FACULTY OF AGRICULTURE

Minia J. of Agric. Res. & Develop.
Vol. (43), No.2, pp 115- 130, 2023

BIOCHEMICAL IMPROVEMENT INDUCED BY SILYMARIN AND NANO SILYMARIN IN EHRLICH ASCITES CARCINOMA BEARING MICE

Sally M. Abd El-Aziz El Shafei, Esam A. El-Malt, Emad S. Shaker, Nihal K. Sabra, Atef A. Abd El-Rahman*
Department of Agricultural Chemistry, Faculty of Agriculture, Minia University, El-Minya 61519, Egypt
*Correspondence: atef.ahmed@mu.edu.eg; atefnagi2000@yahoo.com; Tel: 01019695527

Received : 10 January 2023 Accepted: 25 June 2023

ABSTRACT

Silybum marianum (L.) was regarded as one of the wonder plants through the last decades. The main bioactive compound in *Silybum marianum* is silymarin. Therefore, the present study aimed to evaluate the Silymarin and Nano silymarine activities on mice with induced Ehrlich Ascites Carcinoma (EAC). Sixty Swiss albino female mice (weigh 20–22 g) were randomly divided into equal four groups, 15 rats each. The first one didn't receive any treatment and served as the standard control. Each of the three remaining groups received 2.5 x 10^6 EAC cells intraperitoneally. The second group didn't receive further treatment and served as the EAC control. Silymarine and nano silymarine were administered orally at 100mg/kg b.w dailly for 6 weeks to mice in the third and fourth groups, respectively. Biochemical parameters, including liver and kidney functions as well as lipid profile, were evaluated in all experimental groups. Results revealed that, mice with induced EAC displayed adverse influence on the biochemical parameters. The animals treated with silymarine and nano silymarine extracts exhibited significant improvements in the biochemical parameters following treatment, with a stronger impact in case of nano silymarine.

Key words: Ascites carcinoma, ehrlich, Mice, silymarin, nano silymarin

INTRODUCTION

One million deaths every year have been representing the mortality ratio from the liver cancer worldwide (**Lee *et al*., 2005**). Natural products proved to have a leading effect with the molecular approaches through the last two decades (**Harvey, 1999**). One of the oldest and most thoroughly researched plants used in the treatment of liver and gallbladder disorders is the plant *Silybum marianum*, popularly known as milk thistle, which is a member of the Asteraceae family **(Pradhan *et al*., 2006)**. Seeds of *Silybum marianum* showed a good protector and chemopreventive source comparing to commercial drugs (**Shaker *et al*., 2010**). These seeds have historically been utilized as a natural cure for several diseases and malignancies. The active component of this plant is silymarin, which is an extracted standardised from silymarin flavonolignans (silybin A and B, isosilybin A and B, silydianin, and silychristin) and flavonoids (taxifolin and quercetin), with the remaining 20 to 30% being a chemically undefined fraction made up of polymeric and oxidized polyphenolic compounds **(Kren *et al*., 2005)**. Silymarin has numerous biological and pharmacological properties, including antioxidant activities. **(Koksal *et al*., 2009)**, acceleration of protein synthesis, cell regeneration **(Fraschini *et al*., 2002)**, and significant anticancer actions **(Singh and Aggarwal, 2004)** against a number of human carcinoma cell lines. In comparison to commercial silymarin, *S. marianum* extract had better results, achieving a double DPPH rate **(Hadaruga and Hadaruga, 2009)**. Silymarin's application is limited by poor water solubility, chemical stability, and bioavailability. For these reasons, nano-delivery systems were the first solution to improve the functional performance **(Zhang et al., 2022)**. The bioavailability of silymarin nanoencapsulated by ultrasound has been demonstrated. It was shown that nanoencapsulated silymarin reduced the oxidative stress that led to hepatotoxicity (**Yousefdoost *et al.* 2019)**. Otherwise, the biological activities of silymarin that support human health were examined. Their nutraceutical component in functional foods and supplements has also been researched. In order to measure the silymarin performance, they also used nano delivery methods **(Zhang *et al.* 2022)**. The current study evaluated the protective action of silymarin and nano silymarin extracts against liver damage using chemical analytical tests for the liver, kidney, and lipid profile in Ehrlich ascites carcinoma mice model.

MATERIALS AND METHODS

Plant seeds and extraction

S. marianum seeds were obtained from Harraz (Natural Herbs Market), Cairo, Egypt. They were then, dried in the shade, cut, and extracted (100 gramme) using ethanol 95% (250 ml). The ethanol was removed under decreased pressure to obtain the dried extract (2.1% yield w/w) which was formulated into aqueous suspension.

