Flavonoids of Honey and Propolis: Characterization and Effects on Hepatic Drug-Metabolizing Enzymes and Benzo[*a*]pyrene−DNA Binding in Rats

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The influence of dietary sunflower honey, propolis, and a flavonoid extract of propolis was examined on drug-metabolizing enzyme activities in rat liver and on microsome-mediated binding of benzo[*a*]pyrene to DNA. Characterization of flavonoids present in sunflower honey and propolis was achieved in order to assess the relative effects of different components of honey and propolis. Honey and propolis contained the same major flavonoids, pinocembrin, chrysin, galangin, and pinobanksin. The concentration of flavonoids was higher in propolis. Sunflower honey produced no significant changes on phase I and phase II enzyme activities and no modification of *in vitro* binding of benzo[*a*]pyrene to DNA. Propolis treatment produced an increase of ethoxyresorufin deethylase, pentoxyresorufin depentylase, ethoxycoumarin deethylase, glutathione transferase, and epoxide hydrolase activities. A flavonoid extract from propolis slightly enhanced only few enzyme activities, ethoxycoumarin deethylase and epoxide hydrolase. The induction pattern was similar to that observed with pinocembrin (a major flavonoid of propolis) administered solely. Binding of benzo[*a*]pyrene to DNA by microsomes from rats fed with propolis or a flavonoid extract from propolis was not significantly modified. These results contribute to identification of food or foodstuffs that can modify drug-metabolizing enzymes and binding of carcinogens to DNA.

Keywords: *Sunflower honey; propolis; flavonoids; drug-metabolizing enzymes; benzo[a]pyrene−DNA binding*

INTRODUCTION

Flavonoids are polyphenolic compounds that occur naturally in plants. A recent survey of flavonoid content of fruit, vegetables, and beverages showed the abundance and the widespread distribution of these compounds in the human diet (Hertog et al., 1993). Experimental studies showed that flavonoids can inhibit carcinogenesis in rodents (Deschner et al., 1991; Suschetet et al. 1990; Verma et al., 1988; Wattenberg and Leong, 1970). In addition, many flavonoids are known to modify the activities of drug metabolizing enzymes. The effects are complex, depending on the enzyme activity, the structure of the flavonoid, or the method of administration [reviewed by Smith and Yang (1994)]. It has been suggested that this modulating effect could partially explain the chemopreventive effects of flavonoids. Despite the intensive interest in flavonoids as modulators of drug metabolizing, only few studies were conducted with these compounds administered through the diet. In a previous work it was shown that several dietary flavonoids modulated drug-metabolizing enzymes in the rat liver (Siess et al., 1989). Polyhydroxylated flavonoids such as quercetin or chrysin produced no effect whereas less polar flavonoids, flavone flavanone and tangeretin, enhanced different phase I and phase II drug-metabolizing enzymes activities.

The objective of the present study was to assess the effect of sunflower honey, which contains high levels of flavonoids, on drug-metabolizing enzymes and *in vitro* binding of benzo[*a*]pyrene to DNA. In addition, propolis was selected since it contains the same flavonoids but at higher levels than in honey, (Sabatier et al., 1992). Moreover, the effects of a flavonoid extract of propolis and of flavone and pinocembrin, two of the flavonoids found in honey (Berahia et al., 1993), were also determined in order to evaluate the relative effects of different components of honey or propolis. Rats were fed diets containing these different components (sunflower honey, propolis, flavonoid extract) for 15 days. Flavone and pinocembrin were administered solely, by ip injection. Many hepatic phase I and phase II activities were evaluated: ethoxyresorufin *O*-deethylase (EROD) and aryl hydrocarbon hydroxylase (AHH) as markers of P450 1A1, ethoxycoumarin *O*-deethylase (ECOD) as a marker of P450 1A and 2B, pentoxyresorufin depenthylase (PROD) as a marker of P450 2B1,2, erythromycin demethylase (ERDM) as a marker of P450 3A, and laurate ω-hydroxylase (LAH) as a marker of P450 4A. Glutathione *S*-transferase (GST) and UDP-glucuronyltransferases (UGTs) were assayed as enzymes involved in detoxication of xenobiotics. *In vitro* DNA binding of benzo[*a*]pyrene, catalyzed by hepatic microsomes from rats fed these different diets, was also measured.

