high enough concentration to affect the cells and therefore validates the study. No statistically significant increase in micronucleated PCE's was observed at any dose level or harvest time point. The MegaNatural™ GSKE induced no signs of clinical toxicity in any of the treated animals, nor was it cytotoxic to the bone marrow at either time point. GSKE did not induce any statistically significant increase in mouse micronucleated PCE's at any dose level or harvest time point. A summary of the study findings is presented below in Table 8.2. It was concluded that the test articles, MegaNatural™ GSE and MegaNatural™ GSKE, were negative in the mouse bone marrow micronucleus assay under the conditions of this assay.

Table 8.2 Micronucleus Data Summary

Treatment	Dose	Harvest Time (hr)	% Micronucleated PCE's (±SE)	Ratio of PCE:NCE (Mean ±SE)
Vehicle Control	0.5% CMC	24-hours	0.04±0.02	0.53±0.03
		48-hours	0.05±0.02	0.79±0.09
Positive Control	80 mg/kg	24-hours	2.19±0.20*	0.41±0.03**
MegaNatural™ GSE	500 mg/kg	24-hours	0.02±0.01	0.61±0.06
	1000 mg/kg	24-hours	0.02±0.01	0.76±0.14
	2000 mg/kg	24-hours	0.04±0.03	0.55±0.06
		48-hours	0.06±0.02	0.44±0.44**
MegaNatural™ GSKE	500 mg/kg	24-hours	0.04±0.03	0.84±0.06
	1000 mg/kg	24-hours	0.01±0.01	0.75±0.07
	2000 mg/kg	24-hours	0.05±0.03	0.50±0.05
		48-hours	0.03±0.02	0.68±0.08

* Significantly greater than the corresponding vehicle control, $p<0.01$.
** Significantly less than the corresponding vehicle control, p 0.05.

000100

8.1.2 Literature Evaluation of Mutagenicity Studies of Polyphenols

8.1.2.1 Mutagenicity Studies Employing Gravinol Super™

Based upon the compositional comparison of MegaNatural™ GSE and GSKE to the Kikkoman Gravinol Super™ as found in Section 3.1, the following evaluation of Gravinol Super™ is especially relevant in establishing the lack of mutagenic activity in GSE and GSKE. Based on their own observations, Yamakoshi, *et al.*, [2002] concluded that Gravinol Super™ was non-mutagenic in a reverse mutation test, a chromosomal aberration test, and a micronucleus test following oral administration to mice.

The mutagenic potential of Gravinol Super™ grape seed extract was investigated using a *Salmonella tymphimurium* Ames assay, a chromosomal aberration test using Chinese hamster lung (CHL) cells, and a micronucleus test using ddY mice. Strains TA98, TA100, TA 1535, and TA1537 of *S. tymphimurium* were exposed to increasing concentrations of Gravinol Super™ (19-1250 μg/plate in TA98 and TA100, 156-5000 μg/plate in TA 1535 and 1537) with and without S-9 mixture. No increase was observed in the number of revertant colonies occurred in the four strains at any concentration, either in the presence or absence of the S-9 mix.

In an *in vitro* chromosomal aberration test, Gravinol Super™ was examined for the potential to induce structural chromosome aberrations in cultured CHL cells. In the absence of S-9 mix, cells were exposed to Gravinol Super™ at doses of 9.4, 18.8, and 37.5 μg/ml continuously for 24 or 48 hours. Cells were exposed for 6 hours to Gravinol Super™ at doses of 18.8, 37.5, 75.0, 150.0, and 300.0 μg/ml in the presence of S-9 mix and 18.8, 37.5, and 75.0 μg/ml in the absence of S-9 mix, washed, and cultured for an additional 18 hours. CHL cells were also cultured with dimethylnitrosamine (DMN; 1000 μg/ml), and Mitomycin C (MMC; 0.04 μg/ml) as positive controls. No biologically or statistically significant increases in the frequency of metaphases with aberrant chromosomes were seen in the cultures treated with Gravinol Super™ at either sampling time, either in the presence or absence of S-9 mix.

In a mouse micronucleus assay, Gravinol Super™ was dissolved in distilled water and administered orally at doses of 0, 0.5, 1, and 2 g/kg to male ddY mice twice, with doses coming 24 hours apart. MMC was given to mice as a positive control. Peripheral blood (5 μl) was collected from the ventral tail 24 hours after the final dose and placed on acridine orange-coated glass slides. The frequency of micronucleated peripheral reticulocytes (MNRETs) was counted based on an examination of 1000 reticulocytes per mouse. The frequencies of MNRET's in the Gravinol Super™ treated mice (0.10%, 0.22%, and 0.36% for the 0.5 g/kg, 1.0 g/kg, and 2.0 g/kg treatments, respectively) were not significantly greater than the control frequency (0.13%).

8.1.2.2 Literature Citations of Mutagenicity Studies Employing Individual Components Found in MegaNatural™ Gold GSE and GSKE

Several studies have been reported which examined the mutagenic activity of fractions isolated from extracted wine polyphenols. This activity was reportedly associated with the presence of quercetin compounds in selected fractions. In an attempt to isolate and identify any mutagens found in red wine, an organic extraction process was employed to remove as much of the polyphenols from the red wine as possible [Yu *et al.*, 1986]. The resultant extract was then evaporated and extracted with methanol and evaporated to a final volume of 25 ml. Histidine auxotrophic strains TA98 and TA100 of *Salmonella typhimurium* were employed in conjunction with human fecal glycosidases in the presence or absence of S9 rat liver microsomes. In the initial screening, the phenolic extract was tested over a 3-log concentration range in the presence of S9 and fecalase in both strains. Revertant rates twice that of the spontaneous reversion rate were obtained in both strains. The mutagenicity of the phenolic extract exhibited a dose response effect over a narrower range of concentrations. Using a Sephadex LH-20 chromatographic column, the phenolic extract was then fractionated further into 3 fractions FrI, FrII, and FrIII. Each fraction was then tested in strains TA98 and TA100 again with fecalase in the presence or absence of S9. FrIII was significantly more mutagenic in TA98 in the presence of fecalase than the other fractions and was selected for further fractionation. Employing multi-layer coil counter-current

chromatography, FrIII was broken down into 5 sub fractions designated FrIII-1 through FrIII-5. Previous observation had determined TA98 to be the more sensitive indicator of mutagenicity of the wine components and the five FrIII fractions were then tested in TA98. Most of the mutagenic activity of FrIII was essentially recovered in FrIII-4. Concentration and UV spectrographic analysis determined the mutagenic component to be Rutin (3-rhamnoglucosyl quercetin).

In a second *S. typhimurium* mutagenesis assay, the mutagenic potential of the substances found in red and white wines of Chile were examined because of the high risk of gastric cancer in the region [Bull *et al.*, 1987]. Samples of 50 - 200 mL of wine were concentrated to one tenth of their volume by lyophilization. An equivalent of 10 mL wine was subsequently extracted in 20 volumes of chloroform:methanol (2:1) followed by addition of 5 volumes of water. Both the upper methanolic and lower chloroformic phases were then evaporated at room temperature under nitrogen and the residue was dissolved in 1 ml of dimethyl sulfoxide (DMSO). During preliminary evaluation, substances in the chloroform phase displayed no mutagenic activity. Therefore, only the methanolic phase was employed in the later experiments. The mutagenicity assays were conducted using the methanolic phase in *Salmonella typhimurium* strains TA98 and TA100, both with and without metabolic activation in the form of S9 rat liver fraction microsomes. The results indicated that the fraction isolated from red wines were twice as mutagenic as the fraction from white wines in TA98, whereas activity was about equal in strain TA100. A linear relationship was observed between mutagenic activity and the amount of wine extractant added up to 1 ml. When the tests were conducted in the presence of S9 metabolic activation, mutagenic activity remained the same as that obtained without activation. In this study, no attempt was made to determine which component found in the methanolic fraction taken from red wine was responsible for the mutagenic properties displayed in *S. typhimurium*. In the discussion of their results, the authors cited research postulating the mutagen may be a glycoside of mutagenic flavanols such as quercetin. This would be consistent with the findings of Yu [1986], discussed previously.

