ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Clinicopathological Studies on the Chemotherapeutic Effect of Silymarin and Taurine on N-nitrosodiethylamine induced hepatic cell carcinoma in Rats

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Manuscript Info

Manuscript History:

Received: 14 November 2015
Final Accepted: 15 December 2015
Published Online: January 2016

Key words:
Hepatic cell carcinoma, Silymatin, Taurine, Tumor markers, Oxidative stress, Apoptosis.

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Abstract

This new study was performed to investigate the chemotherapeutic effect of both silymarin and taurine alone or in combination on rats with N-nitrosodiethylamine (NDEA) induced hepatic cell carcinoma. Seventy five adult male albino rats were divided into five equal groups; the first was the negative control group(C). The second was the hepatic cell carcinoma group (HCC).The third was the hepatic cell carcinoma group treated with silymarin (HCC+S), while the fourth was the hepatic cell carcinoma group treated with taurine (HCC+T) and the fifth was the hepatic cell carcinoma group treated with both silymarin and taurine (HCC+S+T). Blood samples were collected after one, two and three months from five animals of each group for biochemical analysis; however liver was collected at the end of the experiment for histopathological examination. Our obtained data confirmed that silymarin in combination with taurine have a powerful chemotherapeutic effect against NDEA induced hepatic cell carcinoma in rats.

Introduction:-

Hepatic cell carcinoma (HCC) considered one of the most commonly spread tumors causing mortalities between populations all over the world , ranking the fifth of the most prevalent malignancies **(Jamilah, 2015)**. Nowadays its occurrence has been widely more than the old decades **(Subbaraj *et al.*, 2013)**. The mean reasons of hepatic cell carcinoma (HCC) in human cases are hepatitis B and C viruses **(Schütte *et al.*, 2009)**. Moreover, many causes as air pollution **(Kim *et al.*, 2014)**, alcoholism **(Sidharthan and Kottilil, 2014)**, as well as several ingested carcinogens, such as aflatoxins **(Hamid *et al.*, 2013)** and nitrosamines **(Kowsalya *et al.*, 2015)** also included.

N-Nitrosodiethylamine (NDEA) is a powerful hepatocarcinogenic N-nitroso alkyl compound; it causes toxicity due to the activation of cytochrome p450 enzymes which produce reactive electrophiles resulting in increasing the oxidative stress level leading to cytotoxicity, mutagenicity and carcinogenicity **(Waris and Ahsan, 2006)**. It is the most important xenobiotic system that induced HCC and is a common screening model in experimental animals to investigate the potential hepatoprotective effect of drugs and herbal medicine especially that with antioxidant activities **(Dakshayani *et al.*, 2005)**.

In recent decades, natural substances have great attention in the scientific research for prevention and treatment of cancer **(Subbaraj *et al.*, 2013)**. Silymarin, an active extract milk thistle (Silybum marianum) plant, is used for the protection against various liver conditions such as hepatitis and cirrhosis in both clinical settings and experimental models. Silymarin has several actions in experimental liver diseases as anti-inflammatory, fibrinolytic, cell membrane stabilizer, immunomodulation and regeneration **(Deep *et al.*, 2008)**. Silymarin can prevent the proliferation of cancer cells in both in vivo and in vitro models through its interfering with the expressions of cell cycle regulators and proteins involved in apoptosis not only in hepatic cell carcinoma but also in lung and breast cancer **(Pavan *et al.*, 2014)**.

Taurine is a sulfur-containing amino acid (non-essential amino acid).It has been found that taurine has noticeable ability on scavenging the oxygen-free radicals, regulation of intracellular calcium homeostasis, maintenance of cell membrane stability and cells protection **(Huxtable,1992)**. It has cardio-protective **(Wójcik *et al.*, 2010)** and antitumor properties **(Wang *et al.*, 2009)**. Recently, protective effects of taurine against anticancer drugs on normal cells were investigated. But anticancer effects of taurine on cancer cells remain poorly understood **(Kim and Kim, 2013)**. Therefore, we investigated the anticancer effects of taurine alone and combination of silymarin with taurine in experimental hepatic cell carcinoma rat model.

