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Isozyme- and gender-specific induction of glutathione *S*-transferases by flavonoids

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Abstract Most dietary flavonoids have antioxidant activity in vitro however, secondary mechanisms such as the ability to influence gene expression with the consequent modulation of specific enzymatic activities involved in the intracellular response against oxidative stress, are being realized. In the following study, we examined the ability of the flavonoids: flavone, morin, naringenin, (+)-catechin, and quercetin to modulate the activity of glutathione S-transferases (GSTs) mGSTA, mGSTP and mGSTM in hepatic tissues of male and female Swiss Webster mice. Subchronic dietary exposure to morin, naringenin, (+)-catechin, and quercetin (2,500 mg/kg diet for 20 days) did not produce statistically significant changes in GST activity. Conversely, gender-, and isozyme-specific induction of mGSTs were observed in animals fed flavone. A sevenfold increase in total mGST activity was observed in female animals whereas a fourfold increase was observed in male animals. Enzyme specific assays indicate that there were greater increases of both mGSTM (eightfold) and mGSTP (fourfold) activities in females as compared to males (sixfold and twofold, respectively). As testosterone is involved in the regulation of GSTs in mice, castrated males were fed flavone for 5 days (2,500 mg/kg diet). In this case, dietary flavone resulted in similar fourfold increases in total GST activity in inact and castrated animals. Isozyme specific studies indicate that increases could be attributed to an induction of mGSTM and mGSTP.

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Introduction

Glutathione transferases (GSTs; EC 2.5.1.18) are the primary mammalian enzyme system responsible for detoxifying chemical carcinogens and by-products of lipid peroxidation (Hayes and Pulford 1995). There is considerable evidence that the induction of phase II enzymes, such as the GSTs, can protect against the development of chemically induced cancers and oxidative stress by increasing the metabolism of electrophilic intermediates and reactive oxygen species (Hayes and Pulford 1995; Benson et al. 1978, 1979; Kensler et al. 1999). Epidemiological studies suggest diets rich in fruits and vegetables protect against cardiovascular disease and vascular dysfunction (Hertog et al. 1993; Schroeter et al. 2006), however are less clear for cancer (Steinmetz and Potter 1996; Scalbert et al. 2005). There are several cellular and molecular mechanisms suggested for the anti-carcinogenic activity associated with fruits and vegetables which have been recently reviewed (Chen and Kong 2005; Moon et al. 2006). Mechanisms include the ability of certain dietary agents to inhibit the initiation stage of carcinogenesis by some combination of inhibiting phase I metabolism and/or inducing phase II metabolism or by acting as suppressing agents, which stop or reverse the promotion and progression of cancer. Isothiocyanates as well as several flavonoids (e.g. quercetin, morin and flavone) demonstrate the ability to induce phase II metabolism (Munday and Munday 2004; Siess et al. 1996; Gandhi and Khanduja 1993; Kawabata et al. 2001). Interpreting these results is not always straightforward, for example, in human hepatoma cells in

culture, quercetin aglycone does not induce GST protein or activity (Musonda et al. 1997), whereas quercetin-4'-glucoside (a natural form in plants) increases the activity of quinone reductase (a phase II marker enzyme) in Hepalclc7 cells (Williamson et al. 1996). Nonetheless, dietary flavonoids capable of inducing phase II metabolism are generally considered good targets for chemopreventative agents due to their low toxicity.

Cytosolic GSTs comprise a large family of detoxification enzymes that function as hetero- or homodimers and are classified by placement into one of seven species-independent gene classes [for recent review see Hayes et al. (2005)]. The most abundant classes expressed in mammalian tissues are Alpha (GSTA), Mu (GSTM) and Pi (GSTP). The pattern of GST isozyme expression varies during development and aging, and is organ-, sex- and species-specific (Egaas et al. 1995; Gopal et al. 2000; Mitchell et al. 1997; Igarashi and Satoh 1989; Singhal et al. 1992). Distinct gender-related differences in GST expression have been shown in the rat, mouse and human. For example, in the mouse basal levels of soluble hepatic GST are higher in males as compared to females (1,136 μg/g vs 1,006 μg/g wet wt) with greater levels of GSTP in males (295 μg/g vs 91 (μg/g wet wt) and higher levels of GSTA in females (474 μg/g vs 387 μg/g wet wt) (Mitchell et al. 1997). Studies of human colon and skin indicate that females contain relatively higher amounts of GSTP as compared to males, while expression of GSTA is approximately twofold higher in male colon than in female colon (Singhal et al. 1992). Gender-related variations in expression of GSTs may contribute to observed sex-related differences in incidences of certain types of cancer (Benson et al. 1989; Million et al. 1989).

