Pergamon

Biochemical Pharmacology, Vol. 47, No. 11, pp. 2063–2068, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)E0072-S

INHIBITORY EFFECTS OF PLANT POLYPHENOLS ON RAT LIVER GLUTATHIONE *S*-TRANSFERASES

KAI ZHANG and NAGARATNAM P. DAS*

Laboratory of Flavonoid Research, Department of Biochemistry, Faculty of Medicine, National University of Singapore, Singapore 0511

(*Received* 13 *October* 1993; *accepted* 29 *December* 1993)

Abstract—Several novel naturally occurring flavonoids and other polyphenols exerted varying degrees of concentration-dependent inhibition on uncharacterized rat liver glutathione *S*-transferase (EC 2.5.1.18, GST) isoforms. The order of inhibitory potencies of the five most potent polyphenols was tannic acid > 2-hydroxyl chalcone > butein > morin > quercetin, and their IC_{50} values were 1.044, 6.758, 9.033, 13.710 and 18.732 μM, respectively. Their inhibitions were reversible, as indicated by dialysis experiments. The optimum pH for the inhibitions by four of the compounds (tannic acid, butein, 2-hydroxyl chalcone and morin) was in the range of pH 6.0 to 6.5, but for quercetin the optimum pH was 8.0. These potent inhibitors possess one or more of the following chemical structural features: (a) polyhydroxylation substitutions, (b) absence of a sugar moiety, (c) for the chalcones, the presence of an open C-ring and hydroxylation at either the C-2 or C-3 position, (d) for the flavonoids, the attachment of the B-ring to C-2, and (e) a double bond between C-2 and C-3. Butein exhibited a non-competitive inhibition toward both glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB). Interestingly, tannic acid showed a non-competitive inhibition toward CDNB but a competitive inhibition toward GSH. The inhibitory potency of tannic acid on rat liver GSTs was concentration and substrate dependent. Using CDNB, *p*-nitrobenzyl chloride, 4-nitropyridine-*N*-oxide, and ethacrynic acid as substrates, the IC_{50} values for tannic acid were 1.044, 11.151, 20.206, and 57.664 μM, respectively.

Key words: glutathione *S*-transferase; polyphenol; flavonoid; tannic acid; inhibition; rat liver

Glutathione *S*-transferases (EC 2.5.1.18, GSTs†) represent a family of multifunctional enzymes with important roles in detoxification processes. They catalyse the conjugation of electrophilic xenobiotics with the sulfhydryl moiety of glutathione (GSH), thus providing less toxic and more water-soluble derivatives [1]. It has been suggested that increased expression of GSTs in tumor cells contributes to the resistance against certain anti-cancer chemotherapeutic drugs by GST-mediated conjugation reactions [2–4]. Therefore, it is interesting to study the effects of inhibitory agents on GST activity and the possibility of sensitization of drug-resistant tumor cells by inhibiting GST activity. It would be beneficial to find suitable GST inhibitors that can cause drug-resistant cells to become sensitive to certain anti-cancer drugs. *In vitro*, sensitization of cultured tumor cells to alkylating agents, such as chlorambucil, has been achieved by adding relatively nontoxic GST inhibitors to the culture medium [5, 6].

The flavonoids form a class of plant-derived polyphenolic substances that have various biological and pharmacological effects [7, 8]. They are generally considered nontoxic [9] and are often found in many traditional herbal medicines [10] and in the human diet (about 1 g/day) [11]. Only a limited number of polyphenolic compounds have been reported previously to inhibit GST activity [12–14]. In view of the important roles that may be exerted by GST inhibitors, we studied the inhibitory effects of eighteen flavonoids and eight other polyphenols on uncharacterized GST isoforms isolated from rat liver. These plant polyphenols exhibited varying degrees of inhibitory potencies, and thus their structure–activity relationships were rationalized. The five most potent inhibitors were selected for further studies to determine their inhibitory characteristics and substrate specificities.