Materials and methods:-

Carcinogenic, protective substances and chemicals:- N-nitrosodiethylamine (NDEA) and taurine were purchased from Sigma Chem. Co., St Louis, Mo, USA. Silymarin was obtained from CID Co., Cairo, Egypt. All other reagents and chemicals used were of analytical grade.

Ethical conditions: All animals were handled, approached and treated according to the National Institutes of Health (NIH) guidelines for the Care and Use of Laboratory Animals, and were comforted by Ethics of animal use in research committee (EAURC), Zagazig University.

Hepatic cell carcinoma induction with N-nitrosodiethylamine protocol:- According the method previously described by **Yoshiji *et al.* (1991)**; Hepatic cell carcinoma was induced experimentally in rats at two months of age through a single intraperitoneal injection of NDEA dissolved in saline (200 mg / 100 g b. wt.). Two weeks after administration of NDEA the carcinogenic effect was activated by phenobarbital (PB) 0.05 % (50 mg / 100 g b. wt. daily) for ten consecutive weeks.

Animal housing and experimental design:- Seventy five adult male albino rats (8-10 weeks age) were obtained from the Laboratory Animal breeding unit, Faculty of Veterinary Medicine, Zagazig University. Rats were allowed for one week prior to the experiment. The animals were housed in an insulated wooden box in under controlled environmental conditions (12/12 h light/dark cycle, 50% humidity, and 30°C), fed on standard rodent diet and water was adequately offered. Rats were divided into five equal groups; each group containing 15 rats.

Group I (C): Control rats kept on normal saline (0.9%).

Group II (HCC): Control hepatic cell carcinoma rats.

Group III (HCC+S): Hepatic cell carcinoma rats treated orally with silymarin (50 mg kg^{-1} b.wt.) for 3 months according to **Fraschini *et al.* (2002)**.

Group IV (HCC+T): Hepatic cell carcinoma rats intraperitoneal treated with taurine (500 mg kg^{-1} b.wt.) for 3 months according to **El-Nahrawy and Heibashy (2011)**.

Group V (HCC+S+T): Hepatic cell carcinoma rats treated with both silymarin and taurine with the previous doses and duration.

After one, two and three months from starting the treatment with silymarin and taurine, five animals from each group were fasted overnight, decapitated and sacrificed for blood collection; the blood sample was transferred into clean tube not containing anticoagulant then centrifuged at 3000 rpm for 15 min to separate sufficient clear non-hemolysed serum for biochemical analysis of liver enzymes, tumor, inflammatory and oxidative stress markers.While, liver was taken only after three months for histopathological examination.

Biochemical analysis:-

Hepatic markers enzymes: Serum alanine transaminase (ALT) and aspartate transaminase (AST) were measured calorimetrically using commercial kits obtained from Diamond Diagnostics, Egypt according to **Reitman and Frankel (1957)**. Alkaline phosphatase (ALP) activity was determined by Randox kit according to **Belfield and Goldberg (1971)**.

Estimation of selective tumor and inflammatory markers:- Serum carcino-embryonic antigen (CEA) and alpha-fetoprotein (AFP) was estimated according to the methods described by **Gold and Freedman (1965); Abelev (1974)** respectively. Moreover, tumor necrosis factor -alpha (TNF – α) was assayed according to **Beutler *et al.* (1985)**. Nitric oxide level (NO) in serum was measured according to **Berkels *et al.* (2004)** using colorimetric kit which provided by Biodiagnostic Company (Cairo, Egypt).

Oxidative stress assaying: Serum total antioxidant capacity (TAC) and total oxidant capacity (TOC) were measured according to **Cao *et al.* (1993); Flohe and Gunzler (1984)** using kits collected from Labor Diagnostika Nord GmbH & Co. (LDN, Germany).

Statistical analysis:- Collected data were summarized and statistically analyzed through one way (ANOVA) using the software statistical program (SPSS, ver.16.00, USA). Data are expressed as the mean ± SD, and results were statistically significant at $P < 0.05$ **(SPSS, 2008)**.