An overview of the beneficial and hazardous effects of phenolic compounds conducted by Stich [1991] indicated that the large variety of phenolic compounds in nature may posses multiple functions and thus some may act as antimutagens, anticarcinogens, and antipromoters, whereas others may act as mutagenic, carcinogenic, and promoting agents. Choi *et al.,* [1994] investigated the mutagenic and antimutagenic activities of several flavonoids, including (+)-catechin and (-)-epicatechin using the Ames test. Both (+)-catechin and (-)-catechin demonstrated a dose-dependent antimutagenic activity against aflatoxin B_1, with a 5.0% solution of the flavonoids inhibiting mutagenicity by 89% and 77% respectively. On the other hand, Hayakawa *et al.*, [1999] reported that catechin, among other polyphenolic compounds, in conjunction with Cu^{2+}, facilitated DNA cleavage. At the molecular level, simple phenolics can induce double strand DNA breaks *in vitro*, as shown in Table 8.3 below [Stich, 1991].

Table 8.3 DNA-Breaking Activity of Phenyl Compounds *In Vitro*

Compound	Substitution Groups				DNA-Breaking Activity
	R1	R2	R3	R4	
Phenol	OH	H	H	H	-
Pyrocatechol	OH	OH	H	H	+++
Resorcinol	OH	H	OH	H	-
Hydroquinone	OH	H	H	OH	+++
Pyrogallol	OH	OH	OH	H	+++
Gallic Acid	OH	OH	OH	H	+++
Caffeic Acid	OH	OH	OH	H	+++
Ferulic Acid	OH	OH	H	H	+
Chlorogenic Acid	OHC_3	OH	H	H	+

000104

In a series of experiments designed to evaluate flavonoid action upon DNA, as both a mediator of antioxidant and pro-oxidant activity, Ohshima *et al.* [1998], examined the effects of 18 flavonoids upon the pBR322 plasmid isolated from calf thymus. In the first assay, the level of single-strand DNA breakage (SSB) was evaluated by incubation of the various flavonoids in the absence or presence of a NO releasing compound, diethylamine-NO. Incubation of the plasmid DNA with 0.1 mM of flavonoid alone for 30

minutes resulted in no significant increase in conversion of the covalently closed circular double-stranded super-coiled DNA (form I) to a relaxed open circle (form II), compared with that of the non-treated plasmid. Incubation with the flavonoid and DEA-NO, also at a concentration of 0.1 mM, increased the formation of form II significantly, although catechin and epicatechin were not regarded to have had a significant effect in this regard. The results of this assay were expressed as the mean number of SSB (±SD) per 10^4 base pairs of DNA after correcting for background and are shown below in Table 8.4.

Table 8.4 DNA Strand Breaks Induced by Flavonoids in Combination with an NO-Releasing Compound

Flavonoid	Single strand breaks per 10^4 bp DNA	
	None	DEA-NO
None	0	0
Catechin	0	0.12±0.10
Cyanidine chloride	0.06±0.05	0.37±0.12
Delphinidine chloride	0.10±0.07	0.49±0.05*
Epicatechin	0	0.08±0.02
Epicatechin gallate	0	0.14±0.04
Epigallocatechin	0	0.27±0.08
Epigallocatechin gallate	0	0.69±0.02*
Myricetin	0	0.99±0.02*
Quercetagetin	0.14±0.03	1.31±0.14*
Quercetin	0	0
Rutin	0	0
Caffeic Acid	0	0
Catechol	0.06±0.04	0.57±0.08*
Gallic acid	0	0.20±0.10
Pyrogallol	0.12±0.04	1.70±0.14*
Catechol in water**	0.06±0.04	1.54±0.02*
Pyrogallol in water**	0.18±0.04	2.92±0.04*

* Significant induction of SSB compared to nontreated DNA ($p<0.05$).
** The experiments were carried out in the absence of 0.1% DMF.

000105

In a second assay conducted by Ohshima *et al.* [1998], 0.5 mM of peroxynitrite was added to a reaction mixture containing flavonoids prepared in dimethyl formamide (DMF) at a concentration of 0.05 mM or 0.5 mM. Each reaction was conducted in duplicate. After having run to completion, each reaction was dried and analyzed for single strand breaks per 10^4 base pairs of DNA. It was determined that flavonoids decreased peroxynitrite mediated SSB in a dose dependant manner as shown below in Table 8.5.

Table 8.5 Effect of Flavonoids on Peroxynitrite Mediated DNA Strand Breaks

Flavonoid	Inhibition (%) of Peroxynitrite Mediated SSB *	
	0.05 mM	0.5 mM
Apigenin	54	55
Baicalein	51	68
Catechin	85	97
Cyanidine	88	92
Delphinidine	29	86
Epicatechin	85	95
Epicatechin gallate	24	87
Epigallocatechin	34	70
Epigallocatechin gallate	64	93
Myricetin	49	82
Quercetin	42	73
Quercetagetin	22	88
Rutin	29	95
Caffeic Acid	91	97
Catechol	89	98
Gallic acid	71	93
Pyrogallol	73	89

* Peroxynitrite at 0.5mM induced formation of 3.69±0.10 SSB per 10^4 bp DNA in the presence of 0.1% DMF.

000106

In a similar assay, the effect of exogenous flavonoids added at concentrations of 0.01, 0.1, and 1mM on the peroxynitrite mediated nitration of guanine in the plasmid DNA was evaluated. Most test compounds at the 1 mM concentration almost completely inhibited 8-nitroguanine formation. Samples were tested by HPLC for the presence of 8-

nitroguanine or 8-oxoguanine. The levels of 8-nitroguanine and 8-oxoguanine in DNA formed with 0.1 mM peroxynitrite were 2.89±0.41 and 0.25±0.08 mM/M guanine, respectively. Decomposed peroxynitrite did not generate 8-nitroguanine but formed 8-oxoguanine at the level of 0.08±0.01 mM/M guanine. Non-treated plasmid DNA contained 8-oxoguanine at the level of 0.05±0.01 mM/M guanine but no detectable 8-nitroguanine. The 8-oxoguanine levels in the present assay were not significantly higher in the calf thymus DNA plasmid treated with peroxynitrite than in DNA treated with decomposed peroxynitrite [Ohshima *et al.*, 1998]. Therefore, no clear effects, either acceleration or inhibition, of the tested flavonoids on the formation of 8-oxoguanine could be determined. The results of HPLC analysis of 8-nitroguanine are presented below in Table 8.6.

Table 8.6 Effect of Flavonoids on Peroxynitrite Mediated Formation of 8-nitroguanine

Flavonoid	Inhibition (%) of Peroxynitrite Mediated Formation*		
	0.01 mM	0.1 mM	1 mM
Apigenin	8	36	77
Baicalein	33	92	100
Catechin	29	88	99
Cyanidine	40	78	100
Delphinidine	20	85	100
Epicatechin	15	79	97
Epicatechin gallate	19	100	100
Epigallocatechin	11	86	94
Epigallocatechin gallate	15	98	100
Myricetin	32	89	99
Quercetin	24	95	100
Quercetagetin	15	100	100
Rutin	23	95	100
Caffeic Acid	43	90	98
Catechol	29	96	98
Gallic acid	12	95	100
Pyrogallol	18	93	100

* Results are presented as percentage of control.

