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International Journal of Pharmaceutical Research & Allied Sciences, 2017, 6(1):175-188

Research Article

ISSN : 2277-3657
CODEN(USA) : IJPRPM

Antitumor Activity and Hepatotoxicity Effect of Sorafenib Incorporated into Nanoemulsion Formulated with Flaxseed Oil

Running title: Antitumor Activity and Hepatotoxicity Effect of Sorafenib

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ABSTRACT

Sorafenib (SRF), an inhibitor of tyrosine kinase used to treat different kinds of cancers, found to have hepatotoxicity effects. The current study aims to fabricate the flaxseed oil in a nanoemulsion containing SRF (SRF-NE) and to evaluate its anticancer activity in vivo. The droplet sizes and charges of SRF-NE were determined by the zetasizer Nano ZS. Five groups (n = 20) of female Swiss Albino mice were used for antitumor activity assessment. Groups I & II served as the untreated mice and mice inoculated with Ehrlich ascietes carcinoma cells (EAC+), respectively. Groups III-V were EAC-bearing mice administered day-by-day via oral gavage with 7 doses of free-NE, 30 mg SRF/kg of mice weight, solubilized in 1:1 ratio of Cremophor and 95% Ethyl Alcohol (SRF-Cremo), and SRF-NE, respectively. The side effect of the subjected formulas on the liver was assessed by determining the relative liver weight, serum biochemical parameters, reactive oxygen species and implementing the histological examination. The z-average diameter and zeta potential of SRF-NE were 77.46 ± 8.28 nm and - 3.4 ± 1.2 mV, respectively. Among all of the treated groups, SRF-NE group has the least tumor volume with increased activity of the lactate dehydrogenase and the greatest survival (28± 2.54 days). Compared to SRF-Cremo, SRF-NE, subjected into the mice, has amended the relative liver weight, decreased the level of alanine aminotransferase and raised the activity of the catalase. In conclusion, encapsulating SRF in a NE formulated with flaxseed oil has improved its antitumor activity and reduced its hepatotoxicity.

***Keywords:** Chemotherapeutic agents; Nanoparticle; Toxicity; Reactive oxidative species; Histology*

INTRODUCTION

It has been recently reported that cancer death rate is 40% higher than the past decade [1]. Cancer treatment by the chemotherapeutic agents is still facing challenges and obstacles due to the side effects associated with the drug administration. SRF is an oral administered chemotherapeutic agent, multi-targeted drug acts as a receptor for tyrosine kinase inhibitors and the rapidly accelerated fibrosarcoma (RAF) kinase inhibitors, and has anti-angiogenic and antiproliferative properties [2].

Although SRF was approved to be used in the treatment of patients with hepatocellular carcinoma, renal cell carcinoma and differentiated thyroid carcinoma, some studies have indicated that SRF might cause severe liver injury and hepatic failure, especially in patients with cirrhosis[3-5]. In addition, SRF has many side effects which include anorexia, nausea, diarrhea, stomatitis/pharyngitis, hand–foot skin reaction, rash/desquamation, pruritus, and alopecia, fatigue and fever [6,7]. Therefore, many research studies are attempting to load SRF in nanoparticles with the aim to ameliorate the SRF's efficacy and reduce its cytotosixicity on the healthy cells. SRF was incorporated into nanostructured lipid carriers [8,9] and it has also been combined with other chemotherapeutic drugs in nanocarriers [10-12].

It could be beneficial to combine SRF with hepatoprotective natural products such as, the flaxseed oil, an essential oil which is naturally high in α-linolenic acid and antioxidants like tocopherols and beta-carotene [13-15]. To optimize the encapsulation of flaxseed oil with SRF, a water-in-oil nanoemulsions (NE) was formulated in the current study by mixing higher fraction of flaxseed oil with low fraction of water in the presence of the surfactant, Tween 80, and the cosurfactant, Span 20 followed by solubilizing an appropriate amount of SRF.

NEs are heterogeneous systems that consist of suspended nanodroplets with diameter sizes fall in the range of 20–200 nm which are fabricated by mixing water and oil by the aid of surfactants and cosurfactants [16]. They are usually used in pharmaceutical industries to improve the bioavailability of the drugs and protect them from degradation. NEs are also applied in food technology to enhance the oxidation stability of the food ingredients.

