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Original Article

COMPARATIVE ANALYSIS OF BIOLOGICAL ACTIVITY OF *SILYBUM MARIANUM* L. FOOD SUPPLEMENTS AVAILABLE ON MARKET: *INVITRO* STUDY

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ABSTRACT

Objective: *Silybum marianum* L. Food Supplements that contain silymarin is widely used as a therapeutic agent in liver diseases. Many brands are available on the market in USA, Egypt, Europe and other countries. The objective of this study was to compare the biological activity in different preparations of silymarin available on the market in USA and Egypt using paracetamol-induced oxidative stress injury on primary cultured rat hepatocytes.

Methods: Forty four silymarin samples available on the market were collected from USA (24) and Egypt (20) and tested for hepat protective antioxidant effects on primary cultured rat hepatocytes. Cytotoxicity was measured by MTT [3-(4, 5-dimethyl-thiazol-2)-2,5-diphenyl tetrazolium bromide] assay and lactate dehydrogenase (LD) leakage into culture medium. Antioxidant effects were determined by glutathione reductase (GR), and Nitric oxide (NO) assays in silymarin, pretreated rat hepatocytes for 2 h followed by incubation with 25 mM paracetamol over a period of 1 h. Therapeutic index was calculated for each tested sample for comparative analysis.

Results: Silymarin preparations significantly decreased toxicity induced by paracetamol in rat hepatocytes, decreased lactate dehydrogenase leakage and prevented GSH depletion ($P<0.01$) and returned NO to basal levels in rat hepatocytes. The therapeutic index was 80, 40 and 20 for samples No. 20, 19 and 5 respectively.

Conclusions: The 44 different silymarin preparations tested in this study exhibited variation in antioxidant capacity and in reducing nitric oxide produced as a result of paracetamol injury. This variation in biological activity did not always correspond to the amount of silymarin recorded on samples.

Keywords: Silymarin, Primary Cultured Rat Hepatocytes, Antioxidant Effect, Glutathione Reductase, Nitric Oxide.

INTRODUCTION

Silybum marianum L. (SM), commonly called milk thistle, is an annual or biennial plant that is native to the Mediterranean and North Africa but has spread to other warm and dry climates in North America, Australia, and Europe. The seed extract of SM is expressed as total silymarin, the active principle, which is approximately 60 % of dried extracts of milk thistle seeds [1, 2]. Traditional medicines, including medicinal herbs and their preparations, are used as part of the primary health care for 70-95 % of the population in the developing world, while over 70 % of the population in developed nations use some form of complementary/alternative medicines [3]. The last few decades have witnessed therapeutic benefits of milk thistle and silymarin in liver necro inflammation and fibrosis given its free-radical scavenging, antioxidant, anti-inflammatory, immunomodulatory, iron chelating, and membrane stabilizing properties as well as its ability to selectively stimulate hepatocyte proliferation [4]. Moreover, being a natural remedy for liver diseases and its entry into NIH clinical trial signifies its hepatoprotective potential [5].

Silymarin is noted for its ability to interfere with apoptotic signaling while acting as an antioxidant [6]. The German Commission E recommends silymarin for the treatment of dyspeptic complaints, toxin-induced liver damage, and hepatic cirrhosis and as a supportive therapy for chronic inflammatory liver conditions [7]. Recently, a systematic review has reported the efficacy and safety of milk thistle products in alcohol, viral or toxin-related liver diseases [8].

Many sole and combined products of silymarin with well-defined preparations are sold under more than 50 different brand names and available market. Silymarin was among the 5 top selling herbal supplements of 2011 in the health and natural foods channel, according to SPINS. Sales of milk thistle in the USA were $108 million in 2011, up 10.2 % from the prior year according to the latest fig. from Nutrition Business Journal [9].

Silymarin is a potent antioxidant composed of several flavolignans. Antioxidants or free radical scavengers are very important in protecting the living cells against any damage, induced by free radicals which are produced continuously in cells, either during phagocytosis or accidentally as by-product metabolites. Each biological system has certain antioxidant mechanisms against the aggregations of such free radicals. The balance of oxidant-antioxidant system must exist in the cell while the disturbance of antioxidant-prooxidant balance causes oxidative stress [10].

Antioxidants are an important endogenous defense mechanism against injury caused by lipid peroxidation and harmful reactions, induced by reactive oxygen species (ROS), which are constantly produced in the body during normal metabolic processes [11]. Antioxidants may act individually or in synergy. Many antioxidant enzymes are sequestered in peroxisomes. Repair mechanisms are also available in the cells as potent mechanisms for removal of oxidized membrane fatty acids [12].

In vitro liver systems represent a convenient system to study screen and compare potential hepatotoxic compounds and to investigate the mechanism by which chemicals induce liver lesions [11]. The best and most successfully applied *in vitro* models are primary cultured mouse/rat.