Cellular regulation of GST expression is complex and influenced by many factors including ligands that activate the antioxidant response element (ARE)/electrophile response element (EpRE) through nuclear factor E2-related factor 2 (Nrf2) and endogenous hormones (Moi et al. 1994). Diet-derived compounds that up-regulate GST include ortho phenols (Fiander Schneider 2000), butylated hydroxyanisole (BHA) (Nijhoff and Peters 1992), sulforaphane (Munday and Munday 2004) and flavone (Nijhoff et al. 1995; Kang et al. 2003; Burns et al. 2004). Endogenous regulation of GST expression in tissues occurs through the action of glucocorticoids (Falkner et al. 2001), growth (GH) and steroid hormones (Srivastava and Waxman 1993; Staffas et al. 1998; Lamartiniere 1981; Hatayama et al. 1986). Sexual dimorphism in the expression of mGSTP in mice is associated with the sexually dimorphic secretion of GH and testosterone (Hatayama et al. 1986). For example, Hatayama et al (1986) demonstrated that the basal levels of mGSTP in males can be decreased to levels found in females by castrating the males, whereas the levels in females can be increased to those of adult males by administration of testosterone. A similar result was also described in rats (Srivastava and Waxman 1993; Staffas et al. 1998). More recently, it has been suggested that 17β-estradiol (E2) and other phytoestrogens represses GST expression in hormone responsive tissues by interfering with ARE-mediated gene transcription (Ansell et al. 2004). The interface between dietary modifiers of metabolism and gender may be a significant consideration when interpreting epidemiological studies relating diet to health or disease status.

The aim of this study was to investigate the influence of several dietary flavonoids on the induction of mGSTA, mGSTM and mGSTP in male and female male mice over a 20-day period. Flavonoids are a structurally diverse group of phenolic antioxidants that are common components of the Western diet. Four classes of flavonoids, highlighting differences in C-ring saturation and B-ring hydroxylation patterns were investigated and included the flavonols quercetin and morin, the flavon-3-ol catechin, the flavanone naringenin, and flavone (Fig. 1). Initial studies demonstrated that flavone produced a marked gender- and isozyme-specific increase in the activity of mGSTP and mGSTM. Because testosterone is involved in the regulation of GST in the mouse, the influence of flavone on GSTs in castrated males was also investigated.

Experimental

Materials

Flavone (2-phenyl benzopyrone; 97%), morin hydrate (3,5,7,2′,4′-pentahydroxyflavone; 95%), and naringenin (5,7,4′-trihydroxyflavone) were purchased from Sigma-Aldrich; (+)-catechin [(+)-3,3′,4′,5,7-flavanpentol; 98%], 1-chloro-2, 4-dinitrobenzene (98%), ethacrynic acid, glutathione reductase (G3664), cumene hydroperoxide (80%), and tris(hydroxymethyl)aminomethane hydrochloride were purchased from Sigma-Aldrich, bromosulfophthalein from ICN; quercetin dehydrate (3,5,7,3′4′-pentahydroxyflavone; 99%) from Acros. Bradford protein dye reagent was purchased from BioRad. All other reagents were analytical grade and purchased from Fisher.

Animal care and sample preparation

Male (25–30 g) and female (18–20 g) Swiss Webster mice were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, MA, USA). Animals were housed in AAALAC-accredited animal care facilities in wire bottom cages and acclimated for 1 week prior to diet manipulation. Within gender, mice were randomly assigned to each

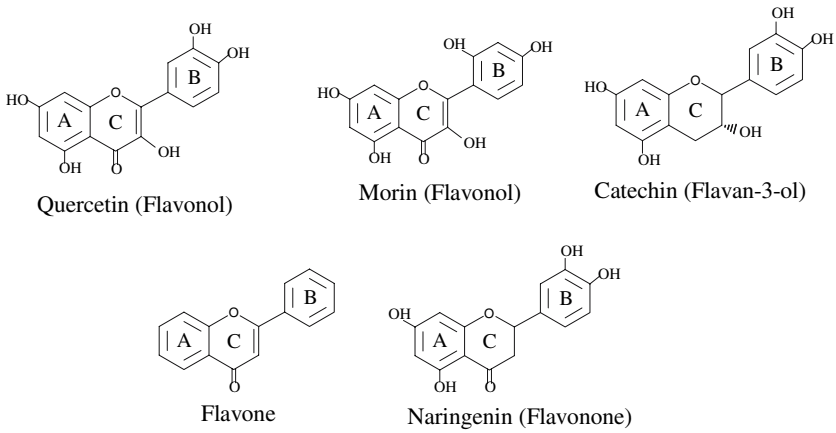

Fig. 1 Flavonoid structures: (1) quercetin (flavonol), (2) morin (flavonol), (3) catechin (flavan-3-ol), (4) flavone and (5) naringenin (flavonone)