MATERIALS AND METHODS

Material. Sunflower honey was collected from different regions in the center of France. Propolis was obtained from Medex (Ljubljana, Slovenia). Flavone (100% purity) was purchased from Sigma Chemical Co. (St. Louis, MO) and pinocembrin (98% purity) from Apin Chemicals Ltd. (Abington, Oxon, UK).

Preparation of Concentrated Honey, Propolis, and Flavonoid Extract. Sunflower honey (100 g) was mixed in

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300 mL of ethanol (96%). Two successive extractions were carried out. The two alcoholic fractions were collected and filtrated through a polycap 75 HD, 5 μm (Whatman). Ethanol was eliminated under vacuum at low temperature (38 °C). The concentrated honey obtained was 50% honey.

Propolis was diluted in ethanol. To obtain the flavonoid extract, propolis was diluted in 2 M NaOH under nitrogen, as previously described (Berahia et al., 1993). Alkaline treatment was carried out for 4 h under nitrogen at room temperature to hydrolyze cinnamic esters. The reaction was stopped by acidification to pH 2 with 4 M HCl. Phenolic acids and flavonoids were extracted with ethyl acetate (v/v, 1/1) three times. The three organic phases were collected, and ethyl acetate was evaporated under vacuum (temperature, 38 °C). The flavonoid extract was made up to 100 mL with ethanol (grade HPLC).

Separation, Characterization, and Quantification of Phenolic Compounds. Phenolic compounds were separated and characterized by HPLC with diode array detection according to Sabatier et al. (1992). A Varian 5500 chromatograph was connected to a Water 990 diode array detector. Separation of phenolic compounds was performed using a Lichrosorb RP18 column (200 × 3 mm, 7 μm) connected to a C-18 guard column (10 × 2.1 mm, 30−40 μm). The mobile phase (flow rate 0.8 mL/min) consisted of acidified distilled water adjusted to pH 2.6 with orthophosphoric acid (solvent A), and acetonitrile (solvent B). The best separation was obtained by the following gradient: at 0 min, 0% B; at 12 min, 9% B; at 20 min, 13% B; at 40 min, 40% B; at 60 min, 70% B; at 85 min, 70% B. The pressure was about 150 atm. The main flavonoids were identified by comparing the retention time and the spectral characteristics of standards purchased from Extrasynthèse (69730 Genay, France) as described previously (Sabatier et al., 1992). The other compounds were characterized by their spectral characteristics and grouped in the three main families: benzoic acids, cinnamic acids and esters, and flavonoids. The quantification of the compounds was carried out by external calibration at 280 nm for benzoic acids and 310 nm for cinnamic acids and esters, and flavonoids. The concentration of phenolic compounds were expressed in milligrams per kilogram or grams per liter equivalent benzoic acid for benzoics, ferulic acid for cinnamics, pinocembrin for flavanones, chrysin for flavones, and galangin for flavonols.

Animals and Dietary Treatments. Male weanling specified pathogen-free (SPF) Wistar rats, obtained from the animal breeding unit of our laboratory, were used. They were housed individually in wire cages maintained at 22 °C with a 12 h-light/dark cycle.

In a first experiment, they were fed a purified diet for 2 weeks. For the next 2 weeks, the concentrated sunflower honey, the propolis dissolved in ethanol, and the flavonoid extract of propolis were incorporated in the diet according to the protocol shown in Table 1. Diet composition was similar to that used in a previous study (Siess et al., 1989), in which energy was provided with a combination of starch and sucrose. The honey diet contained 50% fructose; it differed from the other diets only by the presence of fructose instead of sucrose. Such a difference is not known to have any effect on drug-metabolizing enzymes. The formulas of the different experimental diets are given in the Table 1. The compositions of the mineral mixture and the vitamin mixture were previously described (Siess et al., 1989). Food and water were provided *ad libitum* during the experiment. The animals were killed at the end of the fourth week.

In a second experiment, pinocembrin and flavone, two flavonoid components of honey, were administered by ip injection at the end of a 2-week dietary treatment with the purifed diet. Flavone and pinocembrin were dissolved in corn oil and administered at the dose of 0.3 mmol/kg of body weight daily, for three consecutive days before sacrifice.

