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Phytochemical analysis of the antioxidant properties of *Silybum marianum L*

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SUMMARY

A growing body of evidence supports lipid peroxidation as having a role in the pathogenesis of liver disease. Although the probable cause of damage to human hepatocytes may be multifactorial, free radicals have been implicated in a variety of liver diseases, particularly in the presence of iron overload and toxic substances such as ethanol. Consequently, antioxidants, particularly those of plant origin such as flavonoids, may help to reduce the risk of developing these diseases. *Silybum (S.) marianum*, a medicinal plant widely used in traditional European medicine for the treatment of liver disorders, was evaluated for antioxidant activity. Thin layer chromatography and High Performance Liquid Chromatography analyses of crude extract of the plant confirmed the presence of a number of flavonoids reported in the literature. The antioxidant activity of these flavonoids was measured through inhibition of lipid peroxidation and 1, 1-diphenyl-2- picrylhydrazyl radical scavenging. The crude plant extract showed marked antioxidant activity in both assays. These results suggest that *S. marianum* contains flavonoids with antioxidant activity, capable of inhibiting or scavenging free radicals, thus supporting its traditional use as a hepatoprotective agent.

Key words: Antioxidant; Lipid peroxidation; Flavonoids; Free radical; Linoleic acid

INTRODUCTION

Alcohol-induced liver disease (ALD) poses a major health challenge. More serious ALD includes alcoholic hepatitis and cirrhosis (characterized by progressive hepatic fibrogenesis). Treatment options for either condition are limited, hence the significance of the hepatoprotective properties of *Silybum (S.) marianum*.

S. marianum has been used as a digestive tonic and as a galactogogue in lactating mothers. In Western Herbal Medicine, it is the main remedy used as a hepatoprotective and for the treatment of liver disorders such as hepatitis, jaundice, cirrhosis, alcoholic liver disease and problems associated with the gall bladder. It is also used as an emergency antidote for mushroom-induced liver poisoning (e.g. death-cap mushrooms). The herb has also found use as an anti-hypercholesterolaemic remedy (Saller *et al.*, 2001).

S. marianum contains flavonolignans referred to as silymarin (including silybin, silychristin, isosilybin; silydianin), (Wagner *et al.*, 1984; Bilia *et al.*, 2001; Campodonico *et al.*, 2001); dihydroflavonols (e.g. dehydrokaempferol, dihydroquercetin); flavonols (quercetin, kaempferol); flavones, flavanones and narigin. The plant also contains lipids, sterols and mucilage (Wagner *et al.*, 1984; Grainger-Bisset, 2001; Barnes *et al.*, 2002).

Preliminary phytochemical analyses of the

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plant indicate that the flavanolignan-containing crude extract has promising antioxidant activity on both the 1,1-diphenyl-2- picrylhydrazyl (DPPH) radical and on linoleic acid peroxidation systems, supporting its traditional use in the treatment of liver disease, in particular and its inclusion in the management of degenerative diseases such as osteoarthritis. According to Duh (1998) "antioxidants are believed to intercept the free radical chain of oxidation and to donate hydrogen from the phenolic hydroxyl groups, thereby forming stable radicals which do not initiate nor propagate further oxidation of lipids." As DPPH is known to abstract the labile hydrogen atom of chemical compounds (Ratty *et al.*, 1988) the antioxidant that can scavenge the DPPH radical is expected to depress lipid peroxidation (Aniya *et al.*, 1999). Lipid peroxide assays are often used to establish the antioxidant potential of plant extracts and individual compounds, whereas the DPPH system is generally used for primary screening to test the propensity of a crude plant extract to antioxidant activity.

MATERIALS AND METHODS

Plant material

The plant (*S. marianum L*, Family: Asteraceae (Compositate) was obtained from Proline Botanicals, a leading herbal manufacturer in the U.K.

Preparation of crude plant extract

The crude plant extract was obtained by extracting milk thistle seed (30 g), in a Soxhlet apparatus using 150 ml of 80% ethanol (Markham, 1982; Harborne, 1989) at 25°C. The extracts were filtered and the residue re-extracted under the same conditions. The filtrates were combined and evaporated to dryness under vacuum and the crude extracts reconstituted in (80%) ethanol to give a stock solution of 100 mg/ml. The residue was weighed and re-dissolved in the original solvent to give a stock solution of 100 mg/1 ml, which was stored at a temperature of -18°C.

