasmic vacuolar degenerations were also obvious in the hepatocytes; Kupffer cells were abundant more than normal [30]. Meanwhile, group 3 (G3) treated with "3, 6 diamino-5-cyano-4-(p-chloro phenyl) - Pyrazolo [3, 4- b] pyridine" with Ehrlich at the same time showed a significant amelioration of the hepatic tissue were the hepatic parenchyma appeared like normal but with slightly necrosis that appeared in the form of small necrotic foci. pyrazole derivatives were identified as a potent anticancer agent against cell lines [31].In addition, pyrazol derivatives showed antiproliferative activity in human ovarian adenocarcinoma cells, human lung carcinoma cells, and murine leukemia cells [32].G4 treated with "3, 6 diamino-5-cyano-4-(p-chloro phenyl) - Pyrazolo [3, 4- b] pyridine" after one week from treating with EAC showed moderate disorganization of the hepatic cords with moderate degeneration and necrosis of the hepatocytes. G5 treated with folfox "Oxaloplatin 6mg /kg & 5-fluoro uracil 50mg/kg" after one week from injected with EAC showed moderate degeneration and necrosis of the hepatic parenchyma with moderate congestion within the portal vein in the portal triad., treatment with pyrazolo pyridine (3, 6 diamino-5-cyano-4-(p-chloro phenyl) -

Pyrazolo [3, 4- b] pyridine) show significant improvement in liver mice as antitumor and hepatoprotective effect in comparison to folfox drug as antitumor drug.

Conclusion

Pyrazolopydine derivatives consider as better treatment of liver mice bearing EAC by decreasing the metastasis to the liver and prevent cell cycle proliferation by increasing CDK-inhibitor and prevent cancer.

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Table (1): Primers used in determination of the gene expression of the selected genes:

gene	primers	Expected size	Annealing temp
TNF- α	5'- TACTGAACTTCGGGGTGATTGGTCC - 3' 5'- CAGCCTTGTCCTTGAAGAGAACC -3'	290	65
CDKn2D	5'- GGAGCTGGTGCATCCTGACGC -3' 5'- TGGCACCTTGCTTCAGGAGCTC -3'	312	60
GAPDH	5'- ACCACAGTCCATGCCATCAC -3' 5'- CACCACCCTGTTGCTGTAGCC -3'	450	65

Table 2 Means \pm SE of biochemical analysis in different examined mice group

groups	ALT	AST	T.P	Alb
Group1 (Negative control)	45 \pm 6.7 ^{ef}	205 \pm 9.1 ^{cd}	8 \pm 1.24 ^a	5.2 \pm 0.90 ^a
Group 2 (Positive control)	99 \pm 7.6 ^a	327 \pm 15.20 ^a	4.3 \pm 1.41 ^e	2.4 \pm 1.23 ^d
Group3 Treated with 3, 6 diamino-5-cyano-4-(p-chloro phenyl) - Pyrazolo [3, 4-b] pyridine from the 1st day (35mg/kg) of Ehrlich injection.	49 \pm 2.5 ^e	238 \pm 24.3 ^c	7.2 \pm 0.21 ^{ab}	4.5 \pm 0.22 ^{ab}
Group4 Treated with 3, 6 diamino-5-cyano-4-(p-chloro phenyl) - Pyrazolo [3, 4-b] pyridine after one week (35mg/kg) of Ehrlich injection.	53.5 \pm 1.7 ^d	249 \pm 8.11 ^{bc}	6.8 \pm 0.33 ^b	4.2 \pm 0.32 ^b
Group 5 Treated with Folfox (oxaloplatin 6mg/5-flouro uracil).	47 \pm 6.8 ^{ef}	215 \pm 7.5 ^{cd}	7.4 \pm 0.91 ^{ab}	4.7 \pm 0.25 ^{ab}