000107

Heo *et al.* [1992] investigated the mutagenic and the anti-clastogenic effects of 5 groups of flavonoids, including (-)-epicatechin, in bone-marrow-polychromatic erythrocytes of ICR mice. Mice were given 0.1 mg/kg, 1.0 mg/kg, or 10.0 mg/kg of the respective flavonoids for five days to test the mutagenic potential of each compound. On the fifth day, 150 mg/kg of the carcinogen benzo[a]pyrene was injected intraperitoneally into half of the mice at each treatment level in order to test the anti-clastogenic activity of the flavonoids. The mice were then sacrificed and preparations of bone marrow were carried out to allow for observation of the frequency of micronucleated polychromatic erythrocytes (MPCEs). Epicatechin and catechin, like almost all of the other flavonoids, did not induce a significant increase in MPCEs as compared to controls. The only flavonoid that demonstrated significant clastogenic activity was quercetin. In addition, most of the flavonoids, including (-)-epicatechin, were able to suppress benzo[a]pyrene-induced MPCEs, though (-)-epicatechin did so less potently than the others. Similarly, epicatechin was able to reduce the clastogenic effects of two other carcinogens, ethyl methanesulfonate and 7,12-dimethylbenz[a]anthracene.

At the cellular level, several simple phenolic compounds which are absent or present only at very low levels in the MegaNatural™ GSE and GSKE have proven to be inducers of clastogenic activity through induction of chromosome / chromatid aberrations and sister-chromatid exchanges, as indicated in Table 8.7. This clastogenic activity has not always been matched by an equally strong mutagenic effect in the *Salmonella typhimurium* assay.

000108

Table 8.7 Clastogenic Activity of Monomeric Phenolic Compounds in Chinese Hamster Ovary (CHO) Cells

Compound	Concentration (mg/ml)	Metaphases with Chromosome Aberrations* (%)	Chromatid Breaks per Cell	Chromatid Exchanges per Cell
Catechol	0.05	23.5	0.17	0.67
4-methyl catechol	0.01	20.0	0.10	0.73
Resorcinol	1.6	14.3	0.08	0.95
Phloroglucinol	3.0	10.1	0.03	0.21
Pyrogallol	0.1	22.6	0.14	1.08
p-Hydroxybenzoic acid	25.0	0.0	0.00	0.00
Protocatechuic acid	3.0	20.5	0.04	0.87
Vanillic acid	25.0	4.8	0.02	0.07
Gallic acid	0.05	24.1	0.03	0.70
Syringic acid	3.0	38.6	0.17	1.44
Salicylic acid	25.0	1.5	0.02	0.00
3,4-Dihydroxyphenylacetic acid	0.1	18.5	0.01	1.23
p-Coumaric acid	6.0	3.0	0.01	0.08
Caffeic acid	0.2	33.3	0.08	3.08
Ferulic acid	25.0	7.8	0.12	0.15
Eugenol	0.4	21.5	0.08	0.20

* Concurrent control frequencies for cells receiving culture medium only were 0.7% metaphase plates with chromosome aberrations, with an average of 0.01 breaks per cell and 0.00 exchanges per cell.

The genotoxic activity of phenolic compounds is strongly influenced by a host of modulating factors including varying sensitivities of the different bioassays to the electrophilic metabolites of the phenolics or to reactive compounds generated. However, the *in vitro* bioassays that have been applied to uncover mutagenic and, clastogenic properties of phenols can also be applied to reveal their anti-genotoxic capacities. Phenolic compounds have been shown to inhibit the genotoxic action of a variety of compounds with modes of action that differ widely.

000109

Of particular relevance was a study by Fukuhara *et al*, [1981], which focused on the inhibitory action of phenolics on the mutagenicity of mixtures to which human population

groups are actually exposed, particularly during the pyrolysis of the protein albumin at different temperatures and under nitrogen or air atmosphere in the TA98 strain of *S. typhimurium*. Catechin, as well as chlorogenic acid, pyrocatechol, quercetin, tannic acid, and propyl gallate were all evaluated at 20, 50, 100, or 150μl per plate. Evaluations were also conducted both in the presence of absence of 50μl of S9 liver microsomal fraction. Overall, the polyphenols reduced the mutagenic activity of albumin pyrolyzates to 20-76% that of albumin without additives. In the presence of S9, the mutagenic activity of pyrolyzates of albumin was reduced by the addition of quercetin and catechin at 20, 50, 100, and 150 μl per plate. Some reduction of mutagenicity was observed in the other polyphenols tested.

Toering *et al.* [1996] investigated the ability of (+)-catechin to inhibit plant activation of 4-nitro-*o*-phenylenediamine (NOP) into a mutagenic form and the mechanism through which this is accomplished. Activation of NOP by both the intact plant cell and isolated horseradish peroxidase systems was inhibited by 10 mM (+)-catechin, as was peroxidase, though not as strongly. In addition, (+)-catechin inhibited the direct mutagenicity of NOP in *Salmonella*. Based on these findings, researchers speculated that (+)-catechin inhibits mutagenicity by inhibiting the activity of *O*-acetyltranserase, an enzyme involved in the induction of mutations.

Teal and Castonguay [1992] also investigated the anti-mutagenic effect of polyphenols, including catechin, when the TA1535 strain of *S. typhimurium* was exposed to another carcinogen to which humans are exposed, 80 mM of nicotine-derived nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). The anti-mutagenic efficacies were dose-related between the non-toxic concentrations of 0.1 and 0.5 mmol/dish. At the highest dose, 0.5 mmol, catechin inhibited mutagenesis at a rate of 62%. The overall results of this study indicated that polyphenolic compounds might inhibit the activation of NNK, suggesting the polyphenolic compounds could prevent the initiation phase of the carcinogenic process.

Research by Apostolides [1997] has indicated that polyphenolic compounds may also be effective inhibitors of PhIP, 2-amino-1-methyl-6-phenylimidazo[4,45-*b*]pyridine, which is an important procarcinogen produced during the frying and broiling of fish and meat. The mutagenicity for 10μM of PhIP in *S. Typhimurium* strain TA98 in the presence of S9 metabolic activation was inhibited in a dose-dependent manner by gallate ester of catechin and by tannic acid. The gallate esters of catechin and the tannic acid had low IC_{50} values whereas catechins, methyl gallate, and gallic acid showed no inhibition in this concentration range. The IC_{50} values in this assay were inversely proportional to the molecular weight of the gallate esters, as displayed in Table 8.8.

Table 8.8 Inhibition of Mutagenicity caused by 10μM PhIP by Polyphenolic Compounds

Compound	Molecular Weight	IC_{50} (μM)
Catechin	290	>1000
Epicatechin	290	>1000
Epicatechin gallate	306	>1000
Epigallocatechin gallate	442	500
Monogallates	458	700
Gallic Acid	188	>1000
Methyl gallate	203	>1000
Tannic acid	1701	60

Monteith [1990] investigated the effect of catechin on the metabolism and DNA-binding capabilities of 2-acetylaminofluorene (AAF), a known hepatocarcinogen in rats and a mutagen in microbial assays. Rat parenchymal hepatocytes were isolated from male Sprague-Dawley rats and were cultured in collagen-coated dishes in media containing catechin (1.0, 0.1, or 0.01 mM) in ethanol. At the two lowest doses, catechin significantly increased AAF metabolism (8%, $p<0.025$) by rat hepatocytes, while a 6% reduction in AAF metabolism was seen with a treatment of 1.0 mM catechin. In addition, treatment with this highest dose resulted in a 50% reduction in the conjugation of metabolites, a 53% reduction in the ratio of water-soluble to organic soluble metabolites, and increased binding of AAF metabolites to DNA at a level 1.8 times

above controls. In addition, treatment with catechin approximately doubled the amount of N-OH-AFF metabolite, a carcinogen that was produced by the hepatocytes, and also affected the levels of several of the other AAF metabolites. Researchers also examined the mutagenic ability of catechin in incubations of hepatocytes and *Salmonella typhimurium* cells and observed that catechin itself was not mutagenic at the concentrations tested (0.5, 1.0, and 5.0 mM) and that at concentrations of 1.0 and 5.0 mM it was able to inhibit the mutagenicity of AAF. However, a significant enhancement of AAF-induced mutagenicity was seen with a dose of 0.5 mM catechin.

