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# In-vitro effect of flavonoids from *Solidago canadensis* extract on glutathione S-transferase

Pál Apáti, Peter J. Houghton, Geoffrey Kite, Glyn B. Steventon and Ágnes Kéry

## Abstract

*Solidago canadensis* is typical of a flavonoid-rich herb and the effect of an aqueous ethanol extract on glutathione-S-transferase (GST) activity using HepG2 cells was compared with those of the flavonol quercetin and its glycosides quercitrin and rutin, found as major constituents. The composition of the extract was determined by HPLC and rutin was found to be the major flavonoidal component of the extract. Total GST activity was assessed using 1-chloro-2,4-dinitrobenzene as a substrate. The glycosides rutin and quercitrin gave dose-dependent increases in GST activity, with a 50% and 24.5% increase at 250 mM, respectively, while the aglycone quercetin inhibited the enzyme by 30% at 250 mM. The total extract of the herb gave an overall dose-dependent increase, the fractions corresponding to the flavonoids showed activating effects while those containing caffeic acid derivatives were inhibitory. The activity observed corresponds to that reported for similar compounds in-vivo using rats, thus the HepG2 cell line could serve as a more satisfactory method of assessing the effects of extracts and compounds on GST.

## Introduction

In aerobic organisms, reactive oxygen species (ROS) generated through various endogenous processes and exogenous stimuli, such as the mitochondrial electron transport chain, cytochrome P-450 systems, nitric oxide synthase, inflammation, ultraviolet and ionizing irradiation, may lead to oxidative stress and subsequent damage to cellular macromolecules, including nucleic acids, proteins and lipids (Geoffroy et al 2000; Griffiths et al 2002).

The elimination of free radicals can be achieved through enzymatic and non-enzymatic reactions. The principal defence systems against oxygen free radicals are: superoxide dismutases, glutathione peroxidase, glutathione reductase, catalase and other haemoprotein peroxidases, as well as drug-metabolizing enzymes, such as glutathione S-transferases, glucuronosyl transferases and NAD(P)H:quinone reductase (NOQ), which act by removing compounds capable of generating ROS (Jung et al 1997; Pietta 2000; Pedrielli et al 2001).

Polyphenol compounds are abundant in fruit and vegetables and are proposed as potential chemopreventor agents against carcinogens. Flavonoids belong to a heterogeneous class of polyphenols with chemical characteristics facilitating multiple interactions with organic and inorganic compounds, such as proteins of the cell surface (receptors and enzymes), metals and free radicals (Spencer et al 1988).

Numerous mechanisms have been suggested for potential chemoprevention, with properties including antioxidant action and induction of the glutathione S-transferases. Several papers have indicated that flavononids, especially quercetin derivatives, are capable of inhibiting autoxidation reactions (Pedrielli et al 2001) and scavenging of free radicals (van Acker et al 1995). Although the effect of the flavonoid quercetin on drug-metabolizing enzymes has also been widely demonstrated (Galijatovic et al 2000; Rahden-Staron et al 2001), there are still some areas that remain to be clarified and the effects on other metabolic processes are still not completely understood. This investigation used HepG2 cells, as an alternative to in-vivo methods in animals, since this cell line has been shown to retain much of the drug-metabolizing activity of freshly-isolated human adult hepatocytes (Grant et al 1988). Hep G2 cells contain a mixture of glutathione S-transferases (GSTs) and several previous studies have shown that the major isozymes present are GSTP1 (class pi),

Semmelweis University, Department of Pharmacognosy, Üllöi Str. 26, 1085 Budapest, Hungary

Pál Apáti, Ágnes Kéry

Pharmaceutical Sciences Research Division, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 9NH, UK

Peter J. Houghton, Glyn B. Steventon

Jodrell Laboratory, Royal Botanic Gardens Kew, Richmond, Surrey TW9 3DS, UK

Geoffrey Kite

**Correspondence**: G. Steventon, Department of Pharmacy, King's College London, Franklin-Wilkins Building 150 Stamford Street, London, SE1 9NH, UK. E-mail: glyn.steventon@kcl.ac.uk

GSTM1a (class mu), GSTA1 and GSTA2 (class alpha) (Dierickx 1994). GSTs are an important family of isoenzymes involved in the cell's defence processes against potentially toxic compounds.

