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PROTECTIVE EFFECTS OF SILYMARIN ON EPIRUBICIN–INDUCED HEPATOTOXICITY IN MICE

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ABSTRACT: Hepatotoxicity of chemotherapy is frequent, depending upon the chemotherapeutic regimen and individual factors. The anthraciclins hepatotoxicity was studied on laboratory animals and chemotherapy-treated patients, and it showed clinical, biological, structural and ultrastructural changes at hepatic level. The present study aims to evaluate the hepatic toxic effects of epirubicin in mice and to bring scientific proof on the hepatoprotection capacity of silymarin in liver chemotherapy toxicity. We used six mice lots: control, epirubicin (Epi), Epi+ 50mg/kg sylimarin (SM), Epi+100mg/kg SM, 50mg/kg SM and 100mg/kg SM, and we performed biochemical testing of liver markers, and histological analysis of liver tissue. We also looked at epirubicin within the hepatic tissue in darkfield microscopy with hyperspectral detection. The results revealed the increase of alanine aminotransferase, aspartate aminotransferase and gamma glutamyl transferase in epirubicin treated group, which were improved after silymarin treatment. The liver of epirubicin treated mice showed histological abnormalities, and these were also ameliorated by the treatment with silymarin, especially the 100mg/kg dose. The adjuvant hepatoprotector therapies are especially valuable in the prevention and improvement of liver chemotherapy toxicity.

Keywords: epirubicin, hepatotoxicity, chemotherapy, hepatic serum markers, silymarin

INTRODUCTION:

The pharmaceutical industry brought for the cancer therapy very potent chemotherapeutic agents within the last decades, but whose efficacy is also accompanied by the toxic potential on normal tissues. The modern epidemiological studies have demonstrated that nowadays the drugs are the most frequent causes of liver failure (Jaeschke et al., 2002). The hepatotoxicity after chemotherapy can be induced at standard doses and, besides cardiac and gastrointestinal toxicity, is one of the most frequent causes in reducing the quality of life in the oncological patient.

In the chemotherapy treated patient, the liver disease can have a various etiology, so assigning the toxic cause to chemotherapy is often difficult. The oncological patient can have viral liver infections, tumor liver infiltrates, concomitant medication with a hepatotoxic potential, all of these being events that work together for the determinism of liver disease (Grigorian et al., 2014). Moreover, the toxicity risk increases if poli-chemotherapy regimens are administered, in this case it is difficult to assign the hepatotoxicity to a single agent. So, evaluation of toxicity of an anticancer drug is easier to be studied in experimental research or clinical trials in which that unique drug is given. The liver toxicity can reproduce almost any type of lesion, starting from hepatosteatosis, to necrosis, fibrosis, cholestasis or vascular disease (Boussios et al., 2012). The occurrence of this type of toxicity is one of the reasons why dose-reductions is needed during chemotherapy, chemotherapy administration is postponed or it is even discontinued. Thus it is always justified for having an accurate pre-chemotherapy evaluation of the patient in terms of integrity of the liver function (Kaplowitz et al., 2007) and for finding some adjuvant therapy to support liver function, preventing hepatotoxicity but not interfering with the antineoplastic potential of chemotherapy.

Epirubicin belongs to the class of anthracyclines, antitumor antibiotics and it is metabolized by the liver. Liver toxic effects of this class representatives were studied mostly on doxorubicin, there are few studies that assess the toxicity of epirubicin. The invoked mechanisms for anthracycline-induced hepatotoxicity were proven by histological, ultrastructural, biochemical studies.

Natural antioxidant compounds were studied for their beneficial effect in protecting the liver during and after chemotherapy. Silymarin is a unique substance containing silybin, silidianin and silicristin, which comes from the milk thistle plant and whose effects were studied in lab animals (Jain et al., 2011) with proven hepatoprotective potential. This paper aims to evaluate the hepatic toxic effects of epirubicin in mice and also to bring scientific evidence regarding the hepatoprotective potential of silymarin in liver toxicity. Moreover, this paper is also aiming at bringing evidence that silymarin can hasten the detoxification of epirubicin toxic metabolites in the liver.

MATERIAL AND METHODS:

Animals

We used CD 1 mice, which were fed a standard rodent diet ad libitum. The animals were maintained at 12-h

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light/dark cycle at constant temperature and humidity. All experimental procedures were performed in compliance with institutional guidelines and approved by the Institutional Research Ethics Committee.

Experimental design

For analyzing the influence of silymarin on epirubicin-induced liver damage, mice were randomly divided into six groups: control, epirubicin (Epi), epirubicin+50 mg/kg silymarin (Epi+50SM), epirubicin+100 mg/kg silymarin (Epi+100SM), 50 mg/kg silymarin (50SM), 100 mg/kg silymarin (100SM), of 10 animals/group. The vehicle (0.7% carboxymethyl cellulose solution) was administrated by gavage daily to control and Epi group. The 12 mg/kg cumulative dose of Epi (Alderton et al., 1992), was divided into six single doses of 2 mg/kg and injected intraperitoneally, (i.p.) at two days, starting with the second day of experiment. SM powder was previously dissolved in 0.7% carboxymethyl cellulose. For both co-treated groups (50 mg/kg and 100 mg/kg), SM administration started at 24 h before the first EPI administration and was performed daily by gavage between 1 and 13 day. On 14th day, all mice were sacrificed and liver samples were preserved in a buffered formalin solution for histology techniques.

Biochemistry

The activities of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma glutamiltranspeptidase (GGT) were evaluated by spectrophotometry using the detection kit (Roche reagents, France) according to the producer.

Histology

Liver specimens were fixed in 4% phosphate-buffered formalin, embedded in paraffin and cut into 5 µm thick sections. For histopathological examination, liver sections were stained with Hematoxilin&Eosin and analyzed by light microscopy (Olympus BX43, Hamburg, Germany).

Darkfield microscopy with hyperspectral detection (hsDFM)

Hyperspectral imaging in combination with dark field microscopy was used to assess epirubicin distribution into liver within histology tissue sections. Darkfield microscopy images and hyperspectral plots were acquired using enhanced darkfield transmission optical microscope (Olympus BX43) equipped with hyperspectral imaging spectrophotometer (Headwall, CytoVivaInc, Auburn, AL). Images were acquired using 100x oil immersion lens.

Statistical analysis

Statistical analysis was conducted with a one-way ANOVA using Stata 13 software (StataCorp LP, Texas, USA). A value of $p<0.05$ was considered to be statistically significant.

RESULTS:

Effect of Silymarin on hepatic biochemical markers

Effects of silymarin on serum ALT, AST and GGT activities in mice from all experimental groups are shown in Figures 1-3.

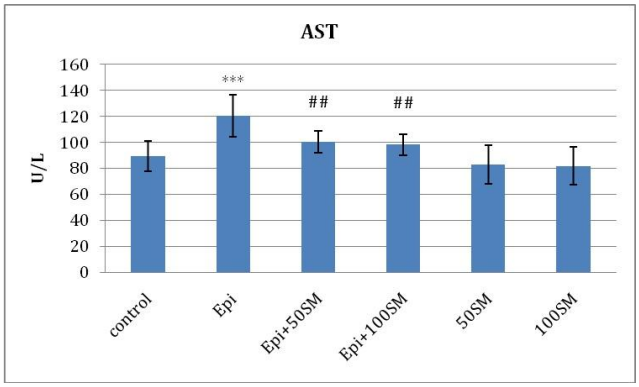

Figure 1: The effects of SM against liver injuries induced by EPI on serum aspartate-amino-transferase (AST). Values are expressed as mean ± SD
* p< 0.05 significantly different from the control group;
# p< 0.05 significantly different from the EPI-treated group.

