

HEPATOTOXIC EFFECT OF 17-OXIMINO-5-ANDROSTEN-3 β -YL BENZOATE ON RATS

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ABSTRACT

Introduction and positioning of the hydroximino group on the parental steroid skeleton has significant effect on the activity profile of steroidal compounds. Earlier reports described the synthesis and evaluation of 17-Oximino-5-androsten-3 β -yl ester derivatives as potential cytotoxic agents on liver cancer cell lines (Hep-2) using Sulphorodamine B⁶ assay. The 17-Oximino-5-androsten-3 β -yl benzoate displayed significant cytotoxicity against liver as compared to standard reference drugs. The current study was undertaken to investigate further the *in vivo* effect of the synthesized derivative on the liver of rats following one month treatment. Male SD rats were divided into two groups viz: normal control and 17-Oximino-5-androsten-3 β -yl ester derivative treated (dose 10mg/Kg body weight, intraperitoneally). After one month of treatment, the hepatotoxicity was assessed by estimating the levels of hepatic marker enzymes, oxidative injury and histopathological studies in the liver of control and treated rats. The study revealed a significant increase in the levels of glutamic oxaloacetic transaminase (GOT/AST), glutamic pyruvic transaminase (GPT/ALT) and alkaline phosphatase (ALP) in the serum and liver of 17-Oximino-5-androsten-3 β -yl benzoate treated rats when compared to control animals. Further, increased lipid peroxidation (LPO) was accompanied by a decrease in glutathione (GSH) level. However, the activities of glutathione-s-transferase, (GST), superoxide dismutase (SOD) and catalase were found to increased significantly in the treated group. Histopathological observations also indicated marked alterations in the histoarchitecture of liver in response to 17-Oximino-5-androsten-3 β -yl ester derivative treatment. In conclusion, the synthesized compounds possess hepatotoxicity and exerts its cytotoxic action by oxidative cell injury.

Keywords: Cytotoxicity, Liver cancer cell lines, Hydroximino steroids, *In Vivo* hepatotoxicity, oxidative stress.

INTRODUCTION

The steroid system, selected by the evolutionary process to perform some of the most fundamental biological functions, has not only inspired the biochemists and endocrinologists, but has also become the basis of the most phenomenal developments in medicinal chemistry. Introduction and positioning of the hydroximino group on the parental steroid skeleton has significant effect on the activity profile of these compounds. Several 6-hydroximino [Fig.1(1-4)],

7-hydroximino [Fig.1(5,6)] and hydroximino derivatives of 16E-arylidensteroids in the androstane and estrane series [Fig.1(7)] have been synthesized and reported as potent cytotoxics and aromatase inhibitors (1-5). Recent work from our laboratory described the synthesis of 17-Oximino-5-androsten-3 β -yl benzoate and its evaluation as antiproliferative agents and 5 α -reductase inhibitors (6). This steroid has displayed significant antiproliferative activity

compared to reference drug finasteride, against human prostate cancer cell line with $3.8\mu\text{M}$ IC_{50} and significant increase in the serum androgen level of testosterone has been found as compared to control (7). These results are consistent with the earlier observation that steroids bearing an oxime group are capable to form a coordinate bond with heme iron of hemoprotein of P-450 enzyme (8).

In vitro cytotoxicity test using cancer cell lines in the preliminary evaluation of cytotoxic agents enables us to select most potent compound, but cytotoxic agents, however, frequently exhibit unspecific toxicity. Nevertheless, the ability to selectively kill the target cell remains a highly desirable property of potential new therapeutic cytotoxic agents (9). *In vitro* toxicity of newly synthesized compound was tested with Red dye uptake (MTT) assay (10). The assay quantifies the viable cells, after 24 h incubation of cells with five different concentrations. The results obtained from MTT assay were statistically significant ($P < 0.001$) and linear equation obtained allowed us to determine toxicity index (LC_{50}). The high oral lethal dose (LD_{50}) to mouse macrophages of $89.4\mu\text{M}$ has been established in wistar rat. Further, as a continuation of our research concerning hydroximino group and its significance, the potential cytotoxic effect on liver cancer cell lines (Hep-2) using Sulphorodamine B⁶ assay has also been reported (11). The aim of the current study is to investigate further the *in vivo* effect of the 17-Oximino-5-androsten-3 β -yl benzoate on the liver function and histology of the liver of the rats following one month treatment.