It has been assumed that organs performing increased metabolism (gastrointestinal and urinary tract) are exposed to continuous oxidative stress, which is responsible for several pathological disorders. The genus *Solidago* L., commonly known as goldenrod, is one of the largest genera of the Asteraceae family, which comprises a large group of medicinal plants with recognized therapeutic applications. The aerial parts of these plants have been used for centuries as an anti-inflammatory diuretic in bladder and kidney disorders, thought to be due to due to their flavonoid and saponin content. Their in-vitro antioxidant and anti-tumour activity has also been demonstrated (Kraus et al 1986; Apáti et al 2003).

In this study we investigated the in-vitro effect of the flavonol quercetin and some quercetin glycosides on the mixture of glutathione S-transferase enzymes found in hepatocytes. Different fractions from a methanolic extract of *Solidago canadensis* herb, as a model of a flavonoid-containing medicinal herb, were also tested.

## Materials and Methods

### Chemicals

All chemicals used were purchased from Sigma-Aldrich Chemical Co. (Gillingham, Dorset, UK).

### Plant material

*Solidago canadensis L.* (Asteraceae) was collected before full flowering state in Csenger, in the north-west of Hungary, in 2000. Aerial parts were used for extractions according to Hungarian Standard (MSZ 12341-1986) for Solidaginis herba. Plant samples were identified in the Department of Pharmacognosy, Semmelweis University, where voucher specimens are deposited (SOC-000826).

### Extraction and fractionation

Air-dried and powdered *S. canadensis* herb (50 g) was extracted at room temperature with $3 \times 150$ mL MeOH–$H_2O$ (1:1). After the evaporation of MeOH the aqueous residue was extracted with $H_2O$-saturated n-BuOH. The organic layers were combined, concentrated and dissolved in a minimum of MeOH. The methanolic solution was fractionated by column chromatography (CC) on Sephadex LH-20, using MeOH as eluant. Six major fractions were collected and, according to HPLC monitoring, a definite difference in chromatographic profile was found in the case of three fractions, therefore fraction 2 (F2), fraction 3 (F3) and fraction 4 (F4) with the crude extract (SH) were chosen for investigation in the Hep G2 cell GST assay. A greenish-yellow material was crystallized from fraction 4 during the evaporation process so it was filtered and the filtrate used as F4a.

### Analysis of extracts

The phenolic composition of the extracts was analysed by HPLC. Air-dried fractions were dissolved in pure MeOH to a final volume of 1 mL and 20 $\mu$L of the sample was injected. Phenolic compounds were separated by RP-HPLC and characterized by their UV spectra as previously described (Apáti et al 2002) and by their mass spectra by electrospray ionization (negative mode) using a Perkin-Elmer API 165 mass spectrometer (Norwalk, CT, USA). External calibration was used to quantify chlorogenic acid, flavonol monoglycosides, flavonol diglycosides and flavonoid aglycones using chlorogenic acid, isoquercitrin, rutin and quercetin, respectively. The quantification of each component was expressed in mg or in mg equivalent of the corresponding external standard and the final composition was expressed in percentage (w/w). A TLC method (silica gel–$CHCl_3$–$CH_3COOH$–MeOH–$H_2O$, 60:32:12:8 using acidic anisaldehyde for detection) was applied to investigate the saponin content of the samples.