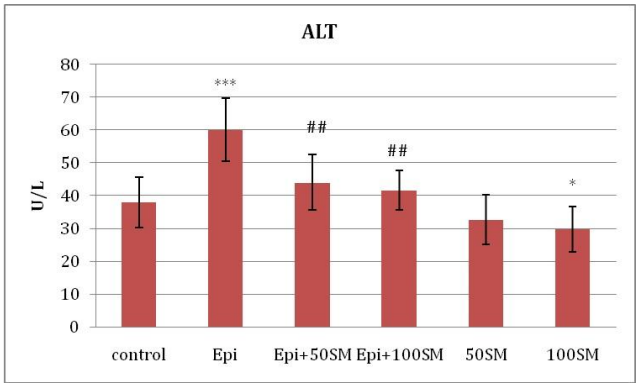

Figure 2: The effects of SM against liver injuries induced by EPI on serum alanine-amino-transferase (ALT). Values are expressed as mean ± SD
* p< 0.05 significantly different from the control group;
# p< 0.05 significantly different from the EPI-treated group.

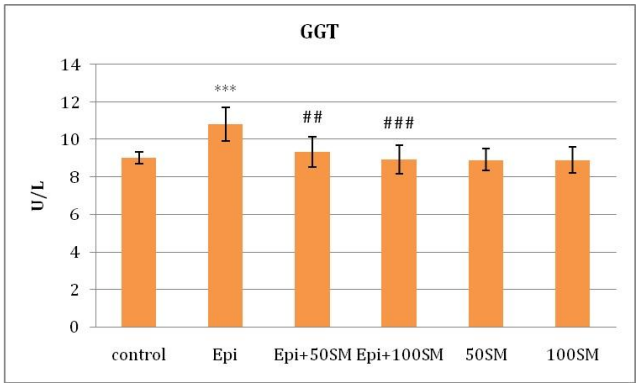

Figure 3: The effects of SM against liver injuries induced by EPI on serum glutamyl transferase (GGT). Values are expressed as mean ± SD
* p< 0.05 significantly different from the control group;
# p< 0.05 significantly different from the EPI-treated group.

After 24 h of Epi treatment, the AST (Fig.1), ALT (Fig.2) and GGT (Fig.3) serum activities significantly increased ($p < 0.001$). Pre-treatment with 100 mg/kg SM decreased significantly the Epi-induced elevation of serum aminotransferases ($p<0.01$) and extremely

significantly for GGT ($p < 0.001$).

Histopathology

Light microscopic evaluation of liver tissues showed normal liver architecture (Fig 4A). In the Epi group, sinusoidal dilatation, inflammatory cell infiltration and hepatocyte steatosis were present (Fig.4B). The treatment with SM, showed mild to moderate accumulation of lipid drops and reduction of inflammatory infiltrates compared to the Epi group, especially for dose of 100 mg/kg SM (Fig.4D).

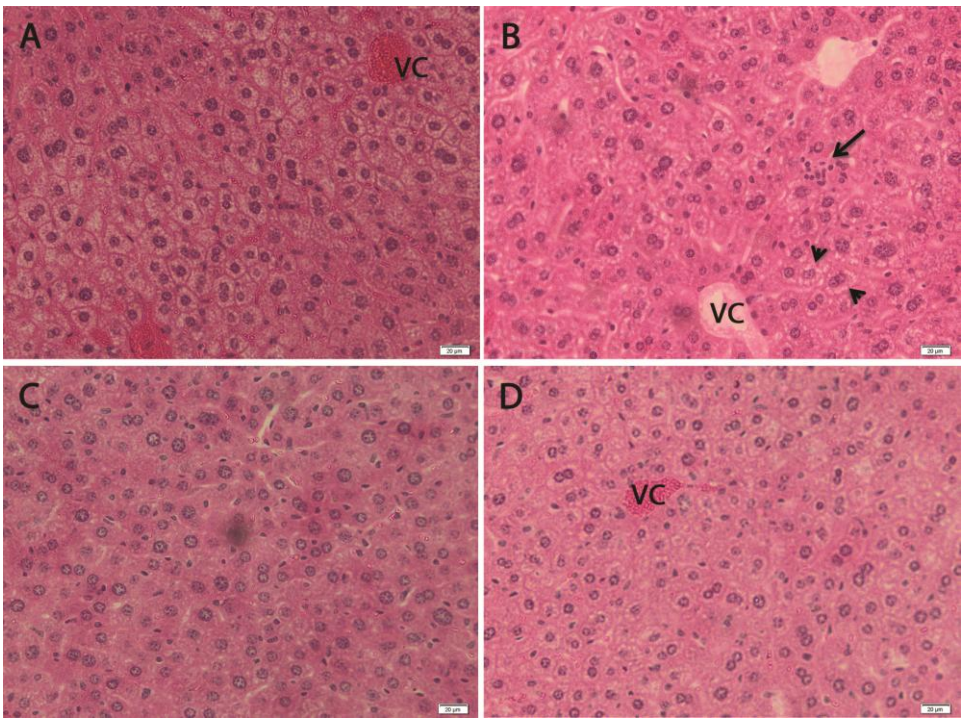

Fig. 4. Hepatoprotective effect of silymarin against liver injuries induced by Epi. A. Control group; B. Epirubicin (Epi) group; C. Epirubicin+ 50 mg/kg silymarin (Epi+50SM); D. Epirubicin+100 mg/kg silymarin (Epi+100SM); inflammatory infiltrates (arrow); hepatocyte steatosis (arrowhead); VC- centrilobular vein

Darkfield microscopy with hyperspectral detection (hsDFM)

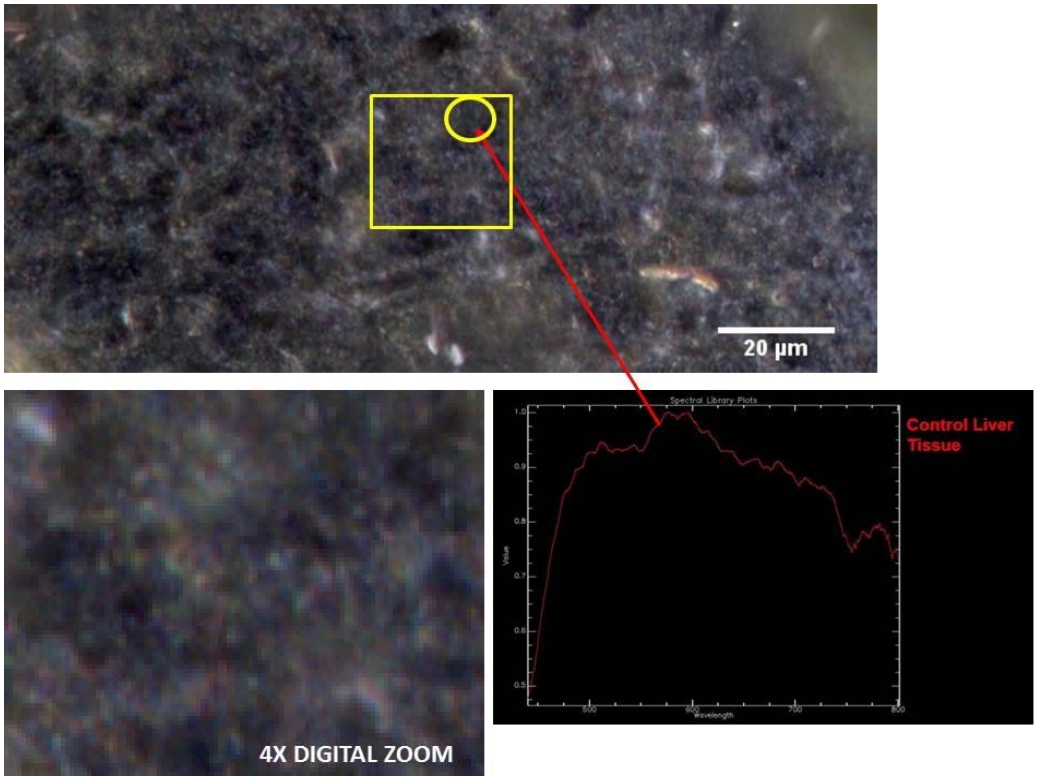

Fig. 5. Hyperspectral image and spectral profile per Control Liver Tissue. The spectral profile is the mean per the region of interest indicated

