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Clinical and Pharmacological Applications of Silymarin Components at Cellular and Molecular Level: A Review

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Abstract: Silymarin, a flavonolignan from 'milk thistle' (*Silybum marianum*) plant is used almost exclusively for hepatoprotection and amounts to 180 million US dollars business in Germany alone. In this review, we discuss about its safety, efficacy and future uses in liver diseases. The use of silymarin may replace the polyherbal formulations and will avoid the major problems of standardization, quality control and contamination with heavy metals or bacterial toxins. Silymarin consists of four flavonolignan isomers namely; silybin, isosilybin, silydianin and silychristin. Among them, silybin being the most active and commonly used. Silymarin is orally absorbed and is excreted mainly through bile as sulphates and conjugates. Silymarin offers good protection in various toxic models of experimental liver diseases in laboratory animals. It acts by antioxidative, anti-lipid peroxidative, antifibrotic, anti-inflammatory, membrane stabilizing, immunomodulatory and liver regenerating mechanisms. Silymarin has clinical applications in alcoholic liver diseases, liver cirrhosis, Amanita mushroom poisoning, viral hepatitis, toxic and drug induced liver diseases and in diabetic patients. Though silymarin does not have antiviral properties against hepatitis virus, it promotes protein synthesis, helps in regenerating liver tissue, controls inflammation, enhances glucuronidation and protects against glutathione depletion. Silymarin may prove to be a useful drug for hepatoprotection in hepatobiliary diseases and in hepatotoxicity due to drugs. The non-traditional use of silymarin may make a breakthrough as a new approach to protect other organs in addition to liver. As it is having a good safety profile, better patient tolerability and an effective drug at an affordable price in near future new derivatives or new combinations of this drug may prove to be useful.

Key words: Cellular therapeutic, herbal drugs, silymarin, antioxidant, price

INTRODUCTION

Physical and pharmaceutical characteristics: Silymarin (SM) was discovered in 1959, a C25 containing flavonoid mixture, could extract from the *Silybum marianum* (milk thistle) plant. Current standardized SM extract (including silibinin, often named silybin) may contain approximately, 65-80% flavonolignans (Silybin A and B, isosilybin A, B, silychristin and silydianin) (Fig. 1) with small amounts of flavonoids (Taxifolin and Quercetin) and approximately, 20-35% of fatty acids and polyphenolic compounds (Surai, 2015b). Silybin as the predominant and primary active ingredient in SM, could be the reason of finding silybin content and silybin antioxidant in the compounds containing milk thistle ingredients, explaining the biological activity of SM (Das *et al*., 2014). In addition, SM has been known as the gold standard medication to treat liver disorders of different etiologies, as traditional herbal remedies ("liver tonics") for almost 2000 years (Surai, 2015b). Thus, the antioxidant and chemoprotective

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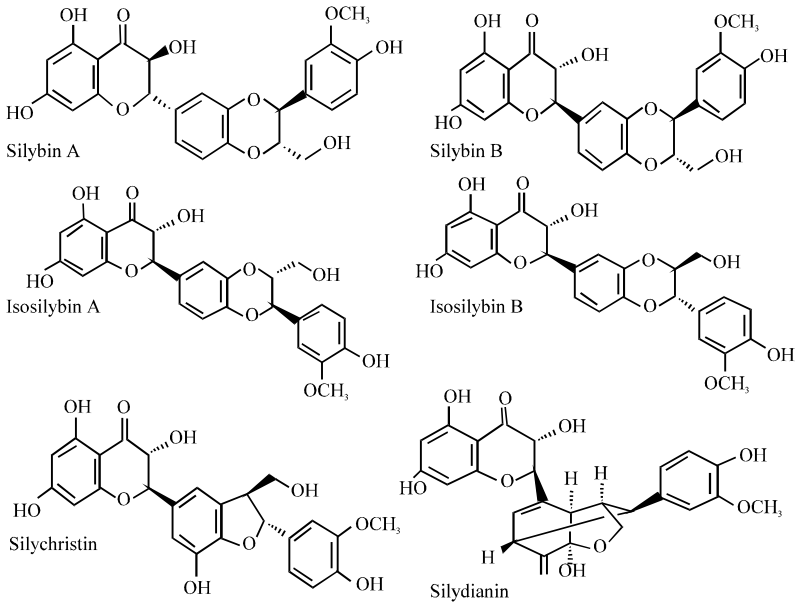