### HepG2 cell culture

The HepG2 cells were grown in monolayer cultures to 80–90% confluency in 75 $cm^2$ flasks with 10 mL of Minimum Essential Medium Eagle (MEM) containing 10% (v/v) fetal bovine serum, 1% (v/v) nonessential amino acid and 1% (v/v) penicillin–streptomycin solution at 37°C with 5% $CO_2$. When the cells reached 80–90% confluency, they were harvested by trypsin (trypsin solution from porcine pancreas – 5.0 g $L^{-1}$ porcine trypsin in 0.2% $Na_4EDTA$, 0.9% sodium chloride) treatment and resuspended in supplemented growth medium. HepG2 cells were seeded at a density of $2 \times 10^4$ cells $cm^{-2}$ into 25 $cm^2$ flasks for GST assay. The cells were then incubated for 48 h in 25 $cm^2$ flasks with 10 mL of growth medium containing the freshly prepared dosing solutions (6.25, 12.5, 25.0, 50.0, 100.0 and 200.0 mM of flavonoid standards, 6.25, 12.5, 25.0, 50.0, 100.0 and 200.0 g $L^{-1}$ of SH and 2, 20 and 200 g $L^{-1}$ of various fractions in dimethyl sulfoxide (DMSO)) in concentration of 0.125% (v/v). Cells were examined microscopically after each treatment for evidence of cytotoxicity, due to saponins or other constituents, or other abnormalities, but no such evidence was seen with any of the cultures.

Cytosol, prepared from detached cells after treatment, was used for the GST assay. The growth medium (containing the dosing solutions) was replaced with pH 6.5 potassium phosphate buffer, and the samples were sonicated to lyse the cells. The lysed suspension was then immediately centrifuged at 10000 *g* for 5 min to separate cell debris from the cytosolic supernatant and the latter was immediately used to determine GST activity and glutathione (GSH) concentration.

### Glutathione S-transferase assay

GST activity was assayed by the spectrophotometric method of Habig et al (1974) optimised to study the enzyme-inducing effect of plant extracts using human hepatoma cell line HepG2. Total GST activity was measured with 1-chloro-2,4-dinitrobenzene (CDNB) as a relatively non-specific substrate for GST activity. Glutathione (250 mM) was prepared in water,

while CDNB (100 mM) was prepared in ethanol. To each well of 96-well plates, 10 $\mu$L of glutathione (8.33 mM) and 10 $\mu$L of CDNB (3.33 mM) were added to make a final volume of 300 $\mu$L in phosphate buffer (pH 6.5). The reaction was initiated by addition of cytosol (60 $\mu$L) and the increase in absorbance at 340 nm was recorded over 10 min at 37°C using a Dynatech microtitre plate reader. Enzyme activity was evaluated from the absorption curve using the extinction coefficient ($\varepsilon$=9.6 mM$^{-1}$ cm$^{-1}$) and expressed in mmol min$^{-1}$ (mg protein)$^{-1}$.

### Protein determination assay

Determination of tissue protein content was performed by the dye binding method of Bradford (1976).

### Data analysis

Statistical data analysis was performed with SPSS 10.0 for both one-way and two-way analysis of variance and Tukey's test. Differences with $P$ values < 0.05 were considered significant, as indicated in the appropriate figure legends.

## Results

### Characterization of fractions

Compounds were characterized by their HPLC retention times, UV spectra and mass spectra, and were identified on the basis of previously published data (Apáti et al 2002). Two groups of phenolic compounds have been identified – phenolic acids, such as caffeic acid, and chlorogenic acid (and possibly caffeoylshikimic acid-glucoside according to the mass spectrum), and flavonoids, such as flavonol diglycosides (quercetin-3-O-$\beta$-rutinoside (rutin), isorhamnetin-3-O-$\beta$-rutinoside (narcissin)), flavonol monoglycosides (quercetin-3-O-$\beta$-D-glucoside (isoquercitrin), kaempferol-3-O-$\beta$-D-glucoside (astragalin), 6″-acetyl-isoquercitrin, isorhamnetin-3-O-$\beta$-D-glucoside) and flavonol aglycones (quercetin, kaempferol, isorhamnetin) (Figure 1). These compounds had chromatographic and spectral characteristics similar to those previously identified in *Solidago canadensis L.* (Budzianowski et al 1990).