Hepatocytes, which generally reproduce the *in vivo* pathophysiology [13, 14]. In contrast to intact rats, dose-dependent glutathione depletion, oxidant stress and cytotoxicity (necrosis) have also been shown in cultured rat hepatocytes [15]. Paracetamol (Acetaminophen) has been used extensively during the last 40 y as a model toxicant, which allows investigation of drug induced cell death *in vivo* and *in vitro*. Many basic concepts of drug toxicity were developed using this model. As a consequence, paracetamol toxicity is one of the most popular models to test potentially hepatoprotective agent's especially natural products [16].

Paracetamol is mainly metabolized in liver to excretable glucuronide and sulfate conjugates. However, the hepatotoxicity of paracetamol has been attributed to the formation of toxic metabolites when a part of paracetamol is activated by hepatic cytochrome P-4 50 to a highly reactive metabolite N-acetyl-p-benzoquinoneimine (NAPQI) [17]. During the detoxification process, glutathione is, directly or indirectly, consumed with subsequent hepatic necrosis. Nitric oxide (NO) is very specific to liver injury and is almost always produced during liver inflammation. Downregulation, NO production is considered to be an indicator of liver protection [18]. Up to the time of writing this study, no published reports were available regarding the comparative analysis of *silybum marianum* food supplements present on market. Therefore, we aimed to compare equal weights of SM food supplements of commercially available brands sold in USA and Egypt for cytotoxicity, hepatoprotection, antioxidant activity and therapeutic index on primary cultured rat hepatocytes.

MATERIALS AND METHODS

Chemicals and reagents

All solvents used for extraction were of chromatographic grade, dimethyl sulfoxide (DMSO) was purchased from Merck. Technical silymarin (>96 % pure) and quercetin were purchased from Sigma Aldrich Inc., and kits were purchased from Biodiagnostic Co., Egypt. All commercial over-the-counter milk thistle food supplements samples were obtained from USA and Egyptian markets. Samples identification numbers, sources and brand names are shown in (table 1).

Preparation of samples

Ten tablets of each commercial sample were randomly taken, crushed and homogenized. Tablets in the form of coated capsules were freed from the capsules cover and their content was homogenized as above. Weight of each 10 tablets was recorded and is presented for each brand sample in (table 1). Twenty mg of each product were extracted in 5 ml of methanol.

Isolation and preparation of rat hepatocytes monolayer culture

A primary culture of rat hepatocytes was prepared according to Seglen, 1976 method [19] and modified by Kiso *et al.*, 1983 [20] using Wistar male rats (250-300 g) obtained from the animal house of the NRC (National Research Center, Cairo). Animal procedures were performed in accordance with the Ethics Committee of the National Research Centre and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Guide, 1985) [21].

LD50 determination on rat hepatocytes monolayer culture

In order to determine LC50, different concentrations were prepared for each sample (100–1000 µg/ml). After two hours incubation of cells with the extract, cell viability was determined using the MTT assay. The assay was performed according to the method of Mosmann, 1983 [22] modified by Carmichael *et al.*, [23]. Absorbance of formasan crystals produced by viable cells was read at 540 and 630 nm dual wave-length using the Automatic Kinetic Microplate Reader (Lab Systems Multiskan RC Reader). Each experiment was repeated three times and the mean absorption of each concentration was calculated.

Lactate dehydrogenate (LDH) leakage

After cell treatment mentioned above, LDH leakage from cell cytosol to the culture medium was measured in culture medium to reflect cell viability. The LDH leakage was measured by an optimized standard method according to the method of King [24].

Evaluation of hepatoprotective activity

The primary culture of rat hepatocytes monolayer was treated with different concentrations (12.5, 25, 50, 100 µg/ml) using serial dilutions technique by dissolving in DMSO (1 % maximum concentration). For each concentration, three replicates were carried out; in addition to a positive control that was 50 µg/ml Silymarin (madawas). The plate was incubated for 2 h at 37 °C and 5 % CO2, then washed twice with PBS. Two hundreds µl of 25 mM paracetamol was added to each well. After one hour incubation with the paracetamol, cell viability was determined using the MTT assay. The concentration of the extract that was able to protect the cells from the hepatotoxic effect of paracetamol by 100 % was considered hepatoprotective. In animal studies, the therapeutic index is the lethal dose of a drug for 50 % of the population (LD50) divided by the minimum effective dose for the population (ED).