Thin layer chromatography (TLC) analyses of crude plant samples

The crude plant extract was analysed for flavonoid content on Macherey-Nagel TLC plates coated with fluorescent Kieselgel 60 F_{254}, eluting with chloroform: acetone: anhydrous formic acid (75 : 16.5 : 8.5). The compounds were detected by visualisation under UV light, developing the plates in 1% methanolic diphenylboric acid-β-ethylamino ester followed by 5% ethanolic polyethyleneglycol 4,000 and subsequently heating on a hot plate (Wagner *et al.*, 1984; British Herbal Pharmacopoeia, 1986). The identified compounds were characterised by their R_f values.

High liquid performance chromatography (HPLC) analyses

HPLC analyses were carried out at the Jodrell Laboratory at Kew Gardens using a Waters 600 HPLC pump with a 600E system controller and a Waters 717 plus autosampler coupled to a Waters 996 photodiode array detector via a solvent gradient system prepared from a solution of methanol and water containing acetic acid (Hostettmann and Hostettmann, 1981; Grayer *et al.*, 2000). Compounds were separated on a 250 mm × 4 mm i.d., 5 μm particle, LiChrospher 100 RP-18e capillary column maintained at 25°C. The solvents were de-gassed with helium. UV-visible spectra were recorded in the range 210 - 400 nm for flavonoids and phenolic acids and 210 - 600 nm for anthocyanins. The crude plant extracts were diluted to 10 mg/ml and filtered prior to HPLC analyses.

Assays for antioxidant activity

The crude plant extract was tested for antioxidant activity by *in vitro* biochemical assays using DPPH and linoleic acid.

Evaluation of antioxidant activity in the DPPH assay

The free radical scavenging activity using the DPPH assay was carried out based on the methods of Pieroni and Aniya. 1 ml of the crude plant extract

was added to 1 ml of 0.1 mM DPPH in 1 ml Tris HCl buffer and shaken. Absorbance was measured at a wavelength of 517 nm, 10 min after adding the extract using propyl gallate and caffeic acid as controls. The antioxidant activity was calculated as [1-(Ai-Aj)/Ac] × 100; where Ai is the absorbance of the crude plant extract mixed with DPPH solution and Tris HCl buffer; Aj is the absorbance of the crude plant extract mixed with ethanol and Ac is the absorbance of a blank solution containing DPPH solution, Tris HCl buffer, ethanol and water.

Evaluation of antioxidant activity in the linoleic acid

Using the ferrous thiocyanate (FTC) method (Duh, 1998), the lipid peroxidation inhibitory effects of the crude extract was measured. A mixture consisting of 4 ml test solution, 4 ml 2.5% linoleic acid in 99.5% ethanol, 8 ml phosphate buffer (pH 7) and 4 ml distilled water was incubated at 37°C for 5 days. The degree of oxidation was then measured by sequential addition of ethanol (10 ml, 75%), ammonium thiocyanate (0.2 ml, 30%), sample solution (0.2 ml) and ferrous chloride (20 mM in 3.5% HCl) solution (0.2 ml). Immediately after the addition of the ferrous chloride, the mixture was stirred for 3 min, and the absorbance measured at 500 nm. The percentage inhibition of linoleic acid peroxidation was calculated as 100 - [(absorbance of sample/absorbance of control) × 100]. n-Propyl gallate and caffeic acid were used as controls and all tests were run in triplicate and averaged.

RESULTS

TLC analyses

TLC analysis of the crude plant extract carried out with the reference compounds silymarin and taxifolin gave R_f values consistent with those reported in the literature (Wagner *et al.*, 1984; Grainger-Bisset, 2001). Three spots were observed each corresponding to the appropriate reference compound. Silymarin was resolved into two yellow spots identified as silybin (R_f 0.6) and silychristin (R_f 0.35). Taxifolin appeared as a pinky orange spot- R_f of 0.4, whilst silydianin appeared as a pale yellow fluorescent zone between taxifolin and silybin with no distinct R_f value.

HPLC analyses

HPLC analyses of the crude plant extract also confirmed the presence of these flavanolignans (Fig. 1).