Materials and Method

Materials and subjects

Flaxseed oil, Tween 80, Span 20, SRF and Cremophor® RH 40 were procured from Sigma-Aldrich (Missouri, USA). Superoxide dismutase kinetic kit, catalase colorimetric kit, lipid peroxide (Malondialdehyde) colorimetric kit and the glutathione peroxidase UV kit and serum analysis kits were supplied from the Bio diagnostic lab for diagnostic and research reagents (Cairo, Egypt). One hundred female Swiss Albino mice, weighing in the range of 25-30 g, were kept in large cages at which each cage contained 5 mice. The animals were acclimatized according to the International Ethical Guidelines and King Abdulaziz University's policy [17].

Methods

Fabrication of nanoemulsion (NE) formulations

The free nanoemulsion formulation (free-NE), SRF-loaded-NE (SRF-NE) and SRF-Cremo were prepared as described by Alkhatib *et al.* [18] and the administered dose of SRF was adopted according to Abd-alhaseeb *et al.*[19] study.

Characterization of the prepared NE formulation

Zetasizer measurements

The z-average diameter, polydispersity index (PDI) and zeta potential of the NE formulations (free-NE and SRF-NE) were measured by using ZetasizerNano ZS (version no MAN0487-2-0, Malvern Instruments, UK).

Inoculation of the EAC cells in the mice

EAC cells were inoculated in the mice as mentioned elsewhere [20]. In particular, a 100 µl of fresh EAC cells, withdrawn from the mouse, was diluted with 9.9 ml of PBS (pH7.0). After that, a 50 µl of trypan blue stain and 50 µl of diluted EAC cells were mixed carefully in Eppendorf tubes. The stained mixture was placed in the hemocytometer. Only the unstained cells, which appeared transparent under light microscope, were counted while the stained ones were considered dead. The total number of cells/ml was calculated using the following equation:

$$\text{Total cell number /ml} = \frac{\text{counted cell from hemocytometer x dilution factor x10,000}}{\text{number of squares}}$$

The mice were weighed and divided into five groups (n =20). The 2.5×10^6 EAC cells/mouse were administered intraperitoneally into all of the groups except group I (Normal) kept untreated. Group II was designated as the positive control which includes EAC-bearing mice. Following 48 inoculation of EAC cells, Groups III-V were subjected into 7 doses of free-NE, SRF-Cremo and SRF-NE, respectively, applied day-by-day via oral gavage.

Following 12 hours fasting of ten mice from each group on the 16^{th} day, mice were weighed, blood was sampled from the retro-orbital plexus for serum analysis, and ascetic fluid was collected for the assessment of the antitumor activity of the drug formulas. After that, mice were slaughtered to isolate their livers for the histological studies. For the antioxidant assays, a small part of each lobe of the excised liver was removed and rinsed in ice-cold normal saline followed by deep freezing at - 80 °C in freezer (Revco™ CxF Series Ultra-Low Temperature Chest

Freezers) in order to be stored for utmost 3 months before performing the experiment. The other ten mice in each group were housed in their cages for the survival study.

Food appetite

One hundred grams of food were added into each cage daily. The amount of food consumed by the mice was calculated by subtracting the amount of the remaining food from the initial amount of the served food. The amount of food consumption was measured every day for around two weeks.

Lactate dehydrogenase activity in the ascetic fluid

The ascetic fluid of the experimental mice was collected to detect the lactate dehydrogenase (LDH) activity, if any. The supernatant of the fluid was prepared as described by Ghosh *et al.* [21]. In brief, the ascetic fluid was centrifuged for 5 min at 800 rpm at 4°C and the supernatant was taken. The assessment of LDH activity was performed according to the protocol of LDH LR (SCE MOD, Cat.No. CZ 908 L) kit which is based on the enzymatic reaction of reducing nicotinamide adenine dinucleotide hydride (NADH) with pyruvate under the effect of LDH as shown in the following equation: $\text{Pyruvate} + \text{NADH} + \text{H} \xrightarrow{LDH} \text{Lactate} + \text{NAD}^+$

Relative liver weight, histological study and serum analysis

The relative liver weight and the histological studies were identified as described elsewhere [18]. For Biochemical assays, Serum, centrifuged at 3000 RPM for 15 min following blood clotting, was employed to determine the liver function. The aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), albumin (ALB), total and direct bilirubin (BIL), total protein (TP) were measured according to the protocol mentioned in the assay kits supplied by the Crescent diagnostics Company (Jeddah, Saudi Arabia).