Glutathione reductase (GR) assay

The content of reduced glutathione was assayed by the spectrophotometric method of Goldberg and Spooner [25]. The assay was performed by using 5, 5 -dithiobis-2-nitrobenzoic acid (DTNB), which reacts with oxidized glutathione reductase (GSH) to form a product (GSSG) with a maximum absorbance at 412 nm. The reduction in GSSG level was determined by reducing GSSG into GSH by adding glutathione reductase and nicotinamide adenine dinucleotide phosphate (NADPH). The resulting GSH was reacted with DTNB. A standard curve of GSH in the culture medium was constructed with concentrations of GSH ranging from 0–100 µM. Glutathione reductase activity was expressed as units of enzyme per 10^6 cells.

Determination of nitric oxide (NO) production

After cell treatment mentioned above, for evaluation of hepatoprotective activity, culture medium was used for measurements of nitrite (a stable metabolite of NO) to reflect NO production by the Griess method [26]. Decrease levels of NO production are indicative of hepatoprotection. Quercetin was used as a reference control.

Statistical analysis

Experiments were repeated at least three times using different hepatocyte preparations. The results are expressed as mean±SD. After testing of normality, the statistical significance was analyzed using one way ANOVA followed by Pearson and Spearman nonparametric correlation analysis with two-tailed significance determined. Statistical significance was considered when value of p was<0.05. Statistical analysis was performed using SPSS, version 13 (SPSS Inc, Chicago, IL, USA).

This study was approved by IRB, National Research Centre, Egypt.

RESULTS AND DISCUSSION

Hepatoprotective effect against paracetamol

Different preparations of SM food supplements investigated in this study revealed little toxic effect on primary culture of rat hepatocytes as assessed by MTT assay. Cell viability was maintained at above 98 % in all preparations up to 25 µg/ml. When the cells were exposed to silymarin preparations at concentration of 50 µg/ml, 14 samples out of 44 revealed reduced cell viability to 67.3 %±2.8. Whereas, the remaining 30 samples started to induce toxic effect at a concentration of 100 µg/ml as indicated by reduced cell viability to 59.7 %±5.7. Cytotoxicity results obtained by MTT assay coincided with results obtained by LDH leakage into the culture medium of rat hepatocytes. Experiments of hepatoprotection effect against paracetamol revealed dose dependent protection. Thirty three samples exerted hepatoprotection against paracetamol at low concentration of 12.5 µg/ml, while 7 samples exhibited hepatoprotection at concentration of 25 µg/ml. The remaining 4 samples showed hepatoprotection at concentration of 50 µg/ml. No toxic effects were noticed either by morphological changes of cells or by LDH leakage into the culture medium at the concentrations that showed hepatoprotection. Calculations of the therapeutic index of the different samples investigated are shown in (table 2). Samples labeled with numbers: 5, 11, 15, 17, 25, 28, 29, 30, 31, 32, 33, 35, 36, 37, 38, 39, 40, 41, 43 and 44 showed the highest therapeutic index of 80 that was similar to that of quercetin. Four of which from USA market and 16 samples were commercially sold in the Egyptian market. Samples labeled with numbers: 4, 6, 12, 14, 16 showed the least therapeutic index of 20. While the remaining samples showed therapeutic index in the middle of value of 40 (samples: 1, 2, 3, 7, 8, 9, 10, 13, 18, 19,20, 21, 22, 23, 24, 26, 27, 34 and 42). Fig. 1 represents results of MTT assay in four samples (5, 11, 15, and 17) and quercetin as a reference control.

Table 1: Commercial milk thistle samples: (Silymarin)

