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Hepatoprotective effects of a self-micro emulsifying drug delivery system containing *Silybum marianum* native seed oil against experimentally induced liver injury

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The main purpose of this study was to certify the effect of native silymarin oil (SM-oil) formulated in a self-microemulsifying drug delivery system (SMEDDS). The optimal formulation was 25 % of SM-oil, 33,3 % of Cremophor RH40, 20 % of Transcutol HP, 16,6 % of Labrasol and 5 % of Capryol 90. In this novel formulation the SM-oil was the active substance and the lipid part. The *in vivo* study examined the preventive effects of SMEDDS containing SM native seeds oil against carbon tetrachloride (CCl_4) induced hepatotoxicity in mice. Determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST) levels and also liver histology investigations have been done. The liver antioxidant status was determined with the concentrations of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), and glutathione (GSH) hepatic lipid peroxidation was examined and expressed in terms of malondialdehyde (MDA) content. The plasma levels of AST and ALT significantly diminished by pre-treatment with 500 mg/kg and 1000 mg/kg SMEDDS. The pre-treatment with 500 mg/kg and 1000 mg/kg SMEDDS increased GSH level by about 6% respectively 24% compared to the CCl4 group. Due to preventive administration of 500 mg/kg and 1000 mg/kg of SMEDDS in the intoxicated animals, MDA levels were reduced by 22% respectively 58%. Also, an insignificant rise by almost 17% and 19% in the animals treated with the both doses of SMEDDS could be noticed. It can be concluded that hepatotoxicity may be avoided by the oral application of our formulation.

1. Introduction

Silymarin, extracted from the milk thistle plant, *Silybum marianum* (SM), has been successfully applied for the treatment of various liver disorders including acute and chronic viral hepatitis, alcoholic liver diseases, toxin and drug induced hepatitis and cirrhosis, fatty liver radiation, and toxicity (Pepping 1999; Flora et al.1998; Luper 1998). However, the therapeutic effects of silymarin are restricted due to its poor water solubility resulting in low oral absorption and bioavailability after oral administration (Pepping 1999). The topical use of silymarin has also received attention because of its antioxidant, anti-inflammatory and immunomodulatory properties which may prevent UVB-induced skin damages or chemically induced skin disorders including erythema, photoaging and skin cancer (Katiyar 2002, 2005). Other effects of the topical application of silymarin have been also reported like prevention of radiodermatitis, skin whitening effect or effectiveness in the treatment of rosaceae (Becker-Sciebel et al. 2011; Rasul et al. 2011; Nield and Ippersiel 2022). Eleven active substances (components) have already been separated from the extract of SM by high performance liquid chromatography/tandem mass spectrometry (HPLC–MS/MS). The major bioactive flavonolignas in silymarin includes silychristins A and B, silydianin, silybin A and B, isosilybin A and B and an unknown compound. Furthermore, three additional components were also detected and partly separated; presumably two silybin stereoisomers and one isosilybin stereoisomer (Kuki et al. 2012).

During the extraction process of SM seeds two phases, a solid powder and an oil extract can be reached. A number of approaches have been used to increase its solubility and thereby bioavailability of the powder extract of silymarin. These include complexation with cyclodextrin (Arcaria et al. 1992) and phospholipids (Yanyu et al. 2006), incorporation in solid dispersion (Chen et al. 2005), solid lipid nanoparticles (He et al. 2007) and also formulation of self-(micro) emulsifying drug delivery system S(M)EDDS (Woo et al. 2007). Most of these studies deal with the internal and external effect of the powder, but not with the therapeutic effect of the native silymarin oil. The oil obtained from the seeds of SM produced very low antioxidant effects according to Batakov (2001). Many formulation approaches were previously employed to increase the solubil-

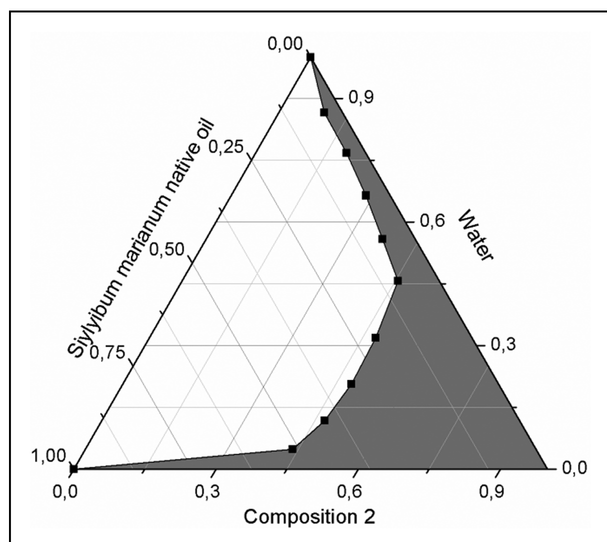

Fig. 1: The pseudoternary phase diagram of composition 2. Shaded area of pseudoternary phase diagram representing the microemulsion.

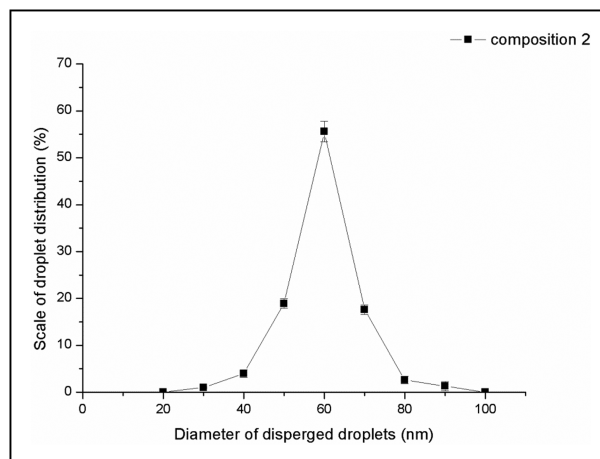

Fig. 2: Evaluated droplet size of selected (composition 2) SMEDDS in water via DLS measurement. Results are reported as means ± SD, n = 5.