MATERIALS AND METHODS

The flavonoids, ellagic acid, tannic acid and other polyphenols were obtained from Extrasynthese-Genay (France). GSH, 1-chloro-2,4-dinitrobenzene (CDNB), *p*-nitrobenzyl chloride, 4-nitropyridine-*N*-oxide and ethacrynic acid were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.).

GST enzymes. Rat liver GSTs (a mixture of isoenzymes having the following activities: 13–16 μmol/min/mg protein using CDNB as substrate, 2.755 μmol/min/mg protein using *p*-nitrobenzyl chloride as substrate, 0.051 μmol/min/mg protein using 4-nitropyridine-*N*-oxide as substrate, and 0.204 μmol/min/mg protein using ethacrynic acid as substrate at 37°) were purified from rat liver by *S*-hexylglutathione affinity chromatography as described by Reddy *et al.* [15].

Protein concentration was determined by the method of Lowry *et al.* [16].

GST inhibition assay. The inhibition studies were carried out at pH 6.5 and 37° using CDNB, *p*-

* Corresponding author. Tel. (65) 7723243; FAX (65) 7791453.

† Abbreviations: GSTs, glutathione-*S*-transferases; GSH, glutathione; and CDNB, 1-chloro-2,4-dinitrobenzene.

nitrobenzyl chloride, 4-nitropyridine-*N*-oxide and ethacrynic acid as substrates. The polyphenols were dissolved in DMSO and stock solutions (10 mM) were freshly prepared. Aliquots of these solutions were then added to the reaction mixture to yield a final concentration range of 0.2 μM to 1 mM. In all assays, the final concentration of DMSO did not exceed 2% (by vol.), and this concentration of DMSO did not affect the rat liver GSTs (data not shown).

Enzyme assay. GST activity was determined according to the method of Habig *et al.* [17]. Enzyme activity was expressed as micromoles of substrate–GSH conjugate produced per minute per milligram of protein at 37°, using extinction coefficients of 9.6, 1.9, 7.0 and 5.0 mM^{-1} cm^{-1} for the substrates CDNB, *p*-nitrobenzyl chloride, 4-nitropyridine-*N*-oxide and ethacrynic acid, respectively. The IC_{50} values were calculated by linear regression of no less than five points in the range of 20–80% inhibition. Each point was the mean of at least two determinations, each performed in duplicate.

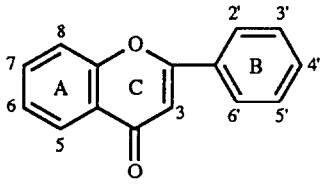
Reversibility of the inhibition. Dialysing experiments were carried out using standard cellulose dialysis tubing having a molecular cutoff in the range of 12,000–40,000 Da. The rat liver GSTs (46 μg) were incubated in aliquots of dialysing buffer (2 mL) containing one of the following compounds: tannic acid (20 μM), butein (200 μM), morin (300 μM), 2-hydroxyl chalcone (300 μM) or quercetin (400 μM) at ambient temperature for 15 min. Aliquots (500 μL) of each incubation mixture were removed and analyzed for GST activities [17]. Additional aliquots of the incubation mixture (1.5 mL) were used to dialyse against the dialysing buffer, which consisted of 0.125 M potassium phosphate buffer (containing 1 mM EDTA), pH 6.5, at 4° for 24 hr. The GST activities were also determined after dialysis. The controls were without inhibitors and were treated the same way as described for the test compounds.

Effects of pH. The effects of pH on inhibition of rat liver GSTs by the five most potent test compounds were determined. The concentrations used for the respective inhibitors were: tannic acid (1 μM), butein (10 μM), morin (15 μM), quercetin (15 μM) and 2-hydroxyl chalcone (15 μM). The GST activities were determined at pH levels of 6.0, 6.5, 7.0, 7.5 and 8.0, respectively.