Preparation of silymarin powder nanoparticles.

Amorphous silymarin powders in nano particle size, was prepared using the Ball milling mechanical method (Model: PQ-N2 Planetary Ball Mill, Gear Drive 4, 220 v). The grinding

process is carried out in National Research Center using equal amounts of stainless steel balls (250 balls between different sizes). The grinding process was conducted under the atmosphere of the chamber in the high energy ball mill with the weight of the ball to powder (10: 1) for 90 minutes with spin speed at 40,000 rpm. The products are filtered to remove impurities and separate the balls. Milled 100 gm nano powder was used **(Loh *et al*., 2015)** to enhance process ability or solubility especially for those with limited aqueous solubility. Alterations in the size, specific surface area and shape of the particles beside mechano-chemical could be fulfilled. As previously mentioned, nano silymarin extract was filtered in whatman paper and the supernatant was concentrated. Silymarin and nano-silymarin ethanolic extracts were applied for transplanted Ehrlich ascites carcinoma cells.

Experimental animals

Ehrlich Ascites Carcinoma (EAC) cells were obtained from the animal house of the National Cancer Institute at Cairo University, and observed their hyperinflation for the female mice **(Vincent and Nicholls, 1967)**. The mice were kept in cages made of polyacrylic measuring 38 cm by 23 cm by 10 cm. Mice were kept in a typical habitat with a 12 hour light/12 hour dark cycle. They were given free access to water and a regular meal. Before the trial began, the mice spent 7 days becoming used to the lab environment.

Transplantation of tumors

The National Cancer Institute in Cairo, Egypt, provided a line of EAC cells, which were then maintained via weekly intra-peritoneal transplantation of 2.5×10^6 cells/mice. Swiss albino donor mice weighing 18–20 g were used to harvest EAC cells, which were then suspended in sterile isotonic saline. Each recipient mice received an intraperitoneal injection of a set amount of viable cells (typically 2×10^6 cells/ 20 g body weight) **(Gothoskar and Ranadive, 1971)**. Trypan blue exclusion experiment determined the cells' viability to be 99%. Ehrlich ascites carcinoma tumor-bearing mice had their ascitic fluid removed 7-8 days after the tumor-bearing cells first appeared. Intraperitoneally, each animal received 0.2 ml of a tumour cell suspension containing 2.5×10^6 tumour cells. Ehrlich ascites carcinoma cells were isolated and suspended in sterile saline (0.9% NaCl, Almotaheda Pharma) from donor female Swiss albino mice weighing 18–20 g. Each recipient mice peritoneal cavity received a set number of viable cells (typically 2.5×10^6 cells/mice) **(Salem *et al*., 2011)**.

Ehrlich ascites carcinoma (EAC) was taken out with a sterile disposable syringe every 0.5 ml, diluted with 4.5 ml of normal saline (0.9%NaCl), and then administered intraperitoneally (i.p.) into each recipient mice. It was permitted for the tumour cells inside the peritoneal cavity to grow **(Hanafy, 2009** and **Abou Zaid et al., 2011)**.

Experimental design. Total 60 female Swiss albino mice weighing 20–22 g were randomly distributed into four groups as follows: **Group 1:** control 15 mice were kept on normal standard diet without any treatment and housed in cages. Mice in this group considered as negative control for non tumor-bearing group. G**roup 2:** positive control 15 mice were received intraperitoneally (0.2 ml of ascetic fluid containing $2.5x10^6$ EAC cells) and were kept on normal

standard diet housed in cages, called tumor-bearing group. Group 3 and Group 4 were received 0.2 ml of ascetic fluid containing $2.5x10^6$ EAC then, e and nano silymarine were administered orally at 100mg/kg b.w dailly for 6 weeks, respectively. Animal blood collection for mice group samples were obtained by puncture of the axillary plexus of mice anesthetizedm, after 6 weeks. The serum Samples were collected to determine and analyze different blood chemistry measurements using Cobas 6000 analyzer series.