Preparation of Microsomal and Cytosolic Fractions. Animals were killed by decapitation after 18-h fasting period. Microsomal and cytosolic fractions were obtained as described previously (Vernet and Siess, 1986). Proteins were quantified by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Table 1. Composition of the Experimental Diets (g/kg of Diet)

			experimental diets		
component	purified diet	control	honey	propolis	flavonoid extract
casein	230	210	210	210	210
D,L-methionine	1	1	1	1	1
sucrose	270	500	0	500	500
wheat starch	360	150	150	150	150
corn oil	50	50	50	50	50
agar	20	20	20	20	20
cellulose	20	20	20	20	20
mineral mixture	50	50	50	50	50
vitamin mixture					
concentrated honey[a]		0	500	0	0
propolis[b]		0	0	2	0
flavonoid extract[b]		0	0	0	2
water added[c]	400	250	90	250	250

[a] Honey water content was 17%. [b] Added as an ethanolic solution (flavonoid concentration was 73 mg/mL for propolis and 97 mg/mL for flavonoid extract). Ethanol (27 mL/kg of dry matter) was added to the control and honey diets to equalize the ethanolic intakes. [c] In order to equalize the texture of the different diets, the dry matter was mixed with water containing the vitamin mixture.

Enzyme Assays. Cytochrome P450 content, NADPH-cytochrome P450 reductase, NADH-cytochrome b_5 reductase, EROD, PROD, ECOD, AHH, and EH activities were determined as described previously (Siess et al., 1992). ERDM was assayed by measuring formaldehyde production using the Nash's reagent, according to Watkins et al. (1985). The reaction system contained 125 mM Tris-HCl (pH 7.5), 0.5 mM erythromycin, 1.25 mM NADPH, and 1 mg of microsomal protein/mL, the final volume being 1 mL. The reaction was carried out at 37 °C and stopped after 10 min by addition of TCA. LAH was determined by a radiometric method as described by Salaün et al. (1978).

UGT activities were determined by the method of Mulder and Van Doorn (1975) using 3 mM UDP-glucuronic acid and 0.3 mM aglycon (*p*-nitrophenol or *p*-hydroxybiphenyl). Microsomes were activated with Triton X-100 such that ratio of Triton concentration to protein concentration was 0.2. Cytosolic GST was assayed using 1-chloro-2,4-dinitrobenzene as substrate, as described by Habig et al. (1974).

Microsome-Catalyzed DNA Binding of [^{3}H]Benzo[*a*]-pyrene. The method used has been described before (Le Bon et al., 1992a). In brief, 40 μM [^{3}H]benzo[*a*]pyrene was incubated with 1 mg of calf thymus DNA in a medium containing 1 mg of microsomal proteins, 0.36 mM NADPH, and 3 mM $MgCl_2$, in a total volume of 2 mL of 0.1 M phosphate buffer (pH 7.4) for 30 min. After isolation and purification of DNA, the specific activity of benzo[*a*]pyrene−DNA binding was determined.

Statistical Analysis. Statistical differences between treatments were determined by analysis of variance followed by Dunnett's test (comparison of the mean of each treatment to the control mean). $P \leq 0.05$ was chosen as indicating significance.

RESULTS

Identification of Phenolic Compounds in Sunflower Honey, Propolis, and Extract of Flavonoids. Phenolic compounds were determined in sunflower concentrated honey, in propolis and in a flavonoid extract from propolis. Sunflower honey and propolis contained three main classes of phenolics structures: benzoic acids, cinnamic acids and esters, flavonoids (Table 2). Propolis contained the same major flavonoids as honey but the concentration was higher than in honey. The flavonoids present in higher levels were pinocembrin, chrysin, galangin, and pinobanksin (Figure 1). All these flavonoids were aglycons with a B ring without hydroxyl groups.

Table 2. Flavonoid and Phenolic Content of Hive Products[a]

phenolic	honey (mg/kg)	propolis (g/L)	flavonoid extract (g/L)
benzoic acids (equiv benzoic acid)	289	13	0
cinnamic acids (equiv ferulic acid)	25	62	0
flavonoids	30	73	97
flavanones (equiv pinocembrin)		31	38
flavones (equiv chrysin)		29	28
flavonols (equiv galangin)		13	31

[a] See Materials and Methods for details on extraction and quantification of phenolic compounds.