S. No.	Sample ID #/Description/Sources & Average weight of each tablet	S. No.	Sample ID #/Description/Sources & Average weight of each tablet
1	Swanson Superior Herbs® Milk Thistle 80 % Silymarin/USA (0.5504 g/tablet)	25	SEDICO® Silymarin Plus, Dietary Supplement/Egypt
2	Ortho Molecular Products® Silymarin Forte/USA (0.4502 g/tablet)	26	Hepamarin® 140 mg Hepatoprotective/Egypt
3	Metabolic Response Modifiers® Silymarin with Bio Sorb/USA (0.4975 g/tablet)	27	Hepacure/Mepaco/Egypt
4	Advance Physician Formulas®((0.3319 g/tablet) Milk Thistle (Standardize Silymarin 80 %)/USA	28	Hepaticum® Cyclodextrin enhanced formula/Egypt Liver support, Soft gelatin capsules/Egypt
5	Pure Encapsulations®(0.2430 g/tablet) Silymarin Milk Thistle Extract/USA	29	Ursoplus®MINAPHARM Silymarin 70 %/Egypt
6	Thorne Research T. A. P. S® Dietary Supplement/USA (0.6789 g/tablet)	30	SELECTIVAL® Dietary Supplement/Egypt
7	Metagenics® Silymarin 80/USA (0.2450 g/tablet)	31	Silipex® Dietary Supplement/Egypt
8	Himalaya Liver Care® Liv.52/USA (0.3768 g/tablet)	32	MARIAGON® Hepatoprotective/Egypt
9	Jarrow Formulas®/USA (0.2786 g/tablet) Milk Thistle (Standardize Silymarin Extract 30:1)	33	Liverin® Improvement of liver function/Egypt
10	Metabolic Maintenance® Silymarin/USA (0.5696g/tablet)	34	**Leaglon® 70** Silymarin/Germany
11	Life Extension®(0.9040 g/tablet) Mega Silymarin with isosilybin B/USA	35	**Leaglon® 140** Silymarin/Germany
12	Purintin's Pride® Silymarin Milk Thistle/USA (0.3466 g/tablet)	36	Levatech® Silymarin/Egypt
13	Natural Wellness®/USA (0.5063 g/tablet) Maximum Milk Thistle, SilybinPhytosome 240 mg	37	MEPASIL® Silymarin, Liver support/Egypt
14	Enzymatic Therapy® Super Milk Thistle/USA (0.3262 g/tablet)		
15	Advanced Beta Glucon Therapy®(0.4867 g/tablet) Bio-Silymarin, Aloha Medicinal Inc./USA	38	Livit® Liver support, Soft gelatin capsules/Egypt
16	Futurebiotics® Silymarin Plus/USA (1.0514 g/tablet)	39	Hepanox® Cap. Napha food support/Egypt
17	Planetary Herbals® (0.7060 g/tablet) Full Spectrum Silymarin 80™/USA	40	Hepato-Forte® Liver Supplement/Egypt
18	Wonder Laboratories® Advanced B-12 Sublingual/USA (0.3443 g/tablet)	41	Levatone® Food Supplement/Egypt
19	21st Century®(0.4508 g/tablet) 200 count Milk Thistle Extract/USA	42	Hepatic Forte® Silymarin/Egypt
20	Source Naturals® Silymarin Plus/USA (0.9847 g/tablet)	43	Hipamax Plus® Dietary Supplement/Egypt
21	Now® Silymarin 100 V caps/USA (0.5090 g/tablet)	44	Liver Albumin Plus® Dietary Supplement/Egypt
22	Good'N Natural® Milk Thistle Extract 250 mg/USA (0.6765 g/tablet)	45	Quercetin/Sigma
23	TwinLab® Silymarin/USA (0.1610 g/tablet)		
24	Nature's Bounty®/Natural Whole Herbs Milk Thistle1000 mg/USA (0.3939 g/tablet)		

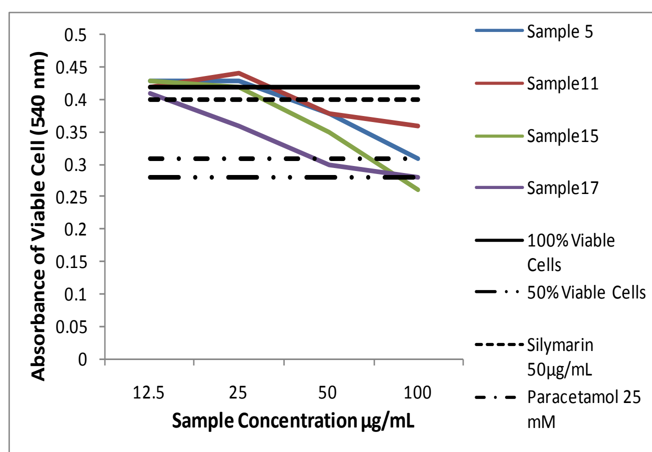

Fig. 1: Viable cell absorbance showing the hepatoprotective effect of some samples against the hepatotoxic effect of paracetamol (25 mM) using MTT assay. Data represents mean of 3 replicates

Table 2: Effect of Silymarin Preparations on LDH Leakage, LD50, Initial Effective Dose (ED), Therapeutic index (TI), Glutathione Reductase and Nitric Oxide Levels in Culture Medium of Rat Hepatocytes