AST, ALT, total bilirubin, total protein, albumin, urea, creatinine, total cholesterol, triglycerides, **high density lipoprotein HDL, and low density lipoprotein LDL concentrations were deremined using automated diagnostic Cobas** 6000 **analyzer.** The Cobas 6000 analyzer series is a completely automated, random-access, software-controlled device for immunoassay and photometric analysis designed for qualitative and quantitative in vitro determinations using a variety of tests. A strong instrument for full diagnostic laboratory automation is the Cobas 6000 analyzer series. Globulin concentration was estimated by subtracting albumin values from total proteins values. The albumin/globulin (A/G) ratio was calculated by dividing albumin by globulin concentations **(Doumas *et al*., 1971).** The triglyceride value was divided by five to determine VLDL. **Non HDL values were calculated by subtracting HDL values from total cholesterol values. Total cholesterol was divided by HDL, and LDL was divided by HDL to calculate the cholesterol/HDL and LDL/HDL ratios (Friedewald *et al*., 1972)**

Statistical analysis

One-way analysis of variance (ANOVA) and Tukey's multiple comparison tests **(Tukey, 1951)** were used in the statistical analysis, which was done using the GraphPad Prism 8 programme and had a 95% confidence interval of $P \leq 0.05$. Results are shown as the mean SEM. The distinction between the treatment and control groups was considered statistically significant.

Ethics Committee Approval

Ethics Committee for the care and use of animals, microorganisms and living cell cultures in education and scientific research at Faculty of Agriculture, Minia University granted consent for all procedures, which were carried out in accordance with the guidlines for the care and use of laboratory animals and assigned an approval number: MU/FA/010/12/22.

RESULTS AND DISCUSSION

Liver function measurements

The serum concentrations of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), are displaying in Figures 1a and b respectively. After receiving an EAC inoculation, mice showed significantly higher serum levels of the enzymes aspartate transaminase (AST) and alanine transaminase (ALT). The ALT and AST activities in nano-silymarine group (Group 4) was lower then in PC group (Group 2) by 35.3 and 51.2 % respectively. The improvement of nanosilymarine in ALT value was by

26.6%, where it was by 7.9% in AST. Serum ALT enhancement levels were more obvious than that for serum AST levels. EAC mice showed and diagnosed liver disease and that was clear in the results for this positive control group as shown in Figure 1 a and b.

In a similar study conducted by **Shaker *et al*., 2010,** the treatment with silymarin showed lower enhancement for ALT and AST as 33.8 and 29.7%, respectively. While using CCl_4 to cause liver injury for rats, silymarin improved GOT into 32.5 and GPT into only 18.6% compared to positive control. The normal catabolic process produces bilirubin from heme breaks, which are waste materials created when old or aberrant red blood cells are destroyed. The heme molecule is separated from the haemoglobin molecule first, and it then travels through the body parts where the breakdown takes place. Elevated levels indicate certain diseases according to the body organ **(Smith and Morton, 2010 & Pirone *et al*., 2009).**

As seen in Figure 2, the EAC mice (Group 2) displayed high values of total bilirubin, reaching 1.56 mg/dl, however these values were reduced by 50% after receiving silymarin treatment and by 56.25% after receiving nano silymarin treatment.

A safe chemopreventive strategy against diseases caused by oxidative stress, such as atherosclerosis, cancer, diabetes, or inflammatory conditions, can involve the modification of bilirubin metabolism by well-defined natural polyphenols. In a recent study condcuted by **Suk *et al*., (2019)**, serum bilirubin concentrations were improved by the naturally occurring silymarin flavonolignans found in milk thistle and similar flavonols. This effect could be a part in the silymarin's hepatoprotective properties, which have been seen in a number of clinical investigations. All cells and tissues depend on proteins, and one of these components, albumin, transports molecules in the blood and prevents fluid from seeping out of blood vessels. (**Pella *et al*., 2022**). When compared to the levels in the control group, the serum total protein and albumin levels in the EAC group (Group 2) decreased significantly ($P \leq 0.05$) by 29.46 and 66.23%, respectively, indicating altered liver function. Similar deficits in albumin and total protein levels were seen in EAC mice in the study by **Kapoor *et al*., (2014)**. In the Silymarine and nano silymarine groups (group 3 and 4), the values of total protein and albumin often revert to normal (Figure 3 a and b)

Similar findings were discovered by **Yassin *et al*., 2022**, who discovered that the serum total protein and albumin levels rose in the groups treated with *Silybum marianum* total extract (STE), silymarin (S), and silibinin (Sb), as opposed to the groups exposed to diethylnitrosamine (DEN) / 2 acetylaminofluorene (AAF) / carbon tetrachloride (CCl_4) alone.