Fig. 1: The chemical structure of silybin A and B, isosilybin A, B, silychristin and silydianin

effects of SM on liver is very well-known that's why it is often prescribed and self-prescribed as a complementary and alternative hepatoprotective medicine (Marzio and Fenkel, 2014). Due to SM's strong antioxidant and tissue regenerative properties, there are many studies on its hepato, neuro, nephro and cardio-protective ingredient (Dayoub *et al*., 2013). However, it seems likely that direct Antioxidant (AO) activity of polyphenols does not contribute directly to the antioxidant defence of the body and only limited work has been carried out to explore SM/silybin impact on the induction of cellular antioxidant defence via the modulation of various transcription factors, including Nrf2 and NF-κB and respective gene and protein expressions. Potential molecular proliferative signaling targets for anti-cancer activity of silibinin include the receptor tyrosine kinase, STAT and rogen receptor and NF-κB pathways; however, anti-cancer activity of SM is beyond the scope of the present review. Therefore, this review focuses on evaluating recent studies on SM (or silibinin) antioxidant effects in various *in vitro* and *in vivo* model systems in the context of its contribution to the antioxidant systems regulation and participation in cell signaling. Oxygen free radicals and other Reactive Oxygen Species (ROS) such as superoxide anion radical O_2, Hydrogen peroxide (H_2O_2), alkoxyl (RO), peroxyl (ROO), hydroxyl radical (OH) and hypochlorous acid (HOCl) as well as Reactive Nitrogen Species (RNS) such as Nitric Oxide (NO) and peroxynitrite are known to damage living tissues and cellular components. In biological systems this process is called oxidative stress or oxidative damage and has become a significant topic in the field of environmental toxicology (Halliwell and Gutteridge, 2015) (Fig. 1).

Moreover, an imbalance between the oxidative forces and antioxidant defense systems may cause oxidative injury, implicated in different diseases such as atherosclerosis, diabetes mellitus, cancer, liver cirrhosis, and so on (Bullon *et al*., 2014). ROS are in fact continuously generated in physiological conditions andeffectively eliminated by several intracellular and extracellular antioxidant systems (Gammella *et al*., 2016). It should be noted that most of the hepatotoxic chemicals damage of livercells mainly initiate by inducing lipid peroxidation and other oxidative damages (Subbaraj *et al*., 2013). In fact, liver could pose a uniquemetabolism and may play a pivotal role in the removal of substances from the portal circulation which it issusceptible to toxicity of drugs, xenobiotics and oxidative stress (Yang *et al*., 2014).

Furthermore, the current treatment for hepatotoxicity could include medications influencing the P450 enzyme mechanism either by inhibiting (amiodarone, cimetidine, ciprofloxacin and so on) or inducing (rifampicin, carbamazepine, phenobarbital, phenytoin) the metabolic activity of enzymes (Sasaki and Shimoda, 2015). SM is the major bioactive constituent of the milk thistle seed extract of the medicinal plant *Silybum marianum* of the family Asteraceae (Khan *et al*., 2013). SM has been widely used as a therapeutic agent fora variety of acute and chronic liver diseases (Inzucchi *et al*., 2012). It has been used

for centuries for the protection ofthe liver from toxic substances, treating liver damage, therapy of hepatitis and cirrhosis (Zhang *et al*., 2013).

Action mechanism, antioxidant properties and clinical applications: SM and its main constituent silibinin sources, metabolism and bioavailability have already been reviewed extensively (Surai, 2015b). It has been shown that after oral consumption silibinin is characterised by comparatively low availability, e.g. in rats it is only about 0.95% (Surai, 2015b). In fact, after the oral administration of the standardized milk thistle extract Legalon, flavonolignans were rapidly absorbed and eliminated with a half-life for silibinin of 6 h. The main biotransformation route of silybin and derivatives was identified to be conjugation. SM can contribute to the antioxidant defenses in different ways. Firstly, by direct free radical scavenging. Secondly, by preventing free radical formation by inhibiting specific enzymes responsible for free radical production or by maintaining the integrity of electron-transport chain of mitochondria in stress conditions. Thirdly, by participating in the maintenance of optimal redox status of the cell by activating a range of antioxidant enzymes and non-enzymatic antioxidants, mainly via transcription factors, including Nrf2 and NF-κB. Finally, by activating an array of vitagenes, responsible for the synthesis of protective molecules, including HSP, Thioredoxin (Trx), sirtuins and so on and providing additional protection in stress conditions (Surai, 2015a).