Sample	%LDH leakage in culture medium after 2h Concentrations in µg/ml				IC_{50} (µg/ml) By MTT assay	ED (µg/ml)	TI	Glutathione Reductase/ 10^6 cells (U/l)	Nitric Oxide (µmol nitrite/10^6 cells)
	125	250	500	1000					
1	19	22	51	78	500	12.5	40	5157.72	11.17±2.2
2	21	24	61	85	500	12.5	40	5583.06	11.17±2.7
3	22	26	49	89	500	12.5	40	5062.60	12.83±3.1
4	18	25	55	97	500	25	20	5721.72	7.47±2.4
*5	17	22	31	60	1000	12.5	80	5917.33	3.08±1.7
6	20	26	29	74	1000	50	20	5147.00	7.67±1.1
7	19	22	47	94	500	12.5	40	5169.10	6.58±1.6
8	18	21	29	83	1000	25	40	5195.54	6.42±3.8
9	17	22	31	78	1000	25	40	5113.52	9.42±0.92
10	18	24	55	102	500	12.5	40	5240.12	7.92±1.6
*11	18	20	27	69	1000	12.5	80	5186.52	7.25±1.8
12	21	22	24	83	1000	50	20	4915.93	6.58±2.6
13	19	21	25	79	1000	25	40	4848.92	7.92±2.5
14	19	22	27	89	1000	50	20	4864.33	4.25±3.1
*15	17	22	28	74	1000	12.5	80	4942.70	5.75±1.4
16	21	24	24	84	1000	50	20	5139.63	7.50±3.3
*17	16	22	26	76	1000	12.5	80	4899.83	9.58±1.8
18	17	22	23	73	1000	25	40	4976.86	6.67±2.4
19	19	21	53	98	500	12.5	40	5047.86	9.50±0.86
20	17	21	49	89	500	12.5	40	5222.69	5.42±0.67
21	17	22	51	105	500	12.5	40	5004.99	6.75±0.47
22	19	23	47	89	500	12.5	40	5011.02	6.0±0.87
23	21	23	47	91	500	12.5	40	5013.03	7.0±0.91
24	21	24	54	97	500	12.5	40	5173.12	4.50±0.68
25	22	24	26	69	1000	12.5	80	4734.38	12.37±1.9
26	19	21	58	94	500	12.5	40	5927.30	6.47±2.3
27	18	20	25	58	1000	25	40	5278.20	6.96±2.1
28	19	21	26	60	1000	12.5	80	6230.70	6.59±2.3
29	18	22	27	59	1000	12.5	80	4773.91	10.93±1.7
30	17	19	22	58	1000	12.5	80	5203.92	118.33±1.1
31	19	21	23	54	1000	12.5	80	4681.42	6.19±1.6
32	19	22	24	55	1000	12.5	80	5390.86	6.42±2.8
33	21	25	26	53	1000	12.5	80	5071.91	11.22±2.33
34	19	23	29	54	1000	25	40	4836.83	7.93±1.8
35	19	23	27	48	1000	12.5	80	5141.67	3.07±1.22
36	22	24	25	55	1000	12.5	80	5060.56	7.07±2.5
37	21	25	27	57	1000	12.5	80	5016.33	3.26±3.1
38	21	22	23	59	1000	12.5	80	5035.11	6.22±1.4
39	19	22	24	62	1000	12.5	80	4896.44	7.96±3.3
40	18	20	24	48	1000	12.5	80	4891.12	51.10±1.8
41	17	21	24	49	1000	12.5	80	4834.55	6.96±2.4
42	19	22	47	89	500	12.5	40	4623.82	5.30±0.86
43	20	23	26	61	1000	12.5	80	4673.44	4.48±0.67
44	21	23	25	53	1000	12.5	80	4723.67	5.67±0.47
*Quercitin	19	22	24	51	1000	12.5	80	5428.33	5.11±1.7

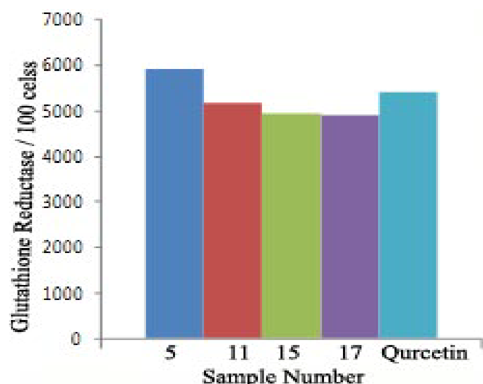

Fig. 2: Activity of glutathione reductase was measured as decreased of NADPH absorbance at 340 nm and expressed as Unit enzyme activity per 100 cells. The results are expressed as mean, n=3

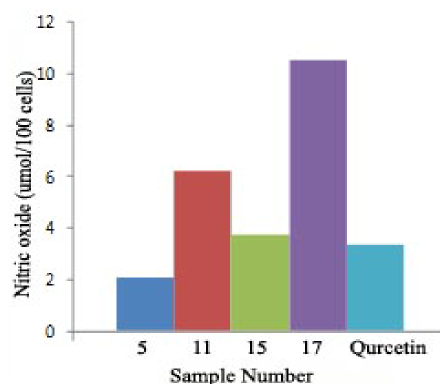

Fig. 3: Bar chart showing the difference in nitrite activity using nitric oxide kit assay of some tested samples. Data represent mean of 3 replicates/point

Effect of silymarin preparations on glutathione reductase (GR) activity

Data from this study revealed that treatment of hepatocytes with paracetamol significantly decreased GR content in the cells compared to normal control untreated cells (P<0.01). The decrease of GR in the cells resulted from depletion of GR during oxidative injury of hepatocytes.