MATERIAL AND METHODS

Chemicals and Equipment

All the chemicals used for the study were of analytical grade. Nicotinamide adenine dinucleotide phosphate-oxidase (NADPH), reduced glutathione (GSH), 1, chloro-2, 4-dinitrobenzene (CDNB) and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), were purchased from Sigma Chemicals Co. The Ultraviolet

spectrophotometer (UV) from Beckman Company was used for the enzymatic estimations.

Chemistry

For the syntheses of compounds (14), 17-oximino-5-androsten-3 β -ol (13) was used as starting material. Recently published reports from our laboratory described the synthesis of 13 from commercially available (25R)-5-spirosten-3 β -ol (Diosgenin) (8) according to the literature (Scheme 1) (12-14). 17-Oximino-5-androsten-3 β -yl benzoate (14) was prepared by treating 3 β -hydroxyl function with benzoic acid in dichloromethane in the presence of dicyclohexylcarbodiimide (DCC). In the esterification reaction, DCC acts as dehydrating agent which forms an O-acylurea called acid-DCC complex, similar to an acid anhydride or acyl halide. This is followed by attack of alcohol on carboxylic carbon of acid-DCC complex, as a nucleophilic catalyst to give esters and dicyclohexyl urea as side product (15).

Animals

Female Wistar rats weighing 150 ± 200 g were procured from the Central Animal House, Panjab University, and Chandigarh. The animals were housed in polypropylene cages in the departmental animal house under hygienic conditions and were acclimatized for at least one week before putting them on different treatments. They were allowed free access to both food and water available *ad libitum*. All procedures were done in accordance with ethical guidelines for care and use of laboratory animals and were approved by the ethical committee on Experimental Animals Committee.

Experiment Design

After acclimatization, the animals were segregated into two different groups with 7 rats in each group. Animals in the group I were fed standard laboratory feed and water *ad libitum* throughout the period of experimentation. Animals in group II received intraperitoneal treatment of 17-Oximino-5-androsten-3 β -yl benzoate (14) at a dose level of 10mg/kg body weight for a duration of 1 month.

Biochemical estimations

Preparation of Tissue Homogenate

After the completion of different treatment duration, the animals were fasted overnight and euthanized under light ether anesthesia. The livers were removed and homogenized in potassium phosphate buffer (pH 7.5) using mechanically driven Teflon fitted Potter Elvehjem homogenizer. Tissue homogenates were centrifuged at 10,000 g for 30' at 4°C and the supernatant was used for the estimation of different enzymes and lipid peroxidation.

Protein

Protein assay was done by the method of Lowry *et al* (16). Briefly, 20µl of samples were diluted with 100 mM phosphate buffer (pH 7.5) to a volume of 0.5 ml. 5 ml of prepared reagent (48 ml of 2% sodium carbonate, 1 ml of 1% copper sulphate and 1 ml of 2% sodium potassium tartarate) was then added and allowed to stand for 10' at room temperature. 0.25 ml of 1N Folin Ciocalteu's reagent was added and allowed to stand for 30' at room temperature. The absorbance was measured at 680 nm on double beam spectrophotometer.

Liver Marker Enzymes

Transaminases

Activity of transaminases i.e aspartate aminotransferase (AST or GOT) and alanine aminotransferase (ALT or GPT) were estimated in serum and liver by the method of Reitman and Frankel (17). For AST/GOT estimation, 100µl of homogenate/serum was added to 0.5 ml solution of buffered substrate (200mM DL-aspartic acid and 2mM α -ketoglutaric acid in 0.2M phosphate buffer, pH 7.4) followed by incubation at 37°C for 60'. Reaction was terminated by the addition of 0.5ml of 1mM 2, 4-dinitrophenyl hydrazine (DNPH) and the tubes were allowed to stay at room temperature for 20'. Finally, 5.0ml of 0.4N sodium hydroxide (NaOH) was added for the development of color, which was read at 510nm. For estimation of ALT/GPT, the assay procedure was essentially the same as that for AST/GOT, except for the use of a different buffered substrate (200mM DL-alanine and 2mM α -ketoglutaric acid in 0.2M phosphate buffer, pH 7.4).

