Clinicopathological Studies on Effect of Doxorubicin Hydrochloride on Heart of Rats

Amany A M Abd-Allah and Hager T H Ismail
Clin.Path.Dept.,Fac.Vet.Med., Zagazig Univ.

ABSTRACT

The present work was performed to study the anticancer effect of Doxorubicin HCl and Propolis and their combination in the treatment of mammary cancer induced by N-methyl-N-nitrosourea beside the ameliorating effect of Propolis against cardiotoxicity of Doxorubicin HCl using the clinicopathological changes.

One hundred and twenty five female white rats (two months old and 100 gm body weight) were divided into 5 equal groups .Starting of experiment by induction of carcinogen N-methyl-N-nitrosourea (MNU) into gps. 2-5 while rats kept as control in gp.1.After incidence of mammary tumor in gps. 2-5 begin the treatment which continue for 69 days. Rats were given carcinogen only, Doxorubicin HCl, Propolis and combination of therapeutic dose from Doxorubicin HCl and Propolis in gps. 2,3,4 and 5 ,respectively. Blood samples were collected after 120 days from carcinogen induction, thirty and seventy days post-treatment.

Gp. 3, showed increasing serum CK and LDH activities and cardiac (MDA) assay in addition to decrease cardiac (CAT) and (SOD) activities thirty and seventy days post-treatment. Those changes were appeared in gp. 5 but to lesser degree.

Gp. 4, showed improvement of the previous parameters. Gp. 2 , showed some changes in these parameters.CA 15.3 showed best value in gps. 3 and 5 especially seventy days post-treatment.

INTRODUCTION

Cancer is a major burden of disease which worldwide *(1)*.Anthracyclines are members of a very important class of antitumor antibiotics that have been used for many years in the treatment of different types of cancer *(2)*. Doxorubicin HCl is first anthracyclines antibiotics and one of the most potent anticancer drugs against most animal and human tumors prescribed alone or in combination with other agents which has the widest spectrum of activity *(3-5)*.Doxorubicin HCl induced cardiomyopathy may result in progressive heart failure after anti-neoplastic therapy, thus limiting the application of this potent chemotherapeutic agent *(6)*.The production of free radicals as by-product of Doxorubicin HCl metabolism is considered to be the main mechanism of Doxorubicin-induced cardiotoxicity*(7)*.

Propolis has attracted much attention in recent years as a useful or potential agent with application in pharmaceutical products *(8)*. .Many of publications lately considering the antitumor action of Propolis and its constituents which indicate their potential for the development of new antitumor agents *(9)*. A cardioprotective effect of Propolis extract can be attributed to direct scavenging properties of flavonoids one of Propolis constituent*(10)*.

The aim of the present work is to evaluate of Doxorubicin HCl as anticancer drug. The efficacy of Propolis as natural anticancer agent when used alone. Combination of Propolis with Doxorubicin HCl to overcome

cardiotoxicity of later. The evaluation was done by studying biochemical and histopathological changes.

MATERIAL AND METHODS

Material

Experimental animals

A total of 125 clinically healthy female white rats (two months old and 100 gm average body weight) were purchased from the laboratory animal housing, Faculty of Veterinary Medicine , Zagazig University. The animals were kept in metal cages, under hygienic conditions , given balanced ration with water *ad-libitum* and observed for 10 days before starting of experiment.

Preparation of the used carcinogen and therapeutic agents

Induction of mammary cancer was done in gps.2-5, by using of N-methyl-N-nitrosourea (MNU) which was injected intraperitoneally 50 mg/kg B.W, as single dose in the beginning of experiment ***(11)***. The therapeutic dose of Doxorubicin HCl was 60-75 mg/m^2 in human ***(12 , 13)*** .This therapeutic dose of human is equal to 10-12.4 mg/kg B.W for rat ***(13)***. While the recommended dose of Propolis in rat was 50 mg /kg B.W ***(14)***.

Methods

Table 1. Experimental design

The experimental design is summarized in Table 1,(n = 25)

Design	Gps.	Induction of mammary cancer (120 days)	Treatments for 69 days after the occurrence of mammary cancer		Blood samples
		N-methyl-N-nitrosourea (MNU) Intraperitoneally (50 mg/kg B.W) as single dose	Doxorubicin HCl Intraperitoneally (10 mg/kg B.W) every 3 weeks B.W)daily daily	Propolis Orally (50 mg/kg B.W)daily	
Control group	1	-	-	-	Blood samples were collected from the retro-orbital venous plexus after 120 days from the induction of mammary cancer , thirty and seventy days from starting treatment from all groups.
Experimental groups	2	+	-	-	
	3	+	+	-	
	4	+	-	+	
	5	+	+	+	

Sample collection

Blood samples were collected from the retro-orbital venous plexus as five ml of blood without anticoagulant in a sterile test tube for separation of serum for biochemical analysis *(15)*.

Biochemical studies

Serum creatine kinase (CK) and lactate dehydrogenase (LDH) activities and cardiac malondialdhyde (MDA) level and cardiac catalase (CAT) and superoxide dismutase (SOD) activities and serum cancer antigen 15.3 (CA 15.3) level were performed according to *(16-21)*, respectively using test kits of Spectrum, Biodiagnostic and Monobind Inc.

Tissue specimens

Mammary glands were collected after 120 days from induction of carcinogenic agent for histopathological examination. While, heart and mammary glands were collected thirty and seventy days post-treatment by Doxorubicin HCl and Propolis and their combination for histopathological examination. Also, pieces of heart were taken and homogenate for some biochemical analysis.

Statistical analysis

The obtained data were analyzed using F-test *(22)* except tumor marker results after 120 days from carcinogen induction was analyzed using T-test*(23)*.

RESULTS

Regarding to some cardiac enzymes tests, Table 2 shows highly significant increase in serum creatine kinase activity in gps. 3 and 5 and highly significant decrease in gp. 4 thirty and seventy days post-treatment .While, serum lactate dehydrogenase activity showed highly significant increase in gps. 2, 3 and 5 thirty and seventy days post-treatment with highest value in gp. 3.

Table 3 shows highly significant increase in cardiac malondialdhyde level in gps. 2,3 and 5 thirty and seventy days post-treatment, the highest value in gp. 3 especially after seventy days of treatment.