Pretreatment of hepatocytes with different concentrations of silymarin preparations for two hoursprior to the addition of paracetamol to the cells provided significant protective effect against this depletion. Tested silymarin preparations modulated the liver against oxidative stress in a dose dependant manner without any cellular cytotoxic changes at treated doses. GR content in the cells of different silymarin samples were elevated corresponding to the addition of various concentrations of the silymarin preparations. Only few samples showed significant difference with the reference control, quercetin as shown in (table 2). Fig. 2 represents results of GSH assay in four samples (5, 11, 15, and 17) and quercetin as a reference control.

Effect of silymarin preparations on the inhibition of nitric oxide (NO) production

Pretreatment of hepatocytes with different concentrations of silymarin samples for two hours prior to the addition of paracetamol to the cells reduced the levels of nitrite, an NO metabolite, inculture medium to mean basal levels. Results revealed dose dependent decrease in the levels of NO in culture medium of hepatocytes. Silymarin preparations showed no cellular cytotoxicity within the indicated concentrations, as evaluated by the release of LDH into the culture medium and trypan blue exclusion by hepatocytes. Tested silymarin samples with numbers: 5, 14, 24, 33, 36 and 42 revealed specific injury protection of hepatocytes, as shown by the inhibition of NO production. Those silymarin samples exhibited the same effect or even better protection exertedby quercetin as shown in (table 2). Fig. 3 represents results of NO assay in four samples (5, 11, 15, 17) and quercetin as a reference control.

DISCUSSION

The fruit of the milk thistle plant (*Silybum marianum* L., Family Asteraceae) contains flavonoids that are proven liver protectants and used in the treatment of liver diseases [27-29]. The standardized extract known as silymarin contains three flavonoids of the flavonol subclass. Silybin predominates, followed by silydianin and silychristin [30]. These constituents likely offer the synergistic benefit of sparing liver cells from destruction [31]. Silybin protects the liver by conserving glutathione, the key antioxidant in the liver, in the cell [32]. Although silybin is the most potent of the flavonoids in milk thistle, similar to other flavonoids it is not well absorbed [33, 34]. Several pharmaceutical manufacturers have developed milk thistle products standardized to a higher content of silymarin (70 %-80 %) than in non-standardized herbal preparations [35], mainly because of silymarin's poor oral bioavailability [36]. As part of pharmaceutical development, several clinical studies have been conducted in Europe to assess the efficacy of oral milk thistle products in liver diseases of diverse etiology [37]. However, to date no comparative analysis of various silymarin brands has been reported regarding biological activity or therapeutic index.

Results from this study revealed effective protection of all tested silymarin tablets of hepatocytes from injury-induced paracetamol. Although equal weight from each tablet was used for extraction, variation was observed among different tablets regarding cytotoxicity, antioxidant activity and therapeutic index. The therapeutic index (TI) calculated for all tested silymarin preparations are far from just being safe as reported for safe drugs to have TI between 5 and 10 [38]. The variation observed in therapeutic index in different samples may be attributed to various concentration of silymarin, variable flavonoids proportions or synergistic effect of other food supplements in the same preparation. A therapeutic index does not consider drug interactions or synergistic effects [39]. Total amounts of silymarin tablets available in USA market vary greatly among the different samples with, 1 sample containing between 100 and 200 mg/g, 3 samples containing between 200 and 300 mg/g, 6 samples containing between 300 and 400 mg/g, 4 samples containing between 400 and 500 mg/g, 4 samples containing between 500 and 600 mg/g and 6 samples containing higher than 600 mg/g as shown in (table 1**).** The amount of silymarin registered on tablets did not always correspond with higher biological activity. For example, sample number 5 (from USA market), demonstrated the least cytotoxicity, the highest biological activity, highest therapeutic index and contained silymarin of only 0.243g/tablet. Samples number 6 and 16 although had large amount of silymarin 0.6789 and 1.0514 g/tablet respectively, revealed the least therapeutic index recorded in this study of 20. These findings raise the assumption of different flavonol subclass proportions with pre dominating the least bioactive isomer. Also the amount of silymarin preparations commercially available in Egyptian market varies among different samples. Sixteen samples out of 20 showed therapeutic index of 80, only 4 samples out of 20 showed therapeutic index of 40. Interestingly, sample number 34 from (Legalon 70/Germany) demonstrated half the therapeutic index of sample number 35 (Legalon 140/Germany) that attributes to concentration of silymarin in the preparation. Regarding glutathione reductase activity was noticed in all silymarin treated primary rat hepatocytes. Three samples showed better results than quercitin. Silymarin samples number 2 (0.4502 g/tablet) and 26 (0.140 g/tablet) although had therapeutic index of 40, lower than other samples, they achieved GR activity better than quercitin. Moreover, these samples had fewer amount of silymarin than other samples and achieved higher GR activity. This raises the assumption of the presence of other flavonoids in the same preparation that synergistically improved the antioxidant capacity of those samples. Sample number 28 had therapeutic index of 80 and showed GR activity better than quercitin. The inhibition of NO production was detected in all preparations. However, some samples revealed more inhibition of NO than others such as sample number 5 which contains silymarin of 0.243 g/tablet, although samples with more silymarin concentration showed less inhibition of NO production. This explains that the variation in activity does not always depends on the amount of silymarin in preparations.

