PERGAMON

Food and Chemical Toxicology 40 (2002) 1731–1743

Food and Chemical Toxicology

www.elsevier.com/locate/foodchemtox

Subchronic 3-month oral toxicity study of grape seed and grape skin extracts

S.S. Bentivegna*, K.M. Whitney

Huntingdon Life Sciences, Mettlers Road, East Millstone, NJ 08875-2360, USA

Accepted 10 June 2002

Abstract

Meganatural™ brand grape seed extract (GSE) and grape skin extract (GSKE), containing proanthocyanidin (PAC) polyphenolic compounds, are intended for use in food as functional ingredients exhibiting antioxidant activity. Proanthocyanidins, as well as the minor constituent phenolic compounds in GSE and GSKE, are present naturally in many foods such as fruits, vegetables, chocolate, tea, etc., and on average people consume 460–1000 mg/day of these combined substances. Although humans have ingested PACs for centuries without reported adverse effects, the current toxicology literature contains relatively little formal evidence regarding their safety. Accordingly, as part of a program to investigate the safety of GSE and GSKE, these products were incorporated into chow and fed to rats for at least 3 months in a GLP-compliant subchronic toxicity study. Groups of CD® (Sprague–Dawley) Crl:CD® IGS BR rats (20 males and 20 females per group) were fed diets containing GSE at concentrations of 0, 0.63, 1.25 or 2.5% (w/w); GSKE was fed at 2.5% (w/w) only. Clinical observations were recorded and body weight and feed consumption measured throughout the study. After 1 month, blood was obtained from 10 rats/sex/group by retrobulbar puncture for interim measurement of clinical pathology. At the end of the study the rats were subjected to a full necropsy, aortic blood samples were collected for clinical pathology, selected organs were weighed and a complete list of tissues was preserved from all animals. Histologic examination was performed on all tissues from control and high-dose GSE and GSKE groups. There were no treatment-related changes that were considered to be of toxicologic significance. Therefore, a dietary concentration of 2.5% GSE or 2.5% GSKE was considered to be a no-observed-adverse effect level (NOAEL). This was equivalent to a time-weighted average dose over the course of the study of approximately 1.78 g/kg body weight/day GSE or GSKE in male rats and 2.15 g/kg body weight/day in female rats.

Keywords: Grape seed; Grape skin; Extract; Subchronic; Rat; Diet; Polyphenol; Proanthocyanidin

1. Introduction

Meganatural™ grape seed extract (GSE) and grape skin extract (GSKE) contain oligomeric and polymeric proanthocyanidins (PACs) as well as lesser quantities of other natural phenolic compounds. PACs are based generally on (+)-catechin and (−)-epicatechin flavan-3-ol monomer units and exhibit antioxidant properties (Ricardo-DaSilva et al., 1991; Hammerstone et al., 2000). Published values indicate estimated dietary intake of flavanoids, catechins and proanthocyanidins by the average American consumer is in the range of 460–1000 mg/day (Santos-Buelga and Scalbert, 2000; Scalbert and Williamson, 2000). Intake arises from the common occurrence of these substances in fruits, juices, tea, coffee, vegetables, and in many other foods and beverages.

The antioxidant activity of GSE and GSKE make them candidates for addition to foods and beverages to retard deterioration; it is possible that the antioxidant activity of GSE and GSKE ingested with these foods would also support physiologic defenses against in vivo-generated free-radical species. (Ricardo-DaSilva et al., 1991; Scott et al., 1993; Hu et al., 1995). It has been conjectured that PACs present in wine consumed by the French population may contribute to their reduced mortality from ischemic heart disease even in the face of consuming a high-fat diet—a situation popularly known

Abbreviations: GSE, grape seed extract; GSKE, grape skin extract; NOAEL, no-observed-adverse effect level; PAC, proanthocyanidin.

* Corresponding author. Tel.: +1-732-873-2550x4500; fax: +1-732-873-3992.

E-mail address: bentives@princeton.huntingdon.com (S.S. Bentivegna).

0278-6915/02/$ - see front matter
PII: S0278-6915(02)00155-2

as the "French Paradox." (Constant, 1997; Kopp, 1998; Das et al., 1999; Law and Wald, 1999). A number of clinical studies (Whitehead, et al., 1995; Maalej et al., 1997; Maxwell, 1997; Nutall et al., 1998; Nigdikar et al., 1998) and non-clinical studies (Vallet et al., 1994; Tebib et al., 1994, 1995; Zhao et al., 1999; Bagchi et al., 2000) have examined potentially beneficial effects on cholesterol and lipoprotein parameters, antitumor activity and general in vivo antioxidant status. While results of these studies suggest potential benefits, this database does not formally address the safety of PACs for human consumption.

Therefore, as part of a program to investigate the safety of GSE and GSKE, a 90-day feeding study in rats was conducted. The highest dietary concentration was chosen to provide significant exposure to the test articles yet avoid potential nutritional interference due to the known ability of tannin substances to complex with and reduce the digestibility of dietary protein. The concentrations selected were 0.63, 1.25 and 2.5% (w/w) of diet.

2. Materials and methods

2.1. Study design

Groups of 20 male and 20 female Crl:CD(SD) IGS BR Sprague–Dawley rats were fed diets containing GSE as summarized in Table 1. Clinical observations, body weights and feed consumption were measured, and at 1 month orbital sinus blood samples were obtained from 10 animals/sex/group for analysis of interim clinical pathology parameters. At the end of the test period, aortic blood samples were taken for clinical pathology, selected organs were weighed and specified tissues from all animals were preserved for subsequent histopathologic examination.

This study was conducted at Huntingdon Life Sciences, East Millstone, New Jersey and designed to meet or exceed United States Food and Drug Administration 1982 Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food. It was also compliant with FDA Good Laboratory Practice Standards (21CRF, Part 58) as well as all appropriate parts of the Animal Welfare Act Regulations.

2.2. Test material

Meganatural™ Gold brand GSE (lot 2501–040157) and GSKE (lot 2511–040060) were supplied by Polyphenolics Incorporated. GSE was composed of approximately 90.5% total phenols while GSKE contained 87.3% total phenols expressed as gallic acid equivalents by colorimetric analysis (Singleton and Rossi, 1965). A representative profile of phenolic components appears in Table 2.

Using Hobart and Patterson Kelly Twin Shell Mixers, appropriate blended with certified Rodent Diet No. 5002, (Meal) (PMI Nutrition International, St. Louis, MO, USA) to achieve the desired concentrations. Fresh diets were prepared at 2-week intervals. Control animals received untreated diet. All prepared diets were analyzed to confirm test article content, homogeneity and stability using standard sampling and analytical techniques.