Concerning the antioxidant enzymes activities, Table 3 shows highly significant decrease in gp. 3 and non significant change in gps. 2,4 and 5 thirty days post-treatment . Highly significant increase in gp. 4 ,highly significant decrease in gps. 2 and 3 ,the lowest value was found in gp. 3 and non significant change in gp. 5 seventy days post-treatment.

Also, Table 3 shows highly significant decrease in cardiac catalase activity in gps. 2 and 3 and highly significant increase in gps. 4 and 5 thirty and seventy days post-treatment.

Regarding to the result of serum tumor marker CA 15.3 , Table 4 shows highly significant increase in animals injected with carcinogen after 120 days from induction of cancer. While, Table 5 shows highly significant increase in serum CA 15.3 in gps. 2 and 4 thirty and seventy days post-treatment with highest value in gp. 2 and non significant change in gps. 3 and 5 .

Table 2.Some cardiac enzymes tests (mean values±SE) in rats in gps.(1-5) thirty and seventy days post-treatment.

Periods	Gps. / Parameters	CK U/l	LDH U/l
Thirty days post-treatment	Control Gp.(1)	292.30 c ±1.04	1612.40 c ±1.29
	MNU(carcinogen) not treated Gp.(2)	293.78 c ±5.63	2341.20 b ±88.60
	MNU(carcinogen) +Doxorubicin HCl Gp.(3)	369.18 a ±5.56	3247.70 a ±1.01
	MNU(carcinogen) +Propolis Gp.(4)	266.40 d ±9.24	1432.80 c ±1.33
	MNU(carcinogen) + Doxorubicin HCl +Propolis Gp.(5)	346.56 b ±4.56	2530.60 b ±42.47
	F-test	**	**
Seventy days post-treatment	Control Gp.(1)	258.46 c ±4.13	1384.40 c ±35.92
	MNU(carcinogen) not treated Gp.(2)	262.00 c ±7.62	1951.80 b ±83.27
	MNU(carcinogen) +Doxorubicin HCl Gp.(3)	886.63 a ±12.25	3287.40 a ±3.22
	MNU(carcinogen) +Propolis Gp.(4)	223.62 d ±1.11	1458.30 c ±1.16
	MNU(carcinogen) + Doxorubicin HCl +Propolis Gp.(5)	305.75 b ±18.07	2393.60 b ±71.79
	F-test	**	**

Means at the same column at the same period followed by different letters were significantly different and the highest value was represented with the letter a

**: Highly significant at 0.01 probability

Table 3. Cardiac (MDA) level and(CAT) and (SOD) activities (mean values ±SE) in rats in gps.(1-5) thirty and seventy days post-treatment.

Periods	Parameters / Gps.	MDA nmol/ g	CAT U/g	SOD U/g
Thirty days post-treatment	Control Gp.(1)	17.42 d ±0.28	13.99 ab ±0.66	5.43 b ±0.12
	MNU(carcinogen) not treated Gp.(2)	19.50 c ±0.43	12.62 b ±0.39	4.78 c ±0.16
	MNU(carcinogen) +Doxorubicin HCl Gp.(3)	23.07 a ±0.58	7.53 c ±0.34	5.01 c ±0.11
	MNU(carcinogen) +Propolis Gp.(4)	18.22 d ±0.43	14.99 a ±0.74	6.05 a ±0.07
	MNU(carcinogen) + Doxorubicin HCl +Propolis Gp.(5)	21.73 b ±0.50	12.33 b ±0.66	5.79 a ±0.06
	F-test	**	**	**
Seventy days post-treatment	Control Gp.(1)	16.59 c ±0.38	10.14 b ±0.32	5.87 b ±0.18
	MNU(carcinogen) not treated Gp.(2)	19.39 b ±0.43	7.89 c ±0.09	4.85 c ±0.13
	MNU(carcinogen) +Doxorubicin HCl Gp.(3)	26.34 a ±0.78	6.72 d ±0.18	4.01 d ±0.10
	MNU(carcinogen) +Propolis Gp.(4)	17.22 c ±0.17	18.23 a ±0.48	6.76 a ±0.17
	MNU(carcinogen) + Doxorubicin HCl +Propolis Gp.(5)	20.15 b ±0.28	10.63 b ±0.32	6.68 a ±0.09
	F-test	**	**	**

Means at the same column at the same period followed by different letters were significantly different and the highest value was represented with the letter a.

**: Highly significant at 0.01 probability.

Table 4. Serum tumor marker(CA 15.3) level (mean values±SE) in rats after 4 months of carcinogen (N-methyl-N-nitrosoure) induction compared with control group.

Parameter \ Gps.	Control	MNU(carcinogen) injected rats
CA 15.3 (U/ml)	1.88 ±0.25	3.38 ±0.17
T- test	**	

Table 5. Serum tumor marker(CA 15.3) level (mean values±SE) in rats in gps.(1-5) thirty and seventy days post-treatment.

Gps. \ Parameter	CA 15.3(U/ml) (After thirty days)	CA 15.3(U/ml) (After seventy days)
Control Gp.(1)	1.88 c ±0.24	1.28 c ±0.16
MNU(carcinogen) not treated Gp.(2)	3.16 a ±0.33	3.80 a ±0.49
MNU(carcinogen) +Doxorubicin HCl Gp.(3)	2.40 bc ±0.12	2.16 bc ±0.39
MNU(carcinogen) +Propolis Gp.(4)	2.92 ab ±0.08	2.84 ab ±0.42
MNU(carcinogen) + Doxorubicin HCl +Propolis Gp.(5)	2.28 bc ±0.21	1.84 bc ±0.26
F-test	**	**

Means at the same column followed by different letters were significantly different and the highest value was represented with the letter a. **: Highly significant at 0.01 probability.