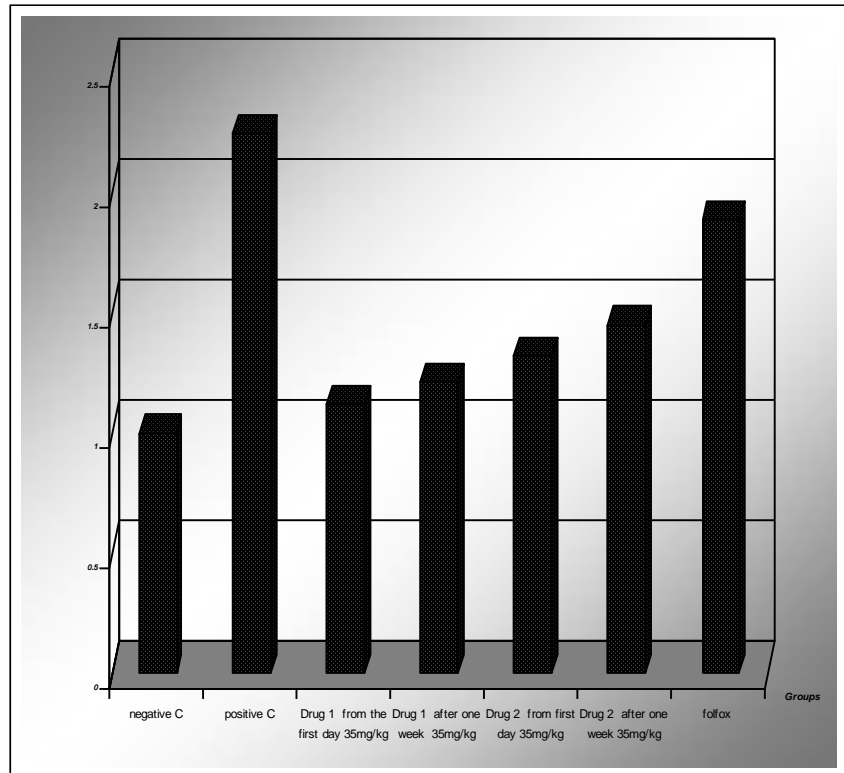


fig 1 mRNA expression of CDKn2d gene in hepatic tissues of male mice

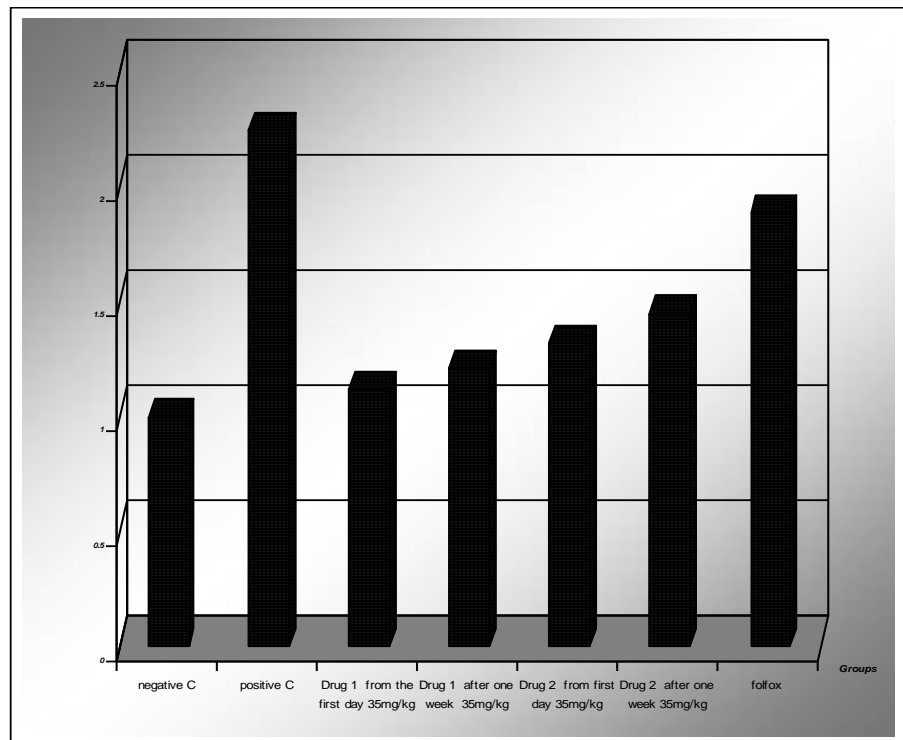


fig2 mRNA expression of TNF-alpha gene in hepatic tissues of male mice

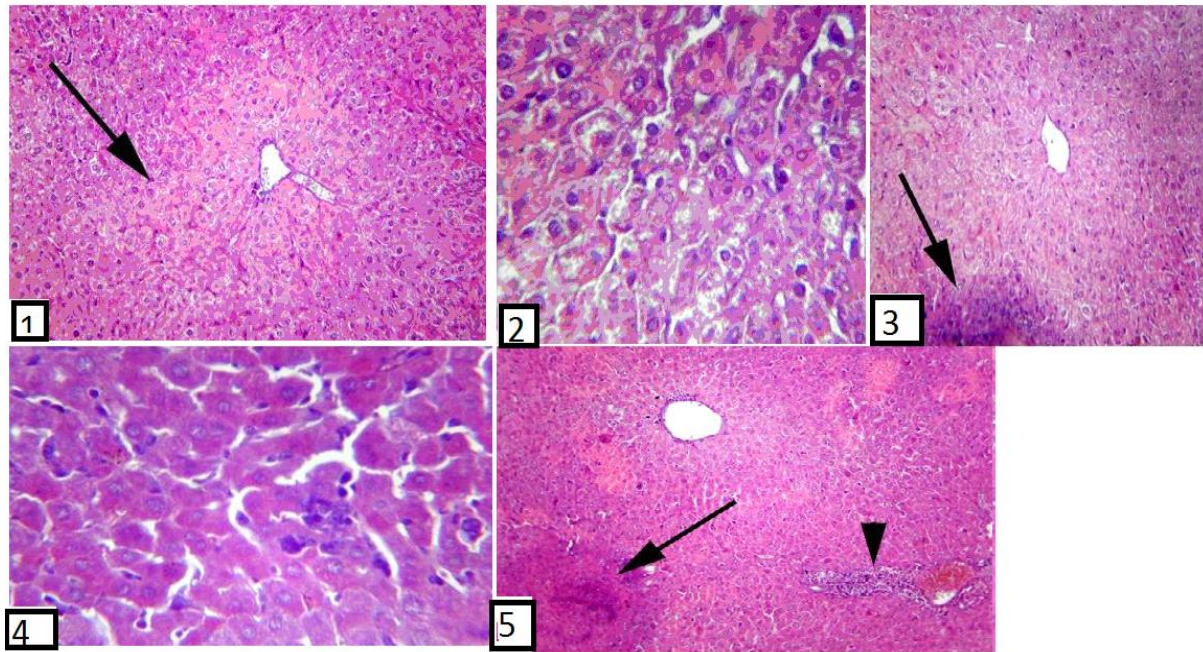


Figure 3: Histopathology of hepatic cells: 1) negative control "G1".2) positive control "G2".3) mice treated with 35mg/kg B.WT 3, 6 diamino-5-cyano-4-(p-chloro phenyl) - Pyrazolo [3, 4- b] pyridine with Ehrlich at the same time "G3".4) mice treated with 35mg/kg B.WT 3, 6 diamino-5-cyano-4-(p-chloro phenyl) - Pyrazolo [3, 4- b] pyridine after one week from injected with Ehrlich"G4". 5) Mice treated with folfox after one week from injected with EAC "G5".