The effects of silibinin on the formation of ROS and eicosanoids by human platelets, white blood and endothelial cells were studied (Karas *et al*., 2016). In addition, the main effect of silibinin was obtained at 50 μM concentration (Anestopoulos *et al*., 2013). Silibinin exerts anti-oxidative and anti-inflammatory effects on monocytes from pre-eclamptic pregnant women may act by inhibiting the *in vitro* endogenous release of ROS and TNF-α production (Fellner *et al*., 2013). It is important to mention that the free radical scavenging activity of pure individual compounds of the SM is reported to vary considerably with silydianin and silychristin being 2-10 fold more active than the silibinin and on a mass basis, SM is shown to be about 8-fold more potent than silibinin as a free radical scavenger (Romero *et al*., 2013). Indeed, SM and silybin (silibinin) are not the same compounds and their AO activities could differ substantially depending on their concentrations in the target tissues. Furthermore, many of the effects of SM/silibinin described *in vitro* may occur at concentrations not currently achievable in humans or animals (Surai, 2015b). Therefore, a direct scavenging ROS by silibinin in biological systems (except the gut) is not likely to substantially contribute to the antioxidant protection.

On other hand, Mitochondria are the primary cellular consumers of oxygen and contain numerous redox enzymes capable of transferring single electrons to oxygen, generating the ROS superoxide ($O_2^{\cdot}$) (Hirst and Roessler, 2015). It is well documented that mitochondrial enzymes known to generate ROS include the Tricarboxylic Acid (TCA) cycle enzymes aconitase and α-ketoglutarate dehydrogenase; the Electron-Transport Chain (ETC) complexes I-III; pyruvate dehydrogenase and glycerol-3-phosphate dehydrogenase; dihydroorotate dehydrogenase; the Monoamine Oxidases (MAO) A and B and cytochrome b_5 reductase. Furthermore, mitochondrial insults, including oxidative damage itself, can cause an imbalance between ROS production and removal, resulting in net ROS production. For example, ROS can induce protein modifications, lipid peroxidation and mitochondrial DNA damage which ultimately results in mitochondrial dysfunction. Many studies have focused on the detrimental effects of ROS but it is now clear that mitochondrially generated ROS are also involved in regulating intracellular signal transduction pathways that result in cell adaptation to stress (Schieber and Chandel, 2014). One of the mechanisms responsible for the decrease in oxidative stress is the protective effect of SM/silibinin on mitochondrial structure and function. Indeed SM protects mitochondria from pathological events by triggering pro-survival cell signaling (Oral *et al*., 2016).

The different behaviour of SM/silibinin in normal and cancerous cells should be mentioned. In particular, SM is shown to have a protective effect against diabetes-induced cardiomyocyte apoptosis as well as apoptosis caused by various toxicants while it causes apoptosis in cancerous cells.

Furthermore, Silymarin significantly inhibits tumor growth and also cause regression of established tumors. It is associated with *in vitro* anti-proliferative, pro-apoptopic and anti-angiogenic efficacy in prostate tumor. Silymarin feeding during the promotion phase of 4-nitroquinoline-1-oxide induced rat tumorigenesis exerts chemopreventive activity against tongue squamous cell carcinoma. The cancer chemopreventive and anticarcinogenic effects of silymarin in long term animal tumorigenesis models and in human prostate, breast and cervical carcinoma cells are also reported. Treatment with silibinin results in a highly significant inhibition of both cell growth and DNA synthesis with loss of cell viability in case of cervical carcinoma cells. Silibinin significantly

induces growth inhibition, a moderate cell cycle arrest and a strong apoptotic cell death in small cell and non-small cell human lung carcinoma cells. Silibinin inhibits the growth of human prostate cancer cells both *in vitro* and *in vivo*. Silymarin and silibinin have strong anti-angiogenesis effect on the colon cancer cell line and effective against chemical-induced bladder carcinogenesis in mice and hepatocellular carcinoma in rats.