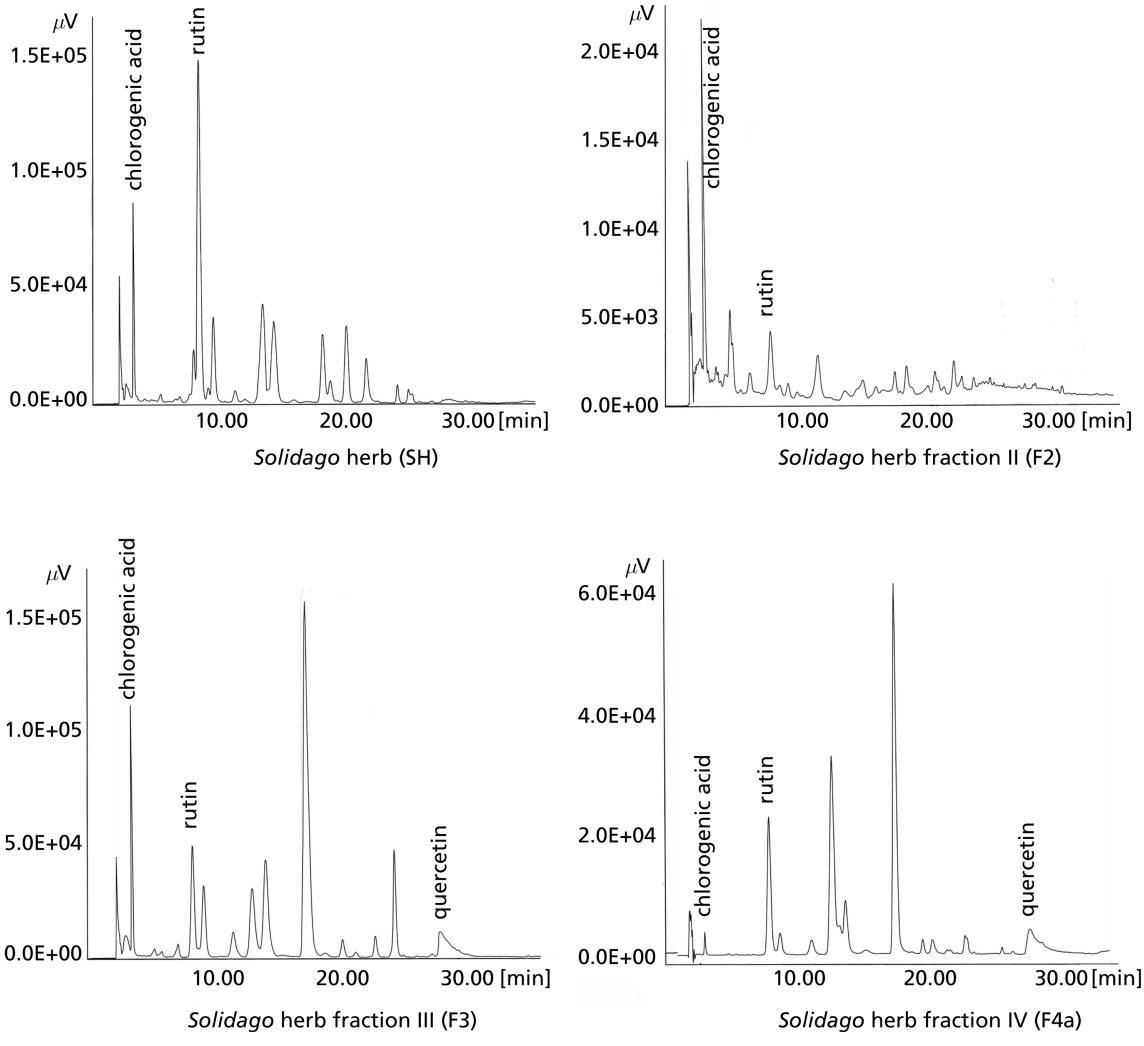


**Figure 1** HPLC chromatograms of polyphenolic compounds in *Solidago* herb.

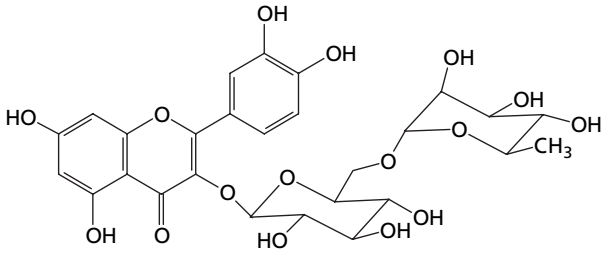

**Figure 2** Chemical structure of rutin.

Among the flavonol diglycosides, only rutin (Figure 2) was found in considerable amounts in the samples; all the remaining flavonoids proved to be monoglycosides, with the exception of quercetin, which was the only flavonol aglycone detected.