Results:-

Changes in hepatic enzymes activities:- Biochemical analysis of liver enzymes activities in serum for different experimental groups was shown in **Fig.1**. After one, two and three months from starting the treatment marked ($P \leq 0.05$) increase of serum ALT, AST and ALP activities was found in hepatocarcinogenic animals group comparing with the control group. On the other hand, treatment with silymarin and taurine alone or in combination caused a great decrease in their activities compared with the control cancer group. The improvement was clearer in the combination group.

Differences in tumor and inflammatory markers:- Data obtained from **Tables 1, 2, 3** illustrate that HCC animals group treated with silymarin and taurine alone or in combination showed a return of serum CEA , AFP, TNF – α and NO levels to the control values. The amelioration was apparent in the combination treated group.

Oxidative stress results:- There was a major ($P \leq 0.05$) increase in the total oxidant capacity with a decrease in the total antioxidant capacity in the HCC animals group when compared with the control group. Meanwhile, marked elevation of TAC and reduction in TOC of HCC group was noticed when treated with both silymarin and taurine that showed obvious improvement when used together **(Tables 4, 5)**.

Histopathological findings:- Microscopical examination of H&E stained liver sections of the control rats (C group) showed normal histological architecture of the liver with normal hepatocytes arranged in cords around the central vein and extend to the portal area **(Fig.2 A , B)**. Meanwhile, liver of rats injected intraperitoneal with NDEA (HCC group) showed prominent proliferation of the fibroblasts in and around the portal area, and extends to the surrounding hepatic cells **(Fig.2 C)** with many eosinophilic hepatic cells containing large and hyperchromatic nuclei plus few mitotic figures **(Fig.2 D)**. Liver showed programmed cell death evidenced by numerous apoptotic bodies **(Fig.2 E)**. Liver of HCC rats treated with silymarin (HCC+S) reveled disassociation of the hepatic cells with moderate proliferation of the fibroblasts around the portal areas with few hepatocytes showing hyperchromatic nuclei **(Fig.2 F)**. While hepatic tissue of HCC rats treated with taurine (HCC+T) showed disassociation of the hepatic cells with marked proliferation of the fibroblasts in the portal area and between the hepatocytes **(Fig.2 G)**.Moreover the liver of HCC rats treated with both silymarin and taurine (HCC+S+T) showed few fibroblasts around the central vein with normal arrangement and histology of the hepatocytes **(Fig.2 H)**.

Discussion:-

Hepatic cell carcinoma (HCC) is one of the famous health problems; the fifth widely spread cancer in the world (**Abeer *et al.*, 2015)** and associated with several changes in gene expression profiles **(Wurmbach *et al.*, 2007)**. The present work investigate the role of both silymarin and taurine alone or in combination in the attenuation and management of hepatic cell carcinoma (HCC) induced by NDEA in male rats.

Regarding to the results of liver enzymes activities and the oxidative status in the current investigation, elevation of the hepatic markers enzymes and total oxidative capacity with total antioxidant capacity depletion were found in the hepatic cell carcinoma bearing rats may be due NDEA-induced oxidative stress through the restoration of antioxidant enzymes **(Ansil *et al.*, 2014)** leading to liver cell damage resulting in liberation of cellular enzymes ALT, AST and ALP into the bloodstream **(Senthikumar *et al.*, 2006**. These results agreed with those obtained by **Tanabe and coworkers (2008); Rasha and Fares (2014); Amal *et al.* (2015)**. The current study showed a significant attenuation in serum ALT, AST and ALP activities following the treatment by silymarin, taurine alone and both silymarin and taurine together when compared to hepatic cell carcinoma bearing group. Our findings agreed with **Hala *et al.* (2011)** who confirmed that that NDEA animals group treated with silymarin showed a significant decrease in MDA level and a significant increase in GSH content and SOD, GPx and GR activities compared to NDEA group. Silymarin also beneficially down-regulated the increase in serum ALT and AST activities induced by NDEA. In contrast, **Ryu *et al.* (2014)** who found that hepatic cell carcinoma bearing rats treated with 0.1 and 0.5% silymarin for 18 weeks and with 0.1% silymarin for 5 weeks respectively showed no significant decrease in serum transaminase levels. Also, taurine at a dose of 5 mg kg^{-1} b. wt./15 days in mercury

intoxicated decline the increased levels of AST, ALT and ALP in serum and lipid peroxidation content and promotes the decreased level of antioxidant system in liver tissue **(Jagadeesan and Sankarsami , 2007)**.