The molecular bases of the anti-inflammatory and anticarcinogenic effects of silybin/SM are yet unknown; they might be related to inhibition of the transcription factor NF-κB which regulates and coordinates the expression of various genes involved in the inflammatory process in cytoprotection and carcinogenesis. In particular, NF-κB contributes to the production of interleukins IL-1 and IL-6, Tumor Necrosis Factor (TNF-α), lymphotoxin, Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) and Interferon (IFN-γ). Furthermore, some of these cytokines, e.g., IL-1 and TNF-α activate NF-κB themselves, thus, creating positive feedback. NF-κB activation occurs on its dissociation from the inhibitory protein I-κB and its subsequent nuclear translocation. NF-κB seems to be a subject to redox regulation, suggesting thus an importantrole of antioxidants in its inactivation. SM was tested in HaCaT (Human keratinocytes) induced with UV light and in $HepG_2$ cells (human hepatoblastoma) induced with okadaic acid and LPS106, 107 and proved to be highly effective in suppressing NF-κB binding activity and its dependent gene expression. Compared to other bioflavonoids tested, SM inhibits transcription factor NF-κB in very low concentrations (12 μg mL^{-1}). Manna studied the effect of SM on NF-κB activation induced by various inflammatory agents. SM blocked TNF-α-induced activation of NF-κB in a dose- and time-dependent manner. This effect was mediated through inhibition of phosphorylation and degradation of inhibitory protein IκBα, an inhibitor of NF-κB. NF-κB-dependent reporter gene transcription was also suppressed by SM. SM also blocked NF-κB activation induced by phorbol ester, LPS, okadaic acid and ceramide, whereas H_2O_2-induced NF-κB activation was not significantly affected. SM also inhibited the TNF-α-induced activation of mitogen-activated protein kinase and c-Jun N-terminal kinase and abrogated TNF-α-induced cytotoxicity and caspase activation. The inhibition of activation of NF-κB and the kinases may provide in part the molecular basis for the anticarcinogenic and anti-inflammatory effects of SM and its effects on caspases may explain its role in cytoprotection. Like SM, the anti-inflammatory drugs sodium salicylate and aspirin are also known to block the activation of NF-κB by preventing the degradation of IκBα. However, SM was effective at a100 fold lower concentration than salicylate, suggesting that it is a potent inhibitor without substantial toxicity. Silybin is also known to induce apoptosis of endothelial cells and to inhibit angiogenesis which is essential for tumor growth and metastasis. Silybin was found to suppress the growth and induce the apoptosis of ECV304 cells (human umbilical vein endotelial cell line). The induction of apoptosis by silybin was confirmed by ladder-patterned DNA fragmentation, cleaved and condensed nuclear chromatin and DNA hypoploidy. Silybin could effectively inhibit a constitutive NF-κB activation as revealed by electrophoretic mobility shift assay and NF-κB dependent luciferase reporter study. Consistent with this, silybin treatment resulted in a significant decrease in the nuclear level of p65 subunit of NF-κB. In addition, silybin treatment caused a change in the ratio of Bax/Bcl-2 in a manner that favors apoptosis. Silybin also induced the cytochrome c release, activation of caspase-3 and -9 and cleavage of poly (ADP-ribose) Polymerase (PARP). These results suggest that silybin may exert, at least partly, its anticancer effect by inhibiting angiogenesis through induction of endothelial apoptosis via modulation of NF-κB, Bcl-2 family and caspases. Series of excellent studies from Agarwal's group has demonstrated high efficiency of silybin in the prostate tumor adjuvant treatment. One possible mechanism of this effect is the indirect potentiation of TNF-α action by silybin inhibition of NF-κB (ref.110). The studies revealed that silybin is able to inhibit constitutive activation NF-κB in human prostate cancer cell line DU145. Silybin also inhibits TNF-α induced activation of NF-κB via IκBα pathway and subsequently sensitizes DU145 cells to the TNF-α-induced apoptosis. SM is known to have an anti-atherosclerotic activity. The mechanism responsible for it can be partly explained by the antioxidative protection of cholesterol transporting lipoproteins but it still partly remains unclear. SM inhibited THP-1 (human monocyte cell line) cell adhesion to Human Umbilical Vein Endothelial Cells (HUVEC). SM also suppressed the TNF-α-induced protein and mRNA expression of adhesion molecules such as VCAM-1, ICAM-1 and E-selectin in HUVEC. Moreover, SM suppressed the TNF-α induced DNA binding of NF-κB in HUVECs (Yu *et al*., 2015). Therefore, part of the SM anti-atherosclerotic activity is mediated by inhibiting the expression of adhesion molecules.