For simple and fast semi-quantitative determination of the phenolic components in the samples, external calibration was used to quantify caffeic acid derivatives, flavonol monoglycosides, flavonol diglycosides and flavonoid aglycones using chlorogenic acid, isoquercitrin, rutin and quercetin, respectively, as external standards. The quantification of each component was expressed in mg, or in mg equivalent of the corresponding external standard, and final composition was expressed in percentage (w/w) (Table 1).

The major component of the crude *Solidago* extract (SH) was rutin (8.93% w/w). Chlorogenic acid and other caffeic acid derivatives were present in the sample together with flavonols. Their concentration was close to 15% w/w of dry matter, corresponding to 149.7 mg equivalents of quercetin-3-O-$\beta$-D-galactoside/mg of SH.

F2 was rich in polar compounds, especially chlorogenic acid, which was the major phenolic acid present, and represented 19.21% w/w of the extract. The majority of flavonoids in F3 were identified as monoglycosides (18.29% w/w) of three aglycones, kaempferol, quercetin and isorhamnetin (Table 1). In addition, other compounds were detected that corresponded to rutin (5.98% w/w), quercetin (5.34% w/w) and caffeic acid derivatives (9.31% w/w).

F4a was shown to contain a high proportion of flavonoid glycosides (51.24% w/w). The low occurrence of phenolic acids (0.31% w/w) and flavonoid aglycones (2.30% w/w) provides a good opportunity to use this sample as a practically pure flavonoid glycosidic mixture.

Since *Solidago* species are known to contain large amounts of saponin glycosides, which may influence the membrane integrity of living cells, determination of saponin content of the samples was considered advisable. According to TLC, the presence of saponin glycosides was confirmed in relatively large amounts in SH and F2, and in detectable amounts in F3, while F4a proved to be free from saponins.

**Table 1** Polyphenol content (% w/w) of various *Solidago* herbal extracts

| Fraction<br>Component | SH<br>4156.19 mg | F2<br>826.38 mg | F3<br>1073.84 mg | F4a<br>436.02 mg |
|---|---|---|---|---|
| Phenolic acid | 7.57 | 23.19 | 9.31 | 0.31 |
| Flavonoid | 15.24 | 2.01 | 29.61 | 51.24 |
| -flavonol diglycoside | 8.93 | 2.01 | 5.98 | 17.35 |
| -flavonol monoglycoside | 6.16 | — | 18.29 | 31.59 |
| -flavonol aglycone | 0.15 | — | 5.34 | 2.30 |

Pure flavonoid standards were investigated for in-vitro GST-inducing activity, using the human hepatocyte cell line HepG2. The results were compared with the effect of different fractions obtained from *Solidago* herb as a model of a flavonoid-containing medicinal herb. $\beta$-Naphthoflavone was used as positive control since it is a well-documented phase II inducer of GST activity (Ioannides & Parke 1993).

HepG2 cells were exposed to the compounds in solution for a period of 48 h. The flavonoid standards and the crude extract of SH were applied in six concentrations (7.82, 15.63, 31.25, 62.50, 125.00 and 250.00 $\mu$M for the standards and 7.82, 15.63, 31.25, 62.50, 125.00 and 250.00 mg $L^{-1}$ for SH), while other plant derived samples were used in three concentrations of logarithmic scale (2.50, 25.00 and 250.00 mg $L^{-1}$) representing extremely low and high concentrations to investigate the possible toxic effects caused by the plant extracts.