Alkaline phosphatase (ALP)

The effect of synthesized compound **14** on the ALP levels was carried out by the method of Bergmeyer (18). Briefly, 50 µl of homogenate/serum was added to 1ml buffered substrate (0.5M Glycine buffer containing 5.5×10^{-3} M p-nitrophenyl phosphate, pH-10.5) and the mixture was incubated for 15 minutes at 37°C. Enzyme reaction was terminated by adding 3.5 ml of 0.1N NaOH and the optical density of the liberated p-nitrophenol was read at 420 nm.

Antioxidant Defense System Enzymes

Lipid peroxidation assay was done by the method of Wills (19). Briefly, 0.5 ml of sample was diluted to 1.0 ml with ice cold 10% TCA (trichloro acetic acid) and centrifuged at 800g for 10 minutes. To 0.5 ml of supernatant, 0.5 ml of 0.67% TBA (thiobarbituric acid) was added and color was developed by boiling at 100° C for 10'. Absorbance was read at 532nm and the amount of malondialdehyde formed was calculated on the basis of molar extinction coefficient of MDA-TBA chromophore ($1.56 \times 10^5 \text{ m}^{-1} \text{ cm}^{-1}$).

Reduced Glutathione (GSH)

Estimation of GSH was performed by the method of Ellman *et al* (20). 0.1 ml of 25% TCA was added to 0.5 ml of sample and centrifuged at 2000 g for 15'. 0.5 ml of supernatant was diluted in a test tube to 1.0 ml with 0.2 M sodium phosphate buffer (pH 8.0). Added 2.0 ml of freshly prepared 10mM DTNB made in 0.2 M phosphate buffer (pH 8.0) and optical density of the yellow coloured complex formed by the reaction of GSH and DTNB (Ellman's reaction) was measured at 412 nm.

Glutathione-S-Transferase (GST)

GST enzyme was determined as according to the method of Habig *et al* (21), using CDNB as a substrate for GST. The increase in absorbance due to the formation of glutathione conjugates by GST was measured in double beam spectrophotometer at 340nm.

Catalase

Assesment of liver function i.e catalase was estimated by a reported method of Luck (22). The reaction mixture contained 50mM potassium

phosphate buffer (pH 7.0), H_2O_2 at a concentration whereby it shows an optical absorbance of 0.5, and appropriate amount of liver homogenate. The blank in reference cuvette lacking H_2O_2 were also run simultaneously. Decrease in absorbance at 240 nm was measured and the amount of H_2O_2 decomposed was calculated using the molar extinction coefficient of H_2O_2 ($0.71 \text{ M}^{-1} \text{ cm}^{-1}$).

Super oxide dismutase (SOD)

The activity of superoxide dismutase was assayed by applying the method of Kono (23). The reaction mixture contained 1.3 ml of 50 mM sodium carbonate solution with 0.1 mM EDTA (pH 10.0), 0.5 ml of 96 μM of nitroblue tetrazolium (NBT) and 0.1 ml of 0.6% Triton-X-100. Reaction was initiated by the addition of 0.1ml of 20 mM hydroxylamine hydrochloride (pH 6.0) and appropriate amount of sample and the rate of NBT reduction was recorded using a spectrophotometer. One unit of enzyme was expressed as inverse of the amount of protein (mg) required to inhibit the reduction rate of NBT by 50%.

Histopathological Studies

For the histopathological observations at light microscopic level, fresh tissue pieces of liver were immersed and fixed in 10% phosphate buffered formalin. Following an overnight fixation, the specimens were dehydrated in ascending grades of alcohol, cleared in benzene and embedded in paraffin wax. Blocks were made and 5-7 μm thick sections were double stained with hematoxylin and eosin and observed under light microscope.

Statistical Analysis

The results are represented as a mean value \pm S.D. The statistical significance of the data has been determined using Independent T-test. P values of $< 0.05\%$ were considered significant.

Observations

The results of all the experiments conducted during the current study are depicted in Tables 1-5. Following table compares the results of treatment group with their normal controls.

Body Weight

The body weights of the control and treated animals are shown in Table 1. It was observed, that the body weights of the normal, control and treated rats increased progressively throughout the study. However, the net body weight of the animals treated with 17-Oximino-5-androsten-3 β -yl benzoate did not show any statistical difference when compared to the control animals. No appreciable change in food consumption was observed among different groups of rats.

