

Biological Evidences of Hepatocellular Carcinoma Treatment with 1,3,4-oxadiazole-2-thiol as Anticancer Agent

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Abstract

Purpose: In recent years, identification of novel potent, selective, and less toxic anticancer agents remains one of the most pressing health problems. This research aimed to *in vivo* illustration of 1,3,4-oxadiazole-2-thiol (OXD-T) anticancer activities as a discovery of the treatment of Hepatocellular Carcinoma (HCC) of Albino rats.

Methods: Hepatocellular carcinoma was induced by 3,3'-Diamino benzidine for three months, three times per week. The post treatment of HCC induced rats was carried out with OXD-T therapeutic (300 mg/kg. b.wt.) and half therapeutic doses (150 mg/kg b.wt.) of administration. Biochemical parameters and comet assay were assessed to evaluate the efficiency of OXD-T treatment on the HCC induced animal.

Results: The administration of 1,3,4-oxadiazole-2-thiol (OXD-T) with therapeutic dose and half therapeutic dose to hepatocellular carcinoma (HCC) induced rats affected the biochemical values as biomarkers; prothrombin induced by vitamin K absence-II (PIVKA-II) and lactate dehydrogenase LDH. Also, serum enzymes; AST, ALT, GGT and Albumin. Furthermore, OXD-T affected the DNA fragmentation parameters (tail length, tail moment, % DNA in tail and % DNA in head of comet). OXD-T therapeutic administration revealed highly significant decreases in the biochemical values and DNA fragmentation parameters more than half dose of OXD-T treatment.

Conclusion: OXD-T antimetabolite with different therapeutic doses of administration affected the growth of hepatocellular carcinoma.

Keywords: 1,3,4-oxadiazole-2-thiol; HCC induced rats; Liver; Biochemical and comet assay

Introduction

Cancer still remains one of the most feared diseases in the modern world [1], in which a group of normal cells proliferates with uncontrolled rules of cell division and growth [2]. Genetic changes can occur at many levels, from gain or loss of entire chromosomes to a mutation affecting a single DNA nucleotide [3]. DNA replication, synthesis and subsequent cell division has been linked to each of the stages of the carcinogenesis process [4,5]. In 2007, cancer caused 7.9 million at about 13% of all human deaths worldwide [6]. Egypt was completely lacking incidence rates at national level. Available statistics were proportions derived from single or multicenter hospital registries that could not be used for calculation of cancer incidence rates [7,8]. The published incidence rates from a cancer registry in Nile delta (Gharbiah governorate), reported in 2002 [9]. There are several traditional approaches to the treatment of cancer; surgery, radiotherapy, chemotherapy, nanotechnology and stem cell therapy. Chemotherapy of human cancers is a viable option for cancer control, especially when chemopreventive intervention is involved during the early stages of the carcinogenesis process [1,10]. Therefore, identification of novel potent, selective and less toxic anticancer agents remains one of the most pressing health problems. Looking for new ethics of treatment and novel chemotherapeutic agents with synthetic or natural origins is one of the hot topics in cancer research laboratories [11].

Material and Methods

Induction of Hepatocellular carcinoma

Inducing agent: 3,3'-Diamino benzidine was injected intra-

protineal as a little modification at 0.5 mg/kg body weight three time/week for two months [12].

Experimental animals

The present study was carried on the albino male rats, aging 12 weeks and weighting 120-150 g. All animals were maintained under the same normal laboratory conditions of temperature, humidity and being given free access of tap water and standard rat food. The first group was kept as a control group. The 2nd group of induced cancer rats with 3,3'-Diamino benzedine. While, the 3rd group of hepatocellular carcinoma induced rats HCC was administered with therapeutic dose (300 mg/kg. b.wt.) of OXD-T. In addition to, the 4th group of Cancerous albino rats was administered with half of therapeutic dose (150 mg/kg b.wt.) of OXD-T [13].

Drug

1,3,4-oxadiazole-2-thiol (OXD-T) was synthesized and investigated with IC50 (3.87 µg/ml) against liver cell line HepG2 respectively [14].

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Biochemical parameters

Human Protein Induced Vitamin K Absence or Antagonist-II (PIVKA-II) ELISA Kit [15,16]. A biomarker Lactate dehydrogenase LDH was determined according to De la Pena et al.; Rohaya et al. [17,18]. Also, routine liver function test parameters aspartate aminotransferase AST, alanine aminotransferase ALT, GGT and Albumin were relevant for monitoring chemotherapy hepatotoxic effects, were performed before and after the completion of the treatment. Their determination was performed using commercial biochemical tests according to Spectrum-diagnostics [19-23]. The values were expressed as the mean \pm SE for the 11 rats in each group. Differences between groups were assessed by one way Analysis of Variance (ANOVA) using [24].