CONCLUSION

All silymarin preparations investigated in this study revealed effective hepatoprotective effect on paracetamol induced liver injury through antioxidant mechanism. The variation in LD50, hepatoprotective effect, antioxidant activity and therapeutic index observed among different samples raise the recommendation to standardized isomer quantification and biological activity assays to be implemented for evaluation of commercial sources of silymarin.

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ABBREVIATION

SM, *Silybum marianum*; LD, lactate dehydrogenase; GR, glutathione reductase; NO, nitric oxide; ROS, reactive oxygen species; NAPQI, N-acetyl-p-benzoquinoneimine; LD50, lethal dose of a drug for 50 % of the population; ED, minimal effective dose; TI, therapeutic index; MTT, 3-(4,5-dimethyl-thiazol-2)-2,5-diphenyl tetrazolium bromide

CONFLICT OF INTERESTS

Declared None

REFERENCES

1. Kvasnicka F, Biba B, Sevcik R. Analysis of the active components of silymarin. J Chromatogr A 2003;990:239-45.
2. Corchete P. *Silybum marianum* (L.) Gaertn. The Source of Silymarin. In: Bioactive Molecules and Medicinal Plants. Ramawat KG, Merillon JM. (eds.); 2008. p. 123-48.
3. Radimer K, Bindewald B, Hughes J, Ervin B, Swanson C, Picciano MF. Dietary Supplement Use by US Adults: Data from