Liver Marker Enzymes

Activity of ALP and transaminases i.e AST/GOT and ALT/GPT has been found to be significantly increased in the serum as well as in the liver of 17-Oximino-5-androsten-3 β -yl benzoate treated rats following 1 month treatment when compared to the control group (Table 3, 4). Further, administration of compound 14 orally to the rats at dose of 10mg/kg body weight for 15 days showed significant increase in ALP and ALT level in serum when compared with the control group (Table 2).

Antioxidant Defense System Enzymes and Lipid Peroxidation

17-Oximino-5-androsten-3 β -yl ester treatment to rats for duration of one month showed a significant increase in hepatic LPO. However, a significant decrease was witnessed in the levels of GSH and activities of SOD, catalase and GST in the liver of rats following 17-Oximino-5-androsten-3 β -yl ester treatment when compared to control group.

Histopathological Findings

Histopathological findings of liver from the treatment group are represented in Fig 1 and 2. Normal control animals revealed clear cut hexagonal array of hepatic plates radiating towards periphery from a central vein. The hepatic sinusoids were quite clear between the laminae. The hepatocytes showed normal polyhedral appearance.

Compound 14 treated animals indicated mild alterations in hepatic histoarchitecture after one month treatment. Though the hepatocytes exhibited lamellar pattern of appearance, still the

sinusoidal spacing was comparatively widened in these animals in comparison to the normal controls. Some degree of hepatic hypertrophy and binucleated cells was also evident.

DISCUSSION

The transaminases/aminotransferases are recognized as the most specific biomarkers of liver injury, that catalyses the reversible exchange of amino acids between alpha-amino and alpha-keto acids and functions at the junction between the metabolism of proteins and carbohydrates (24). During the course of current investigation, a marked increase in the levels of both transaminases i.e aspartate aminotransferase (AST or SGOT) and alanine aminotransferase (ALT or SGPT) has been observed in the serum as well as in the liver of 17-Oximino-5-androsten-3 β -yl benzoate treated. The findings are clearly suggestive of hepatic injury, resulting in the spillage of enzymes into the blood stream, raising the enzyme levels in the blood. Further, the activity of alkaline phosphate alluded a significant rise following compound 14 treatment to rats. This could be due to the activated macrophages including the kupfer cells, which are considered as the cellular source for the increased level of alkaline phosphatase in conditions of liver damage. Also, since the mucosal cells that line the bile system of the liver are the source of alkaline phosphatase, the free flow of bile through the liver and down into the biliary tract are responsible for maintaining the proper level of this enzyme in the blood. This indicates further that the liver or the bile ducts are not functioning properly or are blocked as a result of the damage inflicted by 17-Oximino-5-androsten-3 β -yl benzoate, and thus the enzyme is not excreted through the bile and is released into the blood stream.

In the present study, compound 14 treatment to normal rats indicated a marked increase in the hepatic LPO, implicating the peroxidation of polyunsaturated fatty acids (PUFA), resulting in the degradation of phospholipids, an index of cellular deterioration. The observed increase in the LPO in the present study is concomitant with a remarkable decrease in GSH levels in 17-

Oximino-5-androsten-3 β -yl ester intoxicated animals. Various studies supports the fact that GSH shares an association with lipid peroxidation whereby it has been observed that depletion of GSH enhances LPO (25-27). Since, GSH has a strong electron donating capacity, therefore in conditions of oxidative stress, glutathione is consumed by the glutathione related enzymes to detoxify the peroxides and hence the increased peroxidation in the study is a consequence of depleted GSH stores. Moreover, a statistically significant inhibition in the GST activity was noticed in the 17-Oximino-5-androsten-3 β -yl benzoate. Since, GST catalyze the conjugation of GSH to xenobiotic substrates and thus detoxifies endogenous compounds such as peroxidised lipids and enables the breakdown of xenobiotics, therefore the inhibition in the activity of GST is in conformity with the increased levels of lipid peroxidation, where the GST is participating in mediating the toxicity due to steroidal ester (14) treatment. The observed increase in the levels of SOD following 17-Oximino-5-androsten-3 β -yl benzoate treatment indicates, increased formation of oxygen free radicals, which stimulate SOD activity to thwart the toxicity of these free radicals in conditions of oxidative stress. Further, catalase detoxifies the H₂O₂ derived from SOD and varies its affinity for interaction with substrates. In the current study, we have observed low levels of catalase activity following 17-Oximino-5-androsten-3 β -yl benzoate (14) treatment, which is owed to the utilization of this enzyme activity in converting the H₂O₂ to H₂O. Since at low concentrations, the hydrogen peroxides are preferentially metabolized by GSH-Px because of its lower K_m for H₂O₂, thus, our observed significant inhibition of catalase activity further stipulates that oxidative stress due to compound 14 treatment was immense, that it could acutely suppress catalase activity. In the present study, the hepatic histoarchitecture following 17-Oximino-5-androsten-3 β -yl benzoate treatment showed marked changes viz: increased vacuolization, binucleated cells and widened sinusoidal space in comparison to normal rats. This could be owed to the basis that