Comet assay (Single Cell Gel Electrophoresis (SCGE))

DNA damage was measured using the comet assay under alkaline conditions and dim indirect light. The SCGE assay was performed essentially as described with some modifications [25].

The liver was excised, washed in saline solution and a small fragment of the liver was transferred to a Petri dish kept on ice. The fragment was washed, minced and suspended into 1 ml cold Hank's Balanced Salt Solution (HBSS) containing 20 mM EDTA and 10% Dimethylsulphoxide (DMSO). The fragment was cut into smaller pieces using a disposable microtome razor blade and the solution was aspirated. A fresh mincing solution was added and the liver samples were minced again into finer pieces. The suspension containing isolated cells was transferred to a tube maintained on ice until the preparation of the slides [26]. LMPA (in Ca²⁺ and Mg²⁺ free PBS) to prepare the final cell-agarose suspension. From the final cellagarose suspension, 80 μ l was spread over the microscope, pre-coated with 1% normal melting point agarose. The cells were then lysed in freshly prepared buffer containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 10.0), 1% Triton X- 100 and 10% DMSO for 24 h at 4°C. After lysis, the slides were rinsed three times in deionized water to remove salt and detergent. The slides were placed in a horizontal electrophoresis unit and DNA was allowed to unwind 20 min in alkaline solution containing 300 mM NaOH and 1 mM EDTA, pH > 13. The slides were then neutralized with 0.4 M Tris (pH 7.5), fixed for 5 min in absolute alcohol, air-dried and stored at room temperature. Immediately before analysis, the DNA was stained with 50 μ l ethidium bromide (20 μ g/ml).

Data scoring and photomicrographs

The fluorescent labeled DNA was visualized (magnification 400x) using an automated fluorescence microscope (Carl Zeiss, Germany) and the images were captured on a computer, equipped with Comet Score software (Komet IV). Three parameters were adopted as indicators of DNA damage: tail length (TL in μ m), % DNA in comet tail (% DNA in tail) and tail moment.

Results

Biochemical parameters

Human Protein Induced Vitamin K Absence (PIVKA-II): The current study demonstrated highly significant increase of Human Protein Induced Vitamin K Absence (PIVKA) between the HCC induced rats (5.379 \pm 0.102) and those of the control group (0.967 \pm 0.174) (Table 1 and Figure 1). While, the administration of therapeutic dose (300 mg/kg b.wt.) of OXD-T to adult HCC induced rats resulted highly significant decrease in the rate of PIVKA from the cancerous rats (1.041 \pm 0.246) compared with the cancerous group.

In addition to, the administration of half of therapeutic dose (150 mg/kg b.wt.) of OXD-T to adult HCC induced rats also, affected the rate of PIVKA in rats serums in this group (1.195 \pm 0.335) as they had highly significant decrease from those of the cancerous animals as an improvement of the efficiency of OXD-T on the treatment of Hepatocellular carcinoma.

Lactate dehydrogenase (LDH): Results of the study which were summarized in Table 2 and Figure 2, demonstrated highly significant differences of LDH between the HCC induced rats (3201 U/L \pm 196.6) and the control group (973 U/L \pm 10.4). This indicates that, the cancerous rats were with significant increase rate of tumor marker LDH. Furthermore, the administration of therapeutic dose (300 mg/kg b.wt.) of OXD-T to adult HCC induced rats decreased the rate of LDH

Group	PIVKA Elisa
Normal	0.967 \pm 0.174
Induced	5.379 \pm 0.102 ^a
Dose	1.041 \pm 0.246 ^b
Half dose	1.195 \pm 0.335 ^b

Table 1: Evidences of OXD-T doses administration on level of Human Protein Induced Vitamin K Absence (PIVKA-II).