ity of poorly water-soluble drug substances, either by means of improving the dissolution rate or *via* presenting and maintaining the drug in solution all the while its period in the gastrointestinal tract (Lipinski et al. 2001). SEDDS and SMEDDS (Lawrence and Rees 2000; Humberstone and Charman 1997; Constantinides 1995; Pouton 2000; Gershanik and Benita 2000; Pouton 1997) are composed of poorly soluble drugs, lipoids, surfactants and co-surfactants and they are capable of forming oil-in-water emulsions upon gentle agitation provided by the gastrointestinal motion. The spontaneous formation of a microemulsion advantageously presents the lipophilic drug in a dissolved form, and the resultant small droplet provides a large interfacial surface area for drug release and absorption. Main mechanisms include increasing membrane fluidity to facilitate transcellular absorption, and also opening tight junctions to allow paracellular transport (Humberstone and Charman 1997; O'Driscoll 2002). In the present investigation, an attempt has been made to develop a SMEDDS containing silymarin native oil in order to investigate the effectiveness of the SM-oil and applied surfactants and co-surfactants in this drug delivery system. Our aim was to examine the hepatoprotective effect of SMEDDS containing SM native oil against CCl_4 induced hepatotoxicity in mice.

2. Investigations and results

2.1. *Determination of droplet size of SMEDDS-SM-oil formulation*

By the results of the pseudoternary phase diagram, composition 2 has been selected for microemulsion preparation since maximum microemulsion existing zone was observed as shown in Fig. 1. Our previous cytotoxicity studies were also the aspect for the selection of optimal composition 2. Tensides cytotoxicity of SMEDDS was tested on Caco-2 cell monolayer. Critical micelle concentrations (CMC) were also determined. We found that composition 2 was ideal according to their tensides cytotoxicities and CMCs (Ujhelyi et al. 2012, 2013). The optimal formulation of SMEDDS SM-oil was 25 % of SM native seeds oil, 33,3 % of Cremophor RH40, 20 % of Transcutol HP, 16,6 % of Labrasol and 5 % of Capryol 90 with globule size 57.8 ± 3.32 nm as shown in Fig. 2. SMEDDS spontaneously formed a microemulsion upon mild agitation in purified water at room temperature. Actual emulsifying time was under 20-30 s, and the percentage of transmittance and refractive index of the selected formulation were found to be 97.68 ± 1.31 % and 1.337 ± 0.13 % indicating the transparency as well as the nanosizing of the formulation. Moreover, the developed formulation was stable for 1 month at ambient temperature.

2.2. *Effects of SMEDDS on hepatic function markers*

Effects of the both doses of SMEDDS on plasma AST and ALT levels are shown in Table 1. The elevation of these activities level in the CCl_4-intoxicated group was significantly higher than in the control one. However, this effect was significantly diminished by pre-treatment with 500 mg and 1000 mg SMEDDS. The action of the higher dose was more significant.

2.3. *Antioxidant enzymes level*

Our results showed that intraperitoneal CCl_4 (1 mL/kg b.wt.) injection significantly decreased CAT and GPX enzyme activities in liver homogenate when compared to control group (Table 2). In the case of SOD and GR specific activities an insignificant decrease was observed. The pre-treatment of animals with SMEDDS diminished the damage induced by this toxicant in a dose-dependent manner. So, SOD levels were increased by about 28.5% and 37.5% in the case of of administration of 500 mg respectively 1000 mg SMEDDS compared to individuals IP injected with CCl_4. In the pre-treated individuals with 500 mg and 1000 mg SMEDDS and then exposed to the chemical toxicant, CAT specific activity was amplified by 22.5% respectively 45.5% and GPX one with 18.6% respectively 48.6%, whereas GR one increased by 22.5% respectively 33.5%.
Also in individuals treated only with SMEDDS an increase of all analyzed enzyme activities was noticed compared to control organisms.

2.4. *GSH concentration*

From Fig. 3, a variation of GSH concentration in the liver of different groups can be seen. This biochemical parameter was decreased in the group IP injection with CCl_4 by about 42% compared to control. The pre-treatment with 500 mg and 1000 mg SMEDDS per kg b.w. increased GSH levels by about 6% respectively 24% compared to the lot exposed to toxicant. But the concentration of this tripeptide remained significantly lower in comparison with control. Furthermore, a slight increase of GSH

Table 1: Pre-treatment effect of SMEDDS doses of 1000 mg/kg and 500 mg/kg and CCl_4 (1 mL/kg) intoxication on serum AST and ALT enzymes

	Control	CCl_4	SMEDDS 500 mg/kg	SMEDDS 1000 mg/kg	SMEDDS 500 mg/kg + CCl_4	SMEDDS 1000 mg/kg + CCl_4
AST (IU/L)	80.742 ± 15.09	$2318.225 \pm 166.311^{***}$	$68.614 \pm 2.273_{\#\#\#}$	$62.391 \pm 4.471_{\#\#\#}$	$2163.142 \pm 158.848^{***}/_{\#}$	$1352.714 \pm 69.643^{***}/_{\#\#\#}$
ALT (U/L)	48 ± 4.873	$2384.457 \pm 197.398^{***}$	$45.542 \pm 3.820_{\#\#\#}$	$46.714 \pm 3.039_{\#\#\#}$	$1934.285 \pm 109.809^{***}/_{\#\#\#}$	$1261.542 \pm 108.993^{***}/_{\#\#\#}$

Each value represents the means ± SD for 8 mice and expressed IU/L, and U/L. $^{*}P < 0.05$ significantly different as compared with the control group, $^{\#}P < 0.05$ significantly different as compared with the CCl_4-treated group. Activities were calculated with Two-way ANOVA and Bonferroni post-test.

level was noticed in the animals treated only with SMEEDS at both doses.

2.5. MDA concentration

Figure 4 shows the variation of MDA concentration in the framework of our experiment. Elevations in the levels of end products of lipid peroxidation by 114.5%, in the CCl_4 intoxicated liver compared to control were registered (Fig. 2). Due to preventive administration of 500 mg and 1000 mg of SMEDDS in the intoxicated individuals, MDA levels were reduced by 22% respectively 58%. Also, an insignificant rise by almost 17% and 19% in the individuals treated with the both doses of SMEDDS could be noticed.