Carcino-embryonic antigen and alpha-fetoprotein are mostly used in the diagnosis of HCC **(Stroescu *et al.*, 2006)**. In our study there were an increase in the level of AFP and CEA in HCC bearing animals which agreed with **Ramakrishnan et al. (2007)**. Silymarin administration significantly reduced the elevation of both AFP and CEA may be due to its action on apoptotic cells through decreasing the mitochondrial transmembrane potential by elevation of cytosolic cytochrome c (cyt. c) and upregulating the expressions of proapoptotic proteins, such as p53, Bax, apoptotic protease-activating factor 1, and caspase-3, and downregulating the expressions of antiapoptotic proteins, namely, Bcl-2 and survivin, and proliferation-associated proteins, for example, proliferating cell nuclear antigen, cyclin D1, c-Myc, and β-catenin **(Ramakrishnan *et al.*,2009)** or may be due to the antioxidative role of Silibinin **(Kumar *et al.*,2015)**. Also we found that taurine inhibit tumor markers in the carcinogenic animals by inhibition of tumor proliferation **(Shuo *et al.*,2015)**, enhancement of the antioxidation in the body through the removal of strong oxidizing agents, which subsequently protects normal cells from oxidative damage and induces tumor cell apoptosis **(Mates *et al.*, 2012)**, promotion of the body immunity **(Abd-Rabou *et al.*,2012)** in addition to increasing the efficacy and decreasing the toxicity of chemotherapeutic drugs **(Zhang *et al.*,2014)**.From all the above we concluded that silymarin and taurine have synergistic effect to inhibit the tumor through their antioxidant activities.

