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

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Received: 14 Feb 2015 Revised and Accepted: 10 Sep 2015

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 investigations 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 (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 10⁶ 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).

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. Cytoxicity 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)

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<tr>
<th>S. No.</th>
<th>Sample ID #/Description/ Sources &amp; Average weight of each tablet</th>
<th>Sample ID #/Description/ Sources &amp; Average weight of each tablet</th>
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</table>
| 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 Soft / USA (0.4975 g/tablet) | 27 | Hepacure / Mepaco / Egypt |
| 4     | Advance Physician Formulas® (0.3319 g/tablet) | 28 | Hepaticum®  
Cyclodextrin enhanced formula / Egypt  
Liver support, Soft gelatin capsules / Egypt |
| 5     | Pure Encapsulations® (0.2430 g/tablet) | 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 | Silyta®  
Dietary Supplement / Egypt |
| 8     | Himalaya Liver Care® Liv.52 / USA (0.3768 g/tablet) | 32 | MARIAGON®  
Hepatoprotective / Egypt |
| 9     | Jarrow Formulas® (0.2786 g/tablet) | 33 | Liverin®  
Improve liver function / Egypt |
| 10    | Metabolic Maintenance® Silymarin / USA (0.5696 g/tablet) | 34 | Leaglon® 70  
Improve liver function / Egypt |
| 11    | Life Extension® (0.9040 g/tablet) | 35 | Leaglon® 140  
Silymarin / Germany |
| 12    | Puritan’s Pride® Silymarin Milk Thistle / USA (0.3466 g/tablet) | 36 | Liverin®  
Improve liver function / Egypt |
| 13    | Natural Wellness® / USA (0.5063 g/tablet) | 37 | MEPASIL®  
Silymarin, Liver support / Egypt |
| 14    | Enzymatic Therapy® Super Milk Thistle / USA (0.3262 g/tablet) | 38 | Livit®  
Liver support, Soft gelatin capsules / Egypt |
| 15    | Advanced Beta Glucan Therapy® (0.4867 g/tablet) | 39 | Hepanox®  
Cap. |
| 16    | FutureBiotics® Silymarin Plus / USA (1.0514 g/tablet) | 40 | Hepato-Forte®  
Liver Support / Egypt |
| 17    | Planetary Herbs® (0.7060 g/tablet) | 41 | Levatone®  
Food Supplement / Egypt |
| 18    | Full Spectrum Silymarin 80® / USA | 42 | Hepatic Forte®  
Silymarin / Egypt |
| 19    | Wonder Laboratories® Advanced B-12 Sublingual / USA (0.3443 g/tablet) | 43 | Hipammax Plus®  
Dietary Supplement / Egypt |
| 20    | 21st Century® (0.4508 g/tablet) | 44 | Liver Albumin Plus®  
Dietary Supplement / Egypt |
| 21    | Source Naturals® Silymarin Plus / USA (0.9847 g/tablet) | 45 | Quercetin / Sigma |
| 22    | Now® Silymarin 100 V caps / USA (0.5090 g/tablet) | 38 | Liverin®  
Improve liver function / Egypt |
| 23    | GoodN Natural® Milk Thistle Extract 250 mg / USA (0.6765 g/tablet) | 39 | Hepanox®  
Cap. |
| 24    | TwinLab® Silymarin / USA (0.1610 g/tablet) | 40 | Hepato-Forte®  
Liver Support / Egypt |
|       | Nature’s Bounty® / Natural Whole Herbs Milk Thistle 1000 mg / USA (0.3939 g/tablet) | 41 | Levatone®  
Food Supplement / Egypt |

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

<table>
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<tr>
<th>Sample</th>
<th>%LDH leakage in culture medium after 2h</th>
<th>IC₅₀ (µg/ml) By MTT assay</th>
<th>ED (µg/ml)</th>
<th>TI</th>
<th>Glutathione Reductase/10⁶ cells (U/l)</th>
<th>Nitric Oxide (µmol nitrite/10⁶ cells)</th>
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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 hours prior 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 nitrate, an NO metabolite, in culture 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 exerted by 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 and used in the treatment of liver diseases [27 -29]. 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 and used in the treatment of liver diseases [27 -29].

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 quercetin. 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 quercetin. 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 quercetin. 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 depend 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.

ACKNOWLEDGMENT

The present study is a part of the work of the project entitled "Development of a Natural Product for the treatment of Hepatitis C Virus (HCV) infection" between Industrial Modernization Center (IMC), STDF and National Research Centre (NRC) (2010-2014) and to whom the authors are deeply indebted.

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

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