8.2 Subchronic and Chronic Toxicological Safety

8.2.1 Subchronic Evaluation of MegaNatural™ GSE and GSKE

A 3-month study was designed to specifically assess the potential for toxicity of the MegaNatural™ line of Gold GSE and GSKE products when administered orally via a dietary admixture to 20/sex/group Sprague-Dawley CD rats [Bentivegna and Whitney, 2002]. A summary of this study is provided in the experimental outline as shown in Table 8.9.

Table 8.9 Experimental Outline

Group	Test Article	Dose Level (%)	Number of Animals							
			Total		1-Month & Termination Clinical Laboratory Studies		Week 13 Necropsy		Microscopic pathology	
			M	F	M	F	M	F	M	F
1	Control	0	20	20	10	10	20	20	20	20
2	GSE	0.63	20	20	10	10	20	20	-	-
3	GSE	1.25	20	20	10	10	20	20	-	-
4	GSE	2.50	20	20	10	10	20	20	20	20
5	GSKE	2.50	20	20	10	10	20	20	20	20

000112

Dietary concentrations of 0.63% GSE, 1.25% GSE, 2.5% GSE, and 2.5% GSKE were administered over the 3-month treatment schedule. This correlates to nominal dose concentrations of 6,300, 12,500, 25,000, and 25,000 ppm, respectively. A negative control group of 20 animals received an untreated standard laboratory diet. Analysis of the diet mixtures showed that homogeneous mixtures were produced and that the test articles were stable in the rodent chow diet for 2-weeks. Measured mean concentrations of the test articles during the study ranged from 96% to 106% of nominal concentrations.

Throughout the course of the study, viability checks were performed twice daily to check for mortality and signs of toxic or pharmacological effect. Physical observations and body weight measurements were performed twice pretest and weekly during the study period. All animals survived throughout the study and while some findings were noted across all treatment groups, none were considered to be compound related.

Feed consumption was measured once pretest and then weekly. Body weight gains were similar for all groups. Feed consumption differences were noted early in the study. From day 7, male animals receiving 2.5% GSE or GSKE had increased food consumption by approximately 10%, which was considered small but statistically significant. This elevated consumption continued throughout the length of the study and may have reflected increased dietary intake to compensate for the high concentration of test articles in the diet. Feed consumption in the female treatment groups remained similar to control throughout the study. Table 8.10 lists test article intake.

Table 8.10 Test Article Intake (mg/kg/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

Ophthalmoscopic examinations were performed pretest and weekly during the study. Hematology, coagulation studies, and clinical chemistry were performed on 10 animals/sex/group at one month and at study termination. No effect upon any of these parameters was observed during the consumption of GSE and GSKE.

After at least 92-days of treatment, all animals were sacrificed and a complete macroscopic postmortem examination was conducted. Selected organs were then weighed. No treatment-related abnormalities attributable to either test article were noted. Histopathological examination of selected tissues was conducted upon all animals in the control and two high-dose groups. In those animals receiving 2.5% GSE dietary supplementation, 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, comprised predominantly of lymphocytic interstitial infiltrates, was observed in 11 of 20 animals compared to the occurrence in 4 of 20 control animals. The severity in most cases was minimal. This 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 is corroborated by a comparison with research facility 3-month study historical control data gathered over a period between 2000-2001 and provided below in Tables 8.11, 8.12, and 8.13. Male rats in studies 00-2683 and 00-6126, conducted contemporaneously with the GSE / GSKE study, also experienced renal cortical inflammation in 40% (8/20) and 50% (5/10) of animals, respectively.

Table 8.11 Historical Control Data - Study Data

Study Number	Length of Study	Initial No. On Test (No./Sex)	Route of Administration	Age at Initiation	Initiation Date	Termination Date	Species	Anesthetic
99-2615	3 M	20	Diet	6 W	14-Oct-99	21-Jan-00	CD®	CO_2
99-2628	3 M	10	Dermal	8 W	11-Nov-99	11-Feb-00	CD®	CO_2
99-2610	3 M	20	Diet	6 W	22-Feb-00	22-May-00	CD®	CO_2
99-2643	3 M	10	Diet	6 W	2-Mar-00	2-Jun-00	CD®	CO_2
00-2651	13 W	20	Gavage	6 W	28-Mar-00	30-Jun-00	CD®	CO_2
00-2658	90 D	10	Gavage	6 W	21-Aug-00	22-Nov-00	CD®	CO_2
00-2683	3 M	20	Diet	44 D	20-Feb-01	25-May-01	CD®	CO_2
00-6126	13 W w/4W	10	Inhalation	6 W	06-Feb-01	08-May-01	CD®	CO_2

All rats supplied by Charles River of Kingston, NY.

000115

Table 8.12 Historical Control Data - Male Rats

Microscopic Findings: Kidney	TOTAL	Individual Study Data - Male Animals							
Month/Year of Death		Jan-00	Feb-00	May-00	Jun-00	Jun-00	Nov-00	May-01	May-01
Study Number		99-2615	99-2628	99-2610	99-2643	00-2651	00-2658	00-2683	00-6126
Number Examined	115	15	10	20	10	20	10	20	10
M-nephroblastoma	0	0	0	0	0	0	0	0	0
congestion	0	0	0	0	0	0	0	0	0
acute/subacute inflammation	0	0	0	0	0	0	0	0	0
lymphoid cell infiltrates	1	0	0	1	0	0	0	0	0
acute/subacute pyelitis	0	0	0	0	0	0	0	0	0
subacute(chronic active)/ chronic pyelitis	2	0	0	1	0	1	0	0	0
interstitium: subacute/chronic inflammation	1	0	0	0	0	0	1	0	0
interstitial chronic inflammatory cell infiltrate	0	0	0	0	0	0	0	0	0
cortical scar(s)	1	0	1	0	0	0	0	0	0
cortex: subacute(chronic active)/chronic inflammation	**13**	**0**	**0**	**0**	**0**	**0**	**0**	**8**	**5**
cortex:lymphoid cell infiltrates	0	0	0	0	0	0	0	0	0
cortex: interstitial lymphocytic infiltrates	0	0	0	0	0	0	0	0	0
cortex: lymphoid cell aggregate(s)	7	1	1	0	1	1	3	0	0
cortex: infarct(s)-healing/ healed	2	0	0	1	0	1	0	0	0
cortical tubular basophilia	1	0	1	0	0	0	0	0	0
cortex: proximal convoluted tubular epithelium-basophilic	9	0	0	0	0	0	0	4	5
cortex: proximal convoluted tubular epithelium-eosinophilic/hyaline droplets	0	0	0	0	0	0	0	0	0
cortex: convoluted tubular epithelium-hyperplasia	0	0	0	0	0	0	0	0	0
cortex: convoluted tubules-dilated	1	0	0	0	0	1	0	0	0
cortex/corticomedullary junction: subacute/chronic inflammation	2	0	0	0	0	2	0	0	0
cortex/corticomedullary junction: fibrosis	1	0	0	0	0	1	0	0	0
cortex/corticomedullary junction: mineral deposit(s)	4	0	0	2	0	1	1	0	0
cortex/cortico-medullary junction: tubule(s) dilated	1	0	0	0	0	0	1	0	0
medulla: fibrosis	1	0	0	1	0	0	0	0	0
medulla: mineral deposit(s)	9	2	0	3	2	1	1	0	0
medulla: tubular lumen(s)-granular cast(s)	1	0	0	0	0	1	0	0	0
medulla: tubule(s) dilated/cyst(s)	12	3	0	4	2	3	0	0	0
tubular cyst(s)	1	0	1	0	0	0	0	0	0
pelvis: dilated	8	2	0	0	2	4	0	0	0
pelvic calculi	0	0	0	0	0	0	0	0	0
pelvis: mineral deposits	1	1	0	0	0	0	0	0	0
pelvis: inflammatory cells/cell debris	0	0	0	0	0	0	0	0	0
urothelium: hyperplasia	3	2	0	0	0	1	0	0	0
urothelial/suburothelial mineral deposit(s)	0	0	0	0	0	0	0	0	0
suburothelial mononuclear cell infiltrate	1	0	0	0	0	0	0	1	0
proximal tubular epithelium:mallory-heidenhain staining material	10	0	0	0	0	0	0	0	10