CONCLUSION

Milk thistle seeds have been used for over 2000 years as natural remedy for the treatment of several diseases especially for liver and still widely used for the same. The active constituents of milk thistle seed are three isomeric flavonolignans viz. silibinin or silybin, silychristin and silidianin collectively known as SM extracted from the milk thistle seeds, available commercially as standardized extract. SM and its constituents (mainly silibinin) act as antioxidant and hepatoprotective and effective in treating toxin poisoning, hepatitis, cirrhosis and fibrosis of liver; stimulate liver regeneration. Although, the human studies regarding management of alcoholic cirrhosis and hepatitis are equivocal. Milk thistle seed demonstrates anti-inflammatory, immunomodulatory, lipid and biliary effects. It also has antiviral, antitumor, antimalarial and other therapeutic properties. Milk thistle seed preparations are safe, well tolerated and cause no serious side effects in humans except mild gastrointestinal and allergic reactions. Recent achievements in biochemistry and molecular biology, together with epidemiological data have changed our thinking about food. It has become increasingly clear that our diet plays a pivotal role in maintenance of our health and a misbalanced diet can cause serious health-related problems. It seems likely that antioxidants are among the major regulators of many physiological processes and therefore, a redox balance between antioxidants and prooxidants in the diet, gastro-intestinal tract, plasma and tissues is an important determinant of the state of our health. Plants consumed by humans and animals contain thousands of phenolic compounds. Among them, the effects of dietary polyphenols including SM are of great current interest. Indeed, various phytochemicals, including flavonoids are an essential part of our diet which are responsible for turning on and maintaining an optimal status of our antioxidant defenses. Since, flavonoids are not well absorbed in the gut, their active concentration in the plasma and target tissues are comparatively low but probably sufficient for Nrf2 activation and NF-κB suppression as well as vitagene activation. Indeed, it seems very likely that activation of the Keap1/Nrf2/ARE pathway and inhibition of NF-κB pathway, rather than direct free radical scavenging activity, may be the main mechanisms of the health benefits of phytochemicals, including SM. Therefore, consumption of phytochemicals, including SM could have a pre-conditioning effect on the antioxidant system of the body. This could explain the beneficial health-promoting effects of a diet rich in fruits and vegetables as important sources of the aforementioned chemicals (polyphenols and other phytochemicals) maintaining the body's ability to be highly adaptive to various stresses. SM and its main component silibinin are part of the dietary phytochemical mixture responsible for regulation of the antioxidant defences in the gut and in the whole body. It could well be that some dietary constituents which are not well absorbed could have health-promoting properties by maintaining redox balance in the large intestine where concentration of other antioxidants (vitamin E, carotenoids, ascorbate) could be low but prooxidants (iron, oxidized PUFAs, etc.) and substrates of oxidation are still present. This protective effect in the large intestine could be responsible, for example, for bowl cancer prevention. Therefore, there could be a biological reason for some nutrients not being absorbed but still being involved in antioxidant protection in the lower gut. Taking into account high concentrations of phytochemicals in the gut, it could well be that they play an essential part in maintaining an optimal antioxidant-prooxidant balance in the digestive tract responsible for additional health effects of phytochemicals including SM. In animal nutrition and disease prevention strategy SM alone or in combination with other hepatho-active compomds (carnitine, betaine, vitamin $B1_2$, etc.) could have similar hepatoprotective effects as described in humans with similar mechanisms of protective action. In conclusion, there are many possible mechanisms by which SM can improve the antioxidant defense mechanisms in the body. They include direct and indirect SM actions. First of all, a direct scavenging free radicals and chelating free Fe and Cu are mainly effective in the gut. Secondly, preventing free radical formation by inhibiting specific ROS-producing enzymes or improving the integrity of electron-transport chain of mitochondria in stress conditions as a result of SM consumption is of great importance. Thirdly, maintaining an optimal redox balance in the cell by activating a range of antioxidant enzymes and non-enzymatic antioxidants, mainly via Nrf2 activation is probably the main driving force of AO action of SM. Fourthly, decreasing inflammatory responses in the gut and other tissues by inhibiting NF-κB pathways is an emerging mechanism of SM protective effect in liver toxicity and diseases. Fifthly, activating vitagenes, responsible for synthesis of protective molecules, including HSP, Trx, sirtuins, etc. and providing additional protection in stress conditions deserves more attention in future research. Milk thistle seed shows great promise to be a superior herbal drug. Its good safety profile, better standardization and quality control, easy availability and low cost are added advantages. More definitive research is warranted to corroborate its wide range of phytotherapeutic effects. Further research on milk thistle

seed may make a breakthrough as a new approach in disease prevention in addition to liver complications. Finally, effects on the microenvironment of the gut, including SM-bacteria interactions, a wait future investigation.

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