2.6. Histopathology

Liver sections from control mice (Fig. 5A1) showed normal architecture. In the group treated with CCl_4, large areas of extensive, mainly centrilobular necrosis (Fig. 5B1), vacuolar fatty change (Fig. 5B2) have been found. The low dose of SMEDDS (500 mg/kg) did not prevent efficiently the toxic effect of CCl_4, and large necrotic areas were still present (Fig. 5C1). However, the high dose of SMEDDS (1000 mg/kg) prevented liver necrosis and fat accumulation better, showing minimal hepatic damage (Fig. 5D1, D2), statistically significant compared to the 500 mg dose. (Fig. 5E)

3. Discussion

In our novel SMEDDS formulation studies SM-oil was the active substance and the lipid/oil part of the system. Several reported that the property of oil component and the weight percentage of oil in SMEDDS formulation are determining factors in these preparations (Zidan et al. 2007). Formulations may enhance the oils own therapeutic effect and the oral absorption as well (Gang and Yan 2011).

Besides the selection of oil, surfactant and co-surfactant as well as the mixing ratio of oil to surfactant/co-surfactant may play an important role in SMEDDS formulation (Zidan et al. 2007). In our preparation Cremophor RH 40, Transcutol HP were the surfactants. Labrasol, Capryol 90 as non-ionic tensides were the co-surfactants. Biological effects of several nonionic amphiphilic surfactants have been also investigated according to their chemical structure (Ujhelyi et al. 2012). In our previous studies, the possibility of enhanced bioavailability by cell monolayer structural alteration of various surface active agents has been shown (Ujhelyi et al. 2012, 2013). Various surface active agents could improve both the paracellular and transcellular uptake of active ingredients *in vitro* as well (Sha et al. 2005). Presumably, our preparation may also increase the oral bioavailability of SM-oil by the alteration of intestinal cell monolayer.

According to Batakov (2001), the oil obtained from the seeds of SM produced an antioxidant effect on liver tissues of rats poisoned with CCl_4. The oil just in high doses (2000 mg/kg) reduced the level of lipid peroxidation, increased catalase activity but did not reduce the concentration of selenium in the liver (which decreased as a result of CCl_4 intoxication). The oil did not increase the activity of superoxide dismutase in liver tissues. In spite of this study, we found that SM oil has its own hepatoprotective therapeutic effect (Hermenean et al. 2014). The application of SMEDDS increased the effectiveness of SM oil and decreased the dose of SM-oil significantly (500 mg/kg, 1000 mg/kg). Several studies dealt with the therapeutic effect of SM oil. Sorenson et al. (2011) examined native silymarin

Table 2: Pre-treatment effect of SMEDDS doses of 1000 mg/kg and 500 mg/kg and CCl_4 (1 mL/kg) intoxication on enzyme activities in liver homogenate

	Control	CCl_4	SMEDDS 500mg/kg	SMEDDS 1000mg/kg	SMEDDS 500mg/kg + CCl_4	SMEDDS 1000mg/kg + CCl_4
SOD	100 ± 0.032	75.5 ± 0.024	112 ± 0.068	$120 \pm 0.043_{\#}$	104 ± 0.013	113 ± 0.016
CAT	100 ± 0.655	$81.5 \pm 0.388^{*}$	$123 \pm 0.519^{*}/_{\#\#\#}$	$148 \pm 0.346^{**}/_{\#\#\#}$	$104 \pm 0.198_{\#\#}$	$127 \pm 0.605_{\#\#\#}$
GPx	100 ± 0.777	$83.4 \pm 2.406^{***}$	$122 \pm 0.831^{***}/_{\#\#\#}$	$136 \pm 2.220^{***}/_{\#\#\#}$	$102 \pm 0.112_{\#\#\#}$	$132 \pm 4.287^{***}/_{\#\#\#}$
GR	100 ± 0.019	84.5 ± 0.003	$116 \pm 0.003^{*}/_{\#}$	$138 \pm 0.010^{**}/_{\#\#}$	107 ± 0.001	118 ± 0.006

Each value represents the means ± SD for 8 mice and expressed as % from untreated control. $^*P < 0.05$ significantly different as compared with the control group, $^\#P < 0.05$ significantly different as compared with the CCl_4-treated group. Activities were calculated with Two-way ANOVA and Bonferroni post-test.

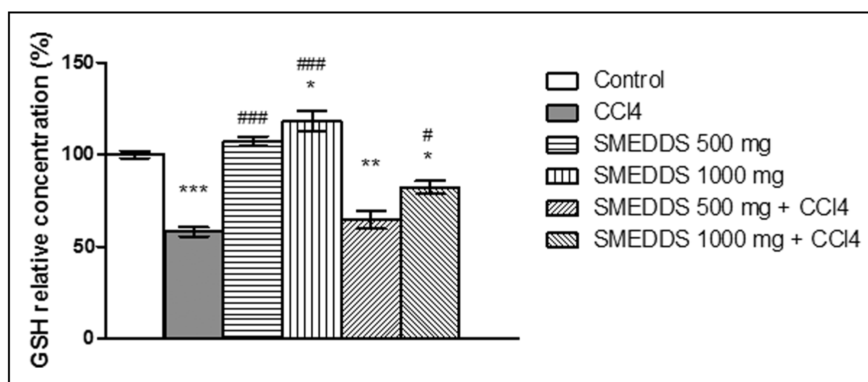

Fig. 3: Pre-treatment effect of *Silybum marianum* oil SMEDDS doses of 1000 mg and 500 mg/kg and CCl_4 (1 mL/kg b.wt.) intoxication on reduced glutathione content in liver homogenate. Values are calculated as means ± SD (n = 8 in each group) and expressed as % from untreated control. $^*P < 0.05$ significantly different as compared with control group, $^\#P < 0.05$ significantly different as compared with CCl_4-treated group. Data were calculated with Two-way ANOVA and Bonferroni post-test.

seed oil for the ability to reduce experimental metastases in mice. In their experimental models, they showed that a single oral dose of *Salmonella enterica* serovar, *typhimurium* expressing a truncated human interleukin-2 gene (Salp IL2) is avirulent, immunogenic and reduces hepatic metastases through increased natural killer cell populations in mice. However, these experiments showed that in SalpIL-2 treated animals the SM oil inhibited the antitumor efficacy of SalpIL2.