treatment group. Animals were fed ad libitum either a casein based control diet (AIN-76A, Dyets Inc.) or the control diet with one of the flavonoids (flavone, quercetin, catechin, morin, or naringenin) incorporated at 2,500 mg/kg diet. The animals were maintained on the diet for 5, 10 or 20 days. Animals were euthanized with carbon dioxide and the livers removed on days 6, 11, or 21 respectively. Control groups were sacrificed at each time point.

Liver tissues from each group (four to six animals/group) were combined and homogenized in 10 mM Tris–HCl buffer (ph 7.4) containing 250 mM sucrose and 1 mM each EDTA and DTT (3:1 buffer:liver). Liver homogenates were centrifuged at 4°C for 20 min at 9,500 g. The supernatant was collected and centrifuged at 100,000*g* for 80 min at 4°C. The resulting supernatant was collected and specific activity was determined immediately following centrifugation.

A subset of animals were used to evaluate the effect of testosterone. Male animals were castrated and fed either the casein based control diet (four animals) or the casein based diet with flavone incorporated (four animals) as described above for 5 days. Liver tissues were treated as described above. Values reported represent an average of the four animals with the group only.

GST enzyme activity

Total GST activity was measured in the pooled liver samples or individual livers of castrated animals using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate (Habig et al. 1974). The specific activity of mGSTM, mGSTP and mGSTA were measured with the substrates bromosulfophthalein, ethacrynic acid and cumene hydroperoxide, respectively (Habig et al. 1974; Paglia and Valentine 1967). Specific activity is defined as μmol product formed per minute per mg protein. The Bradford method was used to determine protein concentration (Bradford 1976). Enzyme activities and protein measurements were performed in triplicate on all samples.

Statistics

Differences between control and treated groups and between genders were determined using the Students *t*-test with a level of significance of $P < 0.05$. The *t*-test was used to determine if there was a statistically significant difference in enzyme activity after flavonoid treatment irrespective of sampling time. With the exception of the castrated animals, the data from the three time points was used to give a $n = 3$ and the CI was calculated (Microsoft Excel, 2002).

Pathology

Hepatic pathology in flavone fed animals was investigated in a subgroup of animals. Livers from male and female animals ($n = 3$/group) fed either the casein based diet or flavone were removed on day 6 and tissues were fixed in 10% phosphate buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Results

An initial goal of this study was to evaluate the time-dependent changes in GST activity after flavonoid administration. However, the pooling of liver samples at each time point resulted in one data point for each treatment time. Nonetheless it appears that maximal activity was reached on day 5 in the male mice and on day 10 for female mice. However, the males may have reached an even higher induction rate than the females between day 1 and 5 but no measurements are available for these time points. While the time-dependent effects could not be clearly discerned, there were subchronic changes in GST activity which could be evaluated. The specific activity of GST isozymes was measured in each diet group after 5, 10, and 20 days of treatment. These three values were used to calculate the average ±_SD for the treatments and

control over the 20-day period and are reported in Tables 1, 2, 3, and 4. Of the flavonoids tested, flavone had the greatest influence on GST activity. Mean values of total mGST activity increased sevenfold in female animals and fourfold in male animals (Table 1). The specific activities of the individual isozymes, mGSTM, mGSTP and mGSTA, are given in Tables 2, 3, and 4, respectively. These data indicate that the flavone-induced increase in total GST activity is due to an increase in the specific activity of mGSTM (eightfold) and mGSTP (fourfold) in females and mGSTM (sixfold) and to a lesser extent mGSTP (twofold) in males (Tables 2, 3). No significant change in the activity of mGSTA was found (Table 4).

The basal level activities of total GST (1.75 μmol/min/mg), mGSTM (0.0039 μmol/min/mg) and mGSTP (0.039 μmol/min/mg) were significantly higher in male animals as compared to total (1.62 μmol/min/mg), mGSTM (0.0029 μmol/min/mg) and mGSTP (0.0.016 μmol/min/mg) in female animals. No gender-difference in the basal level activity of mGSTA was found.