In this experiment, HepG2 cells were exposed to various concentrations of the flavonoid standards quercetin, quercitrin and rutin representing the model structures of flavonol-aglycone, -monoglycoside and -diglycoside, respectively. Application of flavonoid standards caused a dose-dependent change in the activity of GST without any critical influence on cell-viability, assessed by microscopical evaluation. The flavonoid glycosides exerted significant increase in GST activity in HepG2 cells sampled 48 h after treatment, while definite inhibition was observed in the case of the aglycone quercetin (Figure 3).

The results of the GST assay, using HepG2 cells treated with *Solidago* herb, are also depicted in Figure 3. Dose-dependent increase in GST activity with SH can be observed,

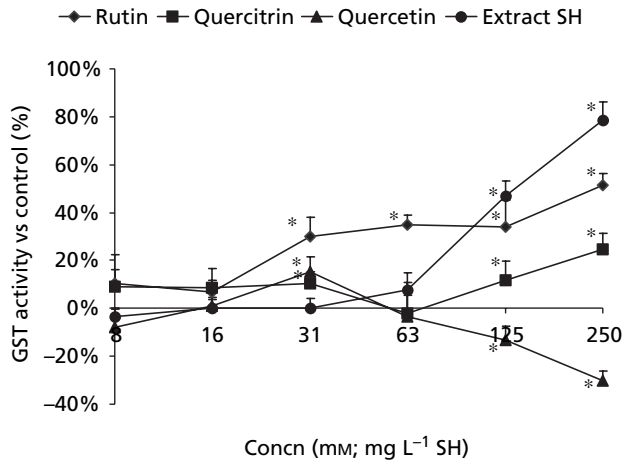


**Figure 3** Concentration-dependent inductive activity of different flavonoid standards (quercetin, quercitrin and rutin) and air-dried methanolic crude extract of *Solidago* herb SH on glutathione S-transferase enzyme in HepG2 cells sampled 48 h after treatment. Values are means ± s.d., n = 8, analysed by two-way analysis of variance. *$P < 0.05$, compared with control.

and the effect is significant at concentrations of 125.00 and 250.00 $\mu$g mL$^{-1}$. The highest amount of the sample applied at this stage of the experiment resulted in low protein concentration, indicating some toxic effects and decreasing cell viability (the shape of the cell wall changed, but no dead cells were noticeable during 48 h incubation), which excluded application of the results for estimating the quantitative effect of SH at this concentration. These concentrations are probably higher than those that would be reached in-vivo but the effect is noteworthy since it indicates the possibility of upregulation, even at lower doses.

Figure 4 depicts the results of experiments in which HepG2 cells were exposed to selected fractions of *Solidago* herb obtained by column chromatography, to determine whether the flavonoids were responsible for the effect of the crude extract. Increasing concentrations of F2 led to a similar, but stronger, activating effect on GST activity. Application of F3 gave a slight dose-dependent inhibition of the enzyme. The results could not be clearly attributed to any of the components but according to Ploemen et al (1993), caffeic acid derivatives present in relatively large amounts seem to be the most likely compounds responsible for the inhibition. As might be expected, fraction F4a, containing mainly flavonol glycosides, caused a slight increase in GST activity, but this was significant only at the highest concentration.