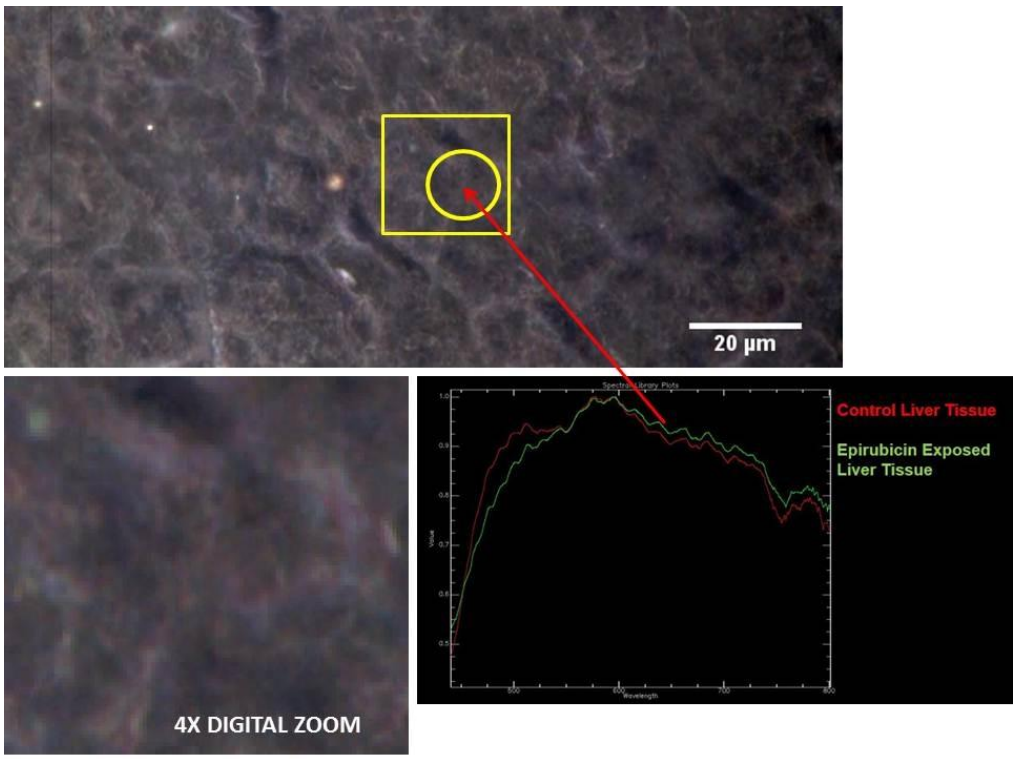

Fig. 6. Hyperspectral image and spectral profile per the Epirubicin Exposed Liver Tissue (Epi). The spectral profile is the mean per the region of interest indicated.

When compared to the Liver Tissue Control spectral profile, it can be observed that there is an increased absorption in the Epirubicin Exposed Liver Tissue spectral profile at approximately 450-550 nm and a marginal increase in reflectance in the Epirubicin Exposed Liver Tissue spectral profile from approximately 625-800 nm (Fig.6).

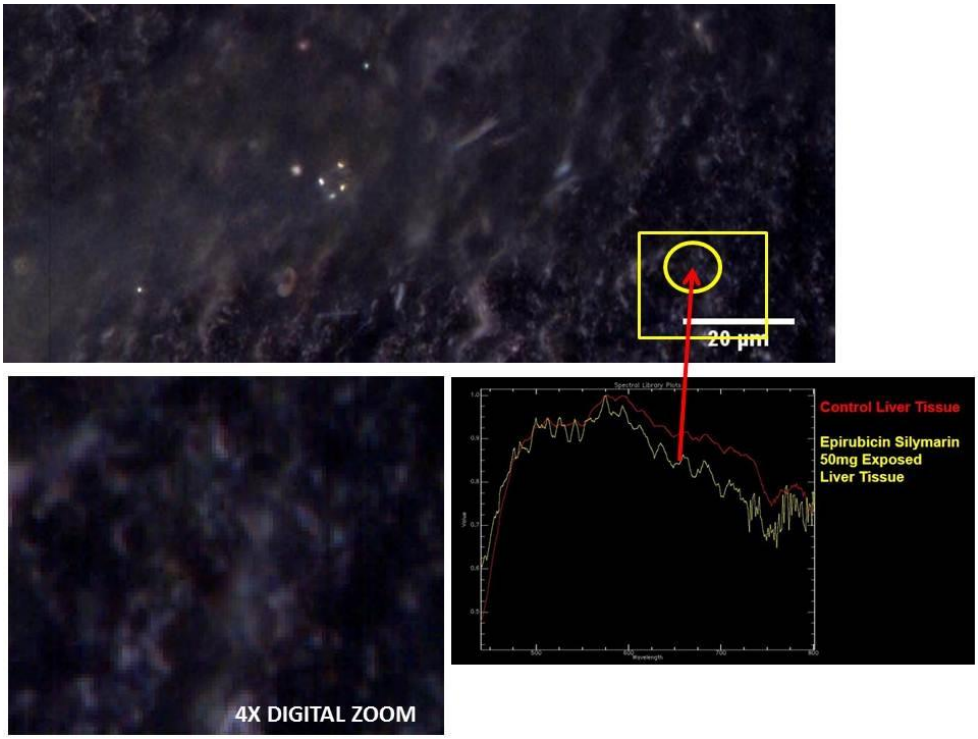

Fig. 7. Hyperspectral image and spectral profile per the Epi+50mgSM Exposed Liver Tissue. The spectral profile is the mean per the region of interest indicated.

When compared to the Liver Tissue Control spectral profile, it can be observed that the Epi+50mgSM Exposed Liver Tissue has a unique spectral profile (Fig.7).

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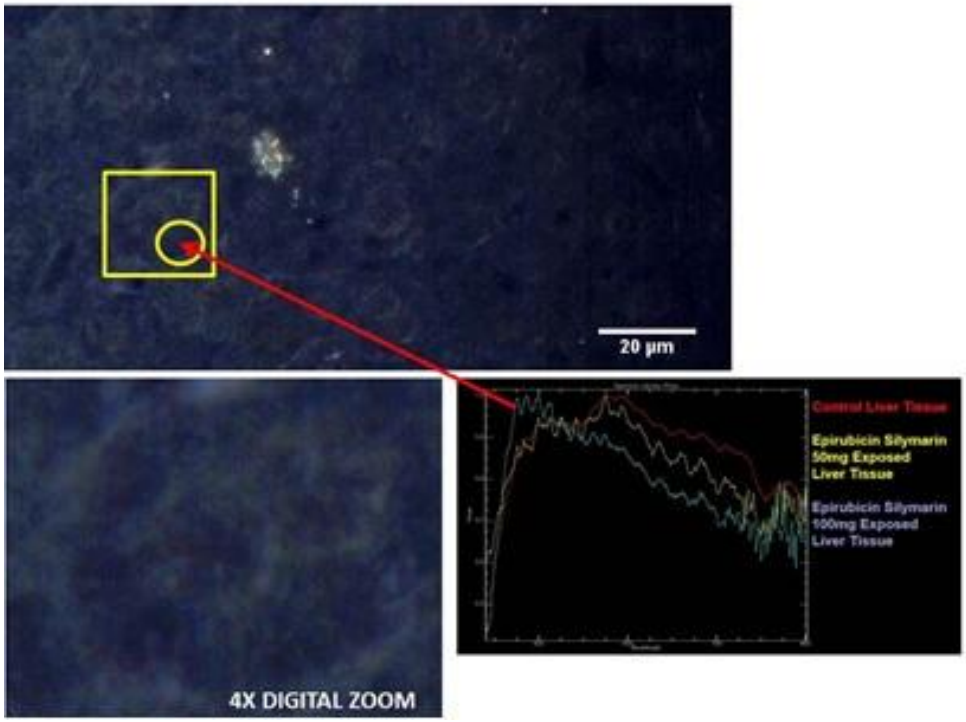

Fig. 8. Hyperspectral image and spectral profile per the Epi+100mgSM Exposed Liver Tissue. The spectral profile is the mean per the region of interest indicated.