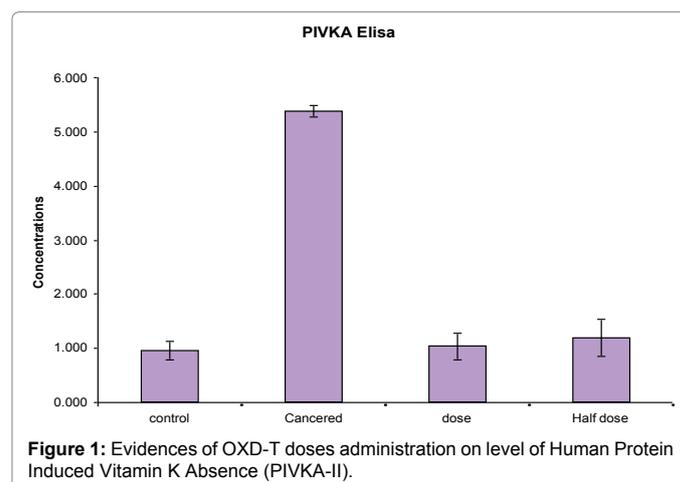


Figure 1: Evidences of OXD-T doses administration on level of Human Protein Induced Vitamin K Absence (PIVKA-II).

Group	LDH
Normal	973 \pm 10.4
Induced	3201 \pm 196.6 ^a
Dose	1737 \pm 123.9 ^{ab}
Half dose	3082 \pm 215.6 ^a

Table 2: Effect of OXD-T treatment on (LDH).

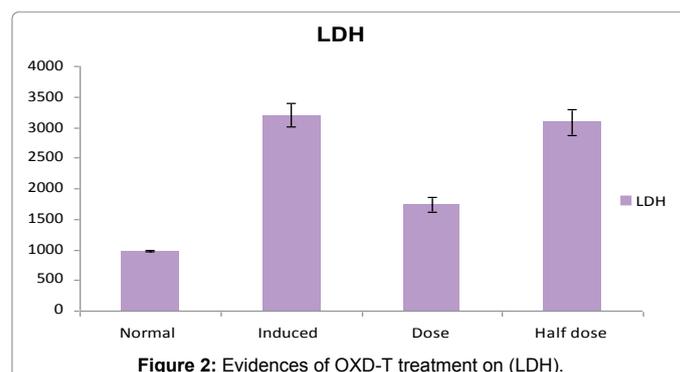


Figure 2: Evidences of OXD-T treatment on (LDH).

in animals serums of this group with significant difference (1737 U/L \pm 123.9) compared with the cancerous group. While, the administration of half of therapeutic dose (150 mg/kg b.wt.) of OXD-T to adult HCC induced rats, didn't affect the rate of LDH in rats serums in this group (3082 U/L \pm 215.6) as they had no significant difference comparing with those of the cancerous group of animals.

Liver functions: Comparison of pre and post-treatment values of tested biochemical parameters in these group of rats has shown that (OXD-T) doses administration led to significant increase in AST, ALT and GGT values of the cancerous rats serums than those of control group. While, the rate of albumin was not significantly affected by the chemical carcinogen (Table 3 and Figure 3).

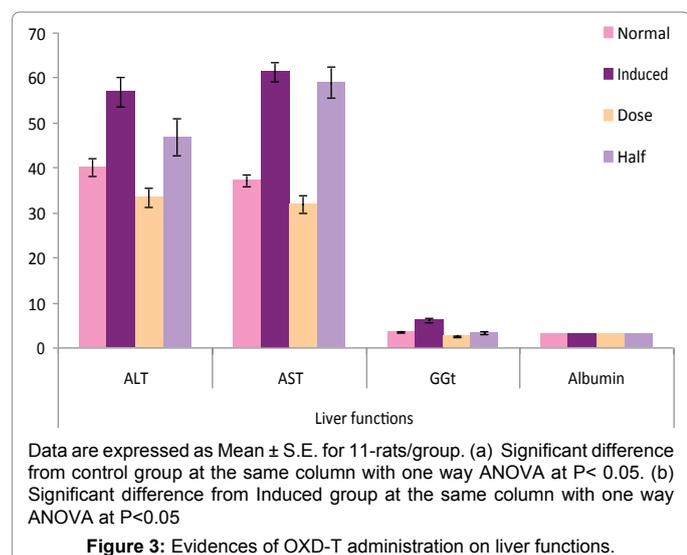
Regarding the HCC induced rats, highly significantly increased mean \pm SE levels of ALT (61.31 \pm 3.23), AST (56.81 \pm 2.28), GGT (6.16 \pm 0.45) and Albumin (2.95 \pm 0.09) of animals serums in this group. While, the administration of therapeutic dose of OXD-T resulted significantly decreased values of ALT (31.83 \pm 2.08), AST (33.4 \pm 1.96), GGT (2.48 \pm 0.15) and Albumin (2.98 \pm 0.09) from those of pre-treatment cancerous rats as a qualify activity of OXD-T treatment. In addition to, the administration of half therapeutic dose (150 mg/kg b.wt.) of OXD-T resulted mild significant difference of liver functions (ALT: 58.94 \pm 4.16, AST: 46.8 \pm 3.38 and GGT: 3.3 \pm 0.19) and Albumin level of (3.00 \pm 0.10).