Table 2
Meganatural™ grape skin extract (GSKE) and grape seed extract (GSE) composition

	GSKE profile	GSE profile
	Average	Average
Catechin % by wt	3.4	4.8
Epicatechin % by wt	4.6	4.4
Gallic acid % by wt	2.4	1
Total phenols % by wt	87.3	90.5
Total anthocyanins % by wt	2.6	NA
HPLC relative profile of monomers (%)	16.7	10.4
HPLC relative profile of oligomers (%)	67.4	74.9
HPLC relative profile of polymers (%)	15.9	14.7
Moisture % by wt	3.7	3.6

Table 1
Experimental design

Group	Test article	Dose level (%)	Number of animals							
			Total		Clinical laboratory studies		Necropsy		Microscopic pathology	
					Month 1 and termination		Week 13			
			M	F	M	F	M	F	M	F
1	Control	0	20	20	10	10	20	20	20	20
2	Meganatural™ GSE	0.63	20	20	10	10	20	20	A.R.	A.R.
3	Meganatural™ GSE	1.25	20	20	10	10	20	20	A.R.	A.R.
4	Meganatural™ GSE	2.50	20	20	10	10	20	20	20	20
5	Meganatural™ GSKE	2.50	20	20	10	10	20	20	20	20

A.R. = as required.

2.3. Test animals

Outbred albino rats of the Crl:CD® (SD) IGS BR strain were received at 28 days of age from Charles River Kingston, Kingston, New York. Animals were acclimated for 14 days during which each animal was examined to confirm suitability for study. Criteria for suitability included acceptable physical examination, body weight and ophthalmoscopic examination. Rats considered suitable for study were distributed into five groups of 20 animals per sex by a computerized random sort program so that body weight means for each group within a sex were comparable. When placed on study, male rats weighed 188–237 g (mean = 211 g) and females weighed 128–172 g (mean = 149 g).

Each rat was identified with a metal ear tag bearing its assigned animal number. The assigned animal number, plus the study number, comprised the unique number for each animal. In addition, each cage was provided with a cage card which was color-coded for dose level identification and contained study number and animal number information.

2.4. Housing and environment

Rats were doubly housed in elevated, stainless-steel, wire-mesh cages during the first week of acclimation and individually housed thereafter. Certified Rodent Diet, No. 502, (Meal) (PMI Nutrition International) or diet containing test article was available without restriction. Diets were dispensed in individual cage cups which were weighed and refilled every 5–7 days. Water, (Elizabethtown Water Company, Westfield, NJ, USA) was likewise available without restriction via an automated watering system. There were no known contaminants in the feed or water which were expected to interfere with the results of the study.

The animal room was maintained on a 12-h light/dark cycle controlled via an automatic timer. Temperature and humidity were monitored and recorded at least daily for maintenance within specified ranges of 18–26 °C and 30–70% relative humidity.

2.5. Clinical observations and ophthalmoscopy

Animals were observed in their cages twice daily for mortality and signs of severe toxic or pharmacologic effects. Each animal was removed from its cage and examined twice pre-test and once weekly during the study period. Examinations included observations of general condition, skin and fur, eyes, nose, oral cavity, abdomen and external genitalia, occurrence of secretions and erections, and autonomic activity (e.g. lacrimation, piloerection, pupil size, respiratory pattern). Changes in gait, posture and response to handling as well as presence of clonic or tonic movements, stereotype (e.g. excessive grooming, repetitive circling) or bizarre behavior (e.g. self-mutilation, walking backwards) were recorded if observed.

An ophthalmologic examination was performed on all animals pretest and at termination of dosing. Eyelids, lacrimal apparatus and conjunctiva were examined grossly; cornea, anterior chamber, iris, lens, vitreous humor, retina and optic disk were examined by indirect ophthalmoscopy.

2.6. Body weight, food consumption and test article intake

Animals were weighed twice pre-test, weekly during treatment and at termination. Terminal, fasted body weights were obtained just prior to necropsy. Feed was available without restriction 7 days/week except for when fasted for clinical pathology studies during week 4. Feed consumption for that week was measured over 5 days. On the same days as body weight was measured, animals were presented with weighed, full feeders. After up to 7 days, feeders were re-weighed and the resulting weight was subtracted from the full feeder weight to obtain the grams consumed per animal over the 5- to 7-day period. Feed consumption was determined weekly, beginning 1 week prior to treatment. Average grams of food consumed per kg of body weight per day (g/kg bw/d) was calculated for each feeding period by first dividing the grams of food consumed by the number of days (5 or 7) in the feeding period. The resultant value for average feed consumed per day was then divided by the average of the current and previous body weight of the animal. Average test article intake values, expressed as mg GSE or GSKE per kg body weight per day (mg/kg/day), were calculated by multiplying the average food consumption value (g/kg bw/day) by the nominal concentration (mg/g) of test article in the diet.

2.7. Clinical pathology

Blood samples for hematology, coagulation and clinical chemistry studies were collected under light anesthesia (CO_2/O_2), via retrobulbar puncture at the interim Month 1 collection and via puncture of the abdominal aorta at termination from 10 animals/sex/group. Animals were fasted overnight prior to each blood collection interval.

Blood for hematology studies was collected (approx. 0.25 ml) into tubes containing ethylenediaminetetraacetic acid (EDTA) anticoagulant. An ADVIA 120 Hematology Analyzer (Bayer Corporation) was employed to measure the following parameters: hemoglobin concentration; hematocrit; erythrocyte count; platelet count; mean corpuscular volume; mean corpuscular hemoglobin; mean corpuscular hemoglobin concentration; total leukocyte count; reticulocyte count; differential leukocyte count. Erythrocyte morphology was evaluated according to Henry (1991).

Blood for coagulation studies was collected (approx. 1.0 ml) into tubes containing sodium citrate anticoagulant. A mechanical clot detection system, STA Compact® (Diagnostic Stago Products) was used to evaluate prothrombin time and activated partial thromboplastin time.

Blood for coagulation studies was collected (approx. 1.0 ml) into tubes containing no anticoagulant, allowed to clot, and centrifuged to obtain serum. A Hitachi 717 Automatic Analyzer (Roche Corporation) was employed to measure the following parameters: aspartate aminotransferase; alanine aminotransferase; alkaline phosphatase; blood urea nitrogen; creatinine; glucose; total cholesterol; triglycerides; total protein; albumin; total bilirubin; sodium; potassium; chloride; calcium; inorganic phosphorus; γ-glutamyl transferase. Globulin (total protein minus albumin) and albumin/globulin ratio values were calculated.

2.8. Pathology

Necropsy was performed on 20 animals/sex/group after animals had undergone treatment for up to 96 days. Animals were fasted overnight prior to necropsy. A necropsy schedule was established to ensure that approximately equal numbers of males and females were examined on each day of necropsy and that examination of animals of both sexes were performed at similar times of the day throughout the necropsy period. All rats were killed by exsanguination following CO_2 inhalation. At necropsy, the following organs were weighed with paired organs weighed together: adrenal glands, brain, heart, kidneys, liver, ovaries, pituitary gland, prostate gland, spleen, testes, thymus, thyroid/parathyroid, and uterus with cervix.