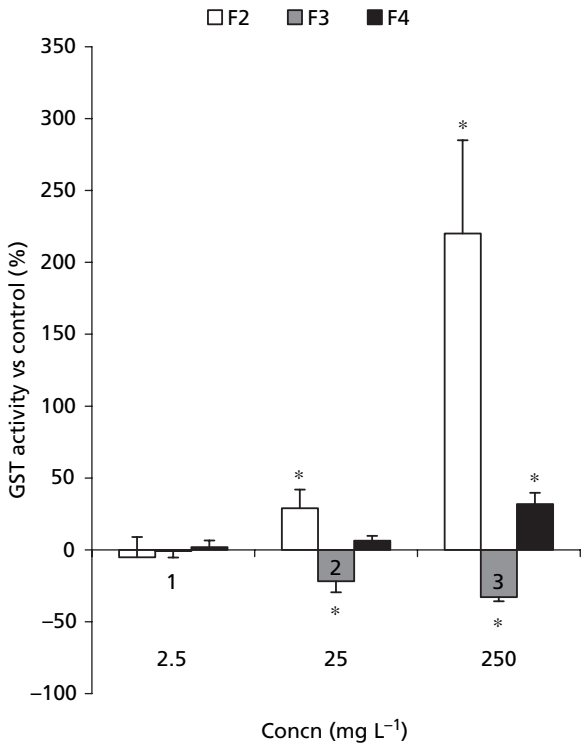


**Figure 4** Effect of F2, F3 and F4 from Sephadex LH-20 fractionation of *Solidago* herb extract SH on glutathione S-transferase using HepG2 cells sampled 48 h after treatment. Three concentrations (2.5, 25.0 and 250.0 mg L$^{-1}$) were studied. Values are means ± s.d., n = 4, analysed by one-way analysis of variance and individual results compared with Tukey's test. *$P < 0.05$, compared with control.

## Discussion

Previous studies have demonstrated that aerial parts of *Solidago canadensis* L. possess considerable antioxidant activity against free radicals, most likely due to the flavonoids and phenolic acids found in the extracts. The aim of this study was to investigate the effect of some flavonoids and plant-derived extracts on the phase II metabolizing enzyme glutathione S-transferase (GST), which plays an important role in the conjugative detoxification of electrophiles, as well as being an essential component of cellular antioxidant defence mechanisms (Yang et al 2001). Since GST exists as a mixture of isozymes, and the profile of these varies with the particular organ, the results reported here give an overall picture of the effect of the flavonoids on the activity of the GST complex in the liver, and other experiments would have to be carried out to determine the effect on the GST in kidneys, gut and platelets. The liver was thought to be the most important organ to study in the first place because of its predominance as a site of metabolic activity.

Quercetin is consumed in the diet predominantly in the form of glycosides. The absorption of quercetin is mainly dependent on the nature of glycoside (Hollman et al 1999). Quercetin is absorbed via the small intestine due to either active transport (Gee et al 1998) or luminal deglycosylation (Day et al 2000). If flavonoid glycosides are absorbed intact, deglycosylation depends on the specificity of cytosolic $\beta$-glycosidases (Day et al 1998), which justifies the investigation of flavonoid glycosides as potential substrates for the GST enzyme.

Current results show that the glutathione S-transferase activating ability of quercetin derivatives depends on the sugar moiety of the molecule, since the glycosides upregulated, while the aglycone inhibited, activity. A similar effect was seen with the *Solidago* herb extract and fractions as a natural source of flavonoid glycosides. It can also be concluded that in-vitro application of flavonoids and flavonoid-containing plant samples is free from toxic effects.

Our results are consistent with the literature data, where small effects on increased GST activity in connection with quercetin derivatives have been reported (Brouard et al 1988), and other results of significant activation (Hirose et al 1999; Teyssier et al 2001) or inhibition (Zhang & Das 1994) have been obtained. Most of these experiments were performed on rats in vivo. The model currently applied is somewhat simplistic so deductions from the process are necessarily somewhat speculative as regards what might occur in-vivo. The pharmacokinetics and metabolism of flavonoids is complex and they appear to exist in a mixture of aglycones, glycosides and glucuronides (Rice-Evans 2004), so making precise prediction of effects difficult.

However, the human hepatoma line HepG2 retains the activity of various phase I and phase II enzymes, which play a crucial role in the activation or detoxification of genotoxic procarcinogens, and so the use of this cell line reflects the metabolism of such compounds in-vivo better than experimental models with metabolically incompetent cells and exogenous activation mixtures (Knasmüller et al 1998).

Plant samples contain a wide variety of constituents, such as trace elements, phenolic acids, flavonoids and saponin compounds, which may influence the xenobiotic metabolising systems. This study has shown that the treatment of HepG2 cells with pure components such as flavonoids was able to affect GST activation and therefore may influence the toxicity and carcinogenicity of environmental chemicals, and act against free radical damage. Although this effect was mild compared with classic inducers (phenobarbital, $\beta$-naphthoflavone), the prevalence of flavonoids in complex plant samples may effectively contribute to the therapeutic potency of other agents.

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