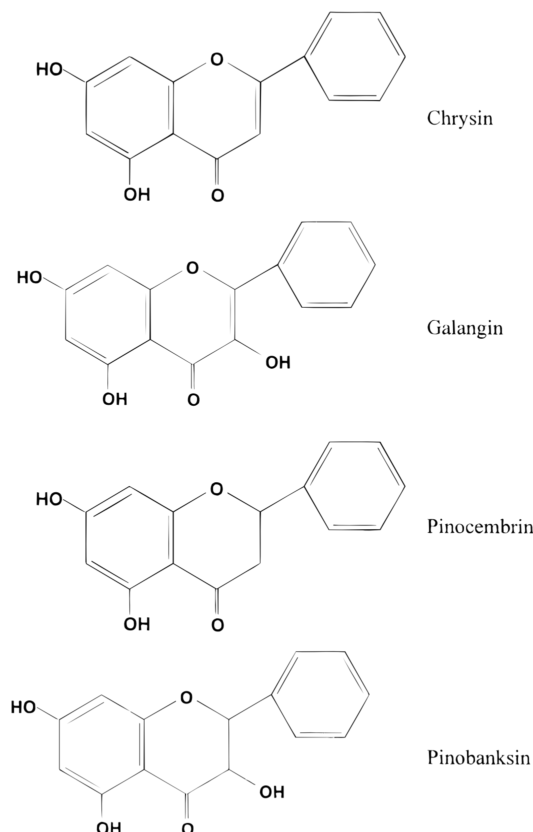

Figure 1. Structures of the main flavonoids present in bee products.

Enzyme Assays and *in Vitro* Binding of Benzo[*a*]pyrene to DNA. Feeding the rats for 15 days with a diet containing concentrated honey did not change any of the measured parameters (Tables 3–5 and 7).

Dietary administration of propolis significantly increased relative liver weights and ECOD (40%), EROD (114%), and PROD (140%) activities. EH and GST were also enhanced by this treatment (Tables 4 and 5).

Incorporation of the extract of flavonoids from propolis in the diet significantly stimulated many enzyme activities: EROD (50%), AHH (50%), ECOD (40%), PROD (55%); ERDM (90%), and EH (7%). The increase was significant only for ECOD and EH activities. In contrast, cytochrome P450 content was reduced by this treatment (Tables 3–5 and 7). Binding of benzo[*a*]pyrene to DNA catalyzed by microsomes from rats fed experimental diets was not significantly modified by any treatment tested (Table 7).

Intraperitoneal administration of pinocembrin at the dose of 0.3 mmol/kg of body weight daily for 3 consecutive days significantly increased ECOD (1.5-fold) and EROD (3.6-fold) activities (Table 6). Flavone administered at the same dose increased a larger range of enzyme activities: ECOD (4.2-fold), AHH (2.7-fold), EROD (18.9-fold), PROD (7.1-fold), EH (3.2-fold), GST (2.2-fold), and *p*-nitrophenol-UGT (2.0 fold). The increase of EROD was the most pronounced. Conjugation of *p*-hydroxybiphenyl was slightly reduced by both flavonoids (Table 6).

DISCUSSION

In previous work we showed that dietary administration of isolated flavonoids induced monooxygenase and transferase activities in rat liver and inhibited microsome-mediated binding of benzo[*a*]pyrene to DNA (Siess et al., 1989; Le Bon et al., 1992a). The purpose of this study was to ascertain whether sunflower honey or propolis, both of which are rich in flavonoids, could produce such effects.

Feeding rats with a diet containing sunflower honey did not produce any effect on drug-metabolizing enzymes activities. Also, *in vitro* binding of benzo[*a*]pyrene to DNA, catalyzed by microsomes from rats fed honey, was not modified. It is likely that either the amount of flavonoids in the honey was too low or the flavonoids found in honey had a low bioavailability. In our experimental conditions, the flavonoid content of the diet was 10 ppm. We have already shown that the threshold dose for an inducing effect of some dietary flavonoids on drug-metabolizing enzymes, was 20 ppm in the diet (Siess et al., 1992). Most of the flavonoids identified in honey were aglycons with hydroxyl groups only on the B ring: pinocembrin, chrysin, galangin, and pinobanksin. Other less polar flavonoids such as flavone or tectochrysin were previously reported in sunflower honey (Berahia et al., 1993), but they were in smaller amounts. It was shown that flavonoids with free hydroxyl groups (quercetin or chrysin) incorporated in a diet produced no effect on drug-metabolizing enzymes (Siess et al., 1989). Other studies showed that hydroxylated flavonoids when ingested orally were extensively metabolized. Ring fission was the major route of degradation of hydroxylated flavonoids, and this was generated by the gastrointestinal microflora (Griffiths, 1982). This could reduce their biological activity.