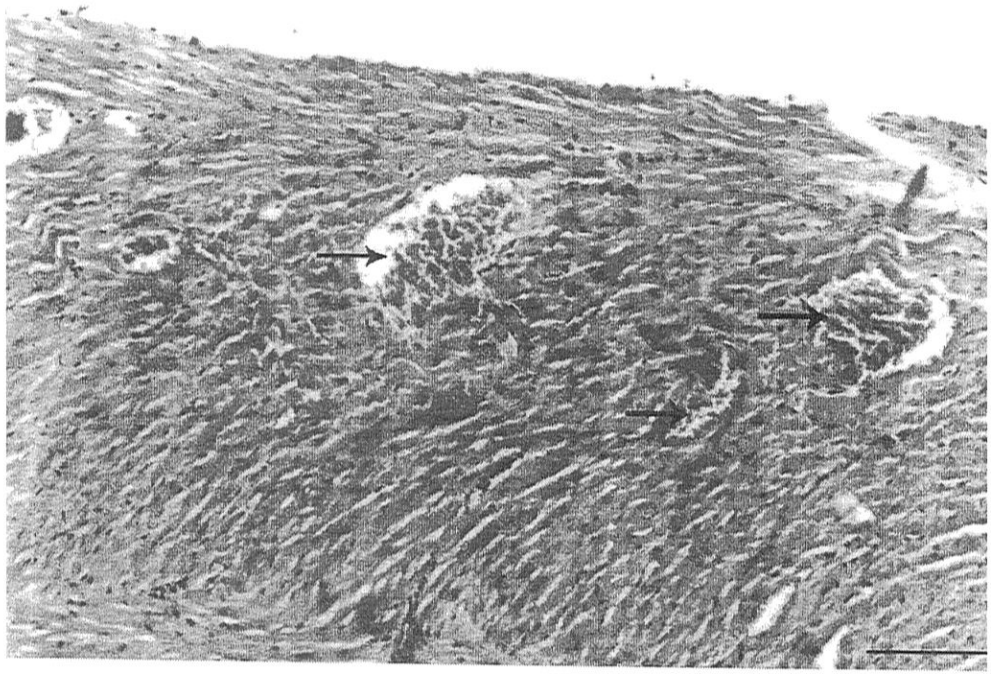

Fig. 1.Photomicrograph of the heart of rat in gp.3 showing hemorrhages among the cardiac muscles (arrows) thirty days post-treatment, HE x 300.

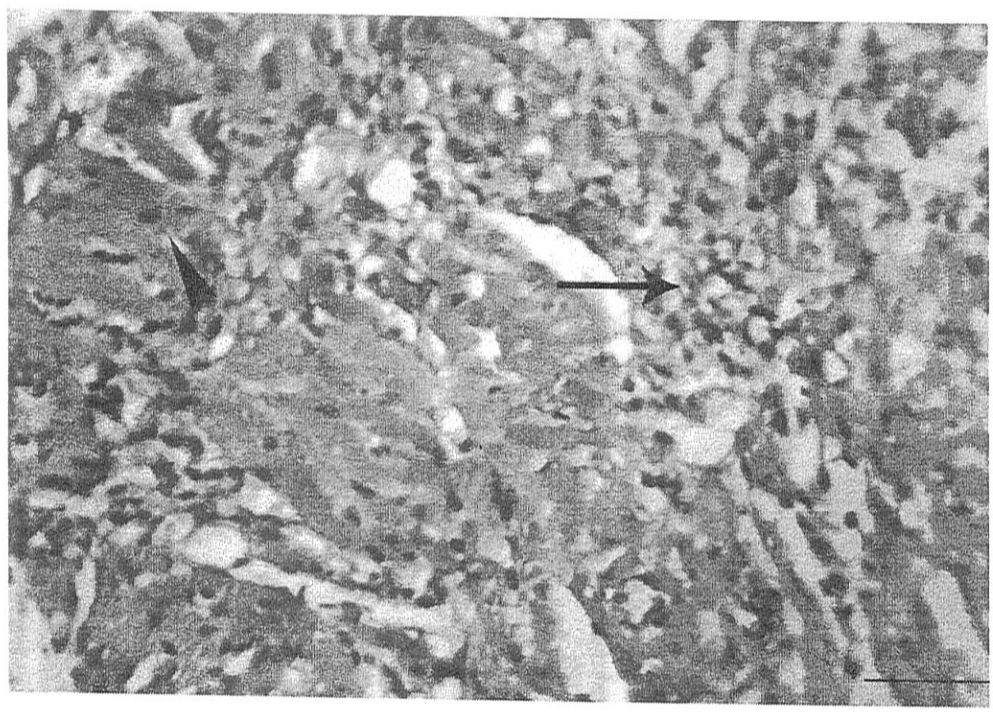

Fig. 2. Photomicrograph of the heart of rat in gp. 3 showing focal coagulative necrosis (arrowhead) and round cells infiltrations (arrow) seventy days post-treatment , HE x 1200.

Fig.3. Photomicrograph of the heart of rat in gp. 5 showing focal hemorrhages among degenerated myocytes (arrow) thirty days post-treatment, HE x 1200.

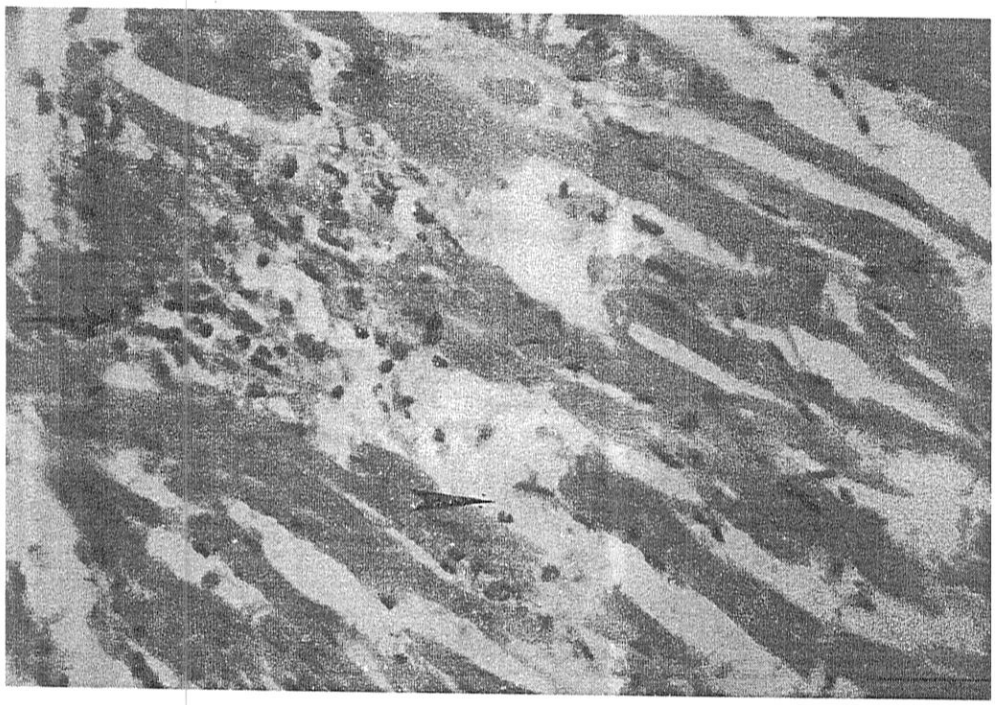

Fig.4. Photomicrograph of the heart of rat in gp. 5 showing focal edema (arrowhead), Zenker's necrosis and few round cells infiltrations among the necrotic myocytes (arrow) seventy days post-treatment, HE x 300.