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Table 8.13 Historical Control Data - Female Rats

Microscopic Findings: Kidney	TOTAL	Individual Study Data - Female Animals							
Month/Year of Death		Jan-00	Feb-00	May-00	Jun-00	Jun-00	Nov-00	1-May	1-May
Study Number		99-2615	99-2628	99-2610	99-2643	00-2651	00-2658	00-2683	00-6126
Number Examined	114	15	10	20	9	20	10	20	10
M-nephroblastoma	1	1	0	0	0	0	0	0	0
congestion	0	0	0	0	0	0	0	0	0
acute/subacute inflammation	0	0	0	0	0	0	0	0	0
lymphoid cell infiltrates	0	0	0	0	0	0	0	0	0
acute/subacute pyelitis	0	0	0	0	0	0	0	0	0
subacute(chronic active)/ chronic pyelitis	0	0	0	0	0	0	0	0	0
interstitium: subacute/chronic inflammation	0	0	0	0	0	0	0	0	0
interstitial chronic inflammatory cell infiltrate	0	0	0	0	0	0	0	0	0
cortical scar(s)	0	0	0	0	0	0	0	0	0
cortex: subacute (chronic active)/chronic inflammation	2	0	0	0	0	0	0	1	1
cortex:lymphoid cell infiltrates	1	0	0	0	0	1	0	0	0
cortex: interstitial lymphocytic infiltrates	0	0	0	0	0	0	0	0	0
cortex: lymphoid cell aggregate(s)	1	0	1	0	0	0	0	0	0
cortex: infarct(s)-healing/ healed	0	0	0	0	0	0	0	0	0
cortical tubular basophilia	1	0	1	0	0	0	0	0	0
cortex: proximal convoluted tubular epithelium-basophilic	2	0	0	1	0	0	0	0	1
cortex: proximal convoluted tubular epithelium-eosinophilic/hyaline droplets	0	0	0	0	0	0	0	0	0
cortex: convoluted tubular epithelium-hyperplasia	0	0	0	0	0	0	0	0	0
cortex: convoluted tubules-dilated	0	0	0	0	0	0	0	0	0
cortex/corticomedullary junction: subacute/chronic inflammation	0	0	0	0	0	0	0	0	0
cortex/corticomedullary junction: fibrosis	0	0	0	0	0	0	0	0	0
cortex/corticomedullary junction: mineral deposit(s)	29	2	1	13	2	2	2	1	6
cortex/cortico-medullary junction: tubule(s) dilated	2	0	0	0	0	2	0	0	0
medulla: fibrosis	0	0	0	0	0	0	0	0	0
medulla: mineral deposit(s)	14	1	0	5	3	1	4	0	0
medulla: tubular lumen(s)-granular cast(s)	0	0	0	0	0	0	0	0	0
medulla: tubule(s) dilated/cyst(s)	2	0	0	0	1	0	1	0	0
tubular cyst(s)	0	0	0	0	0	0	0	0	0
pelvis: dilated	0	0	0	0	0	0	0	0	0
pelvic calculi	0	0	0	0	0	0	0	0	0
pelvis: mineral deposits	1	1	0	0	0	0	0	0	0
pelvis: inflammatory cells/cell debris	0	0	0	0	0	0	0	0	0
urothelium: hyperplasia	2	1	0	0	0	0	1	0	0
urothelial/suburothelial mineral deposit(s)	4	2	0	0	0	1	1	0	0
suburothelial mononuclear cell infiltrate	0	0	0	0	0	0	0	0	0
proximal tubular epithelium:mallory-heidenhain staining material	0	0	0	0	0	0	0	0	0

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The significance of the increased occurrence of this spontaneously occurring lesion in relation to 2.5% GSKE administration was uncertain. Overall, no consistent findings were noted for the lower doses of GSE and no consistent findings were noted for GSKE. There was no observed evidence of toxicity or adverse effects including effects on endocrine and reproductive endpoints. The overall results of this study supported a No-Observed-Effect Level (NOEL) of approximately 2150 mg/kg/day for administration of GSE and GSKE to female rats. In male rats the NOEL for GSE was approximately 1780 mg/kg/day and in GSKE a No-Observed-Adverse-Effect Level (NOAEL) was also determined to be approximately 1780 mg/kg/day. These values represent the time-weighed mean dose rates occurring in the high-treatment groups over the course of the study. Employing a 100-fold safety factor, the observed NOEL of GSE and GSKE can be correlated to a safe human dose of 1075 mg/day for an average 50 kg female. Employing the observed NOEL and NOAEL of GSE and GSKE, respectively, the safe dose for an average 70 kg human male can be correlated to 1250 mg/day. Maximum consumer exposure to GSE and GSKE from their intended use is an additional 10-fold lower than these calculated values for safe human intake.

8.2.2 Pivotal Published Safety Data Based on Substantially Equivalent Grape Seed Extract Products

As previously discussed in Section 3.1 of this document, the compositional profiles of MegaNatural™ GSE and GSKE are similar in content and relative percentage of monomeric flavanols and polymeric proanthocyanidins to that of two other commercially available grape seed extracts marketed as Activin® [Bagchi *et al.*, 2001; Wren *et al.*, 2002] and Gravinol Super™ [Yamakoshi *et al.*, 2002]. The comparative compositions of GSE, GSKE, Activin® and Gravinol Super™ are illustrated in full in Table 3.2 and summarized below in Table 8.14. This provides a basis for comparison so that specific safety data generated using Activin® and Gravinol Super™ may be applied to further illustrate the safety of the MegaNatural™ GSE and GSKE products. It is useful to note that Activin® contains a greater representation of dimer and trimer PACs than do other products (See Table 3.3). Since these less polymerized forms may be more subject to absorption, the Activin® data may be considered "worst case" with respect to the

systemic challenge occurring following ingestion of the products in which the PACs are more highly polymerized.

Table 8.14 Mean Composition of MegaNatural™ Gold GSE and GSKE Relative to Other Commercially Available Grape Seed Extracts by Percentage

	MegaNatural Gold™ Products [a]		Other Products	
	GSE	GSKE	Activin® [b]	Gravinol Super™ [c]
Proanthocyanidins	81.78	80.2	76.3	89.3
Monomeric Flavanols	7.41	5.53	2.8	6.6
Other Polyphenolic Constituents	5.98	8.84		
Subtotal	**95.17**	**94.57**	**79.1**	**95.9**
Moisture	3.74	4.04	5.7	2.24
Ash	0.52	0.82		
Insoluble Matter	0.57	0.42	0.6	0.8
Fatty acids			2.8	
Polysaccharides			10.6	
Amino acids			2.1	1.06
Total (%)	**100**	**99.85**	**100.9**	**100**

[a] Mean value from 6 individual lots of GSE and GSKE, respectively
[b] Data obtained from Wren *et al.*, 2002
[c] Data obtained from Yamakoshi *et al.*,

8.2.2.1 Safety Evaluation of Proanthocyanidin-Rich Extract from Grape Seeds

A proanthocyanidin-rich grape seed extract, Gravinol Super™ as manufactured by the Kikkoman Corporation of Japan, was subjected to a series of toxicological tests, including investigations into its acute and subchronic oral toxicity to Fischer 344 rats in order to document its safety for use in various foods [Yamakoshi, *et al.*, 2002]. To assess its acute oral toxicity, the Gravinol Super™ was dissolved in purified water and administered by oral gavage at doses of 0, 2, and 4 mg/kg. No deaths occurred in either control or treatment groups and no abnormalities were found at necropsy on day 14. The weight of the male rats in the high dose (4 mg/kg) group decreased slightly on

day 1 of the study while the weight of the female rats decreased slightly from day 1 to day 3, but the weights of both sexes increased thereafter.