Several studies demonstrated hepatoprotective activity of different components of SM seeds, both preventive and curative, in CCl_4-intoxicated rats, with differences in the dosage, the route of their administration and their different formulation to increase especially the oral bioavailability (Woo et al. 2007, Abrol et al. 2005, Shaker et al. 2010, Wang et al. 2010). The therapeutic efficiency of silymarin is limited to its poor water solubility and low bioavailability after oral delivery. According to Wei Wu et al. the bioavailability of silymarin was also enhanced greatly by SMEDDS (Wei et al. 2006). Furthermore, Woo et al. also formulated SMEDDS containing silymarin and after its oral administration to rats, the bioavailability of the drug from SMEDDS was 3.6 times higher than the reference capsule (Woo et al. 2007).

In our study, liver protection was achieved by oral application of two doses of SM-oil SMEDDS (500 mg/kg, 1000 mg/kg) before CCl_4 toxic administration. CCl_4-induced hepatic injury is an experimental model frequently used for hepatoprotective drug screening (Weber et al. 2003).

The decrease in serum aminotransferases activity by the high-dose of SMEDDS in CCl_4-intoxicated mice indicates that this SM oil formulation preserves the structural integrity of hepatocellular membrane, which was supported by the histological findings.

In the present study, 7 day pre-treatment with SMEDDS-SM followed by a single dose administration of CCl_4, generated antioxidant protection for mice hepatocytes, evidenced by increased antioxidant enzyme activities (SOD, CAT and GPX) compared to the intoxicated group, in which they were lower compared to control. It was shown that the treatment with CCl_4 in rodents generated ROS (Johnson and Kroenung 1998) and also increased significantly hepatic nitric oxide synthase activity (Tanaka et al. 1999). The reaction of superoxide with nitric oxide occurs despite its SOD-catalyzed dismutation generating peroxynitrite involved in tyrosine nitration and lipid peroxidation (Demicheli et al. 2007, Radi 2004, Radi et al. 1991).

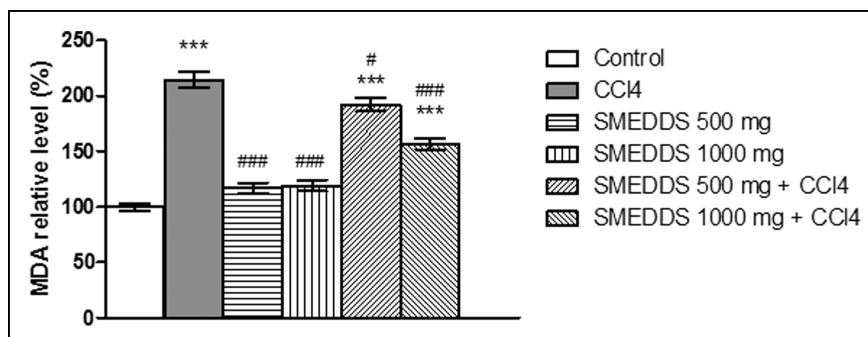

Fig. 4: Pre-treatment effect of *Silybum marianum* oil SMEDDS doses of 1000 mg and 500 mg/kg and CCl_4 (1 mL/kg b.wt.) intoxication on malondialdehyde content in liver homogenate. Values are calculated as means ± SD (n = 8 in each group) and expressed as % from untreated control. $^*P < 0.05$ significantly different as compared with control group, $^\#P < 0.05$ significantly different as compared with CCl_4-treated group. Data were calculated with Two-way ANOVA and Bonferroni post-test.

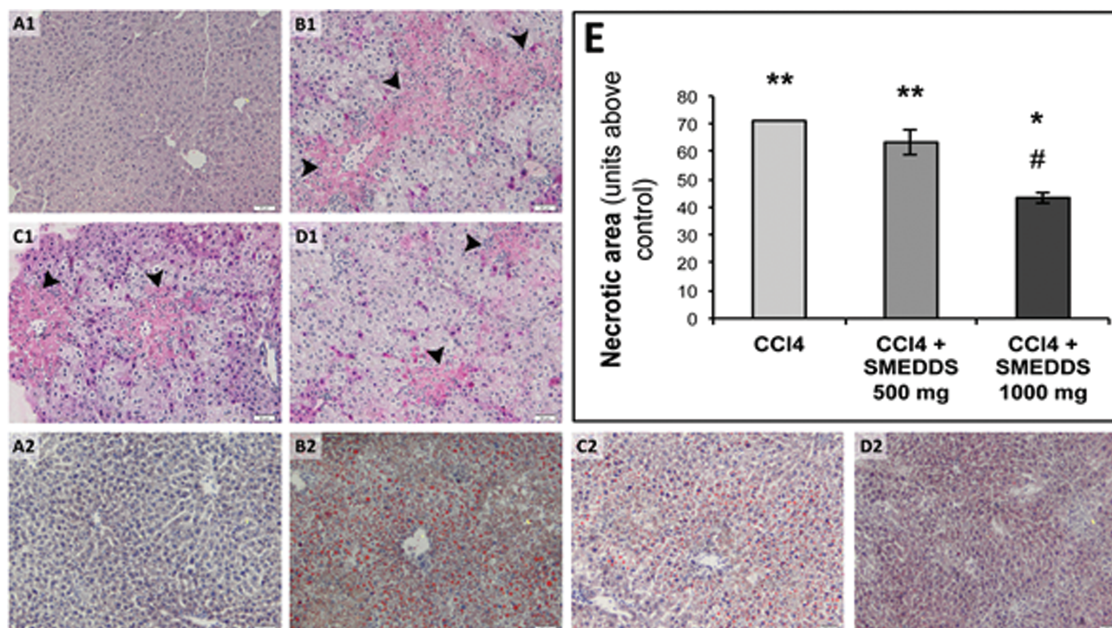

Fig. 5: Effect of *Silybum marianum* oil SMEDDS doses of 1000 mg and 500 mg on histological changes in the liver of CCl_4 – treated mice; (A) Control group; (B) CCl_4 group (C) $SMEDDS_{500} + CCl_4$ group (D) $SMEDDS_{1000} + CCl_4$ group 1.H&E stain (arrowhead-centrilobular necrosis); 2 Oil Red O stain (lipid drops – red); The necrotic area in the livers was calculated as means ± SD (n = 8 in each group) and expressed as % from untreated control. $^{*}P < 0.05$ significantly different as compared with control group, $^{\#}P < 0.05$ significantly different as compared with CCl_4-treated group. Data were calculated with Two-way ANOVA and Bonferroni post-test.