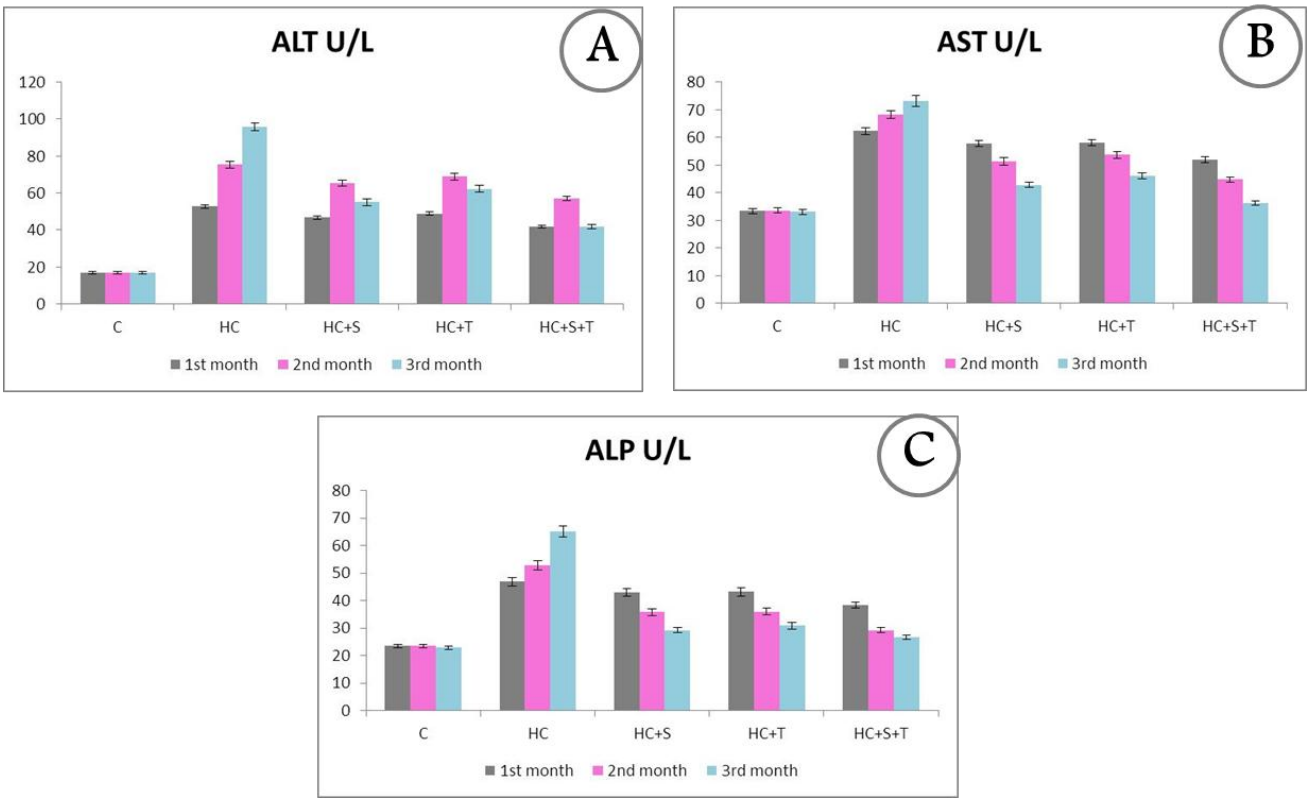

Fig.1: Statistical changes in ALT, AST and ALP (U/L) (A,B,C) after one, two and three months in different treated groups (HCC, HCC+S, HCC+T, HCC+S+T) compared to the control (C).