Globulins from the other view, as an important part in immune system affect on albumin/globulin ratio. In serum protein for control and nano silymerin groups, A/G ratio were 1.3, for the high albumin amount. While, this ratio was 1 for silymarin group. On the other side, A/G ratio was only 0.37 for Ehrlich ascites carcinoma bearing mice (Fig 4 a and b). These results are correlated with that mentioned by **Siems *et al*., (1993)**

who declared the reduction in protein synthesis with EAC bearing tumors. In the same while, high significant increased for EAC was shown in globulin compared with control. Globulin in our result showed very high significant in nano form and high significant in silymarin comparing to EAC. Similar deficits in albumin and total protein levels were seen in EAC mice in the study by **Kapoor *et al*., (2014)**. As well in correlation study, **Yassin *et al*. (2022)**, found that the serum total protein and albumin levels increased in the groups treated with Silybum marianum total extract (STE), silymarin (S), and silibinin (Sb).

Renal function measurements

In comparison to the normal control group, the serum urea and creatinine levels in the EAC positive control group (group 2) were significantly higher. Kidney functions showed improvement values when EAC bearing mice were orally treated with silymarin and nano-silymarin (Figure 5 a and b). The improvement for nano-silymarin for creatinine (30%) was better than with silymarin (28.6%). Similar trend was for urea where improvement were 13.7 and 9.58% for nano-silymarin and silymarin, respectively (Figure 5 a and b).

As been mentioned, the increased levels in urea and creatinine in EAC bearing mice distinguishes it from the negative control mice. The reducing effect of using silymarin and nano silymarin was more obvious on creatinine than urea. In the same time, nano silymarin improvement according with silymarin values were 4.12 and 1.4% for urea and creatinine, respectively (Figure 5 a and b). Similarly, the serum urea and creatinine levels in the EAC control mice in the study by **Kapoor *et al*., (2014)** were significantly higher than those in the normal control group. Additionally, silymarin administration in rats as shown in the study by **Al-Kadi *et al*. (2020)** prevented the increase in creatinine level in septic rats.

Lipid profile

The serum levels of total cholesterol and triglycerides in the EAC control mice (Group 2) were higher than those in the normal control group by 40.08 and 10.23%, respectively. After silymarin and nano silymarin were ingested, the rise in these parameters was slowed down and the levels of cholesterol and triglycerides frequently returned to close to normal (Figure 6 a and b).

Moreover, the good cholesterol (HDL) was improved with 41.4 and 50.4% by silymarin and nano silymarin, respectively comparing to EAC mice group. That was much better than the decreasing happened by those oral ingested in LDL and vLDL values (Figure 7 a , b and c).

In a similar manner, the HDL was improved in the study by **Shaker *et al.* (2010)** with 60% compared to positive control when using silymarin ethanolic extract for rats injected with CCl_4. While this value for LDL in silymarin treated group was 17.2% comparing to positive control. These results were highly agreed with our results for EAC bearing mice. Risk factors measured scientifically well by non-HDL, ratios of Chl/HDL and LDL/HDL showed enhancement by nano silymarin followed by silymarin

compared to EAC bearing carcinoma cells (figure 8 a,b and c). Nano silymarin improved the ratios as 50 and 47.6% while silymarin values were 44 and 41.8%, respectively on Chl/HDL and LDL/HDL. Silymarin improved in our previous work LDL/HDL value for treated positive control CCl_4 by 48.25% in a highly agreed result **(Shaker *et al.*, 2010).**

The primary goal for carcinogen effect for industrial intermediates and carcinogens is the liver. Flavonolignan Silymarin found in the seed of milk thistle used for its ameliorating and hepatoprotective action. The main flavonolignan, silybin is the most active in the mixture with silidianin and silychristine **(Dixit *et al*. 2007).** The safety of silymarin for human at high doses was indicated in previous research by the pharmacological reports **(Devi, 2019)**. Many obstacles face silymarin activity, particularly the very low bioavailability caused by its poor water solubility. Nano formulation of the investigated substance increased the solubility, leading to a hepatoprotective action.

As a free radical scavenger, lipid peroxidation inhibitor, and inducer of endogenous antioxidant mechanisms, silymarin is able to balance tissue oxidant-antioxidant equilibrium, which contributes -at least in part- to its protective effects on the hepatorenal function. Additionally, the anti-inflammatory properties of silymarin are also responsible for its ability to protect the kidney and liver (**Al-Kadi *et al.*, 2020**).

The findings in the current study, showed that nano particle of silymarin enhanced the activity for silymerin with no harmful effect on different chemical analysis. The hepatobiliary carcinogen effect might be found in EAC bearing carcinogen in histological and immune-histological studies. The antioxidant mechanism for nano silymarin might need as well more investigation. Recently, enhancing solubility of of hydrophobic drug silymarin in the mixed micelles showed high solubility than that for pure pluronic micelles (**Garg *et al.*, 2022**). Mixed micellar formulations with maximum silymarin solubilization helped in drug released, and antioxidant potential measured by many methods.