The following tissues were examined in situ and fixed in 10% neutral buffered formalin: adrenal glands, aorta, bone and marrow (sternum and femur), brain (medulla, pons, cerebrum and cerebellum), esophagus, heart, kidneys, lacrimal glands/Harderian glands, large intestine (cecum, colon, rectum), larynx, liver, lungs (with mainstem bronchi), lymph nodes (mesenteric, mediastinal), mammary gland, nerve (sciatic), ovaries, pancreas, pharynx, pituitary gland, prostate gland, salivary gland (submandibular), seminal vesicles, skeletal muscle, skin, small intestine (duodenum, ileum, jejunum), spinal chord (cervical, thoracic, lumbar) spleen, stomach, thymus, thyroid/parathyroid glands, trachea, urinary bladder, uterus with cervix, vagina, Zymbal's gland and tissues with macroscopic findings including tissue masses. Eyes were placed in glutaraldehyde/paraformaldehyde initially and then retained in 10% formalin. Testes and epididymides were placed in Modified Davidson's solution. Smear preparations of marrow from the rib were air-dried and fixed in absolute methanol. After fixation, tissues and organs from all animals in the high-dose GSE and GSKE groups and in the control group were routinely processed, embedded in paraffin, cut at a microtome setting of 3–5 μ, mounted on glass slides, stained with hematoxylin and eosin and examined by light microscopy. Bones were decalcified in formic acid.

2.9. Statistical procedures

The following parameters were analyzed statistically: body weight, cumulative body weight change from baseline, feed consumption, hematology, coagulation, clinical chemistry values and organ weights. The GSKE treated group was compared to control only. The following were used to compare control and GSE-treated groups.

Table 3
Mean analytical concentrations, expressed as per cent of nominal concentrations

Group (test article)	Nominal (dose) concentration ppm (%)	Analytical concentration (% of nominal)
2 (GSE)	6300 (0.63)	106.0
3 (GSE)	12,500 (1.25)	101.8
4 (GSE)	25,000 (2.5)	98.0
5 (GSKE)	25,000 (2.5)	95.6

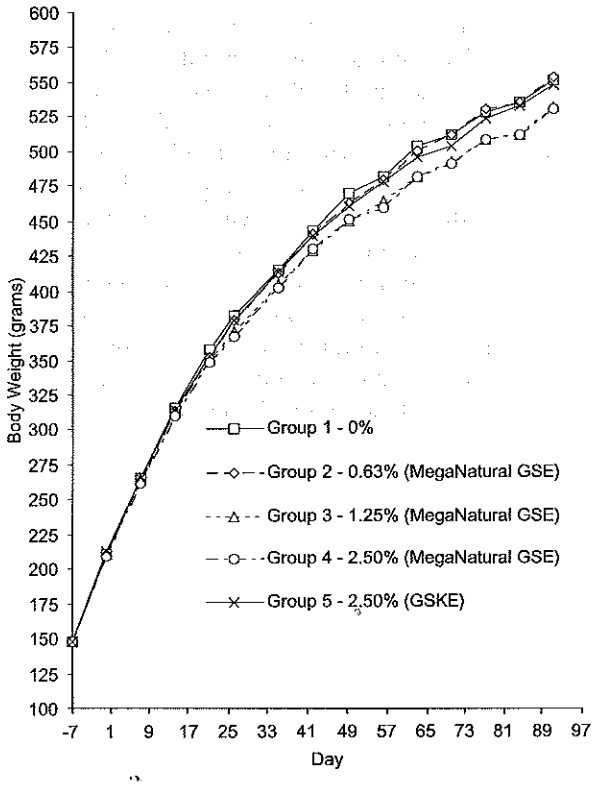

Fig. 1. Group mean body weights of male rats given grape seed extract (GSE) or grape skin extract (GSKE) in their diet for three months.

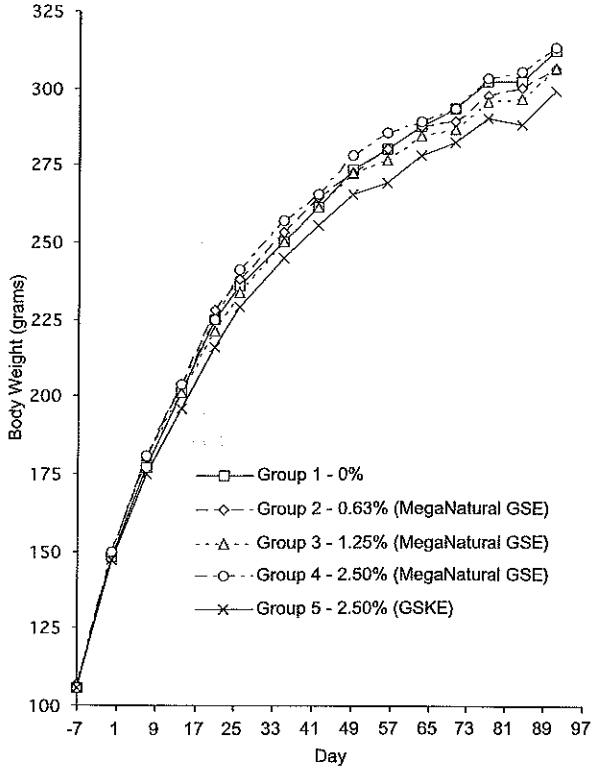

Fig. 2. Group mean body weights of female rats given grape seed extract (GSE) or grape skin extract (GSKE) in their diet for three months.

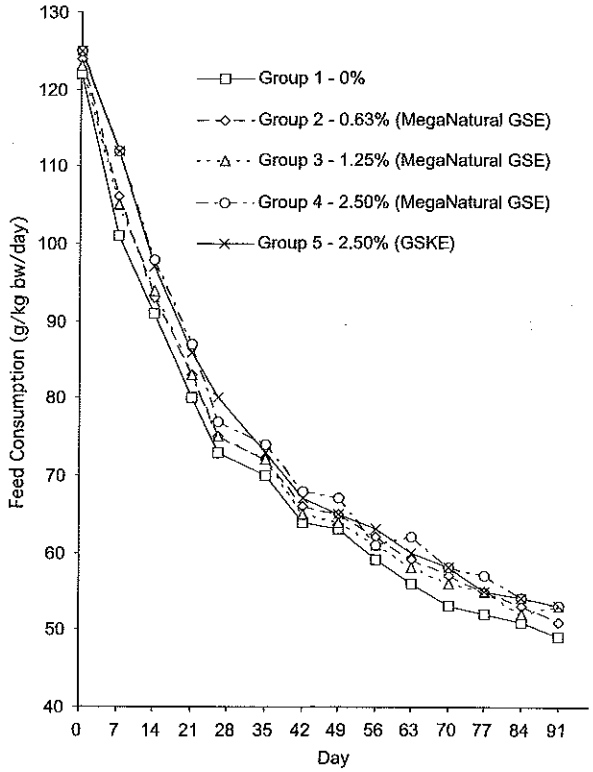

Fig. 3. Group mean feed consumption by male rats given grape seed extract (GSE) or grape skin extract (GSKE) in their diet for three months.