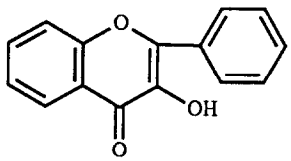
Kinetics. Enzyme inhibitory kinetic studies were carried out using various concentrations of the two

Table 1. Comparison of inhibitory potencies of different flavonoids on rat liver GST activity using CDNB as a substrate*

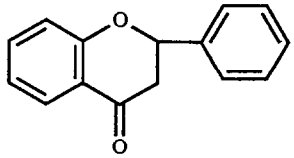
Flavone



Flavonol



Flavanone

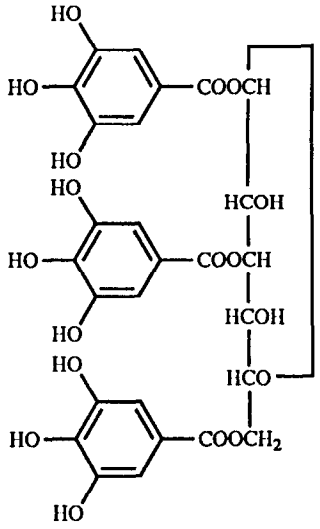
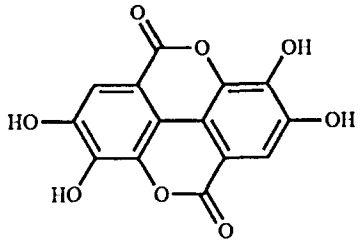
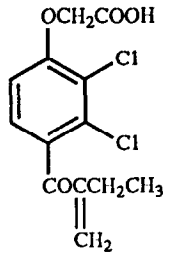
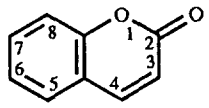
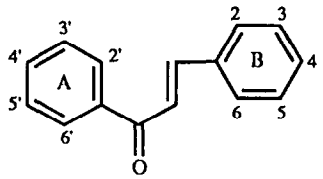


Flavonoids	Class	Hydroxylation pattern	Substitution	IC_{50} (μM)
Morin	Flavonol	3,5,7,2′,4′		13.710
Quercetin	Flavonol	3,5,7,3′,4′		18.732
Isorhamnetin	Flavone	3,5,7,4′	C-3′ = —OCH_3	23.219
Myricetin	Flavonol	3,5,7,3′,4′,5′		24.840
Acacetin	Flavone	5,7	C-4′ = —OCH_3	25.265
Apigenin	Flavone	5,7,4′		29.159
Rutin	Flavonol	5,7,3′,4′	C-3 = rutinose	34.760
Diosmin	Flavone	5,3′	C-7 = rutinose C-4′ = OCH_3	36.909
Myricitrin	Flavonol	5,7,3′,4′,5′	C-3 = rhamnose	41.358
Diosmetin	Flavone	5,7,3′	C-4′ = OCH_3	42.277
Apiin	Flavone	5,4′	C-4 = *O*-glucose-apiose	57.049
Naringenin	Flavanone	5,7,4′		66.083
Quercitrin	Flavonol	5,7,3′,4′	C-3 = rhamnose	76.631
Isoquercitrin	Flavonol	5,7,3′,4′	C-3 = glucose	77,713
Daidzein	Isoflavone	7,4′	B-ring at C-3	84,878
Biochanin A	Isoflavone	5,7	B-ring at C-3	85.173
Naringin	Flavanone	7,4′	C-4′ = —OCH_3 C-5 = rhamnose-glucose	179.84
Catechin†	Flavan-3-ol	3,5,7,3′,4′		983.734

* The flavonoid concentrations used were in the range of 5 μM to 1 mM. The GST activity assay was carried out according to Habig *et al.* [17]. The IC_{50} values were calculated by linear regression of no less than five points in the range of 20–80% inhibition. Each point is the mean of at least two determinations, each performed in duplicate.