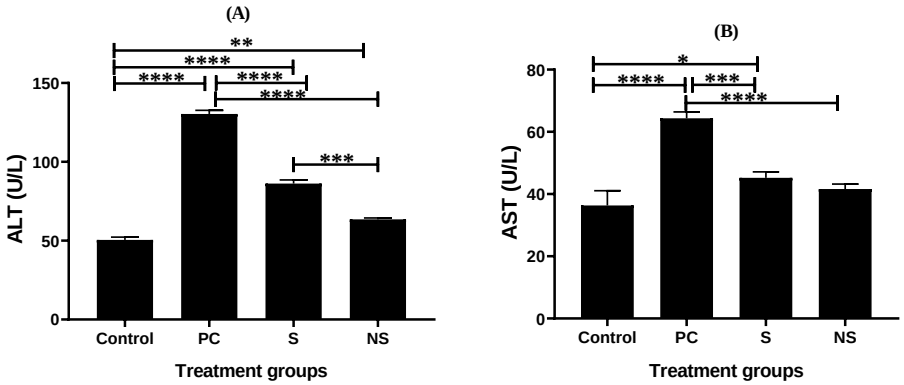

Fig (1): The alanine aminotransferase (ALT) (U/l) and aspartate aminotransferase (AST) activities (U/l) of Ehrlich ascites carcinoma mice ingested silymarin and nano silymarin orally in different experimental groups Values are presented as mean ± SEM, * $P < 0.05$ significant differences, ** $P < 0.01$ highly significant differences, *** $P < 0.001$ very high significant differences, **** $P < 0.0001$ extremely high significant differences. PC = positive control, S = Silymarin, NS = Nano silymarin

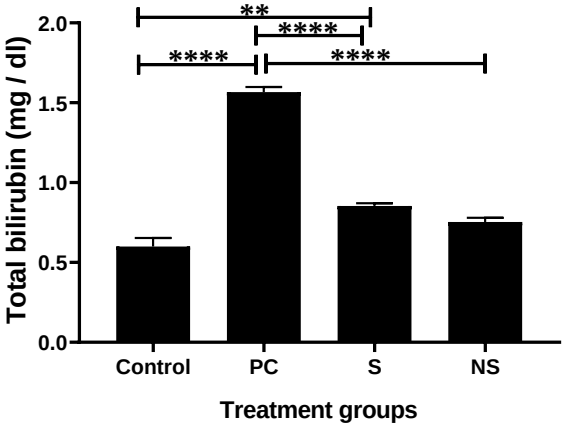

Fig (2): The concentration of serum Total bilirubin (mg/dl) of Ehrlich ascites carcinoma mice ingested silymarin and nano silymarin orally in different experimental groups Values are presented as mean ± SEM, * P < 0.05 significant differences, ** P < 0.01 highly significant differences, *** P < 0.001 very high significant differences, **** P < 0.0001 extremely high significant differences. PC = positive control, S = Silymarin, NS = Nano silymarin

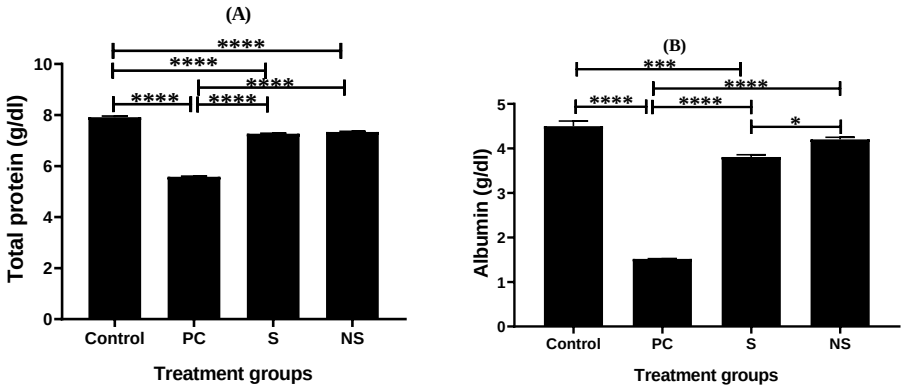

Fig (3): The concentration of serum Total protein (g/dl) and Albumin (g/dl) of Ehrlich ascites carcinoma mice ingested silymarin and nano silymarin orally in different experimental groups Values are presented as mean ± SEM, * $P < 0.05$ significant differences, ** $P < 0.01$ highly significant differences, *** $P < 0.001$ very high significant differences, **** $P < 0.0001$ extremely high significant differences. PC = positive control, S = Silymarin, NS = Nano silymarin