Fig. 5. Photomicrograph of the mammary glands of rat showing adenocarcinoma with proliferation of epithelial and stromal components (arrow) after 120 days from carcinogen (MNU)induction, HE x 1200.

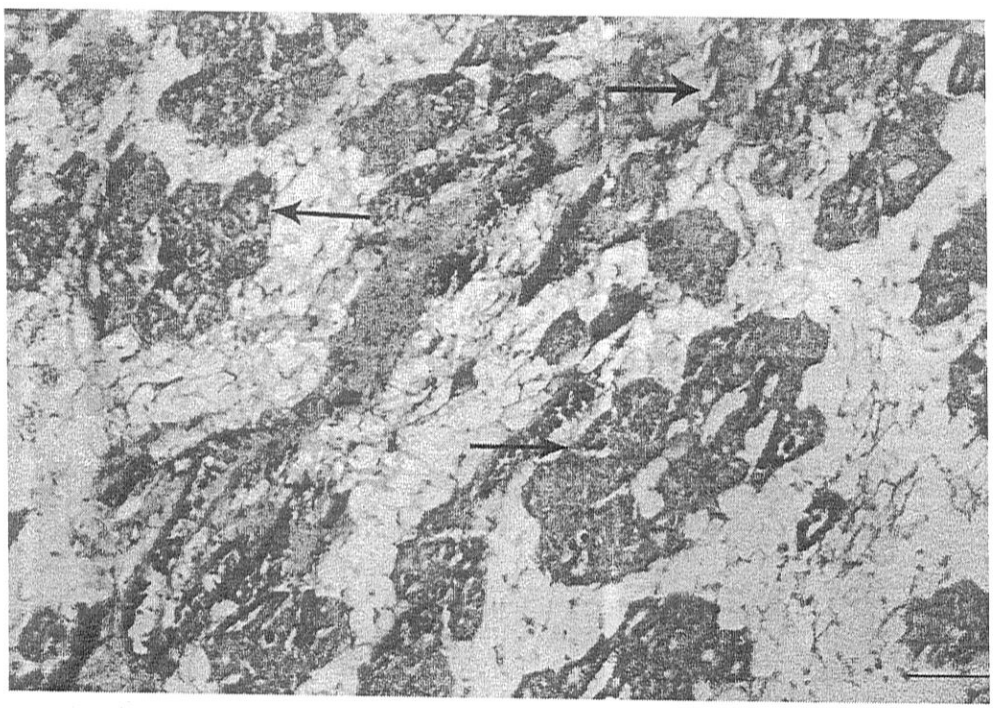

Fig.6. Photomicrograph of the mammary glands of rat in gp. 2 showing multifocal lobular adenocarcinoma (arrows) thirty days post-treatment, HE x 1200.

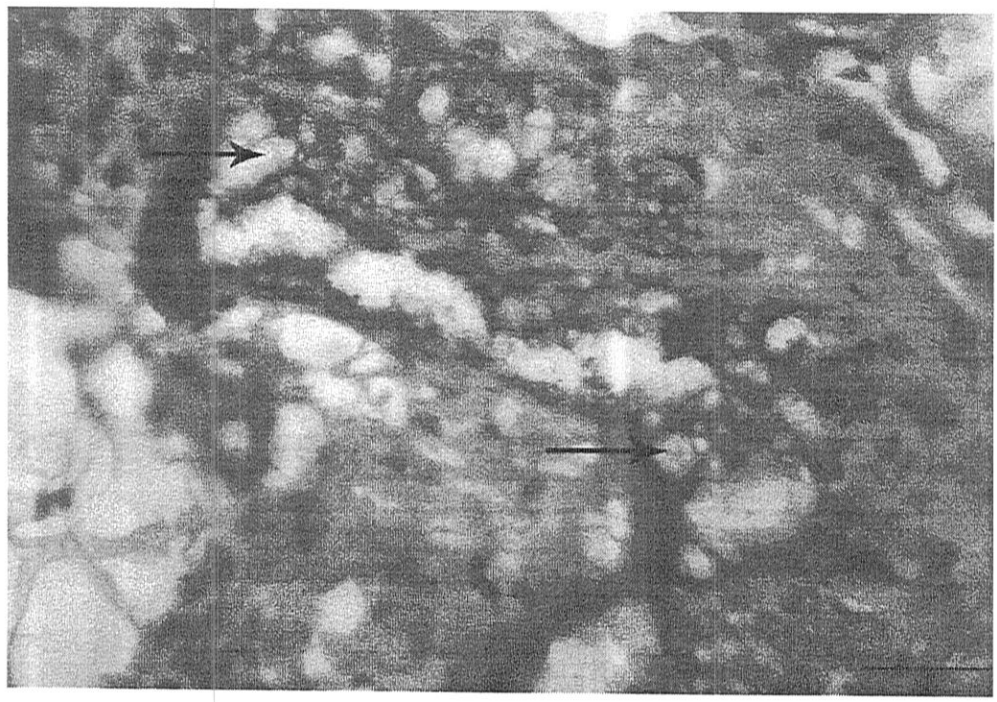

Fig.7. Photomicrograph of the mammary glands of rat in gp. 2 showing low grade and sclerosing ductal carcinomas (arrows) seventy days post-treatment, HE x 1200.