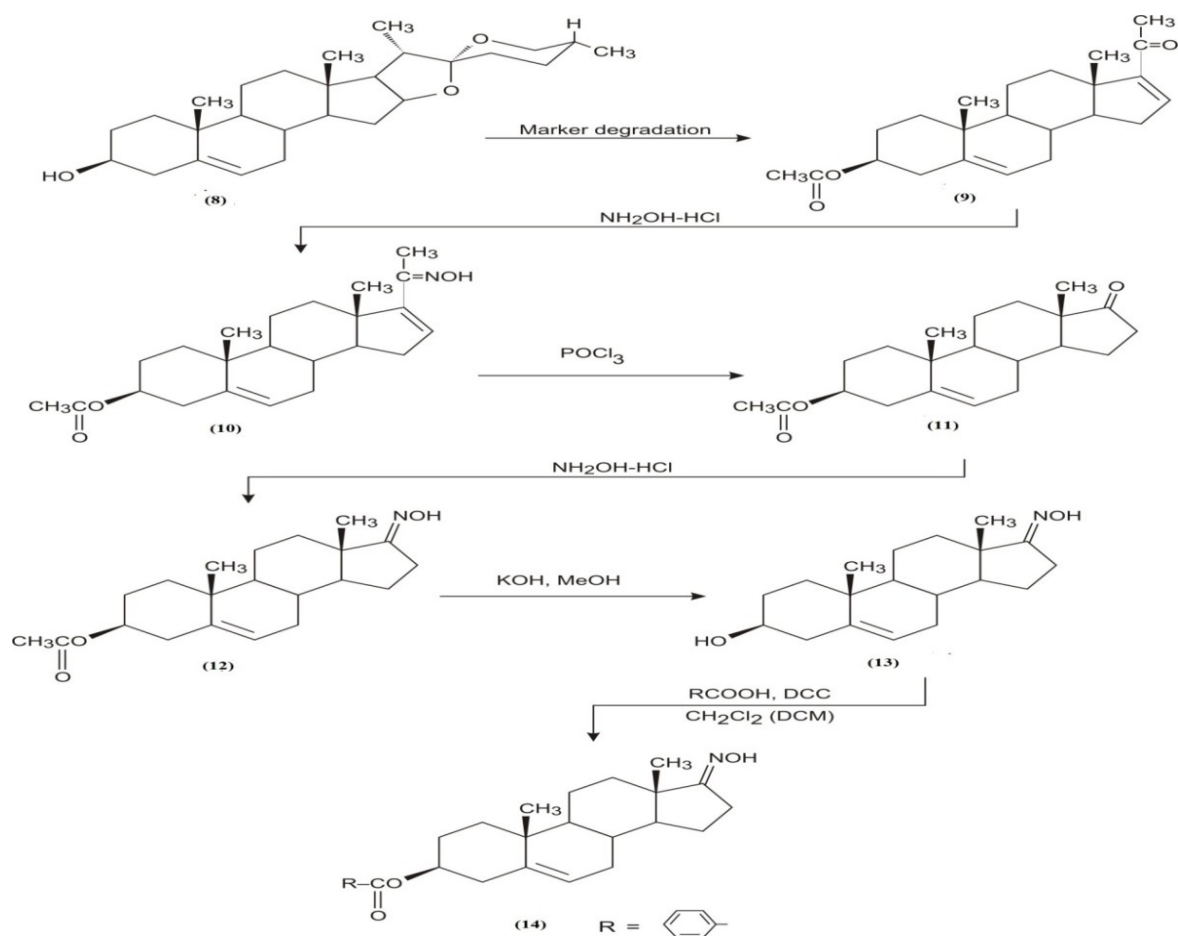
compound 14 manifested its toxic effects primarily by the generation of reactive oxygen species. Because of the increased lipid peroxide levels, the membranous components of the cells got damaged, resulting in the observed necrotic changes. Thus, the altered hepatic histoarchitecture following 17-Oximino-5-androsten-3 β -yl benzoate treatment are in corroboration with the observed biochemical changes, wherein an increased level of lipid peroxidation and hepatic marker enzymes was noticed, which are indicative of hepatic injury.