***In vivo* antioxidant activity**

The lobes of the liver tissue were homogenized in 4 ml of cold PBS at pH 7.0 per gram tissue. Then, the homogenized tissue was centrifuged at 4000 rpm for 15 min at 4° C immediately before the assay. A 1.0 ml of the collected supernatant was added to 0.5 ml of ice-cold extraction reagent and centrifuged at 4000 rpm for 10 min at 4°C. The obtained aqueous upper layer was used immediately for superoxide dismutase (SOD) assay. The remaining supernatant was collected and utilized for the estimation of lipid peroxide (Malondialdehyde, MDA), catalase activity and glutathione peroxidase (GPx). All the antioxidant assays were detected by the optimized UV and colorimetric methods mentioned in the commercial kit.

Survival study

The observation period of the survival study began in the first weeks of age until the study ended at the 8th week of age. The mean survival time (MST) and the increase life span percentage (% ILS) of each group containing ten mice were monitored by recording the daily mortality for 60 days. The MST is the amount of time after which 50% of the mice have died and 50% have survived. The % ILS was calculated using the following equation [22]

$$\% \text{ ILS} = [\frac{(\text{Median survival time of treated group})}{(\text{Median survival time of control positive group})} - 1] \times 100$$

Statistical analysis

The variations between the test samples were examined by the one-way analysis of variance (ANOVA) test and independent sample *t*-test using the MegaStat Excel (version 10.3, Butler University). The significant difference was considered when *p*-value <0.05. All data were expressed as mean ± SD.

Results

Zetasizer measurements

The Zetasizer was utilized to determine the z-average diameters and the zeta potential of the free-NE and SRF-NE. As illustrated in Table 1, the nanoparticles sizes and the negative zeta potentials of both formulas did not significantly differ as the P-values, measured by using the one-factor ANOVA, for the differences between the nanoparticle sizes and zeta potentials were 0.07 and 0.91, respectively. Interestingly, the PDIs of both formulas, calculated through dividing the standard deviation by the average, were less than 0.25 indicating that there were slight discrepancies between the sizes of the nanoparticles of each formula.

Table 1. The physical characteristics of the NE formulations measured by the Zetasizer. Data were expressed as mean ± SD.

Formulation	Z-Average diameter (nm)	Zeta Potential (mV)	PDI
Free-NE	105.9 ± 18.28	- 3.27 ± 1.42	0.173
SOR - NE	77.46 ± 8.28	- 3.4 ± 1.2	0.107

***In vivo* antitumor activity of drug formulations**

Body weight change

As shown in Table 2, the body weights (BWTs) of the experimental animal groups have increased within 16 days. The percentage increase in BWTs of normal, SRF-Cremo and SRF- NE groups were comparatively and significantly less than the change in BWT of free-NE and EAC+ groups.

LDH activity in the ascetic fluid

Table 2 illustrates the effect of the drug formulas on LDH activity in the ascetic fluid. Although the tumor volume in all of the treated groups has significantly decreased compared to EAC+ group, the LDH activity in the ascetic fluid has only considerably enhanced in SRF-NE group.

Food appetite

Table 2 demonstrates the effect of the drug formulations on the food intake of all of the tested groups during 16 days. Among the treated groups, the largest increase in food consumption was observed in the SRF-NE group which was comparable to the normal group. In contrast, the SRF–Cremo group had the least food appetite among all of the tested groups.

Table 2. The effect of the drug formulations on the body weight (BWT) (g), tumor volume, LDH activity of the ascetic fluids and food appetite of the tested groups.

Animal group	% Change in BWT	Tumor volume (ml)	LDH activity in ascetic fluids (U/L)	Consumed food (g)
Normal	7.15 ± 5.49^{b}	-	-	59 ± 2
EAC +	48.52 ± 11.89^{a}	13 ± 3.27	6.43 ± 6.90	44 ± 4
Free-NE	$33.30 \pm 14.42^{a\,b\,d}$	$6.9 \pm 5.5^{\,b}$	7.86 ± 7.56	$59.5 \pm 2^{\,c\,d}$
SOR - Cremo	12.49 ± 11.93^{b}	$4.75 \pm 3.22^{\,b}$	7.86 ± 8.59	$42 \pm 2^{\,a}$
SOR - NE	16.05 ± 4.89^{b}	$2.6 \pm 1.33^{\,b}$	$16.43 \pm 6.90^{b\,c}$	$66 \pm 1^{\,b\,c}$

[a] There is a statistical considerable difference between the tested group and the normal group; **[b]** There is a statistical considerable difference between the tested group and the EAC $^{+}$ group; **[c]** There is a statistical considerable difference between the SOR – Cremo and SOR – NE groups; **[d]** There is a statistical considerable difference between the SOR – Cremo and the free-NE groups.