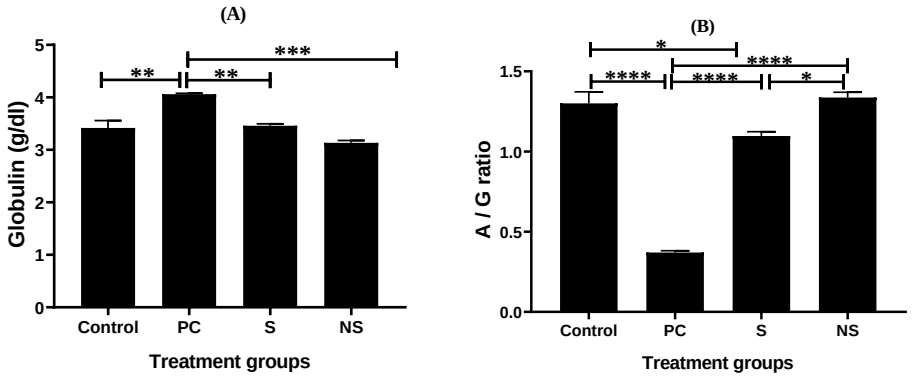

Fig (4): The concentration of serum Globulin (g/dl) and A / G ratio of Ehrlich ascites carcinoma mice ingested silymarin and nano silymarin orally in different experimental groups Values are presented as mean ± SEM, * $P < 0.05$ significant differences, ** $P < 0.01$ highly significant differences, *** $P < 0.001$ very high significant differences, **** $P < 0.0001$ extremely high significant differences. PC = positive control, S = Silymarin, NS = Nano silymarin

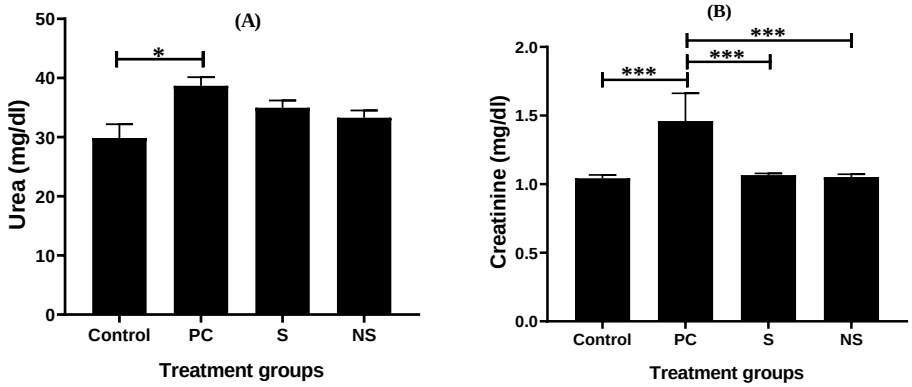

Fig (5): The concentration of Urea (mg/dl), and Creatinine (mg/dl) of Ehrlich ascites carcinoma mice ingested silymarin and nano silymarin orally in different experimental groups Values are presented as mean ± SEM, * $P < 0.05$ significant differences, ** $P < 0.01$ highly significant differences, *** $P < 0.001$ very high significant differences, **** $P < 0.0001$ extremely high significant differences. PC = positive control, S = Silymarin, NS = Nano silymarin

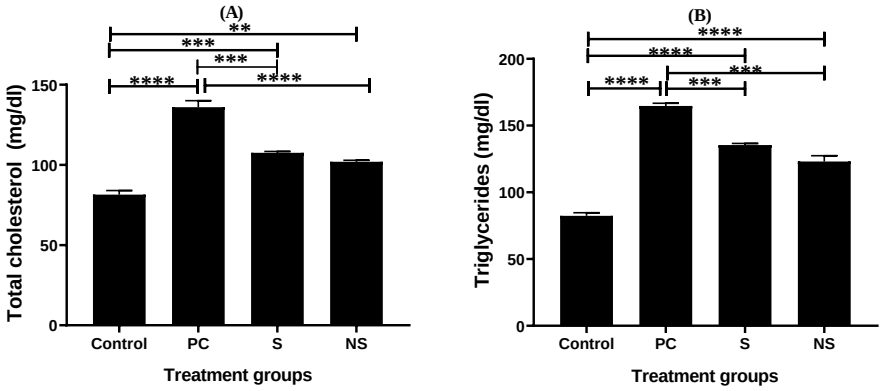

Fig (6): The concentration of serum Total cholesterol (mg/dl) and Triglycerides (mg/dl) of Ehrlich ascites carcinoma mice ingested silymarin and nano silymarin orally in different experimental groups Values are presented as mean ± SEM, * P < 0.05 significant differences, ** P < 0.01 highly significant differences, *** P < 0.001 very high significant differences, **** P < 0.0001

extremely high significant differences. PC = positive control, S = Silymarin, NS = Nano silymarin