† Flavonoids without a carbonyl group at C-4 and a double bond between C-2 and C-3.

Table 2. Comparison of the inhibitory potencies of polyphenols on rat liver GST activity using CDNB as a substrate*

Compounds	Structures	IC_{50} (μM)
Tannic acid		1.044
Ellagic acid		37.075
Ethacrynic acid		6.315
Coumarin		569.466
Chalcone		23.479

	C-2	C-3	C-4	C-2′	C-4′	C-6′	
2-Hydroxyl chalcone	H	H	H	H	H	H	6.758
Butein	H	OH	OH	H	OH	OH	9.033
Phloretin	H	H	OH	OH	OH	OH	68.625
Phloridzin	H	H	OH	O-glucose	OH	OH	204.601

* The polyphenol concentrations used were in the range of 0.2 μM to 1 mM. The GST activity assay was carried out according to Habig *et al.* [17]. The IC_{50} values were calculated by linear regression of no less than five points in the range of 20–80% inhibition. Each point is the mean of two determinations, each performed in duplicate.

substrates, namely GSH and CDNB, and two fixed concentrations of either tannic acid (0.4 and 1 μM) or butein (5 and 10 μM).

Statistical analysis. The results obtained from the dialysis experiments were analysed using Student's *t*-test [18].

RESULTS AND DISCUSSION

Effects of polyphenols on rat liver GST activity. Twenty-six polyphenols examined for their effects on rat liver GST activity showed variable inhibitory potencies. The IC_{50} values for the flavonoids (Table 1) and other polyphenols (Table 2) on the uncharacterized rat liver GSTs were obtained. A high degree of linearity was observed in the concentration–response curves for all the compounds tested, with r^2 values ranging from 0.945 to 0.999.

Generally, most of the natural plant polyphenols exhibiting potent GST inhibition have polyhydroxylations (e.g. tannic acid), but those that have single hydroxylation (e.g. 2-hydroxyl chalcone) have, in addition, other chemical features that affect their potencies (Tables 1 and 2). Merlos *et al.* [14] also reported that the hydroxylation pattern was important for inhibition of GST activity by flavonoid compounds. Flavonoid glycosides showed weaker inhibitory potencies. A similar observation was also reported for a wide range of enzymatic activities [7, 19–23]. In our present study, quercetin, myricetin, naringenin, apigenin, phloretin and morin were at least 2-fold more potent in their inhibitory effects on rat liver GSTs than their corresponding glycosides. Only diosmin was an exception, and this may be due to the presence of $-OCH_3$ in the B-ring. Acacetin and isorhamnetin also possess $-OCH_3$ in the B-ring, and they also displayed increased inhibitory potencies. All of the potent flavonoids (morin, quercetin, isorhamnetin, myricetin acacetin, apigenin) have hydroxylations at C-5 and C-7 in the A-ring, a carbonyl group at C-4, and a double bond between C-2 and C-3. They all belong to either the flavone or flavonol class of flavonoids. Interestingly, when the B-ring of the flavonoid molecule is attached to the C-3 position (i.e. the isoflavone class), their inhibitory potencies decreased (see daidzein and biochanin A). Coumarin (without a B-ring) exerted reduced inhibitory action on the enzyme. The flavan-3-ol (catechin) is also a poor inhibitor, possibly due to the absence of some of the chemical structural features (mentioned above) that are required for inhibitory potencies.

The chalcone, 2-hydroxyl chalcone, and butein (Table 2) were very potent inhibitors. However, they do not possess a C-ring, and their potencies appeared to be associated with the presence of a hydroxyl substitution at either the C-2 or C-3 position (compare with phloretin and phloridzin which lack such substitution and are poor inhibitors). We have reported earlier that the open C-ring type of polyphenols shows increased inhibitory potencies on rat adipocyte cyclic AMP phosphodiesterases [19].