Comet assay (single cell gel electrophoresis, SCGE): DNA damage was measured using the comet assay under alkaline conditions and dim indirect light. The fluorescent labeled DNA was visualized (magnification 400X) using an automated fluorescence microscope (Carl Zeiss, Germany) and the images were captured on a computer with Comet Score software.

Comet parameters were adopted as indicators of DNA damage;

Groups	Liver functions			
	AST	ALT	GGt	Albumin
Normal	40.13 \pm 1.35	37.24 \pm 2.05	3.56 \pm 0.18	3.07 \pm 0.04
Induced	56.81 \pm 2.28 ^a	61.31 \pm 3.23 ^a	6.16 \pm 0.45 ^a	2.95 \pm 0.09
Dose	33.4 \pm 1.96 ^{ab}	31.83 \pm 2.08 ^{ab}	2.48 \pm 0.15 ^{ab}	2.98 \pm 0.09
Half dose	46.8 \pm 3.38 ^b	58.94 \pm 4.16 ^a	3.3 \pm 0.19 ^b	3.00 \pm 0.10

Table 3: Biochemical values of liver functions.



fragmented and non-fragmented nuclei of liver tissues, the percentage of DNA in both head and tail of hepatocytes comet, tail length and tail moment. The present study investigated the parameters of comet assay in pre and post treatment with OXD-T to the HCC induced rats, in comparison with data obtained from normal liver tissues of the control group as shown in Tables 4 and 5 and separated parameters (Figures 4-8).

The percentage of DNA fragmentation in hepatocytes nuclei: The clear decrease in comet assay parameter related to the percentage of intact nuclei had shown in animals with HCC (33%), from normal rats in a control group (93%). The oral administration of therapeutic and half of therapeutic doses of OXD-T to the cancerous rats affected this comet parameter with high increase of non-fragmented nuclei percentage (89%) in therapeutic and (80%) in half dose treatment (Figure 4).

The percentage of DNA in head of comet: The percentage of DNA in comet head different within groups was determined (Figure 5), in comparison to the control animals (7.5%) as well as the rats with HCC had clearly increased in their nuclei components of DNA that improved the active replication of DNA in cancerous hepatocytes (11.29%). While, the post treated cancerous rats with therapeutic OXD-T has decreased rate of DNA in their head of comet (8.96%) from the HCC livers. Orally administration with half dose of OXD-T has almost similar rate of this parameter to that of cancerous hepatocytes (11.0%).

The percentage of DNA in tail comet: The percentage of DNA in tail of comet was investigated to show the fragmentation of nuclei components of DNA within groups (Figure 6). HCC rats were with the higher rate of DNA in their tail of comet than other groups (72%). OXD-T therapeutic administered animals had little decrease in this percentage (70.6%). Also, the half therapeutic dose seems to be similar to the cancerous group at this comet parameter (72.5%).

Tail length and tail moment of comet: Other comet parameters related to the tail; tail length and tail moment were determined (Figures 7 and 8). That exhibited HCC induced rats were with highly difference in these parameters (27.1, 4.54 pixel) from the control group 12.11, 2.58 pixel). The administration of therapeutic dose of OXD-T has better effect on these parameters than those of cancerous rats (22.3, 2.7 pixel). In contrast to half therapeutic administration didn't affect the rate of these comet parameters from the HCC rats (27.5, 4.3 pixel).

Discussion

The searchers daily discover novel antimetabolites to be more effective anticancer agents bind to target DNA base sequences. In recent years, 1,3,4-oxadiazoles have exhibited a wide range of biological properties including anti-bacterial, anti-viral, anti-fungal and anti-tumor agents [27]. This work as *in vivo* determination of anti-hepatic tumor activities of OXD-T that previously had been resulted; 3.87 (μ g/ml) for Liver cell line HepG2 [14]. The potential therapeutic benefits of OXD-T have been fully explored in hepatocellular carcinoma therapy than the half therapeutic dose treatment almost at all parameters of investigation.

In the present study, the biochemical findings supported the histopathological examination of animals liver tissues. Prothrombin induced by vitamin K absence-II (PIVKA-II) is also known as Des-gamma Carboxyprothrombin (DCP) is an abnormal prothrombin protein that is increased in the sera of patients with HCC. Generation of (PIVKA-II) is thought to be a result of an acquired defect in the post-

Groups	Parameters				
	Liver cell without fragmented DNA (%)	DNA in head of comet (%)	DNA in tail of comet (%)	Tail length (pixel)	Tail moment (pixel)
Normal	93 %	7.50 %	67.88 %	12.11	2.58
Induced	33 %	11.29 %	72.89 %	27.10	4.54
Dose	89 %	8.96 %	70.69 %	22.30	2.78
Half dose	80 %	11.00 %	72.50 %	27.0	4.30

Table 4: Effect of different doses of OXD-T on DNA integrities.