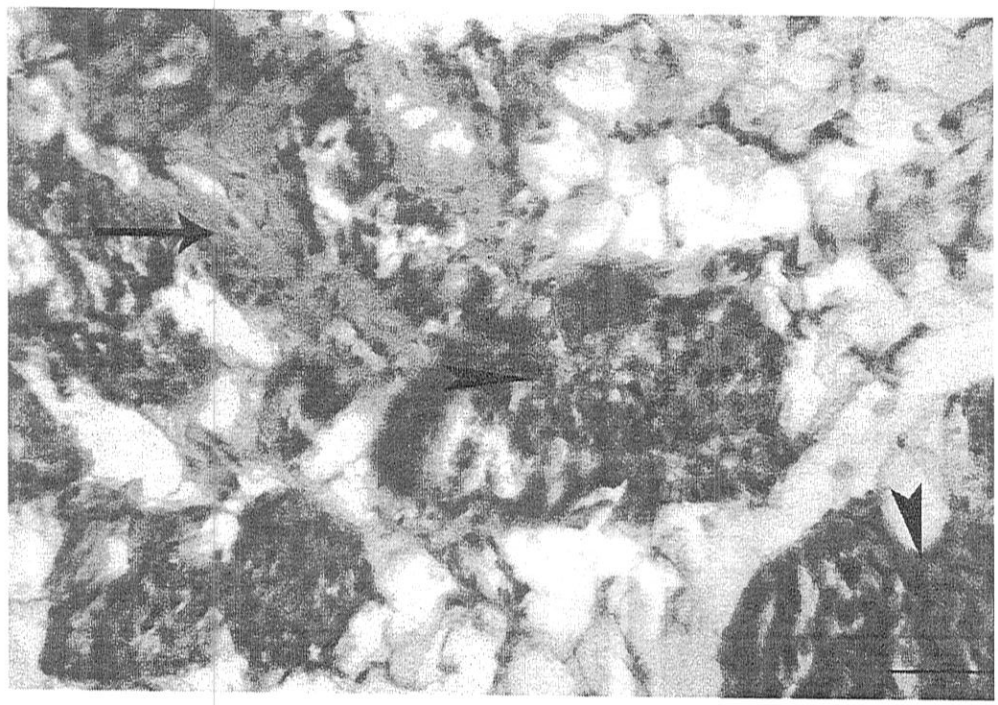

Fig.8. Photomicrograph of the mammary glands of rat in gp. 3 showing adenocarcinoma (arrowheads) and proliferation of stromal tissue (arrow) thirty days post-treatment, HE x 1200.

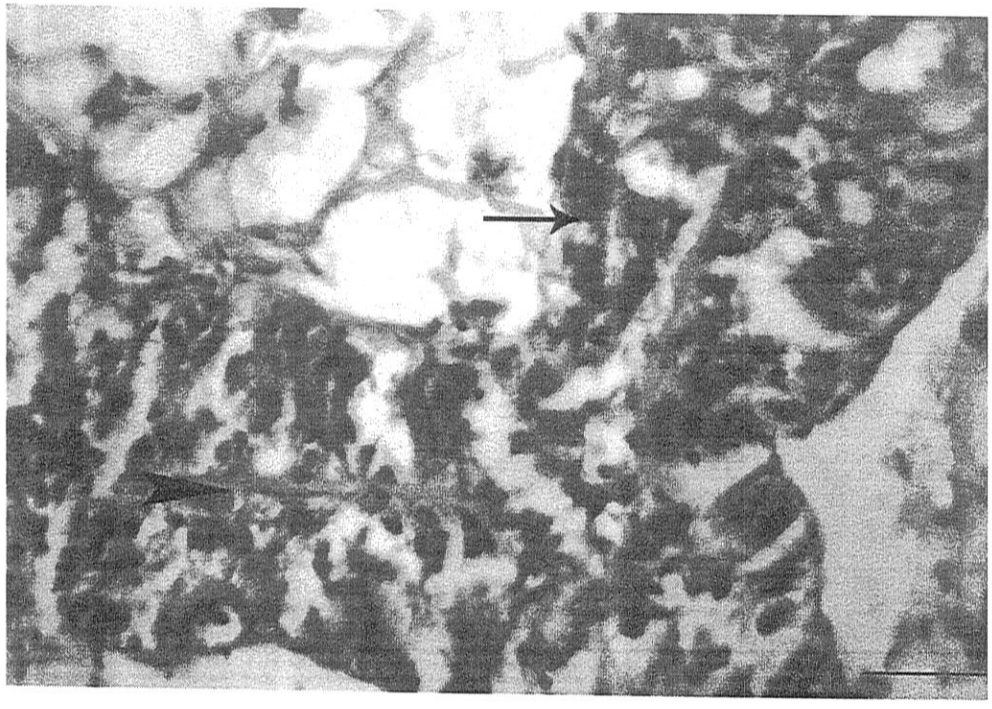

Fig.9. Photomicrograph of the mammary glands of rat in gp. 3 showing adenocarcinoma (arrow) with scattered areas of necrosis and lymphocytes infiltrations (arrowhead) seventy days post-treatment, HE x 1200.

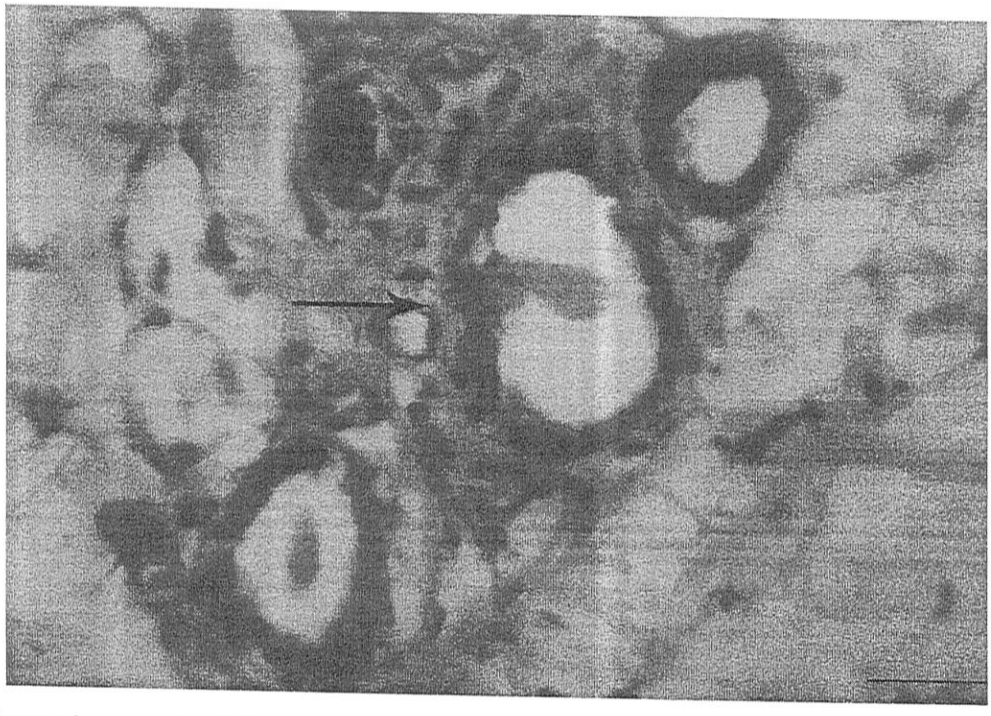

Fig. 10. Photomicrograph of the mammary glands of rat in gp. 4 showing adenocarcinoma in the glandular epithelium of the mammary glands (arrow) thirty days post-treatment, HE x 1200.