Evaluation of equality of group means was made by the appropriate statistical method, followed by a multiple comparison test if needed. Bartlett's test (Bartlett, 1937; Sokal and Rohlf, 1995) was performed to determine whether groups had equal variances. For all parameters except organ weights, if the variances were equal, parametric procedures were used; if not, non-parametric procedures were used. Organ weight data were analyzed only by parametric methods. The parametric method was the standard one-way analysis of variance (ANOVA) using the F ratio to assess significance (Armitage, 1971; Dunlap and Duffy, 1975). If significant differences among the means were indicated, additional tests were used to determine which means were significantly different from the control: Dunnett's (Dunnett,

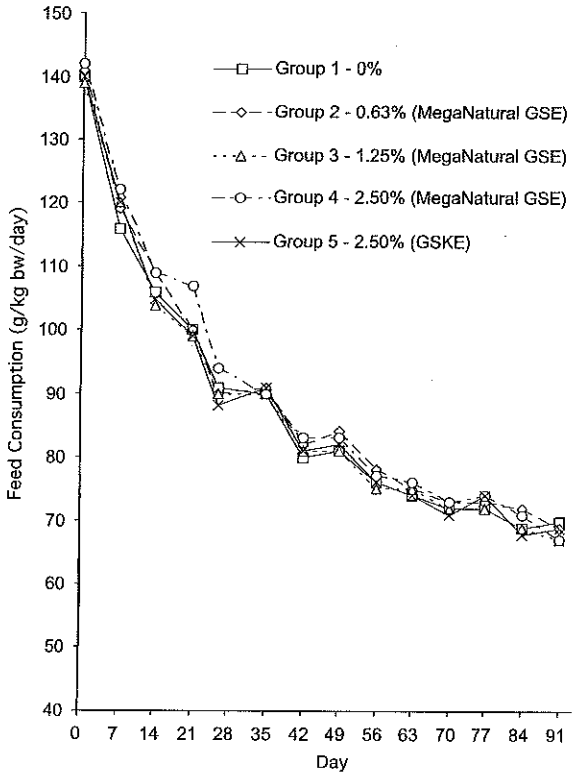

Fig. 4. Group mean feed consumption by female rats given grape seed extract (GSE) or grape skin extract (GSKE) in their diet for three months.

Table 4
Test article intake (mg/kg bw/day)

Dose groups		Day 7	Day 91	Time-weighted mean
Males	0.63% GSE	671	323	434
	1.25% GSE	1308	658	860
	2.50% GSE	2800	1333	1788
	2.50% GSKE	2801	1320	1778
Females	0.63% GSE	751	436	540
	1.25% GSE	1499	841	1052
	2.50% GSE	3026	1677	2167
	2.50% GSKE	2997	1728	2111

Table 5
Terminal hematology: (a) males, (b) females

Parameter		Dietary concentration (%)				
		0 (Control)	0.63% GSE	1.25% GSE	2.5% GSE	2.5% GSKE
(a) Males						
Hemoglobin (g/dl)	Mean	16.6	16.7	16.2	16.8	16.2
	S.D.	0.73	1.11	0.58	0.70	1.77
Hematocrit (%)	Mean	52.1	52.5	51.8	52.9	51.2
	S.D.	1.96	3.82	1.94	2.15	5.12
Erythrocyte count ($10^6/\mu l$)	Mean	9.20	9.28	9.14	9.51	8.76
	S.D.	0.399	0.556	0.389	0.372	1.096
Reticulocyte count ($10^9/l$)	Mean	200.8	209.7	198.7	169.6	231.8
	S.D.	24.98	45.88	38.41	14.45	84.78
Platelet count ($10^3/\mu l$)	Mean	1051	1130	1042	1157	1090
	S.D.	219.0	116.4	227.5	144.9	121.1
Mean corpuscular volume (fl)	Mean	56.7	56.6	56.8	55.6	58.7*
	S.D.	2.01	1.85	2.04	1.07	2.72
Mean corpuscular hemoglobin (pg)	Mean	18.0	17.9	17.8	17.6	18.5
	S.D.	0.84	0.58	0.67	0.49	0.54
Mean corpuscular hemoglobin conc. (g/dl)	Mean	31.8	31.7	31.4	31.7	31.5
	S.D.	0.84	0.64	0.61	0.52	0.64
Total leukocyte count ($10^3/\mu l$)	Mean	12.72	13.78	13.30	13.73	14.18
	S.D.	4.486	2.819	2.261	3.655	2.319
Neutrophils ($10^3/l$)	Mean	1.43	1.69	1.47	1.54	2.42
	S.D.	0.404	0.936	0.374	0.740	2.417
Lymphocytes ($10^3/l$)	Mean	10.60	11.20	11.06	11.38	11.01
	S.D.	4.388	2.263	2.225	2.942	2.281
Monocytes ($10^3/l$)	Mean	0.36	0.50	0.39	0.47	0.45
	S.D.	0.167	0.167	0.080	0.217	0.191
Eosinophils ($10^3/l$)	Mean	0.15	0.17	0.20	0.16	0.12
	S.D.	0.047	0.092	0.181	0.059	0.046
Basophils ($10^3/l$)	Mean	0.08	0.09	0.09	0.08	0.08
	S.D.	0.053	0.031	0.029	0.029	0.042
Large unstained cells ($10^3/l$)	Mean	0.10	0.13	0.10	0.10	0.10
	S.D.	0.062	0.048	0.040	0.054	0.052
Prothrombin time (s)	Mean	11.9	13.0	12.6	12.3	12.6*
	S.D.	0.43	0.90	0.96	0.42	0.75
Activated partial thromboplastin time (s)	Mean	20.9	22.0	21.2	22.5	23.2
	S.D.	2.11	2.06	3.75	2.76	3.69
(b) Females						
Hemoglobin (g/dl)	Mean	15.5	16.2	16.5	15.3	16.8*
	S.D.	1.86	0.90	0.95	1.51	1.42
Hematocrit (%)	Mean	48.1	49.5	50.6	47.1	52.7*
	S.D.	5.44	3.13	3.12	4.73	4.55
Erythrocyte count ($10^6/\mu l$)	Mean	8.10	8.44	8.63	7.97	8.87*
	S.D.	0.767	0.621	0.602	0.847	0.748
Reticulocyte count ($10^9/l$)	Mean	209.7	198.0	220.8	213.1	212.9
	S.D.	34.55	39.43	22.44	85.79	29.20
Platelet count ($10^3/\mu l$)	Mean	1197	1156	1151	1235	1141
	S.D.	357.9	159.6	92.6	214.9	215.8
Mean corpuscular volume (fl)	Mean	59.4	58.7	58.7	59.2	59.3

(*continued on next page*)

Table 5 (*continued*)