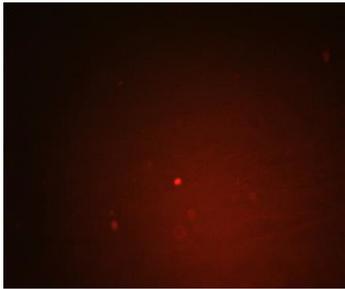
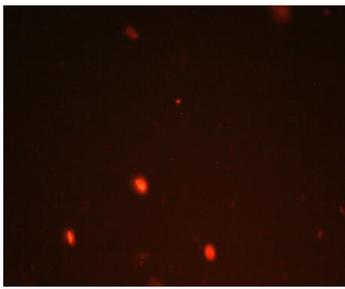
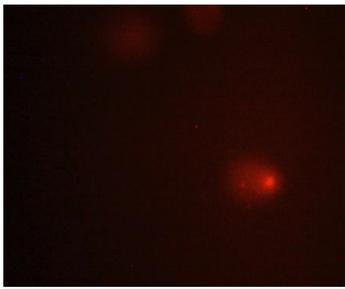
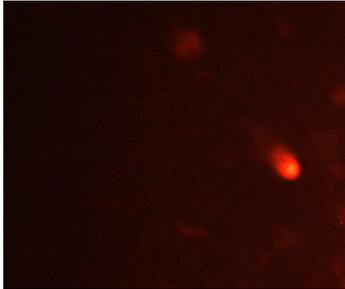
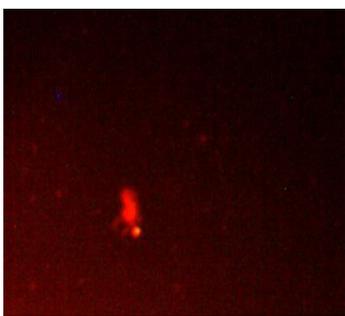