To investigate the influence of testosterone, which has previously been reported to be involved in the regulation of GST in the mouse (Hatayama et al. 1986), castrated males were fed flavone for 5 days (Table 5). Castrated males had lower basal levels of total GST activity (1.11 μmol/min/mg) than the intact males (1.75 μmol/min/mg). Levels were also lower than the mean values reported for female animals (1.62 μmol/min/mg). The baseline activity of mGSTP in the castrated mice (0.014 μmol/min/mg) was lower than in intact males (0.049 μmol/min/mg). Similarly, the basal activity of mGSTM was lower in the castrated male animals as compared to intact males (0.0025 and 0.0037 μmol/min/mg, respectively). In the castrated animals, flavone produced an approximate fourfold increase in total GST activity (Table 5). This increase could be attributed to increases in the mGSTM and mGSTP isozymes. The activity of mGSTA was not influenced by flavone. Male, female and castrated animals all demonstrated a similar approximate fourfold increase in total GST activity in response to flavone; indicating that the induction of GST by flavone is independent of testosterone.

All animals appeared healthy throughout the study. However, the up-regulation of phase II enzymes is a general defense mechanism against biological stress and therefore, hepatic pathology was investigated in a subset of male and female animals fed 2,500 mg/kg flavone for 5 days. The flavone fed animals demonstrated a significant

Table 1 Total GST specific activity (μmol/min/mg protein), CI and fold change of control versus flavonoids in male and female mice following dietary exposure to catechin, flavone, morin, nargengin or quercetin (2,500 mg/kg diet)

	Specific activity			Average[a] ± SD	CI[b]	Fold change[c]
	Day 5	Day 10	Day 20			
Control						
Male	1.75	1.49	1.15	1.46 ± 0.30	1.12–1.80	
Female	1.62	0.85	0.91	1.12 ± 0.43	0.64–1.61	
Flavone						
Male	7.60	6.13	5.26	6.33 ± 1.18*	4.99–7.66	4
Female	6.64	8.22	7.26	7.37 ± 0.79**	6.47–8.27	7
Morin						
Male	1.92	1.08	0.86	1.29 ± 0.56	0.66–1.92	1
Female	1.10	0.92	0.98	1.00 ± 0.09	0.89–1.10	1
Nargengin						
Male	2.00	1.29	1.46	1.58 ± 0.37	1.16–2.00	1
Female	1.09	1.00	0.91	1.00 ± 0.09	0.90–1.10	1
Quercetin						
Male	1.79	1.03	1.11	1.31 ± 0.42	0.84–1.78	1
Female	1.20	0.92	0.82	0.98 ± 0.19	0.76–1.20	1
Catechin						
Male	2.02	1.16	1.22	1.47 ± 0.48	0.93–2.01	1

Significant induction for individual gender control versus diet (Students *t*-test) indicated by $*P = 0.05$ and $**P < 0.001$

[a] Average of specific activity values for day 5, 10 and 20 ($n = 3$)

[b] Confidence intervals (CI) were calculated at a 95% level

[c] Fold change was calculated by the following: average diet SA/average control SA

Table 2 GSTM specific activity (μmol/min/mg protein), CI and fold change of control versus flavonoids in male and female mice following dietary exposure to catechin, flavone, morin, nargengin or quercetin (2,500 mg/kg diet)

	Specific activity			Average[a,b] ± SD	CI[c]	Fold change[d]
	Day 5	Day 10	Day 20			
Control						
Male	0.004	0.004	0.003	0.004 ± 0.0003[§]	0.003–0.004	
Female	0.003	0.002	0.001	0.002 ± 0.0007[§]	0.001–0.003	
Flavone						
Male	0.026	0.027	0.015	0.023 ± 0.007*	0.015–0.030	6
Female	0.023	0.012	0.019	0.018 ± 0.005*	0.012–0.024	8
Morin						
Male	0.002	0.003	0.005	0.003 ± 0.007	0.002–0.005	1
Female	0.002	0.002	0.001	0.002 ± 0.001	0.001–0.002	1
Nargengin						
Male	0.002	0.004	0.004	0.003 ± 0.001	0.002–0.005	1
Female	0.004	0.002	0.002	0.002 ± 0.001	0.001–0.004	1
Quercetin						
Male	0.003	0.003	0.004	0.003 ± 0.000	0.003–0.004	1
Female	0.003	0.002	0.002	0.002 ± 0.001	0.002–0.003	1
Catechin						
Male	0.004	0.002	0.005	0.004 ± 0.001	0.002–0.005	1