Parameter		Dietary concentration (%)				
		0 (Control)	0.63% GSE	1.25% GSE	2.5% GSE	2.5% GSKE
	S.D.	1.82	1.78	1.11	1.23	0.99
Mean corpuscular hemoglobin (pg)	Mean	19.1	19.2	19.1	19.3	19.0
	S.D.	0.65	0.73	0.39	0.48	0.40
Mean corpuscular hemoglobin conc. (g/dl)	Mean	32.1	32.7	32.6	32.5	32.0
	S.D.	0.67	0.65	0.29	0.68	0.42
Total leukocyte count ($10^3/\mu l$)	Mean	12.63	9.78	9.85	10.37	9.26
	S.D.	7.379	2.673	1.967	2.810	3.156
Neutrophils ($10^3/l$)	Mean	2.42	1.06	0.81	0.96	0.80*
	S.D.	4.020	0.554	0.436	0.599	0.263
Lymphocytes ($10^3/l$)	Mean	9.39	8.21	8.54	8.86	7.96
	S.D.	2.846	2.393	1.826	2.626	2.721
Monocytes ($10^3/l$)	Mean	0.53	0.29	0.26	0.28	0.28
	S.D.	0.660	0.155	0.128	0.076	0.158
Eosinophils ($10^3/l$)	Mean	0.12	0.10	0.12	0.12	0.12
	S.D.	0.065	0.030	0.060	0.036	0.068
Basophils ($10^3/l$)	Mean	0.09	0.06	0.06	0.07	0.06
	S.D.	0.106	0.039	0.028	0.059	0.029
Large unstained cells ($10^3/l$)	Mean	0.10	0.06	0.06	0.08	0.05
	S.D.	0.065	0.041	0.021	0.051	0.024
Prothrombin time (s)	Mean	10.8	10.8	10.9	10.7	10.8
	S.D.	0.44	0.25	0.33	0.26	0.29
Activated partial thromboplastin time (s)	Mean	22.0	20.1	19.2	18.0	18.8
	S.D.	8.50	2.44	2.88	2.48	3.20

* Significantly different from control mean: $P \leqslant 0.05$.

1955, 1964; Dunlap et al., 1981), Williams (1971, 1972), or Cochran and Cox's modified *t*-test (Cochran and Cox, 1959). The non-parametric method was the Kruskal–Wallis test (Kruskal and Wallis, 1952, 1953) and if differences were indicated, Shirley's test (Shirley, 1977), Dunn's test (Dunn, 1964) or Pairwise Comparison with Bonferroni Correction (Games and Howell, 1976) were used to determine which means differed from control. Barlett's test for equality of variance was conducted at the 1% significance level; all other statistical tests were conducted at the 5 and 1% significance levels. Dose groups were eliminated from statistical analysis if their standard deviation was 0 and/or *N* (number of animals) in the group was less than or equal to two.

3. Results

3.1. Analysis of diets

Colorimetric analysis of dietary mixes (Table 3) for total phenols, expressed as gallic acid equivalents, confirmed that the preparation procedure used for this study produced homogeneous mixtures and that the test article was stable in the diet, under storage conditions used in this study, for at least 14 days. Analyses conducted during the treatment period confirmed that diets of appropriate concentration were administered.

3.2. Survival, clinical signs and ophthalmoscopy

No unscheduled mortality occurred during this study. Physical observations were generally unremarkable. The only notable observation was occurrence of mild head-tilt in six of 20 female rats treated with 2.5% GSE. The finding was not apparent until the last two observation periods (days 84 and 91) and was not noted in the male animals at any dose of GSE. When the observation was made it was only apparent when the animal was on an open, flat surface; animals in their cages appeared normal. Typically this observation is associated with infection of inflammation of the middle or inner ear; however, there were no macroscopic or microscopic signs of ear infection in these animals. This subtle late-occurring postural change was considered of doubtful relationship to treatment and not relevant to human consumption of this material. There were no findings in the ophthalmic examinations attributable to administration of either test article.

Table 6
Terminal clinical chemistry: (a) males, (b) females

Parameter		0 (Control)	Dietary concentration (%) 0.63% GSE	1.25% GSE	2.5% GSE	2.5% GSKE
(b) Males						
Aspartate aminotransferase (IU/l)	Mean	76	74	76	78	81
	S.D.	11.4	8.0	16.3	10.6	15.3
Alanine aminotransferase (IU/l)	Mean	35	33	37	32	36
	S.D.	7.3	5.1	21.4	5.9	6.7
Alkaline phosphatase (IU/l)	Mean	90	90	83	96	86
	S.D.	14.1	18.3	13.6	13.0	20.9
Blood urea nitrogen (mg/dl)	Mean	12.2	11.7	11.9	12.5	13.0
	S.D.	0.87	1.19	1.30	1.63	1.53
Creatinine (mg/dl)	Mean	0.4	0.3	0.4	0.3	0.4
	S.D.	0.06	0.07	0.05	0.07	0.07
Fasting glucose (mg/dl)	Mean	184	205	207	216	191
	S.D.	39.5	51.1	59.5	47.6	53.8
Cholesterol (mg/dl)	Mean	63	63	59	60	66
	S.D.	12.5	12.2	9.4	14.1	8.4
Triglycerides (mg/dl)	Mean	40	49	45	42	37
	S.D.	14.6	22.8	18.6	12.7	15.8
Total protein (g/dl)	Mean	7.3	7.1	7.0	7.2	7.1
	S.D.	0.31	0.48	0.23	0.25	0.32
Albumin (g/dl)	Mean	4.4	4.3	4.4	4.4	4.4
	S.D.	0.19	0.26	0.11	0.17	0.14
Globulin(calculated) (g/dl)	Mean	2.9	2.7	2.7	2.7	2.7
	S.D.	0.23	0.30	0.18	0.17	0.26
Albumin/globulin	Mean	1.5	1.6	1.7*	1.7*	1.6
	S.D.	0.13	0.16	0.11	0.11	0.17
Total bilirubin (mg/dl)	Mean	0.12	0.11	0.11	0.11	0.11
	S.D.	0.020	0.023	0.027	0.023	0.019
Sodium (mEq/l)	Mean	151	150	151	151	152
	S.D.	1.7	2.0	1.9	1.6	1.4
Potassium (mEq/l)	Mean	6.6	6.4	6.3	6.5	6.7
	S.D.	0.42	1.20	0.52	0.98	0.64
Chloride (mEq/l)	Mean	104	104	105	106**	107**
	S.D.	1.6	1.2	2.1	0.9	1.6
Calcium (mg/dl)	Mean	11.9	11.7	11.9	12.1	12.0
	S.D.	0.47	0.72	0.41	0.76	0.67
Inorganic phosphorus (mg/dl)	Mean	11.0	11.3	12.1	12.5	13.0*
	S.D.	0.91	1.48	2.32	1.40	2.21
γ-Glutamyl transferase (IU/l)	Mean	0	0	0	0.	0
	S.D.	0.0	0.0	0.0	0.0	0.0
(b) females						
Aspartate aminotransferase (IU/l)	Mean	91	156	89	104	93
	S.D.	39.6	149.8	20.9	39.4	22.6
Alanine aminotransferase (IU/l)	Mean	36	80	43	43	43
	S.D.	11.5	93.0	15.4	13.5	13.4
Alkaline phosphatase (IU/l)	Mean	67	49	56	46*	64
	S.D.	34.4	12.6	26.7	11.4	21.9
Blood urea nitrogen (mg/dl)	Mean	15.0	15.3	14.5	15.2	15.0
	S.D.	1.60	1.44	1.57	2.06	2.25