DISCUSSION

The electron-donating properties of flavonoids and many phenolic acids have been repeatedly emphasised as the basis of their antioxidant action (Sugihara *et al.*, 1999). For example, the reported antioxidant action of flavonoids on lipid peroxidation is based on scavenging the principal propagating intermediates, the peroxyl and alkoxyl radicals, by hydrogen donation, halting the radical chain (Bors *et al.*, 1990). This suggests that the flavonoid and polyphenol-containing extracts, could be acting as electron donors, reacting with free radicals to convert them to less reactive compounds, which would terminate a radical chain reaction (Bors *et al.*, 1990). Although the polyphenols and flavonoids may not be the only active compounds, their antioxidant properties may, in part, explain the mechanism of antioxidant activity in the extracts tested.

In this study, all the extracts demonstrated DPPH scavenging activity and it was confirmed that they would also inhibit lipid peroxidation. The action of the plant extracts on DPPH would suggest that they acted as free radical inhibitors and primary antioxidants, which is likely to be the main reason for the inhibition of linoleic acid peroxidation in the lipid peroxide assay. The scavenging effects of the crude extracts of *S. marianum* on the free radical DPPH increased in a dose-dependent manner just as its linoleic acid

BI 12713 Silymarin sp.

Common name	Milk Thistle	Date acquired	1/19/04 5:46:00 PM
Plant part	seed	Sample set ID	1609
Batch number	none	Injection volume	30.00 ul
Extract type	80% EtOH	Injection ID	1620
Supplier	Barbara Pendry	Instrument method ID	1022
Dilution factor	1.00000	Method Set	TCM Authentication