Reversibility of the inhibition. The five most potent polyphenolic inhibitors, tannic acid, quercetin, butein, 2-hydroxyl chalcone and morin, were selected for further studies on the reversibility of their inhibition. Rat liver GSTs were incubated with these inhibitors, after which the inhibitors were removed by dialysis. Although the rat liver GST activities were inhibited up to 76%, the activities recovered significantly after dialysis for 24 hr (Table 3, N = 4, $P < 0.01$). The loss of activity in the controls was only <6% after dialysis. However, the activity could not be recovered to the control level even after 24 hr of dialysis. This finding indicates that, although the inhibition by the test compounds of the rat liver GST enzymes is reversible, the attachment of these inhibitors to the enzymes requires more drastic treatment(s) or a longer period of dialysis to obtain the same level of activity as observed for the dialysed controls.

Table 3. Effects of dialysis on the remaining activities of rat liver GSTs incubated with the five most potent polyphenol GST inhibitors*

Plant polyphenols	Remaining enzyme activities (% of controls)	
	Before dialysis	After dialysis
Tannic acid	45.76 ± 4.14	64.61 ± 0.43
Butein	46.13 ± 3.33	75.82 ± 6.15
Morin	41.33 ± 0.74	59.66 ± 1.64
2-Hydroxyl chalcone	25.47 ± 5.03	65.86 ± 4.26
Quercetin	32.70 ± 1.55	52.55 ± 2.33

* Experiments were carried out as described in Materials and Methods. After dialysis, the GST activities of incubation solutions were significantly ($P < 0.01$, Student's *t*-test) different from the data obtained before dialysis. Control activities before and after dialysis were 10.924 and 10.295 μmol/min/mg protein, respectively. Values are means ± SD, N = 4.

Effects of pH on the inhibition of GSTs by the potent inhibitors. The concentrations for the five most potent inhibitors, tannic acid, butein, 2-

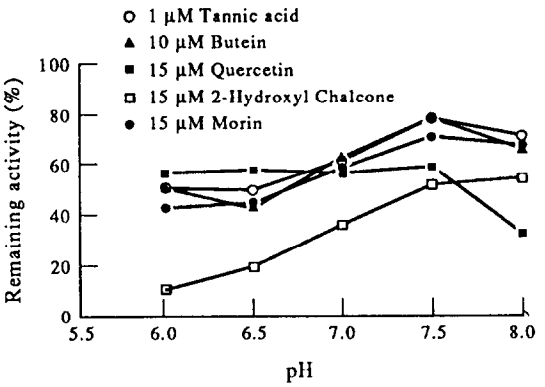


Fig. 1. Effects of pH on the inhibition of rat liver GSTs by five potent inhibitors. Experiments were performed as described in Materials and Methods. Each point represents the mean of two determinations, each performed in duplicate. Control activities (100% remaining activity) were: 10.337, 13.673, 15,058, 12.338 and 10.020 μmol/min/mg protein at pH 6.0, 6.5, 7.0, 7.5 and 8.0, respectively.