CONCLUSION

In conclusion, we described the synthesis of the 17-Oximino-5-androsten-3 β -yl benzoate. The compound was evaluated for biochemical parameters and histopathology of liver. It was observed that the steroidal ester derivative (14) possess a potential for hepatotoxicity and exerts

its cytotoxic action by oxidative cell injury as evident from alleviated levels of enzymes of antioxidant defence system and altered hepatic histoarchitecture. The study compliments the previous *in vitro* findings on the cytotoxic effects of the synthesized derivative and warrants further exploration with regard to other definitive bioassays including protein expression and documentation of specific molecular markers to establish the exact mechanism of 17-Oximino-5-androsten-3 β -yl benzoate mediated hepatotoxicity. Based upon the *in vitro* and *in vivo* findings with 17-Oximino-5-androsten-3 β -yl benzoate, the study also provides a rationale for futuristic trials on its chemotherapeutic potential in experimental liver carcinogenesis.

Declaration of interest statement: The authors report no declaration of interest.



Scheme-1

Table 1: Net body weight of rats subjected to 17-Oximino-5-androsten-3 β -yl benzoate treatment

Groups	Body Weight
Normal Control	206 \pm 29.66
17-Oximino-5-androsten-3 β -yl ester treated	182 \pm 13.03

Table 2: Hepatic marker enzyme status in the serum of rats subjected to 17-Oximino-5-androsten-3 β -yl benzoate treatment (15 days)

Group	ALP (μ moles of p-nitrophenol produced/min/L serum)	ALT (μ mol pyruvate formed /min/L serum)	AST (μ mol pyruvate formed/min/L serum)
Control	26.14 \pm 1.07	41.01 \pm 4.10	25.02 \pm 3.12
17-Oximino-5-androsten-3 β -yl benzoate treated	44.16 \pm 3.25 ^b	52.95 \pm 3.56 ^a	32.25 \pm 5.47

Values are expressed as mean \pm S.D. by Independent T-test ; ^aP \leq 0.01 and ^bP \leq 0.001 in comparison to control group

Table 3: Hepatic marker enzyme status in the serum of rats subjected to 17-Oximino-5-androsten-3 β -yl benzoate treatment (1 month)

Group	ALP (μ moles of p-nitrophenol produced /min/L serum)	ALT (μ mol pyruvate formed /min/L serum)	AST (μ mol pyruvate formed /min/L serum)
Control	32.28 \pm 6.84	54.06 \pm 4.80	40.44 \pm 4.78
17-Oximino-5-androsten-3 β -yl benzoate treated	59.13 \pm 4.96 ^b	72.12 \pm 5.02 ^a	52.18 \pm 4.28 ^a

Values are expressed as mean \pm S.D. by Independent T-test ; ^aP \leq 0.01 and ^bP \leq 0.001 in comparison to control group

Table 4: Hepatic marker enzyme status in the liver of rats subjected to 17-Oximino-5-androsten-3 β -yl benzoate treatment (1 month)