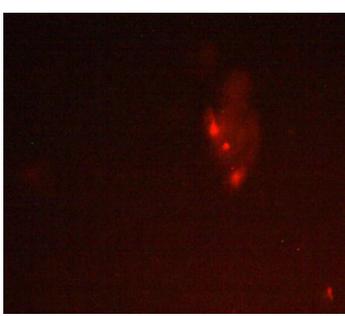
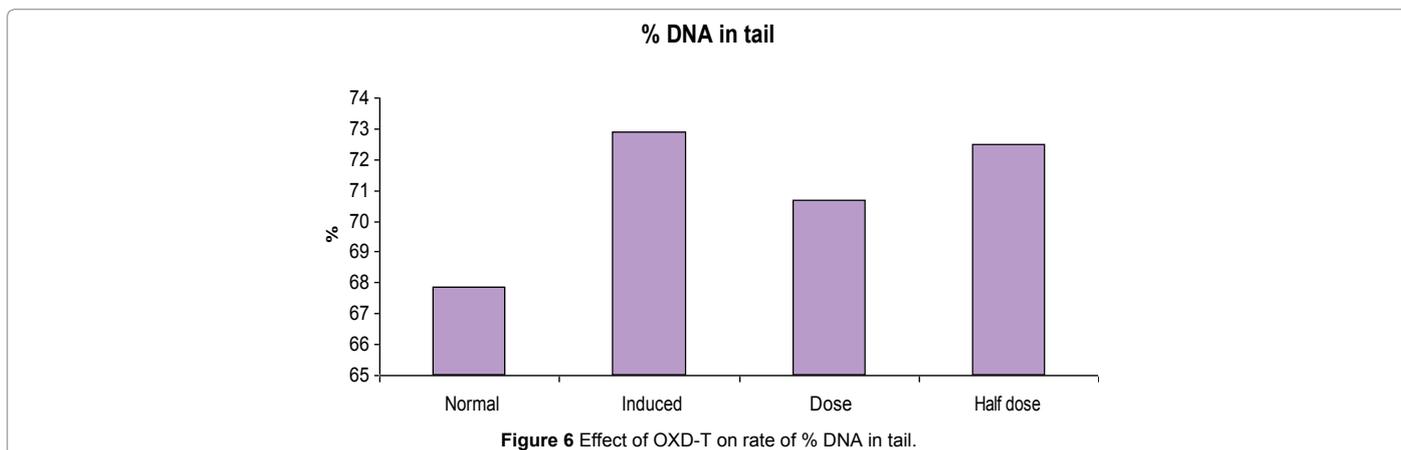
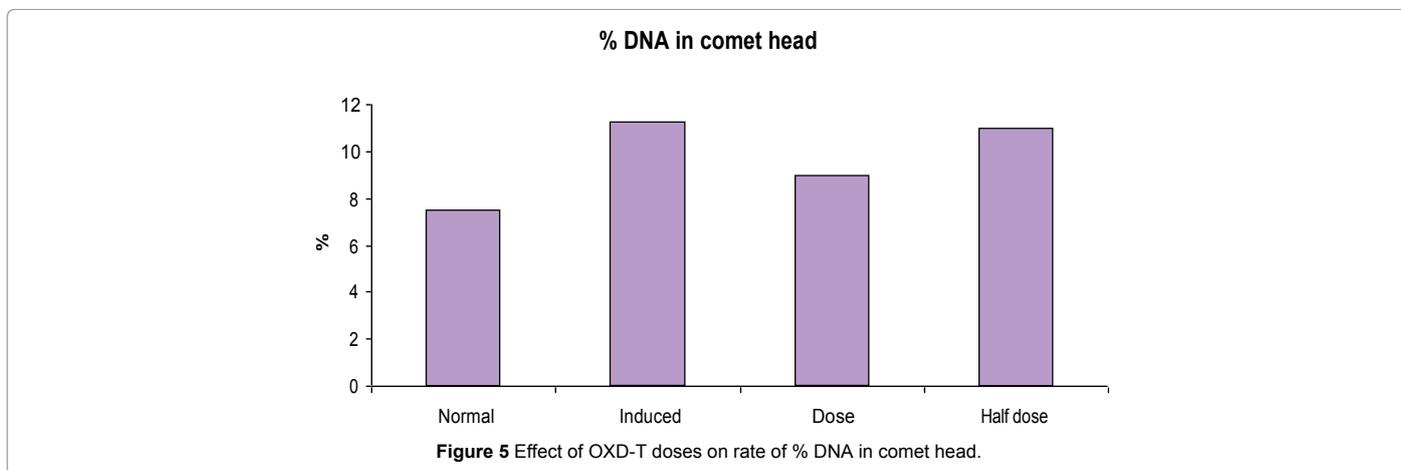
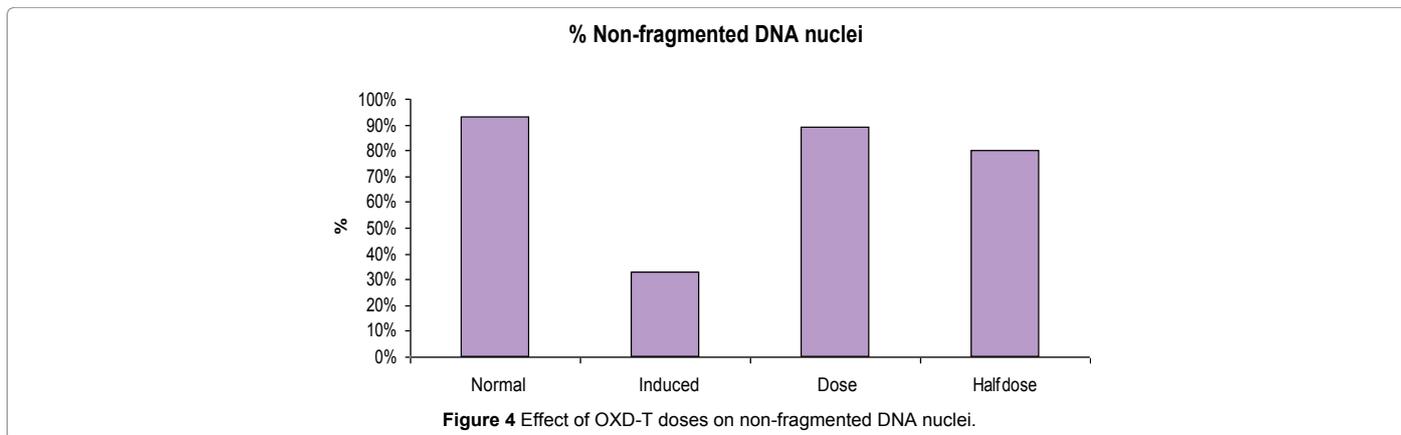
Control		
HCC induced		
Therapeutic dose treatment		
Half dose treatment		