When compared to the Liver Tissue Control and Epirubicin Silymarin 50mg Exposed Liver Tissue spectral profiles, it can be observed that the Epirubicin Silymarin 100mg Exposed Liver Tissue has a unique spectral profile (Fig.8).

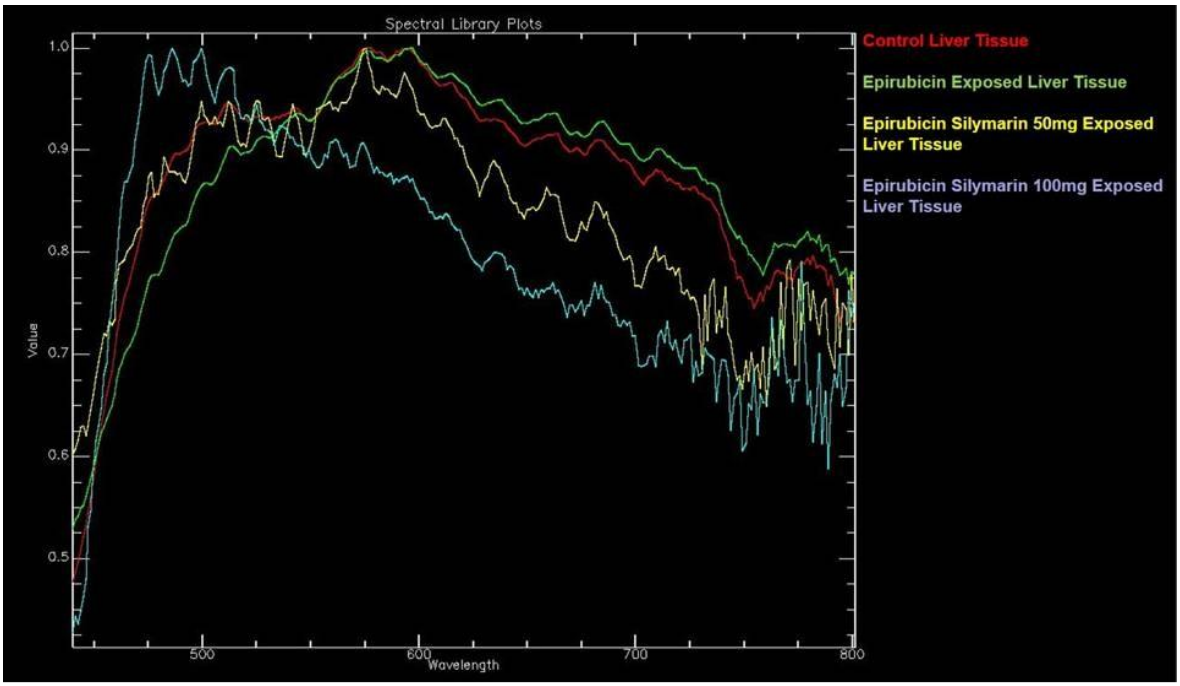

Fig. 9. Hyperspectral microscopy spectral profiles for hepatic tissue in treated mice. (red) Control group. (green) Epi group. (yellow) Epi+50mgSy group. (purple) Epi+100mgSy

DISCUSSION:

The liver has unique metabolic functions and a unique relationship with the gastrointestinal tract, which makes it an important target in terms of drug toxicity, of xenobiotics and oxidative stress. Portal blood brings to the liver drugs or xenobiotics in a concentrated form, directly absorbed from the small intestine. Drug metabolizing enzymes detoxify xenobiotics but can activate the toxicity of others (Jaeschke et al., 2002). The clinician that assesses the hepatotoxicity in the oncological patient receiving chemotherapy must consider the reactions of the body

to antibiotics, antifungals, analgesics, anti-emetics (Kufe et al., 2003), all of these being part of the complex antineoplastic therapy.

Epirubicin belongs to the category of anthracycline antitumor antibiotics and its cytotoxic mechanisms of action are intercalation into DNA and blocking the topoisomerase II, thereby inhibiting both DNA replication and RNA synthesis and causes DNA chain cleavage followed by apoptosis and death of proliferating cells. In addition, epirubicin produces cytotoxic free radicals, which damages the cell membranes (Verret et al., 2013). In the literature there are few studies that relate to epirubicin, because this derivate of doxorubicin is considered to have a lower toxicity risk than doxorubicin. Generally, these studies include patients having polichemotherapy regimens that include at least two chemotherapeutic agents in combination. Epirubicin is mainly used in regimens indicated for the breast cancer (Kufe et al., 2003), where the dose is high, but can be associated with other cytostatic agents for the therapy of hematologic malignancies. In these regimens, the dose is lower, so the toxic effects are expected with lower or null incidence and intensity (Wintrobe et al., 2011).

Some chemotherapeutic agents give dose-dependent hepatotoxicity (Joshi et al., 2014)or depending on the pattern of administration (Boussios et al., 2012).The post-chemotherapy hepatotoxicity appears frequently as an unpredictable reaction, of idiosyncratic type, due to immunological mechanisms or variations in the host's metabolic response (Lee et al., 1995). Despite major advances in understanding the mechanisms that lie behind the toxicity of cytostatics, the pharmacodynamics or the interplay between liver and chemotherapy, clear etiology of liver toxicity for many chemotherapeutic agents remains unexplained (Grigorian et al., 2014). Anthracyclines metabolism occurs predominantly in the liver, and liver antioxidant capacity, including that provided by the production of glutathione may protect against free radical injury (Joshi et al., 2014). It is known that pharmacogenomics can play a key role in determining individual susceptibility to chemotherapy toxic effects (Maor et al., 2013). An important factor contributing to liver damage extension is the capacity of liver regeneration.

The liver has many metabolic functions, yet quantitative assessment of liver function is not available in everyday practice. Liver damage estimate is thus an indirect one (Joshi et al., 2014). Moreover, there isn't yet an accepted system to define liver dysfunction in the patient with cancer (Eklund et al., 2005), although many patients receiving chemotherapy have abnormal results for liver function tests. There are no clinical or histological aspects which are specific to liver toxicity after chemotherapy (Kaplowitz et al., 2007).

The manifestation of hepatotoxicity ranges from asymptomatic, to increase in liver biochemistry tests to fatal liver failure. For anthracyclines, doxorubicin namely, clinical trials and case reports have described the manifestation of liver toxicity as hepatitis (rare), cholestasis (rare), or veno-occlusive disease (rare) (Grigorian et al., 2014). Benjamin described eight patients with impaired liver function, severe mucositis associated with pancytopenia during the administration of doxorubicin therapy, that led to guides for dose-reducing in doxorubicin therapy (Joshi et al., 2014).

The first goal in our study consisted in determination of hepatic serological markers that correlate with liver function, namely alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma glutamyl transpeptidase (GGT). Blood was taken from the mice after their euthanasia and the test results were statistically analyzed. The results revealed statistically significant increased values for each of these tests in the group treated with epirubicin compared to the control, and statistically significant lower values for the groups that had silymarin associated in 50mg/kg or 100mg/kg compared to the epirubicin group (Fig.1,2,3). Improving of liver tests was more evident in the group co-terated with 100mg/kg silymarin compared to the group treated with silymarin 50mg/kg (ALT values 98.225+8.019, p=0.0035 and 100.31+8.239, p=0.0032) (Fig 2).