Yamakoshi, *et al.*, 2002 also investigated the subchronic 90-day oral toxicity of Gravinol Super™ by adding it to a standard diet at concentrations of 0% (control group), 0.02% (low-dose group), 0.2% (middle-dose group), or 2% (high-dose group). The daily Gravinol Super™ consumption for males and females, respectively, was calculated as 13.3 mg/kg and 14.8 mg/kg in the low-dose group, 129.1 mg/kg and 154.0 mg/kg in the middle-dose group, and 1409.8 and 1501.1 mg/kg in the high-dose group, respectively. Food and water consumption and body weight were measured throughout the duration of the study period, and hematology, urine, and blood chemistry were evaluated at the end of the study, and body organs were examined histopathologically. Survival rate was 100% and there were no abnormal clinical signs related to Gravinol Super™ consumption in any of the groups. Nor were there statistically significant differences in body weight, food, or water consumption. There were also no significant differences between groups in hematology values, leukocyte differentials, clinical chemistry values, or urinalysis values. Slight changes were observed upon gross and histopathological examination at necropsy, however, they were not considered treatment-related. The no-observed-adverse-effect level (NOAEL) for the Gravinol Super™ in the diet was 2% or 1410 mg/kg body weight/day in males and 1501 mg/kg body weight/day in females. Researchers concluded that these results indicated a lack of toxicity that supported the safety of proanthocyanidin-rich Gravinol Super™ in various foods.

8.2.2.2 <u>90-Day Oral Toxicity Study of Grape Seed Extract (IH636) in Rats</u>

Wren *et al.* [2002] assessed the 90-day oral toxicity of Activin® grape seed extract in Sprague-Dawley rats. Rats were fed 0. 0.5, 1.0%, and 2.0% Activin® in the diet that resulted in Activin® intakes of approximately 348 and 469 mg/kg/day, 642 and 883 mg/kg/day, and 1586 and 1928 mg/kg/day for males and females in the low-, mid-, and high-dose groups, respectively. Rats were observed for clinical signs of toxicity and changes in serum chemistry and hematological parameters. Organ weights and

histopathological changes were evaluated at study conclusion. All animals survived the duration of the study. Food consumption increased in male and female rats at all dose levels, with the most dramatic changes observed in male rats consuming the 2.0% GSE diet. However, this increased food intake was not associated with increased body weight or absolute organ weight gains, and no significant differences in serum levels of albumin and globulin, or in total serum protein levels, were observed. Slight, statistically significant increases were observed in the serum sodium levels of male rats consuming 1.0 and 2.0% Activin® as compared to controls. Slight but statistically significant decreases in blood urea nitrogen and creatinine in levels of females in the high dose group were also observed. These serum chemistry changes were not accompanied by histopathological findings and were not considered to be toxicologically significant. The Activin® grape seed extract appeared to increase the insoluble fraction of the diet. Male rats consuming the 2.0% test diet exhibited decreased serum iron levels and decreased serum iron/total iron binding capacity ratio compared to controls, though these levels were still within historical limits for Sprague-Dawley rats. No significant treatment-related changes were observed in any of the other parameters monitored. Researchers concluded that administration of Activin® in the diet at levels of 0.5%, 1.0%, or 2.0% for 90 days did not induce any toxicologically significant changes in male and female Sprague-Dawley rats.

8.2.2.3 Activin® – Supporting Safety Data

As part of a review article, Bagchi *et al.* [2000], evaluated the safety and efficacy of Activin® (InterHealth Nutraceuticals Incorporated, Benicia, CA), a commercial water-ethanol preparation of IH636 grape seed PAC extract. Safety studies, conducted in compliance with the U.S. Environmental Protection Agency and Toxic Substances Control Act Health Effects Test Guidelines, 40 CFR §798.4500, demonstrated that the LD_{50} of Activin® was greater than 5000 mg/kg when administered orally via gastric intubation to fasted albino rats. The acute dermal toxicity was found to be greater than 2000 mg/kg in albino rats. This dosage was also considered to be a no-observed-effect

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level (NOEL) for systemic toxicity, though slight erythema and desquamation of the skin was observed in all animals.

The same researchers also observed that Activin® had an 84-98% greater free radical scavenging ability (RSA) against superoxide anion and hydroxyl radical as compared to vitamin E at a dose of 50 mg/l and an RSA 439-575% greater than that of vitamin C at a dose of 100 mg/l against the same free radicals *in vitro*. Activin® was also able to protect cultured neuroactive PC-12 adrenal pheochromocytoma cells and macrophage J774A.1 against oxidative damage induced by H_2O_2 at doses of 50 and 100 mg/l and to protect human oral keratinocyte cells from oxidative stress and apoptosis induced by smokeless tobacco at a dose of 100 mg/l. Pretreatment of mice with Activin® (25, 50, and 100 mg/kg) significantly inhibited the production of reactive oxygen species by mouse peritoneal macrophages and inhibited lipid peroxidation and DNA fragmentation in mouse brain and liver tissues induced by 12-O-tetradeca-noylphorbol-13-acetate (TPA). Chronic use of Activin® (250 ppm) used in combination with chromium and zinc supplements resulted in reduced systolic blood pressure and glycosylated hemoglobin and decreased lipid peroxidation and free radical formation that normally increase with age.

In addition, Activin® was selectively toxic towards cultured human cancer cells (MCF-7 breast, CRL-1739 gastric adenocarcinoma, and A-427 lung cancer cells) at concentrations of 25 and 50 mg/l while enhanced the growth and viability of normal cells. Pre-treatment of liver cells grown *in vitro* with 25 μg/ml Activin® prior to exposure to Idarubicin (30nM) or 4-hydroxyperoxycyclophosphamide (1 μg/ml), two chemotherapeutic agents that are normally toxic to healthy cells, was also examined. There was a significant decrease (>50%) in the number of cells undergoing apoptosis following treatment with Activin®. An increased expression of apoptosis related gene bcl_2 was also observed in Activin® treated cells through Western blot analysis. ACTIVIN® was also thought to down regulate the oncogene p53 in these cells. ACTIVIN® ameliorated the toxic effects associated with these chemotherapeutic agents in normal healthy cells.

Activin® also had short- and long-term protective effects against acetaminophen overdose induced lethality, liver toxicity, hepatic DNA damage, apoptotic cell death, and gene expression in mice, as well as against the pathogenesis of post-ischemic reperfusion injury and ischemic arrest in the heart of rats. Activin® also provided significant protection against both acute and chronic stress-induced oxidative gastrointestinal injury in rats by scavenging reactive oxygen species.

8.3 Supporting Literature Review of Non-Clinical Studies Evaluating Polyphenolic Compounds

8.3.1 Safety and Nutritional Studies

In a 31-day study, grape seed tannins were incorporated into a protein free diet administered to 3 groups of 6 Sprague-Dawley rats at levels of 0.2% or 2.0 % in comparison to a control diet [Vallet *et al.*, 1994]. No significant observations were noted in the group receiving 0.2% tannins. In the high dose group, growth was decreased by approximately 13% compared to control. Food intake and therefore nitrogen, fat, and starch intake remained identical in all three groups due to pair feeding. However, in the highest dose group, enhanced fecal dry weight of approximately 27% percent compared to control and increased fecal nitrogen excretion of 50% compared to control, were observed. No changes in organ weights were observed. This study did not reveal significant toxic effects in the rats except for reduced nitrogen and dry matter digestibility leading to slightly reduced growth levels in the highest dose group. This was thought to be a result of the dietary effect of the condensed tannins within the intestine. The tannins formed less digestible complexes with dietary proteins. They also were shown to directly interfere with mucosal proteins, stimulating the desquamation phenomenon. This observation was corroborated by the increased loss of fecal endogenous nitrogen.