Survival study

The greatest MST was recorded for the SRF–NE group (28± 2.54 days), whereas the MSTs of the free-NE (25 ± 2.07 days) and SRF-Cremo groups (27± 2.81days) did not show any significant difference from the EAC+ group (25 ± 3.27 days).

Toxicity of drug formulations on the liver function

Relative liver weight

Table 3 displays the liver function of the tested groups affected by the drug formulas. It was only the relative liver weight of the SRF-Cremo group that has gotten significantly enlarged when compared to the other groups. In contrast, a considerable decrease in the liver-to-body weight ratio was detected in EAC+ group relative to the normal group.

Serum analysis

Table 3 exhibits the levels of liver enzymes of the experimental animal groups. Relative to the normal and EAC+ groups, AST levels were elevated in both of SRF-Cremo and SRF-NE groups, whereas the levels of ALT were only enhanced in SRF-Cremo group. Interestingly the amounts of ALP, ALB and TP were within the standard ranges and comparable in all of the tested groups. In terms of BIL levels, the level of T.BIL for SRF – Cremo group was significantly greater than the EAC+ group, while the level of D.BIL for SRF – NE group was considerably increased relative to the normal group. Nevertheless, the levels of T.BIL and D.BIL of all of the studied groups were within the standard ranges.

Table 3. The effect of the drug formulations on the liver function of the tested groups.

Liver function	Normal	EAC+	Free-NE	SOR- Cremo	SOR- NE
Liver-to- body weight ratio	0.0459 ± 0.0057^{b}	0.0372 ± 0.0081^{a}	0.0399 ± 0.0085^{d}	$0.0552 \pm 0.0125^{a\,b}$	0.0431 ± 0.0053^{c}
AST (up to17U/l)	7.78 ± 0.93	6.36 ± 0.87	10.98 ± 3.94^{d}	18.37 ± 4.7^{ab}	15.81 ± 3.88^{ab}
ALT (up to17 U/l)	10.64 ± 2.95	8.68 ± 2.38	9.72 ± 5.14^{d}	18.21 ± 2.44^{ab}	11.52 ± 2.90^{c}
ALP (40 -190 U/l)	66.93 ± 1.82	65.22 ± 19.35	52.69 ± 6.30	51.58 ± 9.18	65.8 ± 10.82
ALB (3.8-5.1g/dl)	4.35 ± 0.58	4.53 ± 0.25	4.55 ± 0.81	4.87 ± 0.65	4.94 ± 0.25
T.BIL (up to1.1mg/dl)	0.82 ± 0.09	0.71 ± 0.05	0.79 ± 0.09	0.86 ± 0.08^{b}	0.82 ± 0.01
TP (6.0-8.3g/dl)	6.42 ± 0.27	5.9 ± 0.3	6.12 ± 0.1	5.79 ± 0.84	5.85 ± 0.12

[a] There is a statistical considerable difference between the tested group and the normal group; [b] There is a statistical considerable difference between the tested group and the EAC + group; [c] There is a statistical considerable difference between the SOR – Cremo and SOR – NE groups; [d] There is a statistical considerable difference between the SOR – Cremo and the free-NE groups.

ROS analysis of liver tissue

All tested ROS parameters of the experimental mice groups are summarized in Table 4. The catalase activities of both of the normal and SRF-NE groups were considerably greater than that of EAC+ group. In terms of NADPH consumption, the amount of consumption of the SRF-NE group was comparable to the consumption of the EAC+ and normal groups. In addition, the accumulation levels of MDA were significantly elevated in EAC+ group when compared to the other experimental groups. Interestingly, there were no significant differences in MDA levels between the treated groups and the normal group. Furthermore, the SOD activities of all of the experimental groups were comparable.

Table 4. The ROS analysis of the tested groups treated with different drug formulations in order to detect the liver antioxident. Data were expressed as mean ± SD.