(continued on next page)

Table 6 (*continued*)

Parameter		Dietary concentration (%)				
		0 (Control)	0.63% GSE	1.25% GSE	2.5% GSE	2.5% GSKE
Creatinine (mg/dl)	Mean	0.5	0.4	0.4	0.4	0.5
	S.D.	0.08	0.06	0.07	0.10	0.05
Fasting glucose (mg/dl)	Mean	160	141	158	160	150
	S.D.	57.5	21.6	32.9	27.2	42.5
Cholesterol (mg/dl)	Mean	80	84	75	85	82
	S.D.	12.5	11.3	18.5	20.9	15.9
Triglycerides (mg/dl)	Mean	42	34	35	39	48
	S.D.	11.6	7.6	13.5	11.6	15.5
Total protein (g/dl)	Mean	7.8	7.7	7.4	7.9	8.1
	S.D.	0.55	0.38	0.56	0.77	0.38
Albumin (g/dl)	Mean	4.8	4.8	5.0	5.3	5.3
	S.D.	0.86	0.34	0.49	0.51	0.23
Globulin(calculated) (g/dl)	Mean	3.0	2.8	2.5	2.7	2.8
	S.D.	0.78	0.32	0.35	0.39	0.23
Albumin/globulin	Mean	1.7	1.7	2.1*	2.0*	1.9
	S.D.	0.53	0.28	0.42	0.27	0.15
Total bilirubin (mg/dl)	Mean	0.14	0.14	0.15	0.15	0.16
	S.D.	0.044	0.030	0.031	0.021	0.025
Sodium (mEq/l)	Mean	151	151	155	151	153
	S.D.	2.0	2.4	13.2	2.3	3.5
Potassium (mEq/l)	Mean	6.6	7.4	6.6	7.0	7.7
	S.D.	0.66	2.25	1.23	1.08	2.03
Chloride (mEq/l)	Mean	106	106	105	107	108
	S.D.	1.9	2.0	3.3	1.6	3.3
Calcium (mg/dl)	Mean	12.3	11.6	12.1	12.5	12.2
	S.D.	0.75	1.81	0.93	0.85	1.22
Inorganic phosphorus (mg/dl)	Mean	11.4	11.3	11.3	11.9	13.0
	S.D.	2.59	1.80	2.45	2.14	2.53
γ-Glutamyl transferase (IU/l)	Mean	2	1	0	0	0
	S.D.	3.7	2.5	0.5	0.3	0.5

* Significantly different from control mean: $P \leqslant 0.05$.
** Significantly different from control mean: $P \leqslant 0.01$.

3.3. Body weight, food consumption and test article intake

Mean body weights (Figs. 1 and 2) and body weight gains were similar for all dose groups and both test articles. Feed consumption (Figs. 3 and 4) differences were noted early in the study. From day 7 the male animals receiving 2.5% GSE and GSKE had a small (approx. 10%) but statistically significant increased food consumption compared with controls. This higher consumption continued throughout the study. This may reflect an increased dietary intake to compensate for the high concentration of these test articles in the diet. Male animals consuming 1.25% GSE also had similar statistically significant increases, but at irregular intervals. Feed consumption in the female treatment groups was similar to the control group throughout the study. On a per-unit-body weight basis, test article intake (Table 4) decreased as feed consumption decreased. The multiple between the dose groups was maintained for the duration of the study.

3.4. Clinical pathology

No significant test-article-related changes in hematology or in coagulation parameters were noted at 1 month (data not shown) or at termination in males or females (Table 5a,b). While some statistically significant changes in certain hematology measurements were noted at termination among males and females administered GSKE, the magnitudes were not biologically relevant and the values were within the normal range of rats of

Table 7 (*continued*)

	Dietary concentration (%)				
	0 (Control)	0.63% GSE	1.25% GSE	2.5% GSE	2.5% GSKE
Liver g (S.D.)	8.534 (1.165)	8.267 (0.810)	8.434 (1.366)	8.783 (1.581)	7.886 (0.946)
% of body wt (S.D.)	3.006 (0.394)	2.955 (0.323)	2.996 (0.329)	3.076 (0.429)	2.873 (0.284)
% of brain wt (S.D.)	419.499 (60.620)	405.071 (36.769)	410.733 (72.173)	437.678 (76.218)	389.884 (44.842)
Ovaries g (S.D.)	0.0948 (0.0221)	0.0887 (0.0153)	0.0993 (0.0243)	0.0888 (0.0182)	0.0932 (0.0163)
% of body wt (S.D.)	0.0333 (0.0071)	0.0315 (0.0040)	0.0354 (0.0082)	0.0312 (0.0058)	0.0338 (0.0039)
% of brain wt (S.D.)	4.6576 (1.0613)	4.3324 (0.6543)	4.8303 (1.1918)	4.4197 (0.8470)	4.5918 (0.6773)
Pituitary gland g (S.D.)	0.0189 (0.0043)	0.0199 (0.0053)	0.0200 (0.0089)	0.0222 (0.0092)	0.0173 (0.0040)
% of body wt (S.D.)	0.0066 (0.0014)	0.0071 (0.0018)	0.0070 (0.0026)	0.0077 (0.0028)	0.0063 (0.0012)
% of brain wt (S.D.)	0.9292 (0.2196)	0.9712 (0.2392)	0.9801 (0.4553)	1.1026 (0.4366)	0.8508 (0.1809)
Spleen g (S.D.)	0.581 (0.081)	0.586 (0.059)	0.593 (0.103)	0.552 (0.053)	0.547 (0.076)
% of body wt (S.D.)	0.203 (0.021)	0.209 (0.019)	0.211 (0.031)	0.195 (0.023)	0.199 (0.019)
% of brain wt (S.D.)	28.371 (3.467)	28.639 (2.639)	28.897 (5.333)	27.552 (2.881)	26.981 (3.037)
Thymus g (S.D.)	0.314 (0.070)	0.305 (0.061)	0.328 (0.062)	0.329 (0.087)	0.312 (0.069)
% of body wt (S.D.)	0.111 (0.025)	0.109 (0.025)	0.117 (0.023)	0.115 (0.027)	0.114 (0.024)
% of brain wt (S.D.)	15.431 (3.424)	14.936 (2.859)	15.915 (2.806)	16.355 (4.152)	15.392 (3.200)
Thyroid/parathyroid g (S.D.)	0.0228 (0.0049)	0.0240 (0.0042)	0.0241 (0.0058)	0.0233 (0.0027)	0.0232 (0.0060)
% of body wt (S.D.)	0.0081 (0.0020)	0.0086 (0.0015)	0.0086 (0.0020)	0.0082 (0.0012)	0.0085 (0.0019)
% of brain wt (S.D.)	1.1193 (0.2353)	1.1807 (0.2182)	1.1741 (0.2847)	1.1618 (0.1422)	1.1484 (0.2874)
Uterus g (S.D.)	0.791 (0.279)	0.668 (0.167)	0.710 (0.188)	0.794 (0.296)	0.717 (0.258)
% of body wt (S.D.)	0.282 (0.112)	0.238 (0.059)	0.254 (0.066)	0.285 (0.130)	0.259 (0.079)
% of brain wt (S.D.)	39.052 (14.617)	32.601 (7.350)	34.447 (8.984)	39.676 (15.132)	35.218 (11.362)

**Mean value of group was significantly different from control at $P=0.05$ with Dunnett's test of significance. * (***) Mean value of group was significantly different from control at $P=0.05$ (0.01) with Modified T test of significance.