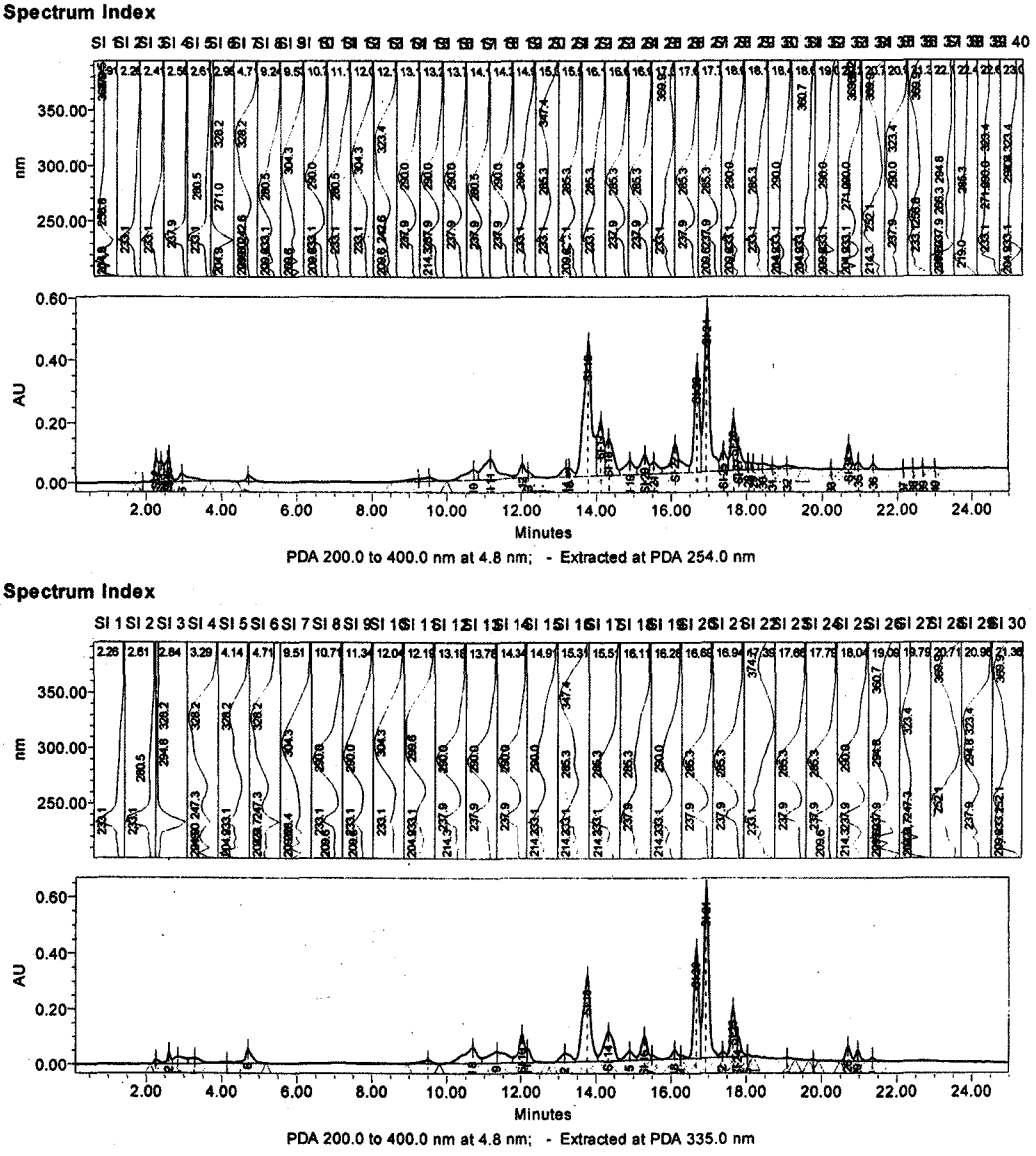

Fig. 1. HPLC chromatograms of *Silybum marianum* seed extracts. Identification of specific flavonoids was done by retention times and by comparing the UV spectra of the peaks with those of the available standards. Where authentic samples were unavailable, general identification of flavonoid types was made by interpretation of the general flavonoid spectrum, which typically consists of two absorption maxima in the ranges of 240 - 285 nm (band II) and 300 - 550 nm (band I), (Markham, 1982; Jodrell Laboratory, 2004).

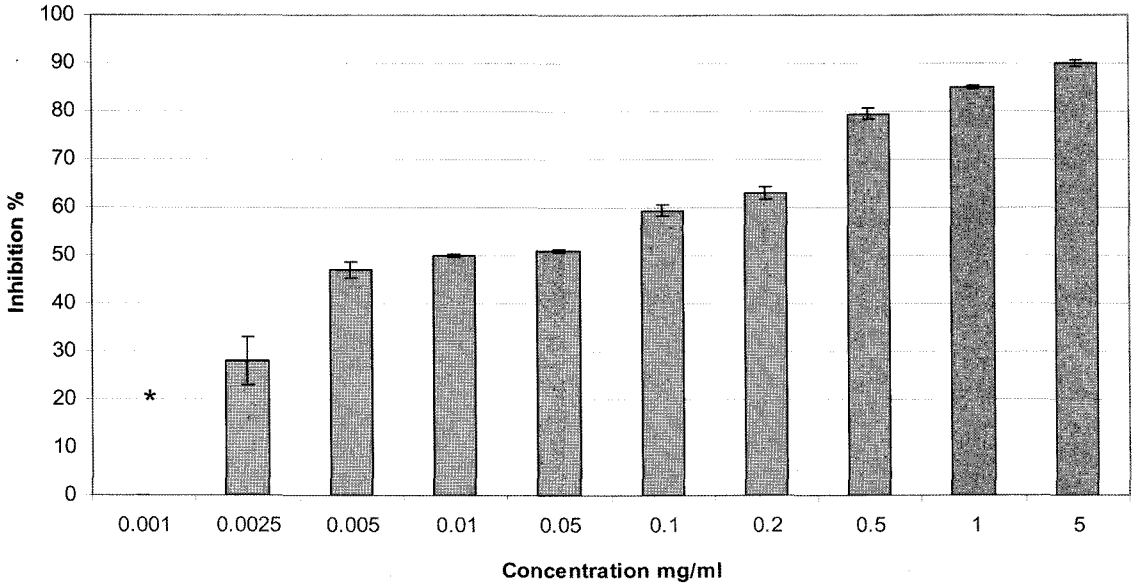

Fig. 2. Evaluation of the inhibition of linoleic acid peroxidation by a range of concentrations of *Silybum marianum* (seed) crude plant extract (80% EtOH) in a lipid peroxide assay to measure antioxidant activity. Each column shows the mean ± S.D. of triplicate determinations except 0.001 mg/ml which represents the data from four experiments.

peroxidation inhibitory effects as illustrated in the graph in Fig. 2.

In both DPPH and lipid peroxidation inhibition assays, the highest level of antioxidant activity was seen at a concentration of 1 mg/ml. The results for the free radical scavenging activity of the crude extracts on the DPPH radical at the concentrations initially tested, i.e. 2.5 mg/ml, 1 mg/ml and 0.5 mg/ml, were comparable with, or higher than, the reference compound propyl gallate. The IC_{50} value for propyl gallate however, was considerably lower than the IC_{50} values of all the crude plant extracts (Table 1).