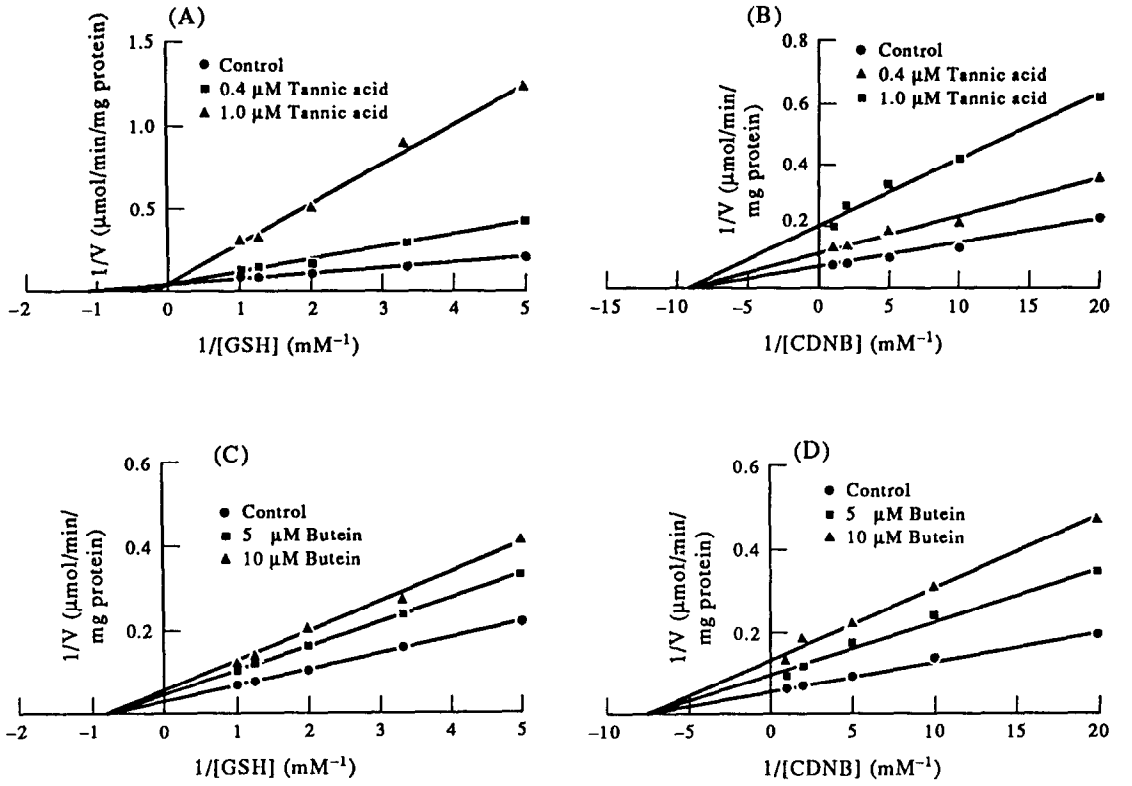


Fig. 2. Enzyme inhibitory kinetics of tannic acid (A and B) and butein (C and D) on rat liver GSTs. Lineweaver–Burk plots showing (i) competitive inhibition toward GSH (A) and non-competitive inhibition toward CDNB (B) by tannic acid, and (ii) non-competitive inhibition toward GSH (C) and CDNB (D) by butein. The assay was carried out as described in Materials and Methods. The substrate concentrations for GSH and CDNB ranged from 0.2 to 1 mM and 0.05 to 1 M, respectively. Values are the averages of duplicate determinations.

hydroxyl chalcone, morin and quercetin, were fixed at 1, 10, 15, 15 and 15 μM, respectively. GST activity was then determined at pH levels of 6.0, 6.5, 7.0, 7.5 and 8.0, respectively, for each inhibitor. Generally, the inhibitory potencies of tannic acid, butein, 2-hydroxyl chalcone and morin, but not that of quercetin, decreased with a rise in pH (6.5 to 7.5, see Fig. 1). However, with a further increase in the pH, tannic acid, butein, morin and quercetin showed a slight increase in their inhibitory potencies, but the action of the 2-hydroxyl chalcone decreased slightly. The optimum pH for the inhibitions by four compounds (tannic acid, butein, 2-hydroxyl chalcone and morin) was in the range of pH 6.0 to 6.5, but for quercetin the optimum pH was 8.0.

Enzyme inhibition kinetic studies of tannic acid and butein. Tannic acid and butein were selected for enzyme inhibitory kinetic experiments. Lineweaver–Burk plots showed that tannic acid exerted competitive inhibition toward GSH (K_m increased while V_{max} remained unchanged; Fig. 2A), but non-competitive inhibition toward CDNB (V_{max} decreased while K_m remained almost unchanged; Fig. 2B). Butein showed non-competitive inhibition toward both GSH and CDNB (V_{max} decreased while K_m remained unchanged; see Fig. 2, C and D).