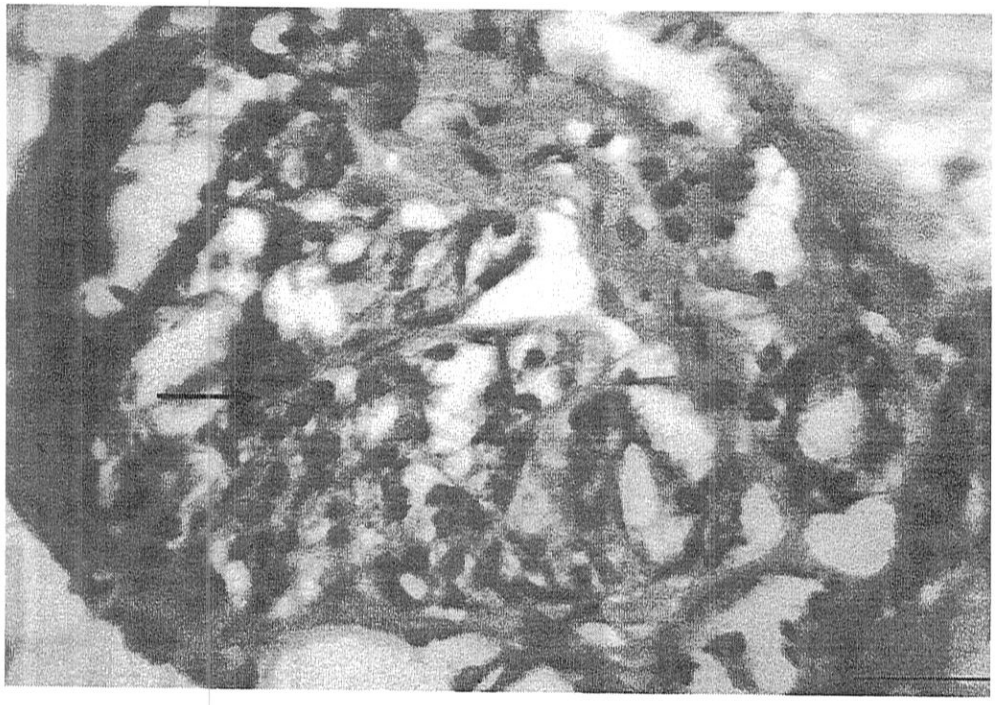

Fig. 11. Photomicrograph of the mammary glands of rat in gp. 4 showing adenocarcinoma with nuclear atypia and mitoses with mild proliferation of stromal tissue (arrow) seventy days post-treatment, HE x 1200.

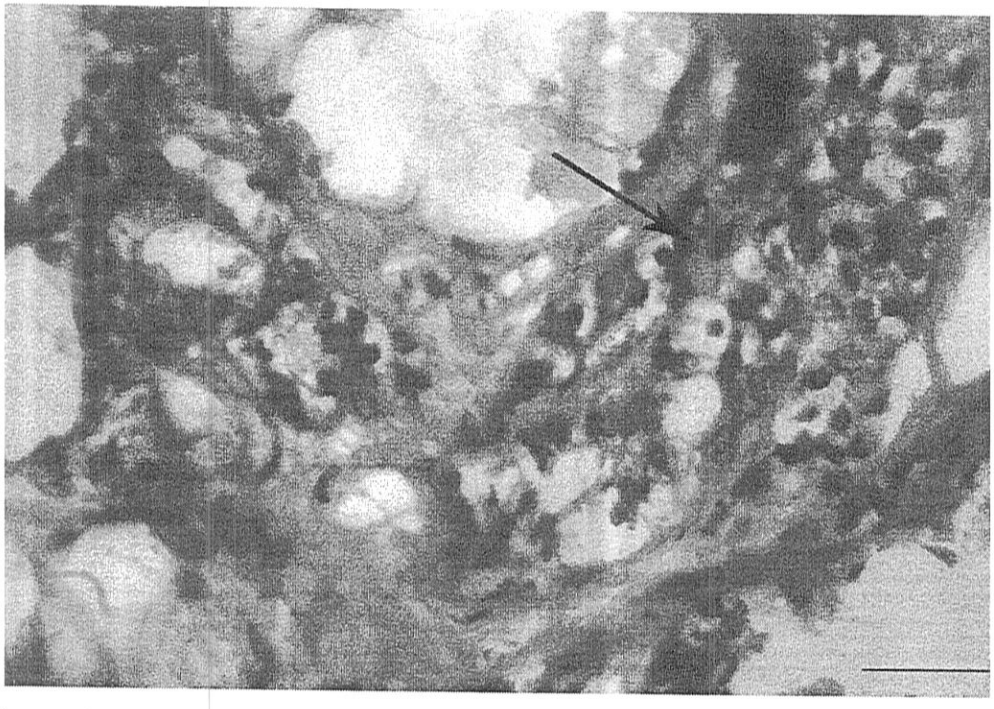

Fig. 12. Photomicrograph of the mammary glands of rat in gp. 5 showing ductal carcinoma with nuclear atypia and mitoses (arrow) thirty days post-treatment, HE x 1200.