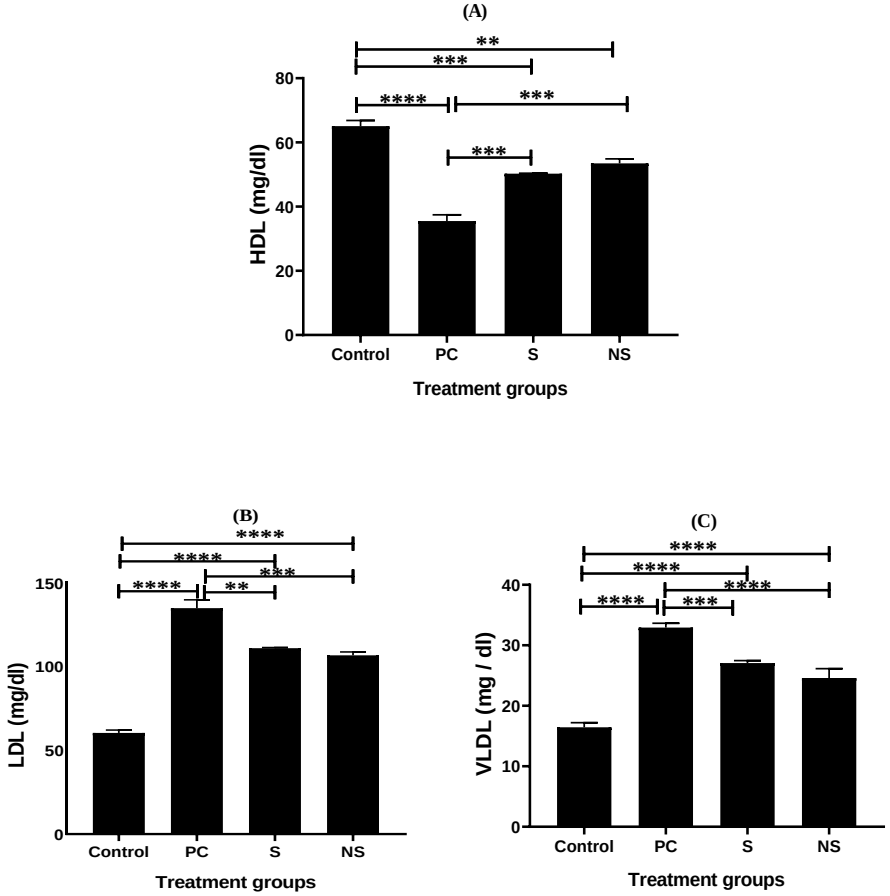

Fig (7): The concentration of serum HDL (mg/dl), LDL (mg/dl) and VLDL (mg/dl) of Ehrlich ascites carcinoma mice ingested silymarin and nano silymarin orally in different experimental groups Values are presented as mean ± SEM, * $P <$ 0.05 significant differences, ** $P < 0.01$ highly significant differences, *** $P <$ 0.001 very high significant differences, **** $P < 0.0001$ extremely high significant differences. PC = positive control, S = Silymarin, NS = Nano silymarin