Significant induction for individual gender control versus diet (Students *t*-test) indicated by $*P = 0.05$

[a] Average of specific activity values for day 5, 10 and 20 ($n = 3$)

[b] Significant difference (P-value 0.05) male versus female for each diet (Students *t*-test) indicated by [§]

[c] Confidence intervals (CI) were calculated at a 95% level

[d] Fold change was calculated by the following: average diet SA/average control SA

($P < 0.05$) increase in liver weight, however no histological changes were detected. Additionally, there was no significant change ($P > 0.05$) in body weight in these animals as compared to controls.

Discussion

Subchronic exposure to dietary flavone produced a large and significant isozyme-specific increase in mGSTP and mGSTM activity and had no significant effect on mGSTA. These results are similar to those we report in a previous study (Burns et al. 2004). In the current study, female animals demonstrated higher increases in total mGST activity (sevenfold) as compared to males (fourfold). Isozyme specific studies indicate that these increases could be attributed to increases in the activity of mGSTM (eightfold in females, fourfold in males) and mGSTP (sixfold in females and twofold in males) isozymes. Increases in GST expression in response to flavone have also been reported for the rat (Nijhoff et al. 1995; Kang et al. 2003). Although we did not observe significant increases in mGSTA activity in the mouse, Kang et al. (2003) reported increased rGSTA2 mRNA expression in H4IIE hepatocyte-derived cells exposed to 10 μM flavone for 18 h. Possible explanations for these contradictory results include the use of different animal models and/or different modes of exposure (i.e. diet verses added to the cell culture medium). Flavonoids undergo extensive intestinal metabolism (e.g. glucuronidation) and thus metabolites of flavone are more likely to be in contact with hepatic cells in vivo than the non-metabolized flavone structure in vitro.

Despite the similar structure of flavonoids tested (Fig. 1), catechin, morin, nargengin and quercetin did not significantly increase the activity of GSTs in hepatic tissues. Although in this study there were no significant effects after morin treatment, Kawabata et al. (2003) showed that dietary morin could elevate GST activities in the hepatic tissue and in the anterior portion of the tongue of male F344 rats and acted as a chemoprotective against tongue carcinogenesis induced by 4-nitroquinoline.

The transcription of phase I and phase II detoxification enzyme expression is mediated through activation of the xenobiotic response element (XRE) and/or the ARE/EpRE; both are *cis*-acting elements found in the promoter region of these genes.

Table 3 GSTP specific activity (μmol/min/mg protein), CI and fold change of control versus flavonoids in male and female mice following dietary exposure to catechin, flavone, morin, nargengin or quercetin (2,500 mg/kg diet)

	Specific activity			Average[a,b] ± SD	CI[c]	Fold change[d]
	Day 5	Day 10	Day 20			
Control						
Male	0.039	0.058	0.048	0.049 ± 0.01[§]	0.038–0.059	
Female	0.016	0.015	0.017	0.016 ± 0.001[§]	0.015–0.017	
Flavone						
Male	0.101	0.067	0.055	0.074 ± 0.024	0.047–0.101	2
Female	0.065	0.059	0.059	0.061 ± 0.003**	0.058–0.065	4
Morin						
Male	0.070	0.039	0.038	0.049 ± 0.018	0.029–0.070	1
Female	0.029	0.012	0.019	0.020 ± 0.008	0.011–0.029	1
Nargengin						
Male	0.060	0.037	0.055	0.051 ± 0.012[§]	0.037–0.064	1
Female	0.024	0.021	0.013	0.019 ± 0.005[§]	0.013–0.026	1
Quercetin						
Male	0.054	0.035	0.029	0.039 ± 0.013[§]	0.024–0.054	1
Female	0.023	0.015	0.016	0.018 ± 0.004[§]	0.013–0.023	1
Catechin						
Male	0.058	0.045	0.032	0.045 ± 0.013	0.030–0.060	1

Significant induction for individual gender control versus diet (Students *t*-test) indicated by $**P < 0.001$

[a] Average of specific activity values for day 5, 10 and 20 ($n = 3$)

[b] Significant difference (*P*-value 0.05) male versus female for each diet (Students *t*-test) indicated by [§]