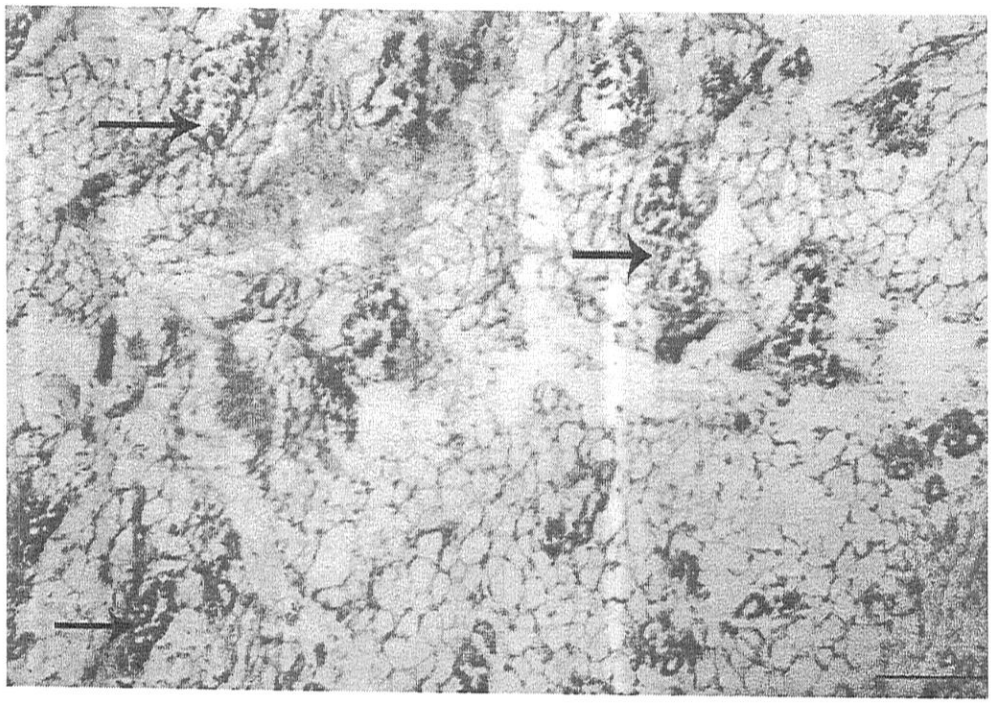

Fig. 13. Photomicrograph of the mammary glands of rat in gp. 5 showing nests of neoplastic cells. The latter were focally necrotic (arrows) seventy days post-treatment, HE x 300.

DISCUSSION

The present study demonstrates that increasing in cardiac enzymes activities in Doxorubicin HCl treated group may be due to severe cardiomyopathy which indicated from the increase in serum activities of cardiac enzymes such as creatine kinase and lactate dehydrogenase *(24 ,25)*. These enzymes are present in sufficiently high content in myocardial tissue so that the death of a relatively small number of tissue results in a substantial increase in measured enzyme activity in serum. Recent studies suggest that mitochondria are the target organelle of Doxorubicin-induced free radical toxicity in myocytes *(26,27)*. An important factor, which can mediate the damaging action of Doxorubicin HCl in myocardial tissues, especially in mitochondria, is high affinity binding of it to cardiolipin, an anionic phospholipid in the inner mitochondrial membrane leading to dissociation of cardiolipin-associated peripheral proteins from the inner mitochondrial membrane, like cytochrome c and mitochondrial creatine kinase resulting in initiation of programmed cell death *(28)*. Our results confirmed by histopathological findings of heart in figures 1 and 2 .

Also, increase in these enzymes in combined treated group of Doxorubicin HCl and Propolis may be due to flavonoids scavenging activity of Propolis has been exploited to obtain protection against the peroxidative damage in heart mitochondria which was induced by the administration of an acute dose of Doxorubicin HCl *(29)*.Our results confirmed by histopathological findings of heart in figures 3 and 4.

Decrease in serum creatine kinase activity in Propolis treated group may be due to the role of caffeic acid phenethyl ester (CAPE) is an active component of Propolis as

antioxidant by scavenging effect for H_2O_2 and tissue stabilizing effect of it *(30)*.

Increase in serum lactate dehydrogenase activity in group which injected with N-methly-N-nitrosourea alone to induce mammary cancer without treatment may be due to malignant tumors inhibit a complex carbohydrate metabolism which differs from non-neoplastic cells with two main paradigms: (1) malignant cells produce large amounts of lactate even in the presence of sufficient oxygen for aerobic glycolysis.(2) intermediates of the tricarboxylic acid cycle (TCA) are used for fatty ,amino and nucleic acid synthesis. Thus, the extensive glucose uptake of cancer cells is needed not only for energy supply but also to provide the components for cellular growth and a high amount of reducing equivalents such as NADPH *(31,32)* high levels of pyruvate are needed which can be introduced either into the TCA, converted into acetyl-COA or degraded to lactate by LDH. By degradation of pyruvate to lactate by LDH, the pool of reductive equivalents on the one hand and the availability of citric acid cycle intermediates for fatty and amino acid synthesis on the other hand is raised. The over expression of LDH_5 in tumor cells supports this theory of a glucose metabolism optimized for cellular growth within malignant tumors *(31,33)*.

Increase in cardiac malondialdhyde level in Doxorubicin HCl treated group especially after prolonged treatment period may be due to increase levels of oxygen species by Doxorubicin HCl which lead to an increase in tissue malondialdehyde (MDA)level which is a breakdown product of lipid peroxidation *(34-36)* .In the presence of transition metal ions, the chain reaction continues and free iron appears to play a particularly important role in Doxorubicin-induced lipid peroxidation *(37)*. Combined treatment of Propolis with Doxorubicin HCl cause restoration of the respiratory chain ratio (RCR)and inhibition of the lipid peroxidation *(38)*.While, increase in malondialdehyde level in group which injected with N-methly-N-nitrosourea alone to induce mammary cancer without treatment may be due to oxidative stress, especially lipid peroxidation is known to be involved in carcinogenesis *(39)*.Increased levels of lipid peroxidation products play a role in the early phases of tumor growth *(40)*.

Concerning the antioxidant enzymes activities in heart, the decrease in catalase activity in Doxorubicin HCl treated group may be due to the heart has relatively low level of CAT activity compared with other tissues and CAT may play only a minor role in defending myocardial cells towards free radical insult *(41)*. Low catalase activity in the heart is responsible for the high sensitivity of this organ to oxidative stress *(42)*. Treatment with Doxorubicin HCl cause a decrease in the antioxidant stores of the heart catalase *(43)*. Decrease in catalase activity in group which injected with N-methly-N-nitrosourea alone to induce mammary cancer without treatment may be due to higher oxygen free radical production and increase oxidative stress in breast carcinogenesis *(44)*.While, increase it in Propolis treated group may be due to flavonoids one of Propolis components may also exert antioxidant abilities through protection or enhancement of endogenous antioxidants *(45)*.