The antioxidant activity of the crude plant extracts on linoleic acid peroxidation compared to propyl gallate showed that in general when tested at a similar concentration, they were equally effective (Table 2).

The differences in the antioxidant activity of propyl gallate in these assays may be explained by the different antioxidant mechanisms being investigated. As already outlined above, DPPH scavenging involves only a single hydrogen-donating mechanism whereas inhibition of linoleic acid oxidation involves a complex sequence of oxidation and reduction processes. At the lowest concentrations tested, 0.001 mg/ml and 0.0025 mg/ml, some of the crude plant extracts were observed to change from antioxidant to pro-oxidant activity. Previous studies have established that besides antioxidant activity, flavonoids can also act as pro-oxidants in vitro depending on conditions that include

Table 1. DPPH radical scavenging activity of the plant extracts expressed as the IC_{50} value (mg/ml)

Plant	DPPH IC_{50} value (mg/ml) ± S.D.	DPPH : concentrations (mg/ml) used to calculate IC_{50} value
Silybum marianum (Milk thistle seed)	0.025 ± 0.945	0.1 : 0.05 : 0.01 : 0.005
Propyl gallate	0.00032 ± 0.471	

Table 2. Inhibition of linoleic acid peroxidation by the plant extracts (concentration 1mg/ml) measured using the lipid peroxide assay

Plant	Lipid peroxide assay % inhibition (1 mg/ml) ± S.D.
Silybum marianum (Milkthistle seed)	85% ± 0.471
Propyl gallate	100 μM final concentration 50% inhibition ± 1.633

Results given as a percentage of the control (oxidizing substrate without antioxidant) are based on the mean of triplicate experiments.

concentration of the antioxidant, the presence of other antioxidants and the presence of transition metals (Boik, 2001). There are two main mechanisms responsible for the pro-oxidant activity of flavonoids (Cos, 2001). The first is their ability to participate in reactions catalysed by transition metal ions such as the Fenton reaction, which produces the highly reactive hydroxyl radical. The second is their polyphenolic structure, which makes them susceptible to auto-oxidation reactions, resulting in the production of reactive oxygen species (Bors *et al.*, 1998). According to Halliwell and Chirico (1993) most biological studies of lipid peroxidation involve transition metal ions, added to, or contaminating, the reaction mixtures. The decomposition of lipid hydroperoxides by transition metals such as iron and copper to give hydroxyl or alkoxyl radicals is also strongly implicated in the generation of free radicals. Lipid hydroperoxides are thought to be reductively cleaved by metal ions in a low valence state to highly reactive alkoxyl radicals, which then remove hydrogen ions from lipids to form new lipid alkyl radicals. For the reduction of the metal ions back from the high valence state to low valency, reducing compounds are required and flavonoids may have the capacity to reduce metal ions directly through autoxidation (Sugihara *et al.*, 1999). ICP analysis confirmed the presence in all the crude extracts of transition metals capable of participating in metal catalysed reactions that can accelerate production of reactive oxygen species.

In spite of laboratory findings confirming the plant's traditional usage as a hepatoprotective, caution is needed when extrapolating from the in vitro results to the in vivo situation, especially since factors such as the bioavailability of flavonoids is still unclear. Furthermore, the antioxidant assays used in this study represent only two antioxidant mechanisms and it may be important to investigate the effects of these plant extracts in other antioxidant systems. It has been shown for example that antioxidants that protect lipid peroxidation against free radical damage may actually accelerate damage to other molecules such as carbohydrates, under certain conditions (Burits and Bucar, 2000). Human-body chemistry is complex and it does not currently appear possible to simulate in vitro studies accurately in vivo. Data on biological markers such as blood levels of flavonoids and their metabolites are not widely available, thus making it difficult to determine the individual or the combined role of the flavonoids and other antioxidants (Pietta, 2000). There is however strong evidence that flavonoid-containing foods such as fruit and vegetables and some medicinal plants have a protective effect against many of the degenerative diseases of ageing where lipid peroxidation is strongly implicated in the pathogenesis (Pietta, 2000). The results of these assays, which have demonstrated antioxidant activity of the crude extract of *S. marianum* indicate that their radical scavenging actions may contribute to their therapeutic effects and that their use in this way may be useful and reasonable.

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