In another rat feeding study, 4 groups of 12 male Sprague-Dawley rats received a normal diet, a hypercholesterolemic diet, or a hypercholesterolemic diet supplemented with 2% grape seed PAC monomers or 2% grape seed PAC polymers, over a course of either 3 or 9 weeks [Tebib, *et al.*, 1994]. The intent was to determine the effect dietary grape seed PAC administration would have upon rat plasma lipoproteins, lipoprotein lipase, hepatic lipase, and aortic and hepatic lipid concentration. Although the goals of this study were predominantly geared towards nutritional endpoints, certain observations can be made about the safety of grape seed PAC monomers and polymers from the results provided. No fatalities or adverse effects attributable to PAC monomer or polymer administration were reported. Weight gain and body fat content in the dose groups receiving a hypercholesterolemic diet were significantly higher in the positive control diet and the diet containing monomers than in the polymer group. This elevated weight gain was attributed to enhanced lipogenesis due to the high lipid level in the diets. The addition of polymeric PACs resulted in weight and body fat content and concentration similar to that seen in the normal dietary group. The results of this study indicated that dietary polymeric grape seed PACs reduced plasma total cholesterol, triacylglycerol and LDL cholesterol concentrations. Significantly lower concentration of plasma VLDL and greater concentration of plasma HDL cholesterol were also observed compared to the control group. In rats fed monomers, lowered triacylglycerol and VLDL concentrations were observed but no effect on HDL or LDL cholesterol concentrations were observed.

Tebib *et al.*, [1995] conducted a similar study in 3 groups of 6 male Sprague-Dawley rats over a 12-week period. A purified diet was provided to a control group whereas the two test groups were pair-fed either monomeric or polymeric grape seed PACs. The PAC concentration of the diets supplying either monomers or polymers was set at 71 mg/kg diet and was related to the daily amount of PACs ingested by a human adult consuming 0.5 L red wine. The purpose of the study was to examine the effects of tannins on cecal fermentation and colonic bacterial enzymes. The results indicated no significant difference in daily food intake due to the pair feeding technique. However, control rats and those fed monomers had greater weight gain than those rats fed

polymers. Cecal weights did not differ between the experimental groups, whereas cecal wall weight and cecal pH was decreased in those rats receiving polymeric tannins in their diet. In this study, all of the bacterial enzyme activities tested were reduced after administration of polymeric tannins in the diet. The authors suggested this could be explained by enhancement of the nitrogen in the colonic content, leading to a dilution of the enzymatic activities. This was regarded as a potentially beneficial result as these same enzymes are responsible for the hydrolysis of glycosides and glucuronides and the reduction of nitro-compounds, which can lead to the generation of toxic and carcinogenic compounds.

8.3.2 Antitumor Activities

Zhao *et al.* [1999] investigated the antitumor promoting effect of the polyphenolic fraction of grape seed extract and the antioxidant activity of several of the individual polyphenols isolated from the extract in a two-stage initiation-promotion model. The polyphenols isolated from the grape seed extract were flavan-3-ol derivatives and included both catechin and epicatechin. To promote tumors, 12-*O*-tetradecanoylphorbol 13-acetate (TPA) was used in 7,12-dimethylbenz[*a*]anthracene (DMBA)-initiated SENCAR mouse skin. Three groups, each containing 20 mice, were treated with 10 μg of DMBA in acetone on the dorsal shaved skin to initiate tumor growth. Two of these groups were then pretreated with 0.5 and 1.5 mg of grape seed polyphenols in 0.1 ml of acetone applied to dorsal skin, with the third group receiving 0.1 ml acetone as a control. Two other groups, one with mice receiving 0.1 ml of acetone alone, and one group treated with DMBA and grape seed polyphenols but without the tumor promoter, also served as controls. The TPA was then applied at a dose of 2 μg in 0.1 ml of acetone to animals in the first three groups. Treatments with grape seed polyphenols and/or TPA were repeated twice a week for twenty weeks. Pretreatment with grape seed polyphenols was found to significantly inhibit TPA tumor promotion in the SENCAR mouse skin, as tumor incidence, tumor multiplicity, and tumor volume were all significantly lower than in the groups treated with DMBA and TPA alone. At ten weeks, treatment with 0.5 mg and 1.5 mg of grape seed polyphenols inhibited incidence by

65% and 80% respectively while at twenty weeks, 35% and 65% inhibition were observed ($p<0.001$, χ^2 test). In addition, tumor volume reduction of 61-83% and 50-87% was also observed in the grape seed polyphenols treated mice. The grape seed polyphenols did not promote tumor growth and topical application at doses of 0.5 mg and 1.5 mg did not result in any incidences of toxicity.

Researchers then isolated and compared the antioxidant activities of individual polyphenols from the grape seed polyphenols in terms of their inhibition of epidermal lipid peroxidation. Nine individual polyphenols were identified including both catechin and epicatechin, which were determined to occur at relatively low concentrations compared with much higher concentrations of several other procyanidins. Most notably, they found that catechin inhibited lipid peroxidation by TPA in a dose-dependent manner, with 0.3 mM resulting in 38% inhibition, 0.5 mM resulting in 61% inhibition, and 0.7 mM inhibiting lipid peroxidation by 90%. Treatment with 1.0 mM catechin completely inhibited lipid peroxidation. Polyphenols with a greater degree of polymerization demonstrated even greater activity in inhibiting lipid peroxidation. In addition, Zhao *et al.* [1999] compared several IC_{50}'s for the polyphenols found in grape seed extract and other known antioxidants. The IC_{50} of catechin was less than that of vitamins C and E but greater than the procyanidins found in the extract. Researchers noted dimers, trimers, and other oligomers of catechin and epicatechin were found in the grape seed polyphenol extract and speculated that these compounds may have increased antioxidant activity and increased tumor inhibition potential as compared to the monomers investigated in this study.

Also looking into the ability of polyphenols to inhibit tumorigenesis, Gali-Muhtasib *et al.* [1998], examined the effect of tannins, including the condensed tannins catechin and epicatechin, on mouse epidermal cells exposed to UVB radiation. Exposure to radiation is known to stimulate sustained hyperplasia and DNA synthesis, an activity that is believed to be related to skin carcinogenesis in humans and is similarly correlated with the tumor-promoting activity of chemical promoters. Researchers determined the rate of DNA synthesis using pulse labeling and measuring the amount of ^{3}H-thymidine

incorporated into the epidermal DNA of hairless mice following exposure to radiation and treatment with various tannins. Pretreatment, consisting of topical application of the catechin monomer prior to exposure to radiation, inhibited DNA synthesis by 27%. Treatment with a condensed tannin sample containing dimers, trimers, and polymers inhibited DNA synthesis by approximately 87%. Treatment with the tannins was only effective when applied a short time before exposure to radiation. The ineffectiveness of treatments much before or after exposure to radiation suggests that tannins do not directly block DNA synthesis and instead act on the mechanism by which UVB radiation stimulates DNA synthesis. The exact mechanism is unknown.