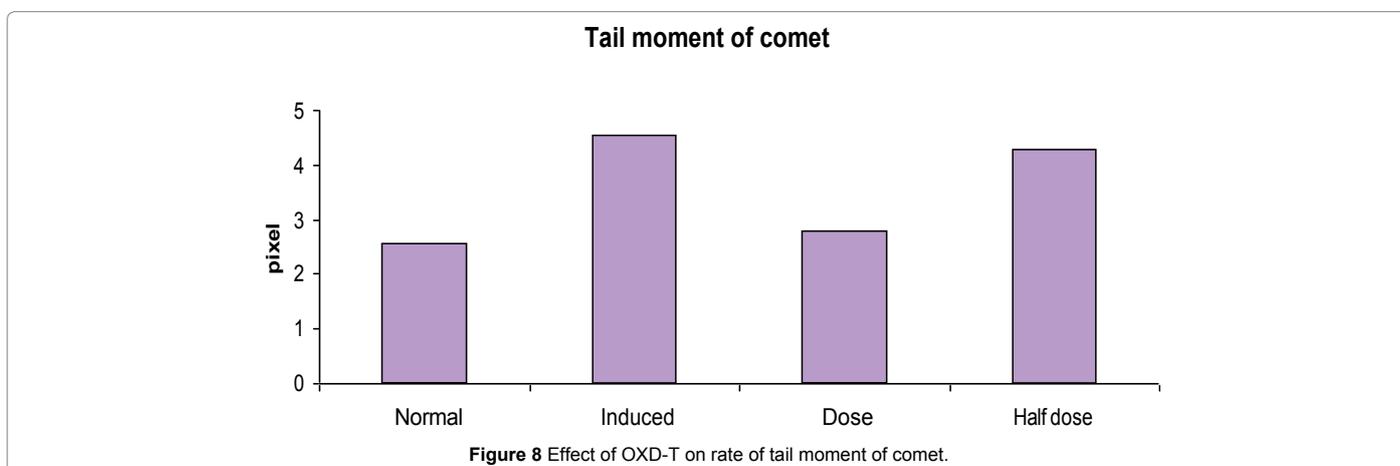
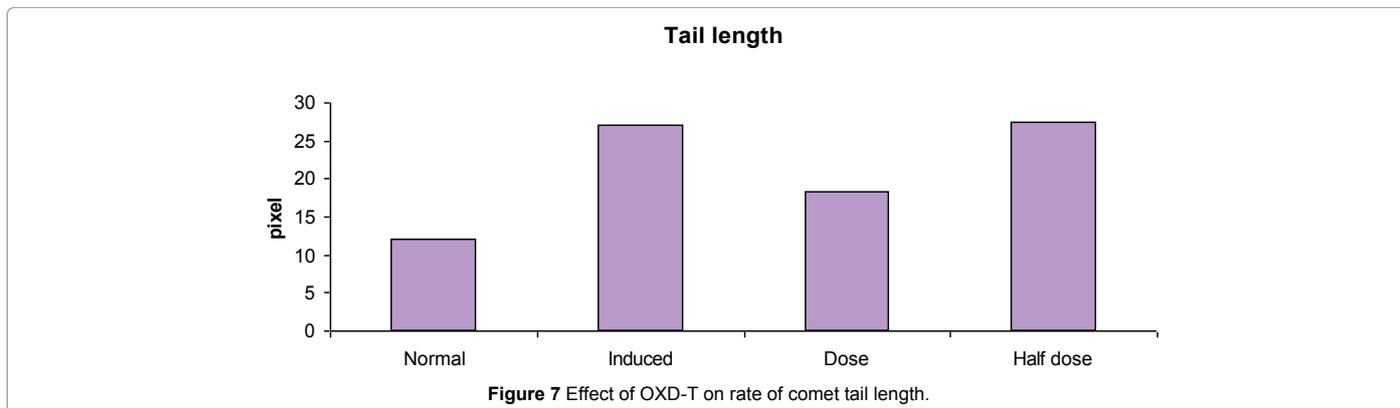
Table 5: Effect of different doses of OXD-T on DNA integrities of rats liver cells.