Inhibitory effects of tannic acid on rat liver GSTs toward different substrates. Tannic acid showed concentration-dependent inhibition of rat liver GSTs for the following substrates: CDNB, *p*-nitrobenzyl chloride, 4-nitropyridine-*N*-oxide and ethacrynic acid (Fig. 3). The IC_{50} values toward these substrates were 1.044, 11.151, 20.206, and 57.664 μM, respectively. These results indicate that tannic acid is able to inhibit GST activity toward a wide range of substrates.

Interest in the use of relatively nontoxic inhibitors of GST activity to enhance the effects of drugs in the treatment of tumors is increasing. Ethacrynic acid has been reported as a GST inhibitor for this purpose [5, 6, 24]. However, chronic exposure to ethacrynic acid could produce cellular alterations that can diminish the potentiation of cytotoxicity initially exerted by the drug. Ethacrynic acid can also induce its own metabolism, and some cells can acquire resistance to it through the higher expression of GSTs [25]. Interestingly, the present results indicate that tannic acid is a more potent inhibitor than ethacrynic acid of rat liver GSTs (Table 2). This compound is a nontoxic polyphenol present in plants and the human diet [9–11]. Therefore, our data allow possible exploitation and further studies

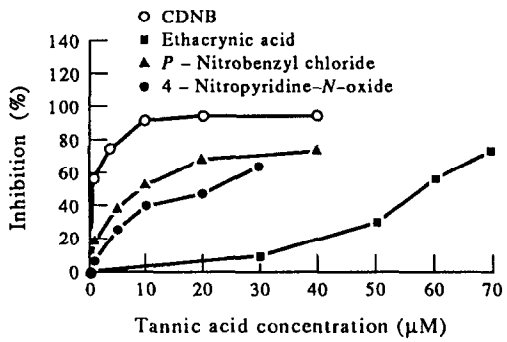


Fig. 3. Inhibition by tannic acid of rat liver GST activity toward different substrates. GST activity was measured according to the method of Habig *et al.* [17]. Values are the averages of two determinations, each performed in duplicate. Control activities (0% inhibition) were: 14.787, 2.755, 0.204, and 0.051 μmol/min/mg protein using CDNB, *p*-nitrobenzyl chloride, ethacrynic acid and 4-nitropyridine-*N*-oxide as substrate, respectively.

of the inhibitory effects of the potent plant polyphenols on various GSTs of human tumor cells as well as the sensitization of these cells to some anticancer drugs.

Acknowledgements—The authors are grateful to the National University of Singapore for the research scholarship awarded to Kai Zhang. We are also very grateful for the research grant received from the Shaw Foundation of Singapore.