Previous work demonstrated that tyrosine nitration was correlated with the decrease of Mn-SOD activity as well as of CAT and GPX ones (Afolayan et al. 2012, Keng et al. 2000, Frank et al. 2003). These could be the reasons for which the specific activities of the three before mentioned enzymes and of GR one were significantly diminished compared to control.

Interesting is that the treatment only with SMEDDS increased all these activities. We suppose that this situation appeared because, possibly, the oxidation of endogenous GSH in the presence of linoleic acid (an important constituent of SM seed oil used for SMEDDS obtaining) catalyzed by lipoxygenase was accompanied by superoxide anions generation (Roy et al. 1995) besides the normal ways in which it is formed: through the one-electron reduction of molecular oxygen by flavoproteins like xanthine oxydase the mitochondrial respiratory chain (McCord and Fridovich 1968, Boveris and Cadenas 1975). Moderate levels of superoxide anions could increase the SOD reaction rate and produce more hydrogen peroxide which could be decomposed in the reactions catalyzed by GPX and CAT. Also the rise of GR activity could be possibly due to conjugated linoleic acid isomers as previoulsly Choi et al. have demonstrated (Choi et al. 2007).

Preventive treatment with SMEDDS obtained from SM seed oil restored the specific activities of all enzymes in the individuals exposed to CCl_4 in a dose dependent manner.

The significant elevation of MDA, the end product of lipid peroxidation, was generally considered a marker of formation of free radicals (Fraga et al. 1987). The decrease of SOD, CAT, GPX and GR activities in the individuals exposed to CCl_4 have generated a lower antioxidant defence capacity in their liver and as a result MDA concentration increased significantly. The pre-treatment by SMEDDS decreased this parameter in the liver of intoxicated mice, which might suggest their antioxidant capacity, which could be due to the presence of tocopherol and ascorbic acid 2,6 dihexadecanoate, present in SM seeds oil. In our opinion the insignificant increase of MDA in mice treated only with the two doses of SMEDDS might appear because content of the polyunsaturated fatty acid, linoleic acid exist in the oil is high.

At the same time, the variation of hepatic GSH content was inversely proportional for the CCl_4 intoxicated group. Pre-treatment with SMEDDS containing SM seeds oil was effective in protecting hepatocytes, abolishing the increasing MDA levels and raised GSH level. These favourable changes could be due to oil antioxidative protective action as well as the capacity of conjugated linoleic acid to enhance GSH content and gamma-glutamylcysteine ligase catalytic subunit in mice (Bergamo et al. 2006).

In the present study, a novel formulation of SMEDDS containing SM native seed oil was formulated. It can be concluded that pre-treatment with SMEDDS-SM oil decreases the CCl_4-induced elevation both in biochemical and morphological parameters. Our results demonstrated that the possible hepatoprotective mechanism of the SMEDDS-SM native seeds oil on CCl_4-induced liver damage in mice might be due to the prevention of lipid peroxidation and stabilizing the hepatocyte membrane. Hepatotoxicity may be avoided by the oral application of our formulation.

4. Experimental

4.1. Formulation of SMEDDS-SM oil

According to our preliminary studies Cremophor RH40, Labrasol, Capryol 90 and Transcutol HP have been selected for SMEDDS formulation.

Self-Emulsifying combinations have been formulated by water dilution method with various previously mentioned tensides and co-tensides. Tenside components were mixed at 37 °C by a Schott Tritronic dispenser combined with a Radelkis OP-912 magnetic stirrer. The applied concentrations of SM native seeds oil have been incorporated in the system at room temperature. To evaluate any signs of phase separation, the mixtures were equilibrated for 24 h. Erweka DT 800 rotating paddle apparatus (Erweka Gmbh, Heusenstamm, Germany) was used to evaluate the efficiency of self-emulsification of different mixtures. One gram of each mixture was added to 200 ml of distilled water with gentle agitation provided by a rotating paddle at 70 rpm and at a temperature of 37 °C. The process of self-emulsification was visually monitored for the rate of emulsification and for the appearance of the produced emulsions. The visual properties registered against the increment of the applied tenside component in ternary triangular diagrams.

Table 3: Composition of the SMEDDS-SM-oil formulations

	SM- oil	Transcutol HP	Cremophor RH 40	Labrasol	Capryol 90
Composition 1	30 v/v %	45 v/v %	20 v/v %	5 v/v %	0 v/v %
Composition 2	25 v/v %	20 v/v %	33,3 v/v %	16,6 v/v %	5 v/v %
Composition 3	16,6 v/v %	16,6 v/v %	16,6 v/v %	33,3 v/v %	16,6 v/v %
Composition 4	16,6 v/v %	16,6 v/v %	16,6 v/v %	16,6 v/v %	33,3 v/v %

Plotting points of preferential combinations selected according to Cartesian coordinate calculation.
The compositions of the SMEDDS-SM native seeds oil formulations are listed in Table 3.

4.2. Determination of droplet size of self-micro-emulsifying systems

The diameter of dispersed phase was investigated by a Cumulant Dynamic Light Scattering (DLS) device. To obtain the diffusion coefficient the intensity correlation function has been analyzed. The measurements have been performed by a Brookhaven Fotometer apparatus. The operation temperature was adjusted to 25° C, the laser detection angle to 90 degree, Lambda to 533 nm, index to 1,334 by Particle Sizing Program 3.1. Diameters of dispersed droplets according to the diffusion coefficient have been evaluated automatically by the computer program.