By contrast, propolis dietary treatment stimulated different enzyme activities: EROD, ECOD, PROD, EH, and GST. The induction pattern of propolis treatment was mixed. Both enzyme probes for cytochrome P450 1A and 2B were induced, and phase II enzymes were also induced. This mixed pattern could reflect the inducing properties of either flavonoids (in the propolis diet the flavonoid content was 20-fold higher than in honey diet) or other components of propolis. Propolis is a mixture of various compounds: phenolic acids, cinnamic acids, terpenes, aliphatic hydrocarbons, sterols, and flavonoids (Greenaway et al., 1990; Marcucci, 1995). Inducing properties of terpenes such as *d*-limonene, geraniol, guaiacol, or cinnamic esters were shown when administered solely (Chadha and Madyastha, 1984; Zheng et al., 1993). Thus, other components of propolis might partially contribute to the overall effect.

Feeding rats with diets containing an extract of flavonoid enhanced only a few enzyme activities by comparison with dietary treatment with propolis. The variation was significant only for ECOD and EH activities. This suggests that flavonoid components of propolis contribute only partially to the inducing effect of propolis. Other components are likely to be more

Table 3. Effect of Experimental Diets on Terminal Body Weights, Relative Liver Weights, Hepatic Cytochrome P450, and NAD(P)H-Cytochrome *c* Reductase Activities[a]

diet	rel liver wt (g/100 g of body wt)	cytochrome P450 (nmol/mg of protein)	NADPH-cytochrome *c* reductase (nmol/min per mg of protein)	NADH-cytochrome *c* reductase (nmol/min per mg of protein)
control	3.48 ± 0.032	1.05 ± 0.039	213 ± 14.9	742 ± 52.2
honey	3.53 ± 0.056	1.10 ± 0.025	199 ± 9.1	734 ± 34.5
propolis	3.72 ± 0.064*	1.02 ± 0.042	225 ± 18.1	793 ± 55.4
flavonoid extract	3.54 ± 0.071	0.88 ± 0.042*	196 ± 16.1	765 ± 30.6

[a] Values are means ± standard error of the mean for eight rats. Those marked with asterisks differ significantly (Dunnett's test) from control value ($P \leq 0.05$).

Table 4. Effect of Experimental Diets on Hepatic Monooxygenases Activities[a]

diet	ethoxycoumarin deethylase[b]	arylhydrocarbon hydroxylase[b]	ethoxyresorufin deethylase[b]	pentoxyresorufin depentylase[b]	erythromycin demethylase[c]	laurate hydroxylase[c]
control	721 ± 54.3	198 ± 37.9	34 ± 1.9	9 ± 0.9	1.0 ± 0.30	8.0 ± 0.49
honey	811 ± 37.7	159 ± 14.7	45 ± 4.6	12 ± 2.2	1.0 ± 0.37	6.9 ± 0.76
propolis	1009 ± 58.0*	267 ± 43.0	73 ± 6.3*	22 ± 3.7*	1.7 ± 0.48	8.4 ± 0.64
flavonoid extract	1009 ± 48.1*	302 ± 59.3	50 ± 4.2	14 ± 1.6	1.9 ± 0.51	8.5 ± 0.68

[a] Values are means ± standard error of the mean for eight rats. Those marked with asterisks differ significantly (Dunnett's test) from control value ($P \leq 0.05$). [b] In picomoles per minute per nanomole of cytochrome P450. [c] In nanomoles per minute per nanomole of cytochrome P450.

Table 5. Effect of Feeding Experimental Diets on Hepatic Epoxide Hydrolase and Transferase Activities[a]

diet	epoxide hydrolase	glutathione transferase	UDP glucuronyl transferase	
			p-nitrophenol	*p*-hydroxybiphenyl
control	8.6 ± 0.25	591 ± 35.6	36.5 ± 5.25	27.6 ± 3.80
honey	8.3 ± 0.34	510 ± 27.0	36.1 ± 2.41	28.5 ± 2.74
propolis	10.9 ± 0.41*	687 ± 36.8 *	39.2 ± 5.23	26.9 ± 1.87
flavonoid extract	9.2 ± 0.35*	569 ± 32.6	36.9 ± 4.29	27.9 ± 1.73

[a] Values (in nmol/min per mg of protein) are means ± standard error of the mean for eight rats. Those marked with asterisks differ significantly (Dunnett's test) from control value ($P \leq 0.05$).