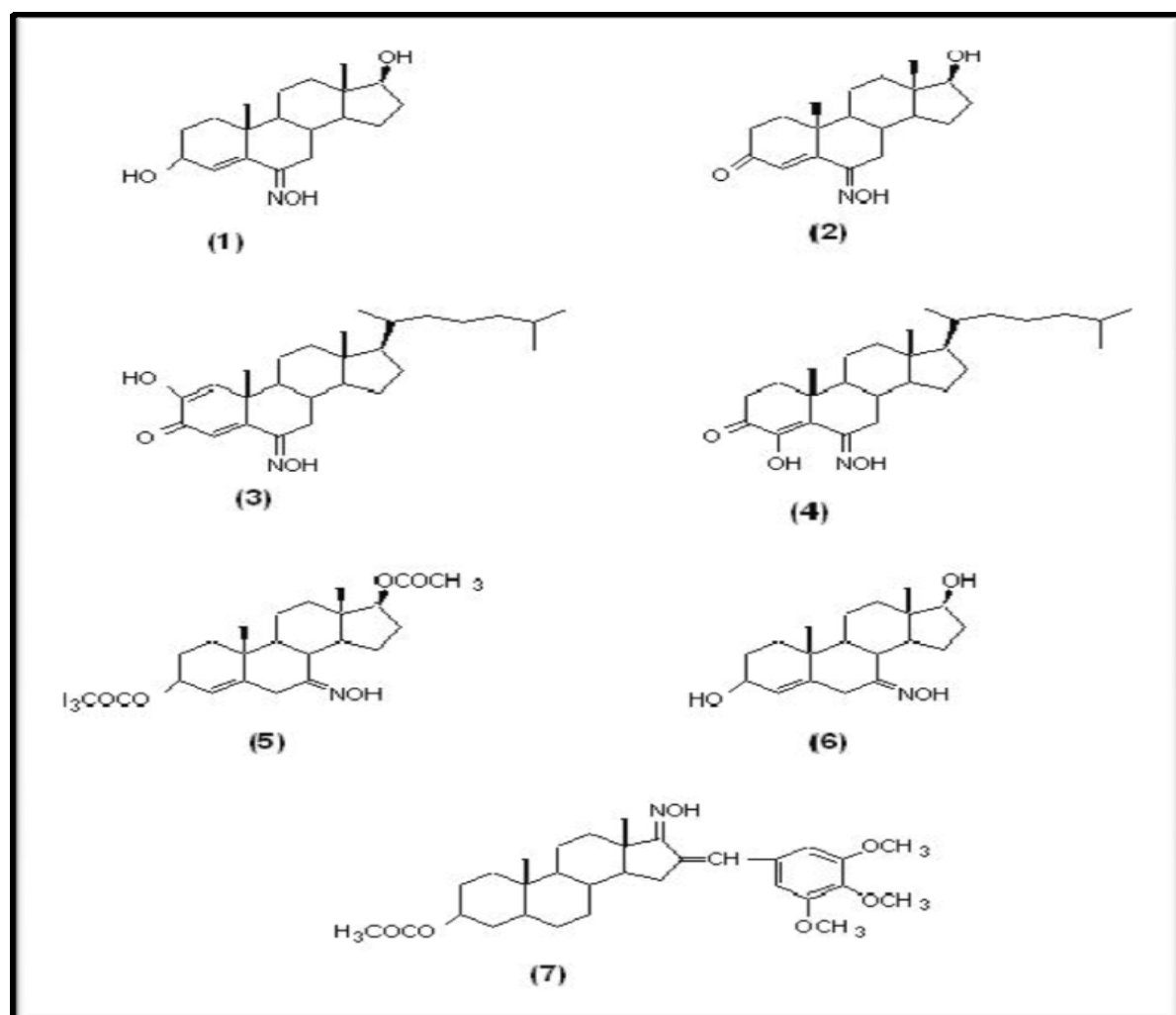
Group	ALP (nmol of p-nitrophenol produced/min/mg protein)	ALT (nmol pyruvate formed min ⁻¹ mg ⁻¹ protein)	AST (nmol pyruvate formed min ⁻¹ mg ⁻¹ protein)
Control	7.93 \pm 1.47	6.06 \pm 1.10	4.66 \pm 1.83
17-Oximino-5-androsten-3 β -yl benzoate treated	13.17 \pm 1.33 ^b	12.2 \pm 2.48 ^c	10.34 \pm 1.06 ^b

Values are expressed as mean \pm S.D. by Independent T-test; ^aP \leq 0.01 and ^bP \leq 0.001 in comparison to control group

Table 5: Hepatic antioxidant status in rats subjected to 17-Oximino-5-androsten-3 β -yl ester treatment (1 month)