4.3. Animals and experimental groups

Swiss male mice (25 ± 3 g), supplied by the Animal House of the Vasile Goldis Western University of Arad, were used. The animals were maintained at a 12 h light/dark cycle, at constant temperature, with free access to food and tap water *ad libitum*. All experimental procedures were approved by the Ethical Committee of Vasile Goldis Western University of Arad. Throughout the experiments, animals were processed according to the suggested international ethical guidelines for the care of laboratory animals.
Forty-eight animals were used for the experiment and divided into six groups, listed in Table 4.
The mice were anaesthetized on 9th day, and blood was collected from *venae cavae* before mice were euthanized by cervical dislocation. Liver samples were used for histopathology and biochemical analyses.

4.4. Preparation of blood plasma and liver protein extract

The collected blood was placed in heparinized tubes and centrifuged for 15 min at 2000 g in order to obtain plasma samples which were used immediately to determine ALT and AST activities. For the preparation of the total protein extract, 0.1 g of liver tissue was suspended in a cold 0.1 M Tris-HCl buffer (pH 7.4) containing 5 mM EDTA and a freshly added protease inhibitor cocktail, and homogenized for 2 min at 16 Hz using a ball mill (type MM 301, Retsch GmbH & Co, Haan, Germany). The homogenate was centrifuged at 8,000 rpm for 30 min at 4 °C to remove the cell debris. The supernatant was collected for enzymatic analyses, as well as GSH, MDA and protein concentration assays.

4.5. Assay of plasmatic hepatic markers

The plasmatic activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were evaluated by the spectrophotometric method using commercially available kits (Roche reagents, France) according to the manufacturer's indication.

4.6. Liver antioxidant status

4.6.1. Superoxide dismutase (SOD) (EC 1.15.1.1)

SOD activity was determined using a spectrophotometric method (Paoletti and Mocali 1990) based on the decrease of optical density at 340 nm due to NADH oxidation by the generated superoxide anion. One unit of enzyme activity is the amount of enzyme required of NADH oxidation inhibition rate of 50%.

4.6.2. Catalase (CAT) (EC 1.11.1.6)

CAT was detected spectrophotometrically at 240 nm, by monitoring H_2O_2 decomposition (Aebi 1974). The CAT activity was expressed as U/mg protein. One unit of enzyme decomposes one μmole of H_2O_2 in a minute at 25 °C and pH 7.

4.6.3. Glutathione peroxidase (GPX) (EC 1.11.1.9)

GPX activity was measured according to Beutler's method (Beutler 1984), through spectrophotometrically changes at 340 nm due to oxidation of NADPH to $NADP^+$ by tert-butyl hydroperoxide. The concentration of NADPH was calculated using a molar extinction coefficient of $6.22 \times 103\ M^{-1} \bullet cm^{-1}$ and the activity was expressed as U/mg. One U of activity is defined as that quantity of enzyme responsible for the oxidation of one μmole of NADPH per minute.

4.6.4. Glutathione reductase (GR) (EC 1.6.4.2)

GR activity was assayed by the decrease in the optic density at 340 nm, due to NADPH oxidation, as result of the enzymatic reduction of the oxidized glutathione (GSSG). The activity of this enzyme was expressed as U/mg. One unit of enzyme oxidizes one μmole of NADPH in a minute in defined conditions (Goldberg and Spooner 1983). The enzymatic activities were reported to protein concentration, and expressed as % of controls.

4.6.5. GSH concentration

GSH was detected in tissue homogenate after deproteinization with 5 % sulfosalicylic acid using the Detect X® Glutathione colorimetric detection kit, according to manufacturer's instructions. The method involved a kinetic analysis in which amounts of GSH caused a continuous reduction of DTNB reagent 5,5'-dithiobis(2-nitrobenzoic acid)] to TNB and further oxidized glutathione formed was restored by glutathione reductase and NADPH. The yellow chromophore formed was quantified at 412 nm using a 10 mM GSH calibration cure. The GSH levels were calculated as nmoles/mg protein.

4.7. Lipid peroxidation assay

Hepatic lipid peroxidation was assayed by a fluorimetric method described by Del Rio et al. (2003) and expressed in terms of malondialdehyde (MDA) content. The liver homogenate (200 μL) was incubated with 700 μL of 0.1 M HCl for 20 min at room temperature. A volume of 900 μL of 0.025 M thiobarbituric acid was added, and the mixture was incubated at 37 °C for 65 min.

Table 4: Experimental groups

Group	Pre-treatment for 7 days	ISS every day for 1 week	IP injection of CCl_4 on the 8th day(1.0 ml/kg)	Euthanized by cervical dislocation on the 9th day
Group 1 control	–	+	–	+
Group 2 CCl_4 control	–	+	+	+
Group 3	SMEDDS p. o. 500 mg/kg	–	+	+
Group 4	SMEDDS p. o. 1000 mg/kg	–	+	+
Group 5	SMEDDS p. o. 500 mg/kg	–	–	+
Group 6	SMEDDS p. o. 1000 mg/kg	–	–	+

ISS = isotonic saline solution, IP = intra peritonial injection.

Further, the samples were subjected to fluorescence analysis (λ_{ex} = 520 nm; λ_{em} = 549 nm) (Spectrofluorimeter FP-6300 JASCO) and the concentration of malondialdehyde was estimated using 1,1,3,3-tetramethoxypropane as standard. The results were expressed as nmoles of MDA/mg protein.

4.8. Protein concentration measurement

The total protein concentration in the liver tissue samples was measured spectrophotometrically at 660 nm according to method of Lowry et al. (1951).