Table 6. Effect of ip Administration of Flavone and Pinocembrin on Hepatic Monooxygenase and Transferase Activities[a]

enzyme	control	flavone	pinocembrin
ECOD	1243 ± 133.2	5264 ± 397.4*	1860 ± 192.4*
AHH	378 ± 50.5	1040 ± 122.2*	336 ± 60.6
EROD	50 ± 6.1	944 ± 125.2*	182 ± 66.9*
PROD	20 ± 2.7	142 ± 44.8*	23 ± 4.1
ERDM	0.43 ± 0.07	0.49 ± 0.16	0.62 ± 0.10
LAH	7.2 ± 0.45	8.7 ± 0.94	7.3 ± 0.64
EH	7.2 ± 0.19	22.9 ± 0.60*	6.4 ± 0.39
GST	611 ± 66.9	1330 ± 226.1*	620 ± 61.3
p-nitrophenol-UGT	52.2 ± 4.71	107 ±.5 5.66*	50.2 ± 4.51
p-hydroxybiphenyl-UGT	45.5 ± 4.85	29.2 ± 1.55*	25.7 ± 1.79*

[a] Activities of ECOD, AHH, EROD, and PROD are expressed as picomoles of substrate formed per minute per milligram of protein and for ERDM, LAH, EH, GST, and UGT as nanomoles per minute per milligram of protein. Values are means ± standard of the mean for four rats. Those marked with asterisks differ significantly (Dunnett's test) from control value ($P \leq 0.05$).

Table 7. Effect of Dietary Treatments on Rat Hepatic Microsome-Mediated Binding of [^{3}H]Benzo[*a*]pyrene to Calf Thymus DNA[a]

treatment	benzo[*a*]pyrene−DNA adducts
control	19.4 ± 1.47
honey	24.8 ± 1.64
propolis	22.7 ± 0.91
flavonoid extract	22.6 ± 1.53

[a] Values are expressed as picomoles of benzo[*a*]pyrene bound per milligram of DNA in 30 min on a per milligram of protein basis. Values are means ± standard error of the mean for four rats.

effective than flavonoids. The induction pattern produced by the mixture of flavonoids appeared to be similar to that observed when pinocembrin was administered isolated. Pinocembrin contributes probably more than other flavonoids to the overall inducing effect of the mixture of flavonoids. Under our experimental conditions, the average daily consumption of flavonoids from the extract was 200 mg/kg of body weight (mean-daily dry matter consumption per rat, was 20 g, and the flavonoid concentration in the diet was 2000 ppm). The group of flavanones accounted up to 40% of the total flavonoids (Table 2). Thus the average daily consumption of flavanones was estimated at 80 mg/kg of body weight. This dose was in the scale of the dose of pinocembrin administered ip (0.3 mmol/kg of body weight per day). Flavone administered at the same dose (0.3 mmol/kg of body weight per day) induced a broad spectrum of phase I and phase II enzyme activities. However, the amount of flavone in the extract of flavonoids was probably too low to contribute to the overall effect of the mixture of flavonoids on drug-metabolizing enzyme activities.

The microsomes of the rats fed with propolis or flavonoids did not modify the binding of benzo[*a*]pyrene to DNA. In a previous study, propolis and flavonoid extract from propolis did inhibit the adduct formation when they were directly added to the incubation medium (Le Bon et al., 1992b). In such a situation, propolis constituents or flavonoids could either inhibit the P450 enzymes responsible for activation of benzo[*a*]pyrene or trap reactive metabolites. Conversely the no-effect observed in the present study suggested that the *in vivo* levels of these constituents were too low to modify the ability of the hepatic microsomes in metabolizing benzo[a]pyrene. As evidence, AHH was not modified by any treatment.

This study emphasized the complexity of identifying the active components of food or foodstuffs which could modify drug-metabolizing enzyme activities. Further studies are needed to evaluate the effects of single

compounds and the consequence of combining them. A study has suggested that very small doses of inducers, while ineffective alone, provide an effect when used in combination (McLean and Driver, 1977).

ABBREVIATIONS USED

AHH, aryl hydrocarbon hydroxylase; ECOD, ethoxycoumarin *O*-deethylase; EH, epoxide hydrolase; ERDM, erythromycin *N*-demethylase; EROD, ethoxyresorufin *O*-deethylase; GST, glutathione *S*-transferase; LAH, laurate ω-hydroxylase; PROD, pentoxyresorufin *O*-depentylase; SPF, specified pathogen free; UGT, UDP-glucuronyltransferase.

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