GROUP	LPO (nmol of MDA formed /min/mg protein):	GSH (μ mol GSH/gm tissue)	GST (μ mol of CDNB conjugates/min/mg protein)	SOD (IU)	CATALASE (mmol of H ₂ O ₂ decomposed/min./mg protein)
control	0.43 \pm 0.09	4.61 \pm 0.76	0.58 \pm 0.09	9.46 \pm 1.42	3.44 \pm 0.66
17- Oximino-5- androsten- 3 β -yl ester treated	0.91 \pm 0.10 ^c	3.18 \pm 0.55 ^a	0.42 \pm 0.07 ^a	12.19 \pm 1.04 ^b	2.24 \pm 0.52 ^b

Values are expressed as mean \pm S.D. by Independent T-test; ^aP \leq 0.05, ^bP \leq 0.01 and ^cP \leq 0.001 in comparison to control group

**Figure 1: Structure of some reported potent steroidal oximes**

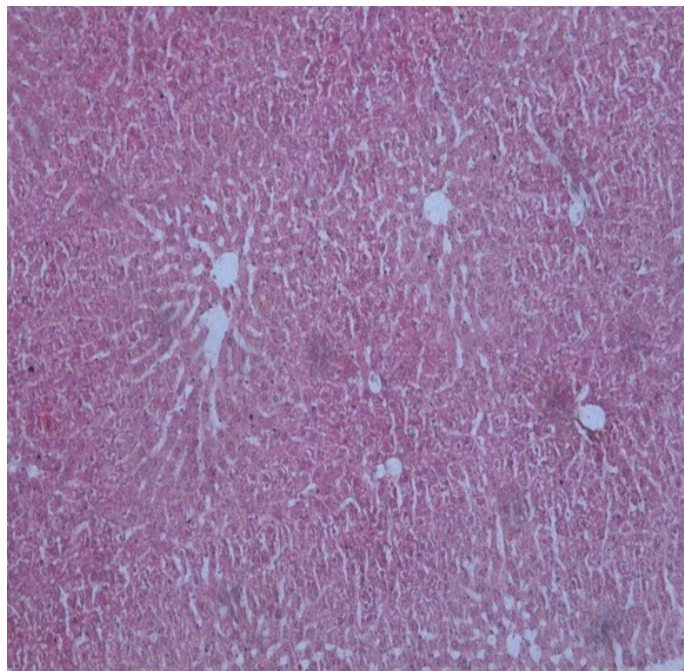


Figure 2: Light micrograph of a normal control animal revealing clear hepatic lobules with hexagonal array of hepatic plates radiating from a central vein.

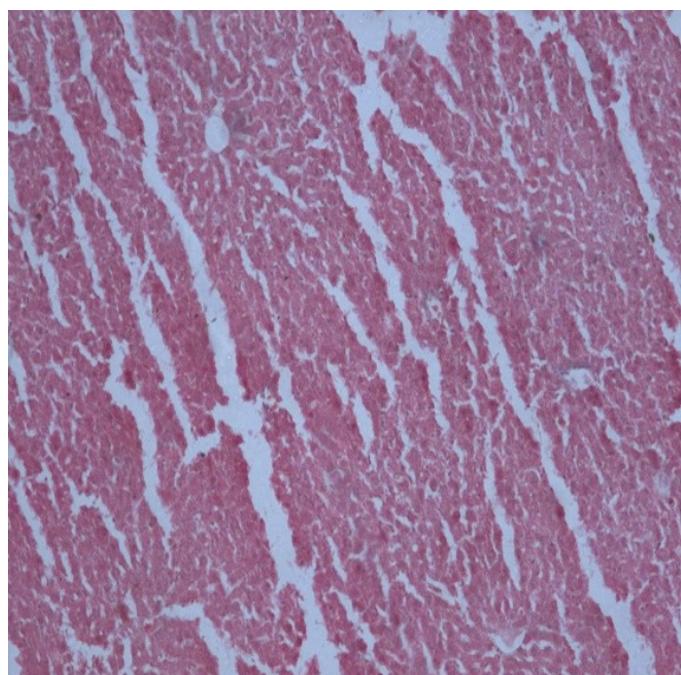


Figure 3: Light micrograph of a 17-Oximino-5-androsten-3β-yl benzoate treated animal revealing disruption of hepatic cords, increased vacuolization with a resultant widening of the sinusoidal spaces and some degree of hepatic hypertrophy

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