Cardiac superoxide dismutase activity increase in Propolis treated group may be due to Propolis constituents include the antioxidant trace elements iron, zinc and selenium which are essential cofactors for the enzymatic antioxidant defense system production represented by superoxide dismutase and other antioxidant enzymes *(46)*. In a combined treated group of Doxorubicin HCl and Propolis a highly significant increase may be due to the effect of Propolis which overcome oxidative stress effect of Doxorubicin HCl. Decrease in SOD activity in Doxorubicin HCl treated group may be due to Doxorubicin HCl in its quinone form with concomitant production of superoxide anion radicals. This process is called redox cycling. Superoxide radicals can dismutate either enzymatically catalyzed by superoxide dismutase or albeit with a lower rate, spontaneously. From this dismutation hydrogen peroxide is formed *(7)*.

Also, SOD activity decreased in group which injected with N-methly-N-nitrosourea alone to induce mammary cancer without treatment may be due to it is the only enzyme that disrupts superoxide radicals and is present in all cells with high amounts in erythrocytes *(47)*. It protects the cells against superoxide- and hydrogen peroxide-mediated LPO. The malignant cells of different cancer types exhibit heterogeneity in the levels of oxidative stress, associated with various expression levels of SOD. Decreased SOD activity was observed in various cancerous conditions *(48 , 49)*. The source of hydrogen peroxide is mainly SOD-mediated dismutation of superoxide radical which is generated by various enzyme systems as well as by non enzymatic pathways. Several reports have cited decreased activities of SOD in various carcinogenic conditions *(50 , 51)*.

Elevated serum CA 15-3 level was found in breast cancer cases as primary diagnosis. There is clear correlation between tumor marker , tumor size and nodal involvement with significantly higher concentrations in cases with larger tumors or in patients with nodal involvement *(52-54)*.

Increase in serum CA 15.3 level in rats which injected with N-methly-N-nitrosourea alone to induce mammary cancer without treatment may be due to a clear correlation between CA 15-3 and disease progression. Propolis treated group also revealed increasing in CA 15-3 level but with lesser degree. The more advanced the disease, the higher the value of CA 15-3 *(55)*. CA 15.3 level showed non significant change in groups treated by Doxorubicin HCl alone and after combination it with Propolis may be due to reduction in CA 15-3 levels following treatment was a favorable predictive factor for time to disease progression during systemic therapy *(56)*. Our results confirmed by histopathological findings of mammary glands of gps.(2,3,4 and 5) in figures (5,6,7,8,9,10,11,12and 13) respectively.

It could be concluded that

1-Doxorubicin HCl caused cardiomyopathy.

2-Doxorubicin HCl has cumulative side effects (increasing side effects by increasing duration).

3-Using Propolis alone has no side effects with lower anticancer activity than Doxorubicin HCl.

4-Combination of Doxorubicin HCl with Propolis decreasing cardiotoxicity of Doxorubicin HCl and increasing anticancer activity of Propolis .

It is recommended that

Using Doxorubicin HCl together with Propolis with therapeutic doses to minimize cardiotoxic effect of Doxorubicin HCl and increasing anticancer activity of Propolis.

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الملخص العربي

دراسات باثولوجية أكلينيكية على تأثير الدوكسوروبيسين هيدروكلوريد على القلب في الفئران

أماني أحمد محمد عبد الله ، هاجر طارق حسن إسماعيل
قسم الباثولوجيا الإكلينيكية – كلية الطب البيطرى – جامعة الزقازيق

أجريت هذه الدراسة على تأثير الدوكسوروبيسين هيدروكلوريد والبروبليس وخليط منهما في علاج سرطان الثدي الناجم عن مادة ن –ميثيل- ن- نيتروزويوريا الى جانب التأثير المخفف للبروبليس ضد تسمم نسيج القلب بالدوكسوروبيسين هيدروكلوريد .وقد أجريت هذه الدراسة على عدد مائه وخمسه وعشرين من أناث الفئران البيضاء عند عمر شهرين ووزن مائه جرام و تم تقسيم هذه الفئران الى خمس

مجموعات متساويه وبدأت التجربه بحقن الماده المسرطنه ن –ميثيل- ن- نيتروزويوريا للمجموعات من الثانيه الى الخامسه وبقيت الفئران في المجموعه الاولى كمجموعه ضابطه للتجربه. بعد احداث سرطان الثدي في هذه المجموعات بدأ العلاج والذي استمر تسعه وستين يوما. المجموعة الثانية تم اعطاؤها الماده المسرطنه فقط.أما المجموعة الثالثة تم اعطاؤها الدوكسوروبيسين هيدروكلوريد و المجموعة الرابعة تم تجريعها بالبروبليس و المجموعة الخامسة تم اعطاؤها خليط من الجرعات الدوائية لكل من الدوكسوروبيسين هيدروكلوريد والبروبليس. تم أخذ عينات الدم بعد مائه وعشرين يوما من حقن الماده المسرطنه و ثلاثين وسبعين يوما من بدايه العلاج. وقد وجد ان الدوكسوروبيسين هيدروكلوريد سبب زياده في الكرياتين كيناز و الاكتيت ديهيدروجيناز في السيرم والمالونالديهيد في نسيج القلب الى جانب انخفاض في الكاتليز و السوبر أوكسيد ديميوتاز في نسيج القلب هذه التغيرات ظهرت في المجموعه التى تعاطت خليطا من الجرعات الدوائيه للدوكسوروبيسين هيدروكلوريد و البروبليس ولكن بصوره أقل بينما أظهرت المجموعه الرابعه تحسنا في هذه العوامل.وقد أظهرت دلالات الاورام أحسن النتائج في المجموعتين الثالثه والخامسه وخاصه بعد سبعين يوما من العلاج .

لذلك يوصى بأستخدام الدوكسوروبيسين هيدروكلوريد مع البروبليس بالجرعات الدوائيه لتقليل التأثير السمي على نسيج القلب بالدوكسوروبيسين هيدروكلوريد و زياده نشاط البروبليس كمضاد للسرطان .