4.9. Histopathology

Liver specimens were fixed in 4% phosphate buffered formalin, embedded in paraffin and cut into 5 μm thick sections. Sections for histopathological examination were stained with hematoxylin & eosin stain using a standard procedure. Frozen sections were cut at 8 μm with the SLEE MNT cryotome, fixed in 10% buffered formaldehyde and stained with Oil Red O kit according to the methods of Bio-Optica staining kits. Mounted slides were examined under a light microscope (Olympus BX43 microscope) and photographed using a digital camera Olympus XC30.

4.10. Statistical analysis

Results were analyzed using GraphPad Prism 5 software. Statistical significance of control samples and the treated one with CCl_4 and different content of SMEDDS were assessed by two-way ANOVA and Bonferroni post-test. Values were expressed as mean ± standard deviation (SD). The values of significance were evaluated with "P values". $P \leq 0.05$ was considered statistically significant, highly significant at $P < 0.01$ and extremely significant at $p < 0.001$ in each group (n = 8)

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References

Abrol S, Trehan A, Katare OP (2005) Comparative study of different silymarin formulations: formulation, characterisation and *in vitro/in vivo* evaluation. Current Drug Deliv. 2: 45–51.

Aebi H (1974) Catalase. In: Bergmeyer HV, editor. Methods in enzymatic analysis. Vol 2, New York: Acadamic press; pp. 674–684.

Afolayan AJ, Els A, Teng RJ, Bakhutashvill I, Kaul S, Davis JM, Konduri GG (2012) Decreases in manganese superoxide dismutase expression and activity contribute to oxidative stress in persistent pulmonary hypertension in the newborns, Am J Physiol Lung Cell Mol Physiol 15: L870–L879.

Arcaria M, Brambilia A, Brandt A, Caponi R, Corsi G, Di Rella M, Solinas F, Wachter W.P (1992) A new inclusion complex of silibinin and beta.cyclodextrins: in vitro dissolution kinetics and *in vivo* absorption in comparison with traditional formulation. Boll Chim Farm 131: 205–209.

Batakov EA (2001) Effect of Silybum marianum oil and legalon on lipid peroxidation and liver antioxidant systems in rats intoxicated with carbon tetrachloride. Eksp Klin Farmakol 64: 53–55.

Becker-Schiebe1 M, Mengs U, Schaefer M, Bulitta M, Hoffmann W (2011) Topical use of a silymarin-based preparation to prevent radiodermatitis. Results of a prospective study in breast cancer patients. Strahlenther Onkol 187: 485–491.

Bergamo P, Luongo D, Maurano F, Mazzarella G, Stefanile R, Rossi M (2006) Conjugated linoleic acid enhances glutathione synthesis and attenuates pathological signs in MRL/MpJ-Fas (Ipr) mice. J Lipid Res 47: 2382–2391.

Beutler E (1984) In: Red cell metabolism (eds). A manual of biochemical methods. New York: Grune and Stratton. Pp. 68–73.

Boveris A, Cadenas E (1975) Mitochondrial production of superoxide anions and its relationship to the antimycin insensitive respiration. FEBS Lett 54: 311–314.

Chen W, Xia H, Wu W (2005) Optimized preparation of silymarin dripping pills by central composite design-response surface method. Chin Trad Herb Drug 36: 679–683.

Choi JS, Koh IU, Jung MH, Song J (2007) Effects of three different conjugated linoleic acid preparations on insulin signalling, fat oxidation and mitochondrial function in rats fed a high-fat diet. Brit J Nutr 98: 264–275.

Constantinides PP (1995) Lipid microemulsions for improving drug dissolution and oral absorption: physical and biopharmaceutical aspects. Pharm Res 12: 1561–1572.

Del Rio D, Pellegrini N, Colombi B, Bianchi M, Serafini M, Torta F, Tegonim SM, Musci M, Brighenti F (2003) Rapid fluorimetric method to detect total plasma malondialdehyde with mild derivatization conditions. Clin Chem 49: 690–692.

Demicheli V, Quijano C, Alvarez B, Radi R (2007) Inactivation and nitration of human superoxide dismutase (SOD) by fluxes of nitric oxide and superoxide. Free Rad Biol Med 42: 1359–1368.

Flora K, Hahn M, Rosen H, Benner K (1998) Milk thistle (Silybum marianum) for the therapy of liver disease. Am J Gastroenterol 93: 139–143.

Fraga C, Leibovitz B, Tappel A (1987) Halogenated compounds as inducers of lipid peroxidation in tissue slices. Free Rad Boil Med 3: 119–123.

Frank J, Lambert C, Biesalski HR, Thews O, Vaupel P, Kelleher DK (2003) Intensified oxidative and nitrosative stress following combined ALA-based photodynamic therapy and local hyperthermia in rat tumors. Int J Cancer 107: 941–948.

Gang Y, Yan L (2011) Preparation, characterization and evaluation of self-microemulsifying drug delivery systems (SMEDDSs) of Ligusticum chuanxiong oil. Biomed Prevent Nutr 1: 36–42.

Gershanik T, Benita S (2000) Self-dispersing lipid formulations for improving oral absorption of lipophilic drugs. Eur J Pharm Biopharm 50: 179–188.

Goldberg DM, Spooner RJ (1983) Glutathione reductase. In: Bergmeyer HU, Bergmeyer J, Graßl M (Eds.) Methods of Enzymatic Analysis, 3rd (eds); Verlag Chemie: Weinheim, Germany; Volume 111, p 258–265.

He J, Hou S, Lu W, Zhu L, Feng J (2007) Preparation, pharmacokinetics and body distribution of silymarin-loaded solid lipid nanoparticles after oral administration. J Biomed Nanotech 3:195–202.

Hermenean A, Stan M, Ardelean A, Nagy L, Deák G, Zsuga M, Kéki S, Bácskay I, Fenyvesi F, Costache M, Dinischiotu A, Vecsernyés M (2014) Antioxidant and hepatoprotective effects of milk thistle (*Silybum marianum* L. Gaertn.) seed oil in a mouse model of acute hepatic injury. Cent Eur J Biol.(Accepted manuscript, doi in progress).

Humberstone AJ, Charman WN (1997) Lipid based vehicles for the oral delivery of poorly water soluble drugs. Adv Drug Deliv Rev 25: 103–128.