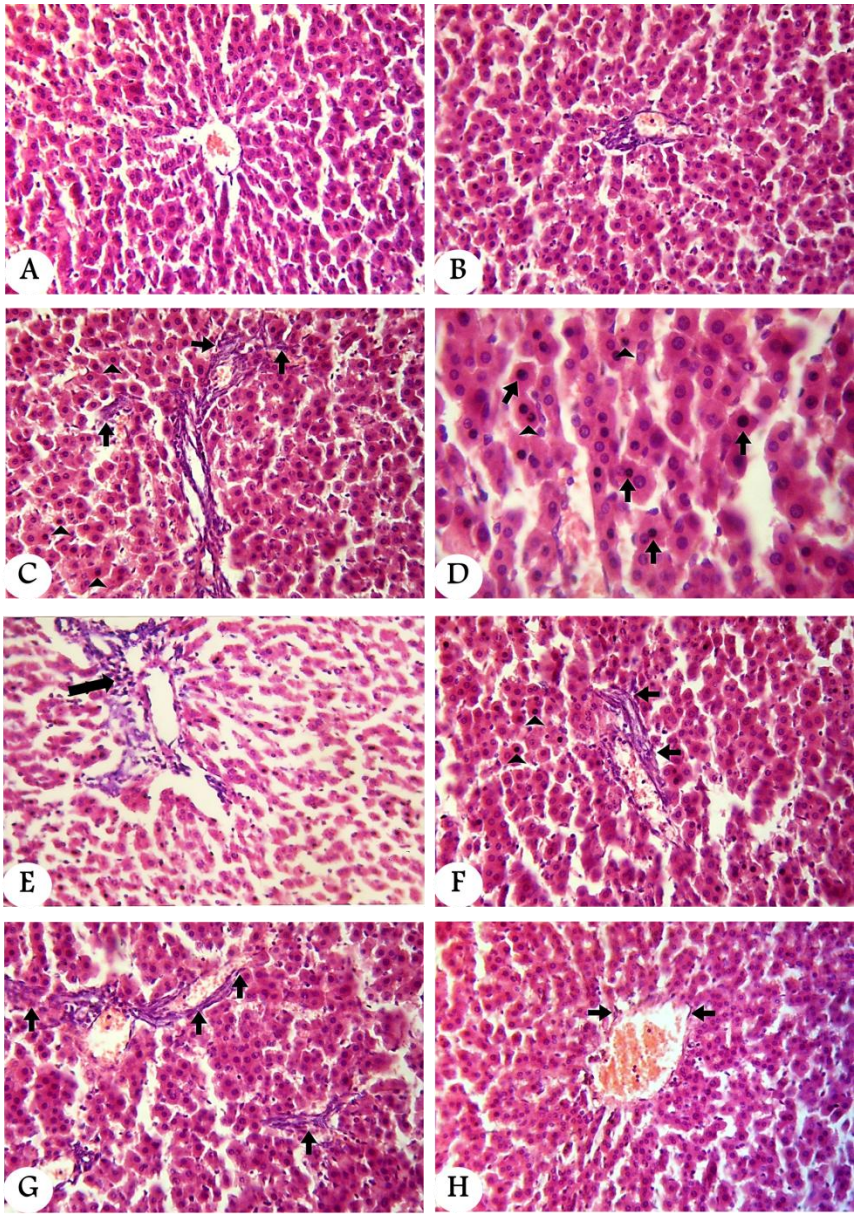

Fig.2: Photomicrograph of H&E stained liver sections of the control rats (C group) showed normal histological architecture of the liver (A , B).Meanwhile, liver of rats in HCC group showed fibroblasts proliferation in and around the portal area (arrow), and the hepatic cells (C) with many eosinophilic hepatocytes containing hyperchromatic nuclei (arrow head) plus few mitotic figures (D) also programmed cell death evidenced by numerous apoptotic bodies (E). HCC+S revealed disassociation of the hepatic cells with moderate fibroblasts proliferation around the portal areas (arrow) and few hepatocytes showing hyperchromatic nuclei (arrowhead) (F). HCC+T group showed disassociation of the hepatic cells with marked proliferation of the fibroblasts in the portal area and between the hepatocytes (arrow) (G).Moreover HCC+S+T GROUP showed few fibroblasts around the central vein (arrow) with normal arrangement and histology of the hepatocytes(H).

Table1: Effect of silymarin (50 mg kg^{-1} b.wt.), taurine (500 mg kg^{-1} b.wt.) and their combinations on CEA(ng/mL),AFP(ng/mL),TNF- α (pg/mL) and NO(µmol/L) in hepatic cell carcinoma bearing rats comparing to the control after one month following the treatment.

	CEA (ng/mL)	**AFP (ng/mL)**	**TNF-α (pg/mL)**	**NO (µmol/L)**
C	0.036 ± 0.005^{d}	1.602 ± 0.069^{e}	1.099 ± 0.071^{d}	58.741 ± 1.134^{d}
HCC	1.348 ± 0.047^{a}	13.815 ± 0.783^{a}	3.776 ± 0.092^{a}	157.327 ± 2.632^{a}
HCC+S	0.927 ± 0.035^{b}	7.328 ± 0.519^{c}	3.142 ± 0.078^{b}	128.934 ± 2.109^{b}
HCC+T	1.004 ± 0.041^{b}	7.945 ± 0.557^{b}	3.154 ± 0.083^{b}	130.327 ± 2.117^{b}
HCC+S+T	0.734 ± 0.036^{c}	4.205 ± 0.463^{d}	2.718 ± 0.069^{c}	104.658 ± 1.871^{c}

Each value represents the mean of 5 rats ± S.E. All data having different letters are differ significantly at $p \leq 0.05$. Carcino-embryonic antigen (CEA) ,alpha-fetoprotein (AFP) , tumor necrosis factor -alpha (TNF – α) ,Nitric oxide (NO)

Table 2: Effect of silymarin (50 mg kg^{-1} b.wt.), taurine (500 mg kg^{-1} b.wt.) and their combinations on CEA(ng/mL),AFP(ng/mL),TNF- α (pg/mL) and NO(µmol/L) in hepatic cell carcinoma bearing rats comparing to the control after two months following the treatment.

	CEA (ng/mL)	**AFP (ng/mL)**	**TNF-α (pg/mL)**	**NO (µmol/L)**
C	0.034 ± 0.004^{e}	1.576 ± 0.078^{d}	1.116 ± 0.081^{e}	59.041 ± 1.127^{d}
HCC	2.364 ± 0.078^{a}	20.88 ± 0.941^{a}	4.847 ± 0.136^{a}	189.294 ± 2.978^{a}
HCC+S	0.597 ± 0.31^{c}	5.094 ± 0.329^{b}	2.348 ± 0.067^{c}	110.792 ± 1.731^{b}
HCC+T	0.702 ± 0.35^{b}	5.197 ± 0.334^{b}	2.986 ± 0.081^{b}	112.006 ± 1.835^{b}
HCC+S+T	0.263 ± 0.36^{d}	3.289 ± 0.279^{c}	1.829 ± 0.054^{d}	91.63 ± 1.436^{c}