Groups	Catalase activity (u/g)	NADPH consumed by GPx(1nmol/min/ml)	MDA (nmol/g.tissue)	SOD activity (u/g tissue)
Normal	2.55 ± 0.51 [b]	2.09 ± 0.62[b]	123.35 ± 33.9[b]	909.23 ± 274.42
EAC+	1.8 ± 0.25	0.85 ± 0.78 [a]	256.62 ± 95.36 [a]	879.29 ± 199.5
Free-NE	2.15 ± 0.45	0.73 ± 0.23[a d]	99.39 ± 15.54[b]	731.48 ± 193.2
SOR-Cremo	2.36 ± 0.34	2.21 ± 0.36 [b]	137.74 ± 48.45 [b]	879.37 ± 188.58
SOR-NE	2.48 ± 0.64[b]	1.50 ± 1.02	176.46 ± 70.54 [b]	1026.63 ± 113.27

[a] There is a statistical considerable difference between the tested group and the normal group; [b] There is a statistical considerable difference between the tested group and the EAC + group; [c] There is a statistical considerable difference between the SOR – Cremo and SOR – NE groups; [d] There is a statistical considerable difference between the SOR – Cremo and the free-NE groups.

Liver histology

Figure 1 (A-E) exhibits the photomicrographs of the liver tissue sections of the experimental animal groups. The liver section of the control negative group, shown in Figure 1A, displayed the normal hepatic structure with normal strands of hepatocytes organized into anastomosing cords or plates that are separated by hepatic sinusoids. In contrast, the hepatic tissue structure of the EAC+ group (Figure 1B), showed excessive dilated sinusoids and the appearance of more Kupffer cells. The treated group with free-NE showed liver section similar to EAC+ group with comparable plates of hepatocytes and central vein as presented in Figure 1C.The hepatic microscopic tissues of the mice treated with SRF-Cremo, shown in Figure 1D, exhibited excessive sinusoidal spaces, activation of Kupffer cells and alteration in the appearance of the hepatocytes whereas the hepatic tissue section of the mice treated with SRF-NE displayed narrow sinusoids channels, presence of few Kupffer cells and normal structure of the hepatic tissue around the central vein (Figure 1E).