Table 8
Incidence of renal cortical inflammation in rats receiving GSE or GSKE in the diet

	Males			Females		
	Control $N=20$	2.5% GSE $N=20$	2.5% GSKE $N=20$	Control $N=20$	2.5% GSE $N=20$	2.5% GSKE $N=20$
Total incidence	4	4	11*	2	2	2
Severity:						
Minimal	4	2	9	2	2	2
Slight	0	1	2	0	0	0
Moderate	0	1	0	0	0	0

* Significantly different from control: $p \leq 0.05$, Fisher's exact two-tailed test.

this strain and age. Similarly, a statistically significant change was seen in prothrombin times of male animals in the GSKE groups. The magnitude of the difference was small and not considered clinically relevant. There were no changes in clinical chemistry parameters in males or females at 1 month (data not shown) or at termination (Table 6a,b), that were attributed to administration of the test articles.

An isolated statistically significant ($P<0.05$) decrease was noted for absolute and relative heart weights (Table 7a,b) in female rats treated with GSKE at 2.5% in the diet; females in the 1.25% GSE group exhibited a decrease in heart/body weight ratio only. These observations are not considered treatment related since they were not observed in males, did not exhibit a dose–response among GSE-treated groups and there was no correlated microscopic pathology. No other potentially treatment-related effects were seen in organ weights and ratios. No test-article-related abnormalities attributable to either test article were noted at necropsy.

Histopathological examination of scheduled tissues was conducted on all animals in the control and two high-dose groups. In those animals receiving 2.5% GSE in the diet, no microscopic lesions attributable to treatment were observed. In male rats receiving 2.5% GSKE, a significant ($P<0.05$) increase in the occurrence of a common renal cortical inflammation, comprising predominantly lymphocytic interstitial infiltrates, was observed in 11 of 20 animals compared

with occurrence in four of 20 control animals (Table 8). The severity of most cases was minimal. The increased frequency was not observed in female rats of the same treatment group. However, this lesion is commonly seen in male rats, increasing in frequency and severity with age. It is considered a component of the entity of chronic nephropathy and therefore not treatment related. This was corroborated by comparison with observations made in two 3-month studies conducted in the same laboratory contemporaneously with the present study using the same strain and source of animals. Control male rats in those two studies exhibited renal cortical inflammation in 40% (8/20) and 50% (5/10) of animals, respectively. The remaining lesions observed in rats receiving 2.5% GSKE were present at a frequency comparable to that of control animals, and were considered to be incidental and representative of changes commonly observed in young rats.

4. Discussion

GSE and GSKE were tolerated well and did not produce any general organ or systemic toxicity when fed to male and female rats at dietary concentrations as high as 2.5% (mean time-weighted daily doses equivalent to approximately 1780 mg/kg body weight in males and 2150 mg/kg body weight in females) for a period of at least 90 days. Males receiving 2.5% of either GSE or GSKE maintained normal growth but had consistently increased food consumption throughout the study compared with control, possibly indicating a compensatory response to the high dietary concentration of these materials. No other changes in body weight gain, food consumption, or survival were found. There were no significant effects on clinical signs or organ weights, no macroscopic observations at necropsy and no histological changes considered to be related to treatment. The occurrence rate of mild renal cortical inflammation in male rats treated with 2.5% GSKE, while increased in comparison to male controls in this study, was consistent with the occurrence rate in contemporaneously conducted 3-month studies and is considered to be an incidental finding unrelated to administration of GSKE. In addition, there were no adverse or clinically relevant changes in clinical chemistry, hematology or blood coagulation parameters at either the 1-month or terminal measurement time points.

The antioxidant activity of GSE and GSKE PACs make them candidates for addition to foods and beverages to retard deterioration, as well as when ingested to potentially support physiologic defenses against in vivo generated free radical species. Although humans have ingested PACs for centuries without reported adverse effect and current dietary intake by US consumers is estimated to be in the range of 460–1000 mg/day, the current toxicology literature contains relatively little formal evidence regarding their safety. Studies reported in the literature have primarily investigated nutritional effects. Tebib et al. (1994) investigated the effects of grape seed PACs fed at 2% of the diet for up to 9 weeks to rats maintained on hypercholesterolemic diets, and reported no adverse effects related to safety in addition to potentially beneficial changes in serum cholesterol and triglycerids parameters. The same author reported a subsequent 12-week study (Tebib et al., 1995) which investigated cecal fermentation and colonic bacterial enzymes in rats fed PACs at a low dietary concentration of 71 mg/kg of diet; again no adverse effects were noted. Bagchi et al. (2000), reported results of a series of studies with Activin, a commercial grape seed PAC extract. These animal studies primarily examined endpoints related to efficacy but did include an acute oral intubation rat study that demonstrated an LD_{50} of greater than 5000 mg/kg, the highest dose employed.

A potentially adverse nutritional effect on protein digestibility was reported by Vallet et al. (1994), who fed grape seed tannins (polymeric high molecular weight PACs and related compounds) for 31 days at concentrations of 0.2 and 2.0% to Sprague–Dawley rats maintained on a "protein free" diet. Growth in the high-dose group was reported to have decreased significantly while fecal nitrogen excretion and fecal dry weight increased. This was thought to be a result of the ability of tannins to form less digestible complexes with dietary and intralumenal protein. These effects were not observed when dietary tannin content was 0.2% or in the previously cited studies using protein-adequate diets. The present study employed a standard rodent chow containing significant protein and is not expected to have been affected by this phenomenon.

Results of the current study strongly support the safety of GSE and GSKE as dietary components for human consumption. The no-observed-adverse-effect level (NOAEL) was considered to be approximately 2150 mg/kg bw/day for administration of GSE as well as GSKE to female rats while approximately 1780 mg/kg/bw/day was considered a NOAEL in male rats. These values represent the time-weighted mean dose rates occurring in the high-dose groups over the course of the study.

References

Armitage, P., 1971. Statistical Methods in Medical Research. Blackwell, Oxford.