REFERENCES

1. Mannervik B, The isoenzymes of glutathione-*S*-transferases. *Adv Enzymol* **57**: 357–417, 1985.
2. Sato K, Glutathione *S*-transferases as markers of preneoplasia and neoplasia. *Adv Cancer Res* **52**: 205–255, 1989.
3. Dulik DM, Fenselau C and Hilton J, Characterization of melphalan-glutathione adducts whose formation is catalyzed by glutathione transferases. *Biochem Pharmacol* **35**: 3405–3409, 1986.
4. Schecter R, Alaoui-Jamali MA and Batist G, Glutathione *S*-transferases in chemotherapy resistance and in carcinogenesis. *Biochem Cell Biol* **70**: 349–353, 1992.
5. Hansson J, Berhane K and Castro MV, Sensitization of human melanoma cell to the cytotoxic effect of melphalan by the glutathione transferase inhibitor ethacrynic acid. *Cancer Res* **51**: 94–98, 1991.
6. Tew KD, Bomber AM and Hoffman SJ, Ethacrynic acid and piriprost as enhancers of cytotoxicity in drug resistant and sensitive cell lines. *Cancer Res* **48**: 3622–3625, 1988.
7. Havsteen B, Flavonoids, a class of natural products of high pharmacological potency. *Biochem Pharmacol* **32**: 1141–1148, 1983.
8. Kuppusamy UR and Das NP, Effects of flavonoids on several venom hyaluronidases. *Experientia* **47**: 1196–1200, 1991.
9. Yugarani T, Tan BKH, Teh M and Das NP, Effects of polyphenolic natural products on the lipid profiles of rats fed high fat diets. *Lipids* **27**: 181–186, 1992.
10. Haslam E, Lilley TH, Ya C, Martin R and Magnolato D, Traditional herbal medicine. The role of polyphenols. *Planta Med* **55**: 1–8, 1989.
11. Kuhnau J, The flavonoids: A class of semi-essential food components: The role in human nutrition. *World Rev Nutr Diet* **24**: 117–121, 1976.
12. Das M, Bickers DR and Mukhtar H, Plant phenols as *in vitro* inhibitors of glutathione *S*-transferases. *Biochem Biophys Res Commun* **120**: 427–432, 1984.
13. Das M, Singh SV, Mukhtar H and Awasthi YC, Differential inhibition of rat and human glutathione *S*-transferase isoenzymes by plant phenols. *Biochem Biophys Res Commun* **141**: 1170–1176, 1986.
14. Merlos M, Sanchez RM, Camarasa T and Adzet T, Flavonoids as inhibitors of rat liver cytosolic glutathione *S*-transferases. *Experientia* **47**: 616–619, 1991.
15. Reddy CC, Burgess JR, Gong ZZ, Massaro ET and Tu CP, Purification and characterization of the individual glutathione *S*-transferases from sheep liver. *Arch Biochem Biophys* **224**: 87–101, 1983.
16. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
17. Habig WH, Pabst MT and Jacoby WB, Glutathione-*S*-transferases: The first enzyme step in mercapturic acid formation. *J Biol Chem* **249**: 7130–7139, 1974.
18. Snedecor GW and Cochran WG, *Statistical Methods*, pp. 172–186. Iowa State University Press, Ames, IA, 1971.
19. Kuppusamy UR and Das NP, Effects of flavonoids on cyclic AMP phosphodiesterase and lipid mobilization in rat adipocytes. *Biochem Pharmacol* **44**: 1307–1315, 1992.
20. Kuppusamy UR, Khoo HE and Das NP, Structure–activity studies of flavonoids as inhibitors of hyaluronidases. *Biochem Pharmacol* **40**: 397–401, 1990.
21. Ratty AK and Das NP, Effects of flavonoids on non-enzymatic lipid peroxidation: Structure–activity relationship. *Biochem Med Metab Biol* **39**: 69–79, 1988.
22. Moroney MA, Alcaraz MT, Forder RA, Carey F and Hoult RS, Selectivity of neutrophil 5-lipoxygenase and cyclo-oxygenase inhibition by an anti-inflammatory flavonoid glycoside and related aglycone flavonoids. *J Pharm Pharmacol* **40**: 787–792, 1988.
23. Vernet A and Siess MH, Comparison of the effects of various flavonoids on ethoxycoumarin deethylase activity of rat intestinal and hepatic microsomes. *Food Chem Toxicol* **24**: 857–861, 1986.
24. Smith MT, Evans CG, Doane-Setzer P, Castro VM, Tahir MK and Mannervik B, Denitrosation of 1,3-bis (2-chloroethyl)-1-nitrosourea by class mu glutathione transferases and its role in cellular resistance in rat brain tumor cells. *Cancer Res* **49**: 2621–2625, 1989.
25. Kuzmich S, Vanderveer LA, Walsh ES, Lacreta FP and Tew KD, Increased levels of glutathione-*S*-transferase transcript as a mechanism of resistance to ethacrynic acid. *Biochem J* **281**: 219–224, 1992.