The cytotoxicity and genotoxicity of doxorubicin may be mediated by free radicals derived from the drug, produced by a variety of mechanisms including the synthesis of reactive oxygen species, alkylation of cellular macromolecules, DNA intercalation and cross-link, lipid peroxidation, damage of the cell membrane, production of ceramides and p53 induction in various tissues (Hozayen et al., 2014; Quiles et al., 2002; Lorenzo et al., 2002). These free radicals can damage the hepatic membranes (Domitrović et al., 2009), by phospholipid activation and lipid peroxidation, that increases the intracellular Ca2 + and the release of ALT, followed by apoptotic cell death (Ogawa et al., 1987; Li et al., 2002). ALT, AST and GGT are released in the same way from the hepatic cell, after cell membrane destruction. Studies assessing the liver toxicity by anthracyclines in human patients showed that patients treated with combined chemotherapy generally develop transient mild or moderate increases of liver tests, even if each cytostatic individually taken has a minimal or no liver toxicity risk, this being due to drug interactions. In patients receiving chemotherapy, transient increases in liver function tests without clinical hepatotoxicity are frequent. But when the toxicity is clinically proven, its diagnosis and discontinuation of the causative drug are imperative. Although the dose adjustments for doxorubicin are based on total bilirubin, according to studies on patients receiving chemotherapy, the epirubicin clearance correlates with serum levels of AST (Eklund et al., 2005). Hepatocellular sensitivity to chemotherapy depends on each antineoplastic agent (Joshi et al., 2014).

A retrospective study on breast cancer patients treated with doxorubicin in association reported changes in laboratory liver tests in 85% of patients, the tests were normalized by 1 year after completion of chemotherapy (Larroquette et al., 1986). Another paper described six patients on combination doxorubicin chemotherapy, in which increase in AST, ALT and bilirubin was observed shortly after chemotherapy

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administration, having also an inflammatory infiltrate and steatosis on liver biopsy. This reaction was considered idiosyncratic (Joshi et al., 2014).The model of liver injury is defined as hepatocellular, with an initial increase in ALT, cholestatic, when the concentration of alkaline phosphatase (FAL) increases, or mixed, in which both tests are elevated. An increase in ALT of three times the normal upper limit and bilirubin greater than 2 times the normal upper limit are used to define clinically significant abnormalities in humans (Navarro et al., 2006).

Various other studies have reported elevated liver enzymes after anthracycline therapy, namely doxorubicin, in different percentages: 1-2% of patients (Joshi et al., 2014; Larroquette et al., 1986; Anandakumar et al., 2007; Ajith et al., 2008; Indu et al., 2014; Salouege et al., 2014), and at different time intervals after chemotherapy, with values becoming normalized between 3 weeks and 1 year after chemotherapy. Chen described a significant increase in ALT, AST, FAL, GGT and total bilirubin post administration of doxorubicin, in contrast with control groups that didn't have these changes (Chen et al., 2004). These can be explained by accumulation of doxorubicin in the liver, with toxic side effects, cell destruction and increased permeability of liver cell (Hozayen et al., 2014).

The results obtained in our study showed elevated ALT similar to those obtained in other studies conducted by Indu (Fig. 2), although the doxorubicin dose used was of 25mg / kg and the epirubicin dose used in our study was 12 mg / kg (75.94 + 7.27 when administered doxorubicin versus 60.187+ 9.53 in our study with epirubicin) (Indu et al., 2014).The medical literature mentions that, in order to produce the same toxic effect, a higher dose of epirubicin should be given compared to the doxorubicin dose. The ratio of doxorubicin and epirubicin for similar toxicity is 1: 1.2 for hematologic toxicity and 1: 1.5 for non-hematologic toxicity (Levine, 2000). However results of our study showed that low-dose epirubicin can cause liver toxicity.

If in terms of increased transaminases, this phenomenon can be observed even transient and without clinical significance, it remains that morphological evaluation to decide whether there are really toxicity injuries due to epirubicin treatment. Thus, histological examination showed lesions that overlap with those described by other researchers, in studies conducted on patients or on laboratory animals, although some authors describe epirubicin as having no toxic effects on liver (Joshi et al., 2014). The histological changes in our study, which are in line with the biochemical findings, are the following: sinusoidal dilatation, inflammatory cell infiltration and hepatocyte steatosis (Fig. 4).

Regarding the results of Verret's research on laboratory animals it has been shown that after embolization with doxorubicin microsphere, there were observed lesions in the bile ducts and liver parenchyma (Verret et al., 2013). The lesions had an aspect of necrosis, the necrosis areas being surrounded by tissue remodeling, characterized by the presence of macrophages and spindle cells in a collagen matrix. The edges of necrosis, at the boundary between normal tissue and necrotic tissue, were sometimes infiltrated by inflammatory cells, predominantly polymorphonuclear neutrophils and some macrophages. One week after initial lesions, the stages of healing and repair followed, through a remodeling and profibrogenic process, by activating the spindle cells, which are the main source of liver pathological fibrosis (Friedman et al., 2008). Angiogenesis is activated in order to develop the vasculature and the blood supply needed for the hypertrophied liver parenchyma and the necrosis repair area (Kaplowitz et al., 2007).Other changes described in the literature after administration of doxorubicin show veno-occlusive disease, changes in hepatic terminal venules (Kaplowitz et al., 2007), cholestatic hepatitis (Patakfalvi et al., 1987), even fatal acute hepatitis in a patient with non-Hodgkin lymphoma treated with doxorubicin and radiotherapy. Our experiment on mice didn't show any signs of liver fibrosis in HE staining (Fig.4.B,C,D), sinusoidal dillatations are likely the result of a process begun early in the first days of the application of chemotherapy. If we would have assessed the mice's livers later it is possible that we found fibrotic changes.

At structural level, other studies describe sinusoidal injury, ranging from sinus dilatation to sinusoidal obstruction syndrome (veno-occlusive disease), which can progress to nodular regenerative hyperplasia (DeLeve et al., 2002). Injury of the endothelial cells lining the sinusoids is the initial event and leads to subintimal thickening and erythrocyte extravasation in subendothelial Disse space (perisinusoidal space). Sinosoidal endothelial cells and the red cells block the sinusoids, arresting the venous flow, leading to hepatic congestion and sinus dilatation. In more advanced stages, there is a fibrotic response in sinusoides leading to obliteration of central venules and to hepatic sinusoidal obstruction syndrome (Maor et al., 2013). Following doxorubicin therapy within the rat liver there are changes in the architecture of the liver trabeculae, cell swelling, sinus atrophy and focal necrosis, reduction of Kupffer cells number, liver weight reduction, according to studies of Martinel Lamas (Martinel Lamas et al., 2015).Optical microscopy also showed that high doses of doxorubicin caused major hepatotoxicity, including liver cords desintegration, which looked as empty vacuole aligned along hepatocyte necrosis, focal inflammation and tissue necrosis. Small doses also produced injury described as: perivascular infiltrates, changes in endothelial cell membrane, periportal fibrosis, marked degeneration of hepatic cords, increased incidence of vacuolar degeneration and apoptotic cell death, liver sinusoidal dilatation and periportal fibrosis. Moreover, many hepatocytes showed pyknotic nuclei indicating caryomegaly and apoptosis. The tendency to liver fibrosis was manifested by the presence of many focal granulomatous cell injury areas. These changes were confirmed at ultrastructural level, where the description revealed rough vesicle endoplasmic reticulum and atrophied mitochondria having ill-differentiated

cisternae, dense macrophages, lymphocytes and fibroblasts collections, with collagen fibrils, as early manifestation of fibrosis (El-Sayyad et al., 2009).

By optical microscopy of liver tissue in the control group we noticed polygonal large normal cells, having prominent round nuclei and eosinophilic cytoplasm, and hepatic sinusoid between hepatic cords with correct positioning of Kupffer cells (Fig. 4 A).