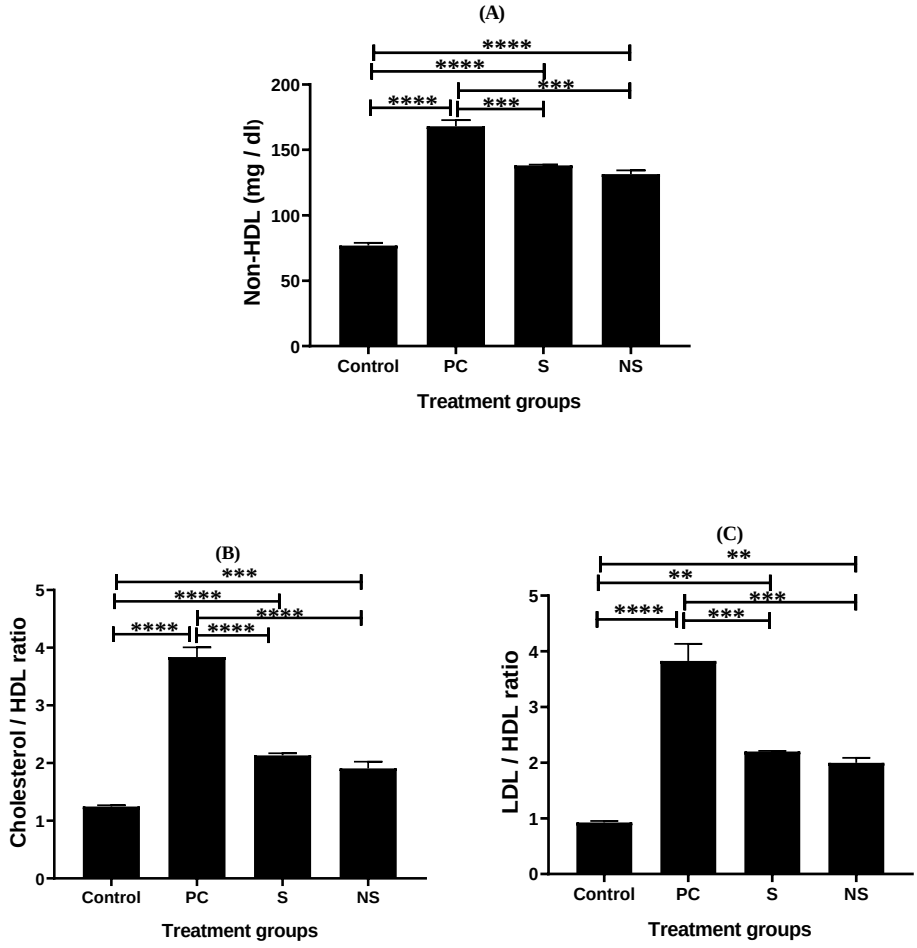

Fig (8): The concentration of Non-HDL (mg/dl), Cholesterol / HDL ratio and LDL /HDL ratio of Ehrlich ascites carcinoma mice ingested silymarin and nano silymarin orally in different experimental groups Values are presented as mean ± SEM, * $P < 0.05$ significant differences, ** $P < 0.01$ highly significant differences, *** $P < 0.001$ very high significant differences, **** $P < 0.0001$ extremely high significant differences. PC = positive control, S = Silymarin, NS = Nano silymarin

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الملخص العربي

التحسين البيوكيميائي الناجم عن السيليمارين والنانو سيليمارين في الفئران الحاملة لسرطان استسقاء إيرليش

سالي محمد عبد العزيز الشافعي – عصام أحمد الملط – عماد صبري شاكر
نهال كامل صبره – عاطف عبد المحسن عبد الرحمن
قسم الكيمياء الزراعية – كلية الزراعة – جامعة المنيا – المنيا – ٦١٥١٩ – مصر

أعتبر نبات شوكة الجمل احد النباتات المميزة خلال العقود الماضية. السليمارين هو المركب الرئيسي النشط بيولوجيا في هذا النبات لذلك ، هدفت الدراسة الحالية إلى تقييم أنشطة سيليمارين ونانو سيليمارين في نموذج الفئران Ehrlich Ascites Carcinoma (EAC). تم اختيار أربع مجموعات تجريبية بشكل عشوائي من ٦٠ أنثى من الفئران السويسرية البيضاء. كانت المجموعة الأولي بمثابة المجموعة الضابطة. أما المجموعات الثلاثة الأخرى فقد عوملت ٢,٥ × ١٠^{٦} خلايا EAC داخل الغشاء البروتوني للبطن. أما المجموعة الثانية فكانت بمثابة مجموعة ضابطة EAC. تم إعطاء سيليمارين ونانو سيليمارين عن طريق الفم بمعدل ١٠٠ مجم / كجم من وزن الجسم يوميًا للفئران في المجموعتين الثالثة والرابعة لمدة ٦ أسابيع على التوالي. تم تقييم القياسات البيوكيميائية ، والتي تشمل وظائف الكبد والكلى بالإضافة إلى خصائص الدهون ، في جميع المجموعات التجريبية. أظهرت النتائج أن المعاملة بخلايا EAC داخل الغشاء البروتوني في الفئران أظهر تأثيرًا سلبيًا على القياسات البيوكيميائية. تم تحسين القياسات البيوكيميائية للحيوانات بشكل ملحوظ بعد العلاج بمستخلصات سيليمارين ونانو سيليمارين. بالإضافة إلى ذلك ، أظهر مستخلص النانو سيليمارين تأثيرًا أقوى من سيليمارين على تحسين القياسات البيوكيميائية.