translational carboxylation of the prothrombin precursor in malignant cells [28]. The validity of PIVKA-II as a tumor marker for HCC patients has been reported by many investigators [29].

The current work was designed to determine the level of prothrombin induced by vitamin K absence-II (PIVKA-II), the most common predisposing factor for HCC. This work demonstrated highly significant increase of (PIVKA) of HCC induced rats from the control group. The administration of therapeutic dose of OXD-T to adult HCC induced rats resulted highly significant decrease in the rate of PIVKA from the cancerous rats. While, half dose of OXD-T treatment

to adult HCC induced rats also, had highly significant decrease from those of the cancerous animals as an improvement of the efficiency of OXD-T on the HCC treatment. This discovery is in agreement with a correlation study which demonstrated the concentration of PIVKA-II was markedly reduced within 2 weeks after treatment in patients with HCC showing a favorable response [16]. Also, other work conducted on 72 patients and 11 healthy individuals as control, patients were initially subjected to complete clinical examination and abdominal ultrasonography. That study determined the direct relation between the level of PIVKA-II and the size of tumor makes it an attractive tool for early HCC diagnosis and surveillance [30]. Furthermore, multiple



studies demonstrated that PIVKA-II is more accurate than AFP for diagnosis of HCC. PIVKA-II was positive in 96%, 93% and 74% in patients with tumor size larger than 5, 3–5, and less than 3 cm while AFP was positive in 65%, 57% and 48% respectively [31]. Moreover, other investigation reported that PIVKA-II is more sensitive than AFP for differentiating HCC from other benign liver diseases [32].

A biomarker lactate dehydrogenase (LDH) might be a molecule secreted by a malignancy, or it can be a specific response of the presence of cancer. LDH serum levels as prognostic factor investigated in early HCC patients treated with hepatic resection. The current study demonstrated highly significant increase of LDH levels between the HCC rats. Furthermore, the administration of therapeutic dose of OXD-T decreased the rate of LDH in animal's serums from those of HCC induced rats. Otherwise, the half dose of treatment didn't significantly differ from cancerous rats. Other investigation illustrated that patients undergoing trans-arterial chemo-embolization (TACE) had levels of LDH seemed able to predict clinical outcome for HCC. The correlation between LDH levels and tumor angiogenesis investigated patients with high LDH levels may be optimal candidates for clinical trial [33].

Highly significant increase in liver functions was determined in serums of HCC rats, from those of normal animals. The main effect of the therapeutic OXD-T administration in the current work was on the biochemical parameter as it highly decreases the levels of liver functions in serums of rats administered and that decrease was less significant with half dose of drug administration. The present is contrary with that investigation of pre and post-treatment values of tested biochemical parameters in group of patients, conforming that anticancer agents

led to the statistically significant increase in AST, ALT and bilirubin levels. While, the GGT parameter matched with current work as well as it showed statistically decreased values in patients serums than those pretreated with anticancer agent [34].

Moreover, several lines of evidences discovered some oxadiazole derivatives which acted as inhibitors and activators of ALT and AST activities, this action showed not proportionate with the increase of their concentrations [35]. While, other retrospective study on 200 patients treated with curative hepatic resection, evaluated several serum and clinical factors collected at baseline before treatment. Patients were divided according to the median Recurrence Free Survival (RFS) in Early Recurrence group (ER) and non-early recurrence group (non-ER). At multivariate analysis, independent adverse prognostic factors; LDH, AST/ALT ratio, alpha-fetoprotein AFP identified significant differences for early recurrence [36].

Current therapeutic OXD-T treatment prevented active DNA replication of hepatocytes nuclei, affecting the growth of the disease. Comet assay parameters investigation improved the effect of OXD-T administration on the percentage of the fragmented and non-fragmented nuclei matched the control group.

Also, the percentage of DNA in comet heads was investigated with high level in HCC rats, decreased with drug administrations. Comet tail parameters of tail length, tail moment and % DNA in tail also investigated in this study with significant decreased values compared to HCC animals. The percentage of DNA in tail is the most preferred parameter because it covers a wide range of damage. While, the half dose of OXD-T had no significantly decrease from cancerous hepatocytes.

Our findings understand that demonstration of compounds with a specific arrangement of the heteroatoms in the oxadiazole ring, showed positive effects in at least one strain. Based on those investigations it was possible to select pharmacologically active structures without mutagenic liability [37]. In addition to, previously stated DNA fragmentation (apoptotic phenomenon) in Hep-2 cells treated with oxadiazole derivative, the staining procedures using ethidium bromide and propidium iodide showed apoptotic bodies in cells of oxadiazole derivative treatment [38].

Conclusion

1,3,4-oxadiazole-2-thiol (OXD-T) antimetabolite with therapeutic dose of administration affected the growth of hepatocellular carcinoma. Oxadiazole derivatives still novel anticancer agents as well as their biological effects on the health of populations have to be much discovered.

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