The epirubicin treated group showed hepatic steatosis (Fig. 4 B,C,D). Other studies published by Joshi, after doxorubicin administration, described changes in hepatic immune cell function. During chemotherapy, up to 85% of patients develope hepatic steatosis that points to a malfunctioning lipid metabolism by altering the level of hepatocyte lipoprotein synthesis. Increasing of the lipid content within the liver cell leads to increased vulnerability of the cell, which over time can lead to irreversible hepatocellular abnormalities by attracting inflammatory cells (Joshi et al., 2014). Protection mechanisms against oxidative stress are significantly altered in steatosis (Bilchik et al., 2005). Regeneration is delayed in the steatosis liver (Garcea et al., 2009), which in turn leads to a prolonged hepatic dysfunction (Veteläinen et al., 2007). The studies conducted by Boussios and El Sayyad also showed that the anthracycline therapy may cause focal infiltration with inflammatory cells and liver steatosis (Boussios et al., 2012; El-Sayyad et al., 2009). In epirubicin treated groups we observed inflammatory infiltrate (Fig. 4 B,C,D), more pronounced in the group that received only epirubicin (Figure 4.B,C,). These inflammatory cells in the hepatic parenchyma are placed in groups.

Our experiment showed a biological cholestatic syndrome, reflected in the increase in GGT (Fig. 3). In cholestatic disease, bile acids endogenously produced induce hepatocellular apoptosis (Jaeschkeet al., 2002), by stimulating the Fas translocation from the cytoplasm to the plasma membrane where self-aggregation initiates and triggers apoptosis, documented by reducing the cell size, and condensation and lobulation of nuclei, caspase activation, DNA fragmentation and phosphatidylserine externalization. Kupffer cell activation and neutrophil infiltration enhances the toxic injury. Kupffer cells and neutrophils are proinflammatory cytokines and chemokines sources, as well as reactive oxygen and nitrogen species sources, that sustain oxidative stress in toxic injury induced by toxicity, through local inflammation (Jaeschke et al., 2002; Lasin et al., 2001).

Another aspect observed in studies on liver toxicity after chemotherapy is that although histological appearance in optical microscopy can have minimal changes, there is still significant damage, visible by electron microscopy, described even since the 80s (Harb et al., 1983). Studies show that doxorubicin can cause at liver subcellular level the following changes: polymorphic mitochondria, cytoplasm vacuolation and accumulation of lipid droplets (Verret et al., 2013).

Inconstant appearance of significant liver injury after chemotherapy with anthracyclines may be explained by the fact that chemotherapeutic drugs target rapidly proliferating cells and the hepatocytes have a slow turnover. Another explanation would be that the liver is protected by intracellular detoxification pathways that protect it from electrophilic metabolites and oxidative stress drug-induced (Kaplowitz et al., 2007). The metabolites may be chemical electrophilic compounds or free radicals which reduce the level of glutathione, covalently bind to proteins, lipids or nucleic acids, or induce the peroxidation of lipids. The consequences are hepatocellular necrosis, apoptosis and sensitization to cytokines or inflammatory mediators produced by non-parenchymal cells.

Non-parenchymal liver Kupffer cells, sinusoidal endothelial cells, the stellate cells (Ito) and leukocytes, neutrophils and monocytes newly recruited, all contribute to the pathogenesis of hepatotoxicity. The role of phagocytes is to remove dead cells and cell debris considering the preparation for liver regeneration. Due to the nature of toxic mediators generated by these phagocytes, healthy cells can also be affected, leading to worsening of existing injuries. Neutrophils can be recruited into hepatic vessels by local tissue damage (Lawson et al., 2000) or by systemic exposure to inflammatory mediators, including TNF-alpha (tumor necrosis factor alpha), interleukin 1, complement factors, platelet activating factor and chemokines. In the liver, neutrophils accumulate into sinusoids and adhere to the venular endothelium (Jaeschke et al., 2002).According to Jaeschke, the neutrophil recruitment into sinusoids usualy does not depend on cell adhesion molecules but it seems to result from mechanical sequestration due to rheological changes of neutrophils, from the active vasoconstriction within the sinusoids and from swelling of the sinusoidal cells (Jaeschke, et al., 2002). Neutrophils are rarely cytotoxic when present in the sinusoids and they need to transmigrate into the sub-sinusoidal space in order to cause tissue injury (Chosay et al., 1997). Neutrophils must receive a chemotactic signal to be able to transmigrate and operate. Chemokines generated by hepatocytes can trigger a neutrophil induced injury (Maher et al., 1997). Moreover, lipid peroxidation products are intensively chemotactic and may be responsible for continuing and amplifying the injury(Liu *et al.*, 1994).The hepatocyte apoptosis was identified as a potent stimulus for the growth and extravasation of the endotoxin-induced injury (Jaeschkeet al., 2002). Reactive oxygen species derived from neutrophils can induce an intracellular oxidative stress at hepatocyte level that triggers the cell necrosis injury in less than 1 hour (Jaeschke et al.,1999). In addition, reactive oxygen species promote inflammation by increasing the activation of the transcription factor NF-kB, which controls the formation of cytokines, chemokines and adhesion molecules (Jaeschke et al., 2002). Once chemotactic stimulated, the neutrophils extravasate and adhere to the parenchymal cells, inducing necrotic cell death through the release of reactive oxygen species and proteases.

Studies that searched for the overexpression of certain genes after doxorubicin chemotherapy described genes involved in apoptosis and metabolism of doxorubicin, as well as genes involved in cell

proliferation (growth factors and transcription factors), in tissue remodeling (metalloproteinases and certain types of collagen), in the inflammatory response (interleukins and chemokines) and in angiogenesis (angiogenic factor). They play a role in healing and liver regeneration after injury. The inhibited genes were genes related to liver function, including glucose and lipid metabolism enzymes (Verret et al., 2013). Gene expression analysis supports the hypothesis that anthracyclines produce DNA damage and induce the expression of pro-apoptotic signaling through p53 pathway leading to cell death. Reducing in the expression of genes related to liver metabolism leads to the idea that following chemotherapy the hepatic metabolism becomes impaired and leads to hepatocyte death (Sehata et al., 2005). We haven't noticed apoptosis signs or necrosis in the liver specimens studied.

Primary and secondary mitochondrial dysfunction is an important mechanism for anthracycline induced microvesicular steatosis or hepatitis with cytolysis (Fromenty et al., 1995). Mitochondria are considered a crucial pawn in the doxorubicin toxicity as the main generator of superoxide for initiating the cascade of reactive oxygen species (Hozayen et al., 2014; Martinel Lamaze et al., 2015).

The most common mechanism for producing hepatitis with cytolysis is P450-dependent formation of reactive metabolites that cause direct toxicity or immune reactions. Reactive metabolites can cause DNA damage and overexpression of p53 or Bax as well as depletion of glutathione (Haouzi et al., 2000), activation of caspase 3 (Martinel Lamaze et al., 2015), all these promoting cell death. Chemotherapy induces the Fas ligand expression in hepatocytes to initiate cell death (Müller et al., 1997).

Using spectral microscopy, we saw that in the hepatic tissue of Epi group the spectral profile is different from that of control group, signaling the presence of epirubicin within the hepatic tissue (Fig. 5,6). The image also shows the difference, by detecting the drug in the hepatic cells (Fig. 6). The presence of epirubicin in the tissue in the groups co-treated with silymarin induces a hepatic spectral profile that is different from the control group and the Epi group, that might be explained through the silymarin capacity of hepatic detoxification, through a rapid elimination of epirubicin toxic metabolites (Fig. 7,8,9).

Antioxidants used by cancer patients are described in the literature as a complementary safe medication and are part of clinical trials (Davies et al., 2006).In these cases it is very important that the concomitant medication doesn't interfere with the cytotoxic antitumor effect of chemotherapy, as reducing the tumoricidal potential of antineoplastic drugs (Hoh et al., 2006; Drisko et al., 2003).

Due to the increased incidence of hepatotoxicity post cytotoxic chemotherapy and also due to the necessity of administering of standard doses of cytotoxic chemotherapeutic agents in order to obtain disease remission, many researchers have studied the effects of natural substances on liver during chemotherapy toxicity. A review of the literature showed that the incidence of hepatic adverse events of grade I-IV was 35%, higher than the prevalence observed when chemotherapy is combined with hepatoprotective substances, in this case the incidence was estimated at 20% (Xiaoyuan et al., 2012).