In two tumor models, Azuine and Bhide [1994] investigated the role of catechin in the chemoprevention of cancer using Syrian golden hamsters and Swiss mice. In the initial mouse assay, one group of 20 females received 0.41 mg/ml of catechin in their daily drinking water for eight weeks. Others groups of 20 females received the same amount of catechin, in addition to consuming a diet containing either 2% or 5% of turmeric, a known chemopreventive agent. Additional groups of 20 females each were established to receive no chemopreventive treatment or only a diet containing either 2% or 5% turmeric. Two weeks into the treatment, all groups of mice began receiving 1 mg benzo[a]pyrene (BP), a known carcinogen, in 0.1 ml of peanut oil by intragastric intubation twice a week for four weeks. Four additional control groups consisting of 20 females each were established. These groups received either the 2% or 5% turmeric diet, catechin alone, or peanut oil alone. The administration of catechin in the drinking water continued for two weeks after treatment with the carcinogen had been completed. All of the control mice treated with BP alone, developed tumors of the forestomach. Treatment with catechin alone reduced the incidence of tumor formation to 80%. In addition, catechin appeared to work synergistically with turmeric, as tumor incidence was reduced to 35% in mice receiving catechin and the 2% turmeric diet and completely protected mice against tumor induction when used in combination with the 5% turmeric diet. Treatment with catechin or turmeric diet did not result in tumor induction when BP was not administered. The overall results for this assay are presented below in Table 8.15.

Swiss male mice were also given a single intraperitoneal injection of 2.5 mg of catechin in 0.2 ml peanut oil to measure the effect of catechin on glutathione S-transferase (GST) activity, a detoxifying enzyme, and glutathione (GSH) substrate levels. Catechin had an inductive effect on the levels of GST activity and resulted in a higher level of reduced substrate. Treatment with 2.5 mg catechin per animal increased GSH content in the forestomach by 8% and the liver by 7%. In addition, a 22% increase in GST activity was seen in the forestomach and a 38% increase in activity was observed in the liver.

Table 8.15 Inhibition Of Benzo[A]Pyrene (BP) Induced Forestomach Tumors In Mice

Group	Average Increase In Weight (g)	Number of Animals with Tumor	% Inhibition of Tumor Incidence	Average No. of Papillomas per Mouse	% Inhibition Papillomas per Mouse
BP only	4.9±0.4	20	-	7.0±0.3	-
BP + catechin	4.9±0.3	16	20	5.4±0.6*	23
BP + 2% turmeric diet	4.8±0.4	20	0	4.0±0.3*	43
BP + 5% turmeric diet	4.6±0.5	12*	40	1.3±0.2*	N/A
BP + catechin + 2% turmeric diet	5.1±0.3	7*	65	2.7±0.7*	61
BP + catechin + 5% turmeric diet	6.0±0.4	0	100	0.0±0.0	100

* P< 0.001, compared to BP alone

In the second tumor model assay, groups of female Syrian golden hamsters were given 0.1 mg/ml of catechin in their drinking water alone or in combination with a 5% turmeric diet. The animals were then treated twice a month for six months with 2 mg/kg of another known carcinogen, methyl-(acetoxymethyl)-nitrosamine (DMN-OAC), which was topically applied to the mucosa of the right cheek pouch of the animals. Controls received either DMN-OAC only or DMN-OAC in conjunction with the 5% turmeric diet. Treatment with DMN-OAC alone resulted in 93% tumor incidence in the control group, while the addition of catechin to the drinking water reduced tumor incidence to 27%.

Catechin in combination with the turmeric diet further reduced tumor incidence to 6%. In addition, a 20% higher average body weight and a 60% delay in tumor formation was observed in groups treated with catechin and catechin with turmeric diet as compared to control groups. Over all results for this assay are presented below in Table 8.16.

Table 8.16 Inhibition of DMN-OAC Induced Tumors in Hamsters

Group	No. of Animals	Weight Gain (g)	(%) Total Tumor Incidence (5-13 Months)	Latency Period (Months)	Mean Tumor Burden (mm^3)	% Survivors
DMN-OAC only	15	52.3±2	93 (14)	5	600±72	27
DMN-OAC + catechin	15	63.3±2	27* (4)	8	3.0±0.7*	93
DMN-OAC + 5% turmeric diet	16	61.8±4	19* (3)	8	2.4±1.8*	73
BP + catechin + 5% tumeric diet	16	63.1±1	6* (1)	8	2.2±0.0*	81

* $p<0.001$, compared to DMN-OAC alone.

Data in these assays described catechin and turmeric to be non-mutagenic and non-carcinogenic. Furthermore, the data indicated that an adjuvant chemopreventive regimen consisting of catechin and dietary turmeric administered prior, during, and after carcinogen administration was more effective than the individual agents in the inhibition of tumor formation.

Caderni *et al.*, [2000] examined the effect of polyphenolic compounds on azoxymethane (AOM) induced intestinal carcinogenesis. Male F344 rats were dosed subcutaneously once per week over 10 weeks with 7.4 mg/kg of AOM. They were then allocated into four groups of 22 animals each and received by dietary admixture 50 mg/kg body weight red wine extract, green tea extract, or black tea extract over a period of 16 weeks. The red wine extract contained a phenolic breakdown similar to those found in

GSE and GSKE. HPLC analysis determined that the red wine extract contained 4.4% (w/w) anthocyanins, 0.8% flavonols, 2.0% phenolic acids, 1.4% catechin, 1.0% epicatechin, and 28.0% PAC units consisting of 18.0% epigallocatechin, 13.2% catechin, 65.0% epicatechin, and 3.8% epicatechin gallate. In rats treated with black tea and red wine extract, there were significantly fewer colorectal tumors than in controls. These data are supplied in Table 8.17.

Table 8.17 Number of Tumors and Their Location in AOM-Induced Rats Treated with Different Polyphenol Extracts [Caderni *et al.*, 2000]

Group (*n*)	Tumors per Rat		
	Colon-Rectum	Small Intestine	Ear
Control (22)	2.54±1.6	0.18±0.39	0.27±0.45
Black Tea Extract (22)	1.54±1.4*	0.27±0.55	0.36±0.49
Green Tea Extract (20)	3.2±1.9	0.25±0.44	0.3±0.57
Wine Extract (22)	1.63±1.6*	0.23±0.43	0.27±0.45

* Significantly different (P<0.05) from the control group by Poisson regression.

In addition, significantly fewer rats in the black tea ($p<0.05$) and red wine extract ($p<0.01$) groups had adenomas as compared to controls. The data indicated that black tea and red wine extracts could protect against AOM-induced carcinogenesis through a mechanism possibly involving increased apoptosis in tumors.

8.3.3 Quercetin

Quercetin is a flavonoid compound that is known to occur naturally in a wide variety of plants and is widely distributed in food, including grapes. The estimated average daily intake of quercetin by an individual in the United States is 25 mg [NTP, 1992]. The FDA, nominated quercetin for toxicity and carcinogenicity studies in the rat based on concern that quercetin administered to rats through the diet at levels of up to 4% caused minor body weight effects whereas higher doses produced greater than 10% reductions in body weight gains relative to controls.

A series of NTP studies was initiated including a battery of genetic toxicology assays. Quercetin induced mutations in *S. typhimurium* strains TA100 and TA98 with and without exogenous S9 metabolic activation. Positive results were also obtained in tests with and without S9 for the induction of sister chromatid exchanges and chromosomal aberrations in Chinese hamster ovary cells.

An NTP 2-year carcinogenicity study was initiated and conducted by administering 0, 1,000, 10,000, or 40,000 ppm of quercetin (>95% pure) in the feed to groups of 50 male and female rats for 104 weeks. Body weights of exposed male and female rats given the low and mid-range doses remained within 5% of control throughout the course of the study. Reduced body weight gain in high dose male and female rats was observed by week 15 and the final mean body weights were 87% of control weights at week 104. Survival and feed consumption were similar among exposed and control groups throughout the study. The average amount of quercetin intake per day at week 52 was 40, 400, and 1,900 mg/kg of body weight.

The principal toxic effects associated with dietary administration of quercetin were observed in the kidney of male rats. There were dose-related increases in the severity of chronic nephropathy and a slightly increased incidence of focal hyperplasia of the renal tubule epithelium. Histopathological evaluation of the male rat kidney revealed additional hyperplasia and adenomas in all dose groups. The overall incidence of renal tubule adenoma or adenocarcinoma combined in male rats was 1/50 in controls and 9/