Johnson DE, Kroenung C (1998) Mechanism of early carbon tetrachloride toxicity in cultured rat hepatocytes. Pharmacol Toxicol 83: 231–239.

Katiyar SK (2002) Treatment of silymarin, a plant flavonoid, prevents ultraviolet light –induced immune suppression and oxidative stress in mouse skin. Int J Oncol 21: 1213–1222.

Katiyar SK (2005) Silymarin and skin cancer prevention: anti-inflammatory, antioxidant and immunomodulatory effects (review). Int J Oncol 26: 169–176.

Keng T, Privalle CT, Gilkeson GS, Weinberg JB (2000) Peroxynitrite formation and decreased catalase activity in autoimmune MRL-lpr/lpr mice. Mol Med 6: 779–792.

Kuki Á, Nagy L, Deák Gy, Nagy M, Zsuga M, Kéki S (2012) Identification of silymarin constituents: an improved high performance liquid chromatographic–mass spectrometric Method. Chromatographia 75: 175–180.

Lawrence MJ, Rees GD (2000) Microemulsion-based media as novel drug delivery systems. Adv Drug Deliv Rev 45:89–121.

Lipinski CA, Lombardo F, Dominy BW, Feeney PJ (2001) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Deliv Rev 46: 3–26.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin-Phenol reagents. J Biol Chem 193: 265–275.

Luper S (1998) A review of plants used in the treatment of liver disease: part 1. Altern Med Rev 3: 410–421.

McCord JM, Fridovich I (1968) The reduction of cytochrome C by milk xanthine oxidase. J Biol Chem 243: 5733–5760.

Nield GL, Ippersiel R (2002) Open Evaluation of Silymarin Cream in the Management of Facial Redness Associated with Rosacea. Cosmetic Dermatology. Cedar Knolls, Vol. 15. Issue 2, 15–20.

O'Driscoll CM (2002) Lipid-based formulations for intestinal lymphatic delivery. Eur J Pharm Sci15: 405–415.

Paoletti F, Mocali A (1990) Determination of superoxide dismutase activity by purely chemical system based on NADP(H) oxidation. Methods Enzymol 186: 209–221.

Pepping J (1999) Milk thistle: Silybum marianum. Am J Health Syst Pharm 56: 1195–1197.

Pouton CW (1997) Formulation of self-emulsifying drug delivery systems. Adv Drug Deliv Rev 25: 47–58.

Pouton CW (2000) Lipid formulation for oral administration of drugs: non-emulsifying, self-emulsifying and self-microemulsifying drug delivery systems Eur J Pharm Sci 11: S93-S98.

Radi R (2004) Nitric oxide, oxidants and protein tyrosine nitration. Proc Natl Acad Sci USA 101: 4003–4008.

Radi R, Beckman JS, Bush KM, Freeman BA (1991) Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. Arch Biochem Biophys 288: 481–487.

Rasul A, Akhtar N, Khan B.A., Tari Mahmood, Zaman,S., Atif A., Haji M. Khan S., Parveen S (2011) Assessmentof antierythmic and skin whitening effects of milk thistle extract. Afr J Pharm Pharmacol 5: 2306–2309.

Roy P, Sajan MP, Kulkami AP (1995) Lipoxygenase-mediated glutathione oxidation and superoxide generation. J Biochem Toxicol 10: 111–120.

Sha X, Yan G, Wu Y, Li J, Fang X (2005) Effect of self-microemulsifying drug delivery systems containing labrasol on tight junctions in Caco-2 cells. Eur J Pharm Sci 24: 477–486.

Shaker E, Mahmoud H, Mnaa S (2010) Silymarin, the antioxidant component and Silybum marianum extracts prevent liver damage. Food Chem Toxicol 48: 803–806.

Sorenson BS, Banton KB, Augustin BL, Leonard AS, Saltzman AD (2011) Antioxidant oils and Salmonella enterica Typhimurium reduce tumor in an experimental model of hepatic metastasis. Onco Targets and Therapy 4: 59–69.

Tanaka N, Tanaka K, Nagashima Y, Kondo M, Sekihara H (1999) Nitric oxide increases hepatic arterial blood flow in rats with carbon tetrachloride-induced acute hepatic injury. Gastroenterology 117: 173–180.

Ujhelyi Z, Fenyvesi F, Váradi J, Fehér P. Kiss T, Veszelka Sz, Deli M, Vecsernyés M, Bácskay I (2012) Evaluation of cytotoxicity of surfactants used in self-micro emulsifying drug delivery systems and their effects on paracellular transport in Caco-2 cell monolayer. Eur J Pharm Sci 47: 564–573.

Ujhelyi Z, Róka E, Fenyvesi F, Fehér P, Váradi J, Réti-Nagy K, Vecsernyés M, Bácskay I (2013) Assessment of the hemolytic activity and cytotoxicity of different PEG-based solubilizing agents. Pharmazie 68: 383–384.

Wang Y, Zhang Z, Liu Z, Liu G, Duan C, Jia L, Feng F, Zhang X, Shi Y, Zhang Q, (2010) *In vitro* and *in vivo* evaluation of silybin nanosuspensions for oral and intravenous delivery. Nanotechnology 21: 155104.

Weber, L.W., Boll M., Stampfl A., (2003). Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. Critical Rev Toxicol 33: 105–136.

Wei W, Yang W, Li Q (2006) Enhanced bioavailability of silymarin by self-microemulsifying drug delivery system. Eur J Pharm Biopharm 63: 288–294.

Woo JS, Kim TS, Park JH, Chi SC (2007) Formulation and biopharmaceutical evaluation of silymarin using SMEDDS. Arch Pharm Res 30: 82–89.

Yanyu X, Yunmei S, Zhipeng C, Qineng P (2006) The preparation of silybin-phospholipid complex and study on its pharmacokinetics in rats. Int J Pharm 307: 77–82.

Zidan AS, Sammour OA, Hammad MA, Megrab NA, Habib MJ, Khan MA (2007) Quality design: understanding the product variability of a self-nanoemulsified drug delivery system of cyclosporine A. J Pharm Sci 96: 9.