Reduction of the oxidative damage of DNA by antioxidants was evaluated as a therapeutic approach to reduce injuries from chemotherapy, such as anthracycline (Quiles et al., 2002). Mitochondria are also targets for hepatoprotective antioxidant phytochemical substances (Danz et al., 2009; Sung et al., 2009).Studies with rutin, hesperidin or both, in which these substances were administered pre-therapy, have proven their hepatoprotective efficacity, by significantly improving the AST, ALT, FAL, and GGT levels, levels that were significantly elevated following administration of doxorubicin (Hozayen et al., 2014). Lipid peroxidation was significantly increased following doxorubicin administration, and peroxidase activity, glutathione peroxidase activity and glutathione-s –transferase activity were significantly decreased.

Silymarin can protect from the epirubicin toxicity by multiple mechanisms: at mitochondrial level by stimulating specific anti-ROS proteins, prevention of injury on mtDNA (mitochondrial DNA), stimulation of replication, inhibition of active membrane lipases, protecting the chain of electron transport for generating optimal ATP during energy consumption. Silymarin protects the intracellular microenvironment by preserving mitochondrial-dependent antioxidant components (Tasduq et al., 2005).

In our study, we observed that the therapeutical effects of silymarin were beneficial in protection against hepatotoxicity, demonstrated both clinically (Fig. 1,2,3) by reducing the levels of hepatic tests, and at structural level by reducing the steatosis and the sinusoidal dilatation in hepatic tissue (Fig. 4 C,D). Moreover, the inflammatory infiltrates present in the group treated with epirubicin were found in a much lower ratio than in the groups to which was added silymarin doses of 50mg/kg or 100mg/kg (Fig. 4 B,C,D), proving the effectiveness against epirubicin-inducedtoxicity.

CONCLUSIONS:

Hepatotoxicity after cytostatic chemotherapy is one of the most serious side effects of anticancer therapy. This can lead to chemotherapy dose reduction, to the modification of the timing of chemo administration, which can have serious consequences on achieving disease remission in oncological patients.

Evaluation of hepatotoxicity due to cytostatic chemotherapy is not easy, given the many causes that can induce liver damage in cancer patients. Therefore, clinical trials that evaluate a single drug or experimental research on laboratory animal are very useful. When given epirubicin, an anthracycline, the toxic liver effects were observed on two levels: first by increasing the levels of serological hepatic markers and secondly by structural abnormalities in light microscopy. The benefit that the cancer patient can have by concomitant administration of silymarin

remains to be evaluated in clinical trials, by now it has been proven to protect the liver in both 50mg/kg and 100mg/kg doses, but the maximal benefice is obtained with the 100mg/kg dose, which improved the histological changes and the biochemical hepatic tests.

The injury was observed at therapeutical doses, the doses used for malignant hematological diseases, which are by far lower that the high doses used in breast cancer regimens.

The mechanisms by which the chemotherapy liver toxicity operates are multiple, many of them are probably still unknown, but it is certain that natural antioxidant products act by counteracting the pathways that induce this type of liver toxicity.

REFERENCES:

Ajith TA, Aswathy MS and Hema U, The Protective effect of Zingiber officinale roscoe against anticancer drug doxorubicin-induced acute nephrotoxicity, Food and Chemical Toxicology, 46: 3178-3181, 2008

Anandakumar PP, Malarkodi SP, Sivaprasad TR. and Saravanan GD, Antioxidant DL-alpha lipoic acid as an attenuator of Adriamycin induced hepatotoxicity in rat model, Indian J ExpBiol, 45 (12): 1045-1049, 2007

Bilchik AJ, Poston G, Curley SA, Neoadjuvant chemotherapy for metastatic colon cancer: a cautionary note, Journal of Clinical Oncology, vol. 23, no. 36, pp. 9073–9078, 2005

Boussios S, Pentheroudakis G, Katsanos K, Pavlidis N, Systemic treatment-induced gastrointestinal toxicity: Incidence, clinical presentation and management, Ann Gastroenterol. 25(2): 106–118, PMCID: PMC3959393, 2012

Chen H, Yu YY, Zhang M J, Deng XX, Yang WP, Ji J, Peng CH. Li HW, Protective effect of doxorubicin induced heat shock protein 72 on cold preservation injury of rat livers, World Journal Gastroenterol 10 (9): 1375-1378, 2004

Chosay JG, Essani NA, Dunn CJ, Jaeschke H, Neutrophil margination and extravasation in sinusoids and venules of the liverduring endotoxin-induced injury,*Am. J. Physiol.* 272, G1195–G1200, 1997

Danz ED, Skramsted J, Henry N, Bennett JA and Keller RS,Resveratrol prevents Doxorubicin cardiotoxicity through mitochondrial stabilization and the Sirt1 pathway, Free Radic. Biol. Med, 46; 1589-1597, 2009

Davies AA, Davey SG, Harbord R, Bekkering GE, Sterne JAC, Beynon R, Thomas S. Nutritional interventions and outcome in patients with cancer or preinvasive lesions: systematic review. J NatlCancer Inst 98:961–73, 2006

DeLeve LD, Shulman HM, McDonald GB, Toxic injury to hepatic sinusoids: sinusoidal obstruction syndrome (veno-occlusive disease), Seminars in Liver Disease, vol. 22, no.1, pp. 27–41, 2002.

Domitrović R, Jakovac H, Tomac J and Sain I, Liver fibrosis in mice induced by carbon tetrachloride and its reversion byluteolin, Toxicol. Appl. Pharmacol 241: 311-321, 2009

Drisko JA, Chapman J, Hunter VJ, The use of antioxidants with first-line chemotherapy in two cases of ovarian cancer, J Am CollNutr 22:118–23, 2003

Drug-induced liver disease, edited by Neil Kaplowitz, Laurie D. DeLeve, 2nd ed.ISBN-10: 0-8493-9896-7, by Informa Healthcare USA, Inc., 2007

Eklund JW, Trifilio S, Mulcahy MF, Chemotherapy dosing in the setting of liver dysfunction, Oncology (Williston Park). 2005 Jul;19(8):1057-63; discussion 1063-4, 1069, 2005

El-Sayyad HI, Ismail MF, Shalaby FM, Abou-El-Magd RF, Gaur RL, Fernando A, Raj MHG, Ouhtit A, Histopathological effects of cisplatin, doxorubicin and 5-flurouracil (5-FU) on the liver of male albino rats, Int. J. Biol. Sci. 5(5):466-473, 2009

Friedman SL, Mechanisms of hepatic fibrogenesis. Gastroenterology, 134:1655–1669, 2008

Fromenty B, Pessayre D, Inhibition of mitochondrial b-oxidation as a mechanism of hepatotoxicity, *Pharmacol. Ther*. 67, 101–154, 1995

Garcea G, Maddern GJ, Liver failure after major hepatic resection, Journal of Hepato-Biliary-Pancreatic Surgery, vol. 16, no. 2, pp. 145–155, 2009

Grigorian A, O'Brien CB, Hepatotoxicity Secondary to Chemotherapy, J ClinTranslHepatol. 2014 Jun; 2(2): 95–102, doi: 10.14218/JCTH.2014.00011, 2014

Haouzi D, Lekehal M, Moreau A, Moulis G, Feldmann G, Robin MA,Lettéron P, Fau D, Pessayre D, Cytochrome P450-generated reactive metabolites cause mitochondrial permeability transition, caspase activation, and apoptosis in rat hepatocytes. *Hepatology*32, 303–311, 2000

Harb JM, Kamen BA, Werlin SL, Hepatic ultrastructure in leukemic children treated with methotrexate and 6-mercaptopurine. Am J PediatrHematolOncol. 5: 323-31, 1983

Hoh C, Boocock D, Marczylo T, et al., Pilot study of oral silibinin, a putative chemopreventive agent, in colorectal cancer patients: silibinin levels in plasma, colorectum, and liver and their pharmacodynamics consequences. Clin Cancer Res 12:2944–50, 2006