Bagchi, D., Bagchi, M., Stohs, S.J., Das, D.K., Ray, S.D., Kuszynski, C.A., Johsi, S.S., Pruess, H.G., 2000. Free radicals and grape seed proanthocyanidin extract: Importance in human health and disease prevention. Toxicology 148, 187–197.

Barlett, M.S., 1937. Properties of sufficiency and statistical tests. Proceedings of the Royal Society, Series A 160, 268–282.

Cochran, W.G., Cox, G.M., 1959. Experimental Designs. John Wiley, New York.

Constant, J., 1997. Alcohol, ischemic heart disease, and the French Paradox. Clinical Cardiology 20, 42–424.

Das, D.K., Sato, M., Ray, P.S., Maulik, G., Engelman, R.M., Bertelli, A.A.E., Bertelli, A., 1999. Cardioprotection of red wine: role of polyphenolic antioxidants. Drugs in Experimental and Clinical Research 25 (2/3), 115–120.

Dunlap, W.P., Duffy, J.A., 1975. Fortran IV functions for calculating exact probabilities associates with z, chi-square, t and f values. Behavioral Research Methods and Instrumentations 7, 59–60.

Dunlap, W.P., Marx, M.S., Agamy, G.G., 1981. Fortran IV functions for calculating probabilities associated with Dunnett's test. Behavioral Research Methods and Instrumentations 13, 363–366.

Dunn, O.J., 1964. Multiple comparisons using rank sums. Technometrics 6, 241–252.

Dunnett, C.W., 1955. A multiple comparison procedure for comparing several treatments with a control. Journal of the American Statistical Association 50, 1096–1121.

Dunnett, C.W., 1964. New tables for multiple comparisons with a control. Biometrics 20-3, 482–491.

Games, P.A., Howell, J.F., 1976. Pairwise multiple comparison procedures with unequal n's and/or variances: a monte-carlo study. Journal of Educational Statistics 1, 113–125.

Hammerstone, J.F., Lazarus, S.A., Schmitz, H.H., 2000. Procyanidin content and variation in some commonly consumed foods. Journal of Nutrition 130, 2086. s-2092s.

Henry, J.B., 1991. Clinical Diagnosis and Management by Laboratory Methods, eighteenth ed. W.B. Saunders Co, Philadelphia.

Hu, J., Calomme, M., Lasure, A., et al., 1995. Biological Trace Element Research 47, 327 [cited in: Williams, R.L., Elliott, M.S., 1997. Antioxidants in Grapes and Wine: Chemistry and Health Effects. Old Dominion University Enological Research Facility, Department of Chemistry/Biochemistry, pp. 3–26 (Chapter 9)].

Kopp, P., 1998. Resveratrol, a phytoestrogen found in red wine. A possible explanation for the conundrum of the 'French paradox'? European Journal of Endocrinology 138, 619–620.

Kruskal, W.H., Wallis, W.A., 1952. Use of ranks in one-criterion variance analysis. Journal of the American Statistical Association 47, 583–621.

Kruskal, W.H., Wallis, W.A., 1953. Errata for Kruskal–Wallis (1952). Journal of the American Statistical Association 48, 907–911.

Law, M., Wald, N., 1999. Why heart disease mortality is low in France: the time lag explanation. British Medical Journal 318, 1471–1480.

Maalej, N., Demrow, H.S., Slane, P.R., Folts, J.D.l., 1997. Antithrombotic effect of flavonoids in red wine. In: Wine Nutritional and Therapeutic Benefits. Journal of the American Chemical Society 247–260 (Chapter 19).

Maxwell, S.R.J., 1997. Wine antioxidants and their impact on antioxidant activity in vivo. In: Wine Nutritional and Therapeutic Benefits. Journal of the American Chemical Society 150–165 (Chapter 12).

Nigdikar, S.V., Williams, N.R., Griffin, B.A., Howard, A.N., 1998. Consumption of red wine polyphenols reduces the susceptibility of low-density lipoproteins to oxidation in vivo. American Journal of Clinical Nutrition 68, 258–265.

Nutall, S.L., Kendall, M.J., Bombardelli, E., Morazzoni, P., 1998. An evaluation of the antioxidant activity of a standardized grape seed extract, Leucoselect. Journal of Clinical Pharmacy and Therapeutics 23, 385–389.

Ricardo-DaSilva, J.M., Darmon, N., Fernandez, Y., Mitjavila, S., 1991. Oxygen free radical scavenger capacity in aqueous models of different procyanidins from grape seeds. Journal of Agricultural and Food Chemistry 39 (9), 1549–1552.

Santos-Buelga, C., Scalbert, A., 2000. Proanthocyanidins and tannin-like compounds—nature, occurrence, dietary intake, and effects on nutrition and health (Review). Journal of the Science of Food and Agriculture 80, 1094–1117.

Scalbert, A., Williamson, G., 2000. Dietary intake and bioavailability of polyphenols. Journal of Nutrition 130, 2073. s-2085s.

Scott, B.C., Butler, J., Halliwell, B., Aruoma, O.I., 1993. Evaluation of the antioxidant actions of ferulic acid and catechins. Free Radical. Research Communications 19 (4), 241–253.

Shirley, E., 1977. A non-parametric equivalent of Williams' test for contrasting increasing dose levels of a treatment. Biometrics 33 (2), 386–389.

Singleton, V.L., Rossi, J.L., 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American Journal of Enology and Viticulture 43, 27–43.

Sokal, R.R., Rohlf, F.J., 1995. Biometry. 3rd edition. W.H. Freeman, San Francisco, pp. 369–371.

Tebib, K., Besancon, P., Rouanet, J.M., 1994. Dietary grape seed tannins affect lipoproteins, lipoprotein lipases, and tissue lipids in rats fed hypercholesterolemic diets. Journal of Nutrition 124, 2451–2457.

Tebib, K., Besancon, P., Rouanet, J.M., 1995. Effects of dietary grape seed tannins on rat cecal fermentation and colonic enzymes. Nutrition Research 16 (1), 105–110.

Vallet, J., Rouanet, J.M., Besancon, P., 1994. Dietary grape seed tannins: effects on nutritional balance and on some enzymic activities along the crypt-villus axis of rat small intestine. Annals of Nutrition and Metabolism 38, 75–84.

Whitehead, T.P., Robinson, D., Allaway, S., et al., 1995. The effect of red wine ingestion on the antioxidant capacity of serum. Clinical Chemistry 41, 32–35.

Williams, D.A., 1971. A test for differences between treatment means when several dose levels are compared with a zero dose control. Biometrics 27, 103–117.

Williams, D.A., 1972. The comparison of several dose levels with a zero dose control. Biometrics 28, 519–531.

Zhao, J., Wang, J., Chen, Y., Agarwal, R., 1999. Antitumor-promoting activity of a polyphenolic fraction isolated from grape seeds in the mouse skin two-stage initiation-promotion protocol and identification of procyanidin B5-3′-gallate as the most effective antioxidant constituent. Carcinogenesis 20 (9), 1737–1745.