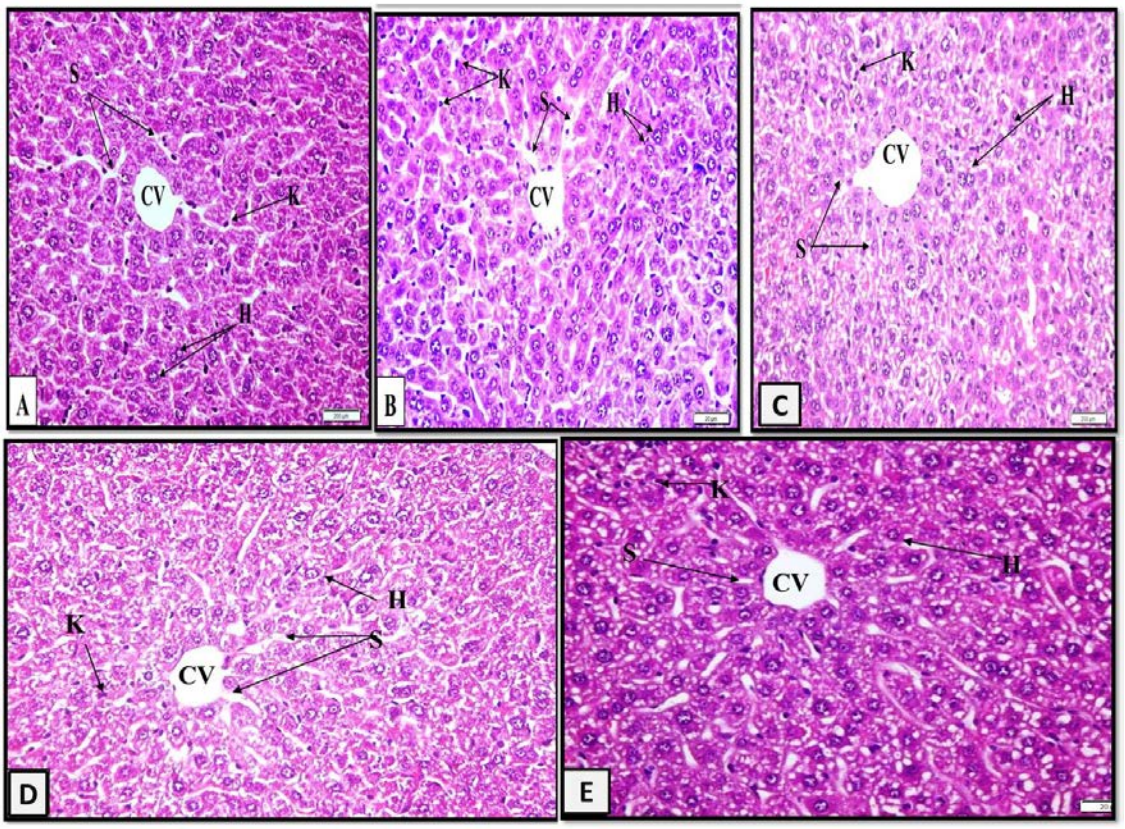

Figure 1. light microscopy images of the hepatic tissues of the A) normal group, showing the hepatocytes (H) that are arranged in cords radiating from the central vein (CV) and separated by blood sinusoids (S) with the presence of few Kupffer cells, B) EAC+ group, showing the central vein (CV) with hepatocytes (H), excessive dilated blood sinusoids (S), and the presence of excess Kupffer cells (K), C) free-NE group, showing the central vein (CV) with hepatocytes (H), excessive dilated blood sinusoids (S), and the presence of few Kupffer cells (K), D) SOR - Cremo group, showing the central vein (CV) with hepatocytes (H), more dilated blood sinusoids (S), and the presence of Kupffer cells (K), and E) SOR – NE group, showing the central vein (CV) with hepatocytes (H), less dilated blood sinusoids (S), and the presence of few Kupffer cells (K). H&E x 400.

Discussion

The anticancer activity of the studied formulas in EAC-bearing mice was evaluated by detecting the efficiency of the administered drug through measuring the BWT, LDH activity in the ascetic fluid, tumor volume, the food intake, MST and %ILS. Among all of the treated groups, SRF-NE group has the least tumor volume, the greatest survival, suggesting that incorporating SRF into the NE has enhanced its antitumor activity. Additionally, the considerable increase of the LDH activity in the ascetic fluid of SRF-NE group indicates that the tumor cells have undergone apoptosis [21].

One of the adverse effects of SRF, administered into hepatocellular carcinoma patient, was the loss of food appetite [23]. In the current study, despite the sharp decline in the tumor volume of SRF-NE group, the percentage change in BWT was higher than the SRF-Cremo group due to the increase in the food intake by SRF-NE compared to the SRF-Cremo group which has lost its appetite. In spite of the increase in food appetite of SRF-NE group, their BWT change did significantly differ from the normal group which could be attributed to the presence of flaxseed oil in NE which was found to enhance the plasma and adipose levels of α-linolenic acid and thereby maintain the expression of leptin protein and prevent excess BWT change [24].

The improvement of the anti-proliferative effect of SRF-NE in mice can be explained by the small droplet sizes of the NE particles and their negative charges. Kotta *et al*. [25] have demonstrated that nanodroplets with negative charges ameliorate the absorption of the drug in the intestinal tract. Honary and Zahir [26] mentioned that the *in vivo* biodistribution of the small and negatively charged nanoparticles were tended to accumulate in tumor more efficiently because they usually don't get adsorbed to the proteins that would be uptaken later by the macrophages.

The side effect of the subjected formulas on the liver was assessed by determining the relative liver weight, serum biochemical parameters, ROS and implementing the histopathological examination. Compared to SRF-Cremo, SRF-NE subjected into the mice has amended the relative liver weight, decreased the level of ALT enzyme and raised the activity of the catalase. In 2013, Shah *et al*. [5] have reviewed the hepatotoxicty of 18 kinase inhibitors, including SRF, and found that ALT was elevated in 21-24% of patients which was correlated to liver injuries in many cases. Another study showed that treating neoplastic patients with tyrosine kinase inhibitors would raise the risk of the liver failure caused by the drug at least two fold [27]. The hepatotoxicity of SRF is attributed to its oxidative metabolism in the liver mediated by CYP3A4 [28]. Flaxseed oil may play a role in the reduction of SRF's toxicity as demonstrated by Naqshbandi *et al.* [29] who found that the hepatotoxicity of the rats,

supplemented for 10 days with adiet rich in flaxseed oil followed by applying a single dose of cisplatin, was considerably reduced. Furthermore, incorporating SRF into the NE has increased the level of catalase which neutralize the production of hydrogen peroxide thereby prevent hepatic cell damage [30].

Conclusion

The antitumor activity of SRF loaded in NE based on flaxseed oil was considerably enhanced. In particular, the survival, food appetite and antiproliferative effect against EAC cells was ameliorated. The toxicity of SRF-NE on the liver was reduced relative to the SRF-Cremo as the level of ALT and liver-to-body weight ratio has decreased and the level of catalase has increased. Additionally, the histological study revealed reduced damage of the hepatocytes of SRF –NE when compared to the other treated groups.

Acknowledgement

This research project (PGP-38-11) was financially supported by King Abdulaziz City for Science and Technology.

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