Each value represents the mean of 5 rats ± S.E. All data having different letters are differ significantly at $p \leq 0.05$. Carcino-embryonic antigen (CEA) ,alpha-fetoprotein (AFP) , tumor necrosis factor -alpha (TNF – α) ,Nitric oxide (NO)

Table 3: Effect of silymarin (50 mg kg^{-1} b.wt.), taurine (500 mg kg^{-1} b.wt.) and their combinations on CEA(ng/mL),AFP (ng/mL),TNF- α (pg/mL) and NO(µmol/L) in hepatic cell carcinoma bearing rats comparing to the control after three months following the treatment.

	CEA (ng/mL)	**AFP (ng/mL)**	**TNF-α (pg/mL)**	**NO (µmol/L)**
C	0.039 ± 0.005^{e}	1.611 ± 0.075^{e}	1.201 ± 0.083^{d}	59.491 ± 1.117^{e}
HCC	2.927 ± 0.091^{a}	32.94 ± 1.139^{a}	6.147 ± 0.182^{a}	212.939 ± 3.372^{a}
HCC+S	0.301 ± 0.064^{c}	3.101 ± 0.344^{c}	1.846 ± 0.217^{c}	82.601 ± 1.034^{c}
HCC+T	0.521 ± 0.075^{b}	3.829 ± 0.351^{b}	2.312 ± 0.209^{b}	86.011 ± 1.175^{b}
HCC+S+T	0.187 ± 0.053^{d}	2.742 ± 0.293^{d}	1.242 ± 0.182^{d}	64.187 ± 0.923^{d}

Each value represents the mean of 5 rats ± S.E. All data having different letters are differ significantly at $p \leq 0.05$. Carcino-embryonic antigen (CEA) ,alpha-fetoprotein (AFP) , tumor necrosis factor -alpha (TNF – α) ,Nitric oxide (NO)

Table 4: Effect of silymarin (50 mg kg^{-1} b.wt.), taurine (500 mg kg^{-1} b.wt.) and their combinations on TOC (mmol/L) in hepatic cell carcinoma bearing rats comparing to the control after one, two and three months following the treatment.

	TOC(mmol/L)		
	1 month	**2 months**	**3 months**
C	0.148 ± 0.006^{e}	0.152 ± 0.007^{d}	0.151 ± 0.006^{c}
HCC	0.469 ± 0.019^{a}	0.627 ± 0.027^{a}	0.949 ± 0.041^{a}
HCC+S	0.389 ± 0.014^{c}	0.342 ± 0.018^{b}	0.246 ± 0.012^{b}
HCC+T	0.407 ± 0.016^{b}	0.345 ± 0.015^{b}	0.252 ± 0.013^{b}
HCC+S+T	0.242 ± 0.011^{d}	0.201 ± 0.009^{c}	0.152 ± 0.008^{c}

Each value represents the mean of 5 rats ± S.E. All data having different letters are differ significantly at $p \leq 0.05$. Total oxidant capacity (TOC).

Table 5: Effect of silymarin (50 mg kg^{-1} b.wt.), taurine (500 mg kg^{-1} b.wt.) and their combinations on TAC (mmol/L) in hepatic cell carcinoma bearing rats comparing to the control after one, two and three months following the treatment.

	TAC (mmol/L)		
	1 month	**2 months**	**3 months**
C	1.178 ± 0.062^{a}	1.167 ± 0.059^{a}	1.171 ± 0.058^{a}
HCC	0.621 ± 0.043^{c}	0.458 ± 0.031^{c}	0.294 ± 0.039^{b}
HCC+S	0.881 ± 0.052^{b}	0.984 ± 0.059^{b}	1.169 ± 0.344^{a}
HCC+T	0.889 ± 0.055^{b}	0.967 ± 0.064^{b}	1.168 ± 0.351^{a}
HCC+S+T	1.165 ± 0.043^{a}	1.173 ± 0.059^{a}	1.182 ± 0.293^{a}

Each value represents the mean of 5 rats ± S.E. All data having different letters are differ significantly at $p \leq 0.05$. Total antioxidant capacity (TAC).

Conflict of Interest Statement:-

We declare no conflict of interest.

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