Holland-Frei Cancer Medicine. 6th edition, Editors: Donald W Kufe, Raphael E Pollock, Ralph R Weichselbaum, Robert C Bast, Ted S Gansler, James F Holland, Emil Frei, Hamilton (ON): BC Decker; ISBN-10: 1-55009-213-8, chapter 154, Laurie D. DeLeve, Hepatotoxicity by Anticancer Therapy, 2003

Hozayen WG, Seif HAS, Amin S, Protective Effects of Ruitn and / or Hesperidin Against Doxorubicin-Induced

Hepatotoxicity, International Journal of Clinical Nutrition, Vol. 2, No. 1, 11-17, 2014

Indu R, Azhar TH, Nair A, Krishnan C, Nair K, Amelioration of doxorubicin induced cardio -

and hepato‑toxicity by carotenoids, Journal of Cancer Research and Therapeutics, Volume 10, Issue 1, January-March 2014

Jaeschke H, Gores G, Cederbaum AI, Hinson JA, Pessayre D, Lemasters JJ, Mechanisms of Hepatotoxicity, Toxicological sciences 65, 166–176, 2002

Jaeschke H, Gores G, Cederbaum A, Hinson A, Pessayre D, Lemasters J, Mechanisms of Hepatotoxicity, Toxicol. Sci. 65 (2): 166-176.doi: 10.1093/toxsci/65.2.166, 2002

Jaeschke H, Ho Y-S, Fisher MA, Lawson JA, Farhood A, Glutathione peroxidase-deficient mice are more susceptible to neutrophilmediated hepatic parenchymal cell injury during endotoxemia: Importance of an intracellular oxidant stress,*Hepatology*29, 443–450, 1999

Jain A, Yadav A, Bozhkov A et al. Therapeutic efficacy of silymarin and naringenin in reducing arsenic-induced hepatic damage in young rats. Ecotox Environ Safe, 74, p. 607–614, 2011

Joshi M, Sodhi KS, Pandey R, Singh J, Goyal S, Prasad S, Kaur H, Bhaskar N, Mahajan S, Cancer Chemotherapy and Hepatotoxicity: An update, Indo American Journal of Pharm Research.2014:4(06), 2014

Larroquette CA, Hortobagyi GN, Buzdar AU, Holmes FA, Subclinical hepatic toxicity during combination chemotherapy for breast cancer, JAMA 256:2988–90, 1986

Laskin DL, Laskin JD, Role of macrophages and inflammatory mediators in chemically induced toxicity. *Toxicology* 160, 111–118, 2001

Lawson JA, Farhood A, Hopper RD, Bajt ML, Jaeschke H, The hepatic inflammatory response after acetaminophen overdose: Role of neutrophils,*Toxicol. Sci.* 54, 509–516, 2000

Lee WM. Drug-induced hepatotoxicity. NEngl J Med . 333: 1118-27, 1995

Levine M, The First Pharmacia & Upjohn Pan European Investigators Forum, CME/CE Released: 6/19/2000

Li Z, Lin H, Yang S, Diehl AM, Murine leptin deficiency alters Kupffer cell production of cytokines that regulate the innate immune system,*Gastroenterology*, vol. 123, no. 4, 2002

Liu P, Vonderfecht SL, McGuire GM, Fisher MA, Farhood A, Jaeschke H, The 21-aminosteroid tirilazadmesylate protects against endotoxin shock and acute liver failure in rats,*J. Pharmacol. Exp. Ther.*271, 438–445, 1994

Lorenzo E, Ruiz-Ruize C, Quesada A, Hernandez G, Rodriguez A, Lopez-Rivas A, Redondo J, Doxorubicin induce apoptosis and CD 95 gene expression in human primary endothelial cells through a p 53-depenent mechanism, J. Biol. Chem 277: 10883-10892, 2002

Maher JJ, Scott MK, Saito JM, Burton MC,Adenovirusmediated expression of cytokine-induced neutrophilchemoattractant in rat liver induces a neutrophilic hepatitis. *Hepatology*25, 624–630, 1997

Maor Y, Malnick S, Liver Injury Induced by Anticancer Chemotherapy and Radiation Therapy, Hindawi Publishing Corporation, International Journal of Hepatology, Volume 2013, Article ID 815105, 2013

Martinel Lamas DJ, Nicoud MB, Sterle HA, Carabajal E, Tesan F, Perazzo JC, Cremaschi GA, Rivera ES, Medina VA, Selective cytoprotective effect of histamine on doxorubicin induced hepatic and cardiac toxicity in animal models, Cell Death Discovery (2015) 1, 15059; doi:10.1038/cddiscovery.2015.59, 2015

Mohamed M. Sayed-Ahmed, Role of carnitine in cancer chemotherapy-induced multiple organ toxicity, Saudi Pharmaceutical Journal, Volume 18, Issue 4, Pages 195–206, Oct 2010

Müller M, Strand S, Hug H, Heninemann EA, Walczak H, HofmannWJ, Stremmel W, Krammer PH, Galle PR, Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (APO-1/Fas) receptor/ligand system and involves activation of wild-type p53,*J. Clin. Invest.* 99, 403–413, 1997

Navarro V J., Senior J R. Drug-related hepatotoxicity. N Engl J Med . 354 (7):731-9, 2006

Ogawa Y, Kondo T, Sugiyama S, Ogawa K, Satake T and Ozawa T, Role of phospholipase in the genesis of Doxorubicin-induced cardiomyopathy in rats,Cancer Res 47: 1239-1243, 1987

Patakfalvi A, Gelencser E, Sipos J, Drug hepatitis of cholestatic type in association with a FAC-regimen for breast Cancer, Acta Med Hung 44: 377-385, 1987

Quiles J, Huertas J, Battino M, Mataix J, Ramirez-Tortosa M, Antioxidant nutrients and adriamycin toxicity,Toxicology 180: 79-95, 2002

Quiles J, Huertas J, Battino M,Mataix J and Ramirez-Tortosa M, Antioxidant nutrients and adriamycin toxicity, Toxicology 180: 79-95, 2002

Salouege I, Ali RB, Saïd DB, Elkadri N, Kourda N, Lakhal M, *et al.*, Means of evaluation and protection from doxorubicin-induced cardiotoxicity and hepatotoxicity in rats, J Can Res Ther 10:274-8, 2014

Sehata S, Kiyosawa N, Atsumi F et al., Microarray analysis of T-2 toxin-induced liver, placenta and fetal liver lesions in pregnant rats,ExpToxicolPathol 57:15–28, 2005

Sung B, Kunnumakkara AB, Sethi G, *et al.*,Curcumin circumvents chemoresistance in vitro and potentiates the effect of thalidomide and bortezomib against human multiple myeloma in nude mice model, Mol. Cancer Ther 8: 959-970, 2009

Tasduq SA, Peerzada K, Koul S *et al.*,Biochemical manifestations of antituberculosis drugs induced hepatotoxicity and the effect of silymarin, Hepatol. Res 31: 132-135, 2005

Verret V, Namur J, Ghegediban SH, Wassef M, Moine L, Bonneau M, Pelage J-P, Laurent A, Toxicity

of Doxorubicin on Pig Liver After Chemoembolization with Doxorubicin-loaded Microspheres: A Pilot DNA-microarrays and Histology Study, CardiovascInterventRadiol36:204–212, DOI 10.1007/s00270-012-0369-1, 2013

Veteläinen R, Vliet AV, Gouma DJ, Gulik TMV, Steatosis as a risk factor in liver surgery, Annals of Surgery, vol. 245, no. 1, pp. 20–30, 2007

Wintrobe's Clinical Hematology Thirteenth Edition, by John P. Greer (Author, Editor), Daniel A. Arber (Editor), BertilGlader(Editor), ISBN-13: 978-1451172683, 2014

Xiaoyuan Li, Jianfeng Zhou, Shuchang Chen, Mei Guan, Yingyi Wang, Lin Zhao, Hongyan Ying and Yanping Zhou, Role of bicyclol in preventing chemotherapeutic agent-induced liver injury in patients over 60 years of age with cancer, Journal of International Medical Research Vol. 42(4) 906–914, 2014