the National Health and Nutrition Examination Survey, 1999–2000. Am J Epidemiol 2004;160:339-49.
4. Saller R, Meier R, Brignoli R. The use of silymarin in the treatment of liver diseases. Drugs 2001;61:2035-63.
5. Fried M, Navarro V, Afdhal N, Belle S, Wahed A, Hawke R, *et al.* Effect of silymarin (Milk Thistle) on liver disease in patients with chronic hepatitis C unsuccessfully treated with interferon therapy. JAMA 2012;308:274-82.
6. Patel N, Joseph C, Corcoran GB, Ray SD. Silymarin modulates doxorubicin-induced oxidative stress, Bcl-xL and p53 expression while preventing apoptotic and necrotic cell death in the liver. Toxicol Appl Pharmacol 2010;245:143-52.
7. Blumenthal M. The complete german commission E monographs: therapeutic guide to herbal medicines. Austin: American Botanical Council; 1998.
8. Mulrow C, Lawrence V, Jacobs B. Milk thistle: effects on liver disease and cirrhosis and clinical adverse effects. Evidence Report/Technology Assessment No. 21 (Contract 290-97-0012 to the San Antonio Evidence-based Practice Center, based at the University of Texas Health Science Center at San Antonio, and The Veterans Evidence-based Research, Dissemination, and Implementation Center, a Veterans Affairs Services Research and Development Center of Excellence). AHRQ Publication No. 01-E025. Rockville, Maryland: Agency for Healthcare Research and Quality; 2000.
9. Robinson M, Zhang X. The world medicines situation 2011. Traditional medicines: Global situation, issues and challenges. *WHO*, Geneva; 2011.
10. Yamamoto Y, Yamashita S. Plasma ratio of ubiquinol and ubiquinone as a marker of oxidative stress. Mol Aspects Med 1997;18:79-84.
11. Krinsky NI. Mechanism of action of biological antioxidants. Proc Soc Exp Biol Med 1992;200:248-54.
12. Baynes J, Dominiczak MH. Antioxidant defenses and reactive oxygen species. In: Medical Biochemistry, Ch. 11, Mosby. 2nd ed.; 1999. p. 133-7.
13. Burke AS, MacMillan-Crow LA, Hinson JA. Reactive nitrogen species in acetaminophen-induced mitochondrial damage and toxicity in mouse hepatocytes. Chem Res Toxicol 2010;23:1286–92.
14. Ni HM, Bockus A, Boggess N, Jaeschke H, Ding WX. Activation of autophagy protects against acetaminophen-induced hepatotoxicity. Hepatology 2012;55:222–32.
15. Yang X, Salminen WF. Kava extract, an herbal alternative for anxiety relief, potentiates acetaminophen-induced cytotoxicity in rat hepatic cells. Phytomedicine 2011;18:592–600.
16. Jaeschke H, Williams CD, McGill MR, Xie Y, Ramachandran A. Models of drug induced liver injury for evaluation of phytotherapeutics and other natural products. Food Chem Toxicol 2013;55:279–89.
17. Jaeschke H, McGill MR, C Williams D, Ramachandran A. Current issues with acetaminophen hepatotoxicity–a clinically relevant model to test the efficacy of natural products. Life Sci 2011;88:737–45.
18. Matsuura T, Kaibori M, Araki Y, Matsumiya M, Yamamoto Y, Ikeya Y, *et al.* Japanese herbal medicine, inchinkoto, inhibits inducible nitric oxide synthase induction in interlukin-1β-stimulated hepatocytes. Hepatol Res 2012;42:76-90.
19. Seglen PO. Preparation of isolated rat liver cells. Methods Cell Biol 1976;13:29-83.
20. Kiso Y, Tohkin M, Hikino H. Assay method forantihepatotoxic activity using galactosamine-induced cytotoxicity in primary-cultured hepatocytes. J Nat Prod 1983;46:841-7.
21. NIH Guide for the Care and Use of Laboratory Animals. NIH *Publication*: US Department of Health, Education and Welfare. Research Triangle Park, North Carolina; 1985.
22. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assay. J Immunol Methods 1983;65:263-71.
23. Carmichael J, DeGraff W, Gazdar A, Minna J, Mitchell J. Evaluation of tetrazolium based semiautomated colourimetric assay: assessment of chemosensetivity testing. Cancer Res 1987;47:936-42.
24. King J. The dehydrogenase of oxidoreductase-lactate dehydrogenase. In: Practical Clinical Enzymology. JC King. Van Nostrand London, UK; 1965. p. 83-93.
25. Goldberg DM, Spooner RJ. Glutathione reductase In: Bergmeyer HU, Bergmeyer J. GraBl M. (ed). Methods of enzymatic analysis. 3rd ed. Vol. 111. Verlag Chemie, Weinheim; 1983. p. 258-65.
26. Montgomery HAC, Dymock JF. The determination of nitrite in water. Analyst 1961;86:414–6.
27. Angulo P, Pate T, Jorgensen RA, Therneau TM, Lindor KD. Silymarin in the treatment of patients with primary biliary cirrhosis with a suboptimal response to ursodeoxycholicacid. Heaptol 2000;32:897-900.
28. Jacobs B, Dennehy C, Ramirez G, Sapp J, Lawrence VA. Milk thistle for the treatment of liver diseases. A systemic review and meta-analysis. Am J Med 2002;113:506-15.
29. Stickel F, Schuppan D. Herbal medicine in the treatment of liver diseases. Dig Liver Dis 2007;39:293-304.
30. Hikino H, Kiso Y, Wagner H, Fiebig M. Antihepatotoxic actions of flavonolignans from *Silybum marianum* fruits. Planta Med 1984;50:248-50.
31. Kidd P, Head C. A Review of the bioavailability and clinical efficacy of milk thistle phytosome: a silybin-phosphatidylcholine complex (Siliphos). Alternative Med Rev 2005;10:193-203.
32. Valenzuela A, Aspillaga M, Vial S, Guerra R. Selectivity of silymarin on the increase of the glutathione content in different tissues of the rat. Planta Med 1989;55:420-2.
33. Savio D, Harrasser PC, Basso G. Softgel capsule technology as an enhancer device for the absorption of natural principles in humans. A bioavailability cross-over randomized study on silybin. Arzneimittelforschung 1998;48:1104-6.
34. Manach C, Scalbert A, Morand C, Remesy C, Jimenez L. Polyphenols: food sources and bioavailability. Am J Clin Nutr 2004;79:727-47.
35. Flora K, Hahn M, Rosen H. Milk thistle (*Silybum marianum*) for the therapy of liver disease. Am J Gastroenterol 1998;93:139-43.
36. Koch HP, Ritschel WA. Bioavailability of silymarin, I: Volumes of distribution of silybin, silydianin, and silychristin from *in-vitro* data (author's transl). Arch Pharm 1981;314:515-7.
37. Feher J, Deak G, Muzes G. Liver-protective action of silymarin therapy in chronic alcoholic liver diseases. Orv Hetil 1989;130:2723-7.
38. Becker DE. Drug therapy in dental practice: general principles part 2-pharmacodynamic considerations. Anesthesia Progress 2007;54:19–24.
39. Muller PY, Milton MN. The determination and interpretation of the therapeutic index in drug development. Nat Rev Drug Discovery 2012;11:751-61.