[c] Confidence intervals (CI) were calculated at a 95% level

[d] Fold change was calculated by the following: average diet SA/average control SA

Compounds that activate both the XRE and ARE/EpRE are termed bifunctional inducers as they up-regulate phase I and phase II detoxification enzymes. Compounds activating only the ARE/EpRE are termed monofunctional inducers, and only up-regulate phase II enzymes. Monofunctional inducers are generally considered to have more potential as chemopreventative agents because they do not up-regulate phase I enzymes responsible for oxidizing xenobiotics. It has been suggested that certain polyphenols influence pathways that regulate ARE/EpRE activation by modifying the release of Nrf2 into the nucleus (Chen and Kong 2005; Masella et al. 2005) or modulate the XRE through interaction with the AhR (Denison et al. 2002). GSTP and GSTA genes carry 5′-flanking regions with copies of the ARE and XRE whereas enhancer regions for GSTM have not yet been characterized (Hayes et al. 2005). High-affinity ligands of the AhR receptor are characterized as being planar, aromatic and hydrophobic molecules. The planar structure and lack of hydroxylation in the A- and B-rings of flavone makes it a good candidate-ligand for the AhR receptor. In recent studies, we determined that dietary flavone increases CYP1A1, CYP1A2 and CYP2B activity in mice which suggests that flavone acts as bifunctional inducer through the XRE (data unpublished). It is reasonable to predict that activation of the XRE by flavone results in an increase in the oxidative metabolism of flavone and subsequent generation of reactive intermediates (e.g. epoxides and quinones) that can activate pathways which regulate the ARE/EpRE and lead to the induction of phase II enzymes.

The gender-related differences observed in basal level activity of GST, (castrated males < females < intact males) clearly demonstrate that testosterone influences the regulation of GST in mice. The lower basal levels of GSTs expressed in female animals may be due in part to the repression of GST expression by E2 bound estrogen receptor alpha (ERα) interference with Nrf2-mediated gene transcription. E2 bound ERα was recently shown to immunoprecipitate with Nrf2 and repress Nrf2-mediated gene transcription of ARE (Ansell et al. 2004). Nonetheless, flavone resulted in similar increases in total GST activity in male, female and castrated animals which can be attributed to primarily an increase in the activity of the mGSTM isozyme. These results indicate that the flavone-mediated induction of the GSTs is independent of sex hormones.

Table 4 GSTA specific activity (μmol/min mg protein), CI and fold change of control versus flavonoids in male and female mice following dietary exposure to catechin, flavone, morin, nargengin or quercetin (2,500 mg/kg diet)

	Specific activity			Average[a] ± SD	CI[b]	Fold change[c]
	Day 5	Day 10	Day 20			
Control						
Male	0.588	0.531	0.470	0.530 ± 0.059	0.463–0.596	
Female	0.569	0.478	0.853	0.633 ± 0.196	0.412–0.854	
Flavone						
Male	0.505	0.433	0.395	0.444 ± 0.056	0.381–0.507	1
Female	0.365	0.373	0.620	0.453 ± 0.145	0.288–0.617	1
Morin						
Male	0.639	0.457	0.541	0.546 ± 0.091	0.443–0.649	1
Female	0.615	0.485	0.851	0.650 ± 0.186	0.440–0.861	1
Nargengin						
Male	0.574	0.475	0.508	0.519 ± 0.051	0.461–0.576	1
Female	0.612	0.508	0.866	0.662 ± 0.184	0.454–0.870	1
Quercetin						
Male	0.582	0.377	0.477	0.479 ± 0.103	0.363–0.595	1
Female	0.571	0.509	0.822	0.634 ± 0.166	0.446–0.822	1
Catechin						
Male	0.540	0.571	0.520	0.544 ± 0.026	0.515–0.573	1

[a] Average of specific activity values for day 5, 10 and 20 ($n = 3$)

[b] Confidence intervals (CI) were calculated at a 95% level

[c] Fold change was calculated by the following: average diet SA/average control SA

Table 5 GST specific activity (μmol/min/mg protein) in castrated and intact males and female animals) of control and flavone challenged (2,500 mg/kg diet) male and female mice and castrated male mice

	Specific activity		
	Total GST	GSTM	GSTP
Control			
Intact male	1.75	0.004	0.039
Castrated male	1.11	0.003	0.014
Female	1.62	0.003	0.016
Flavone			
Intact male	7.60	0.026	0.101
Castrated male	5.03	0.021	0.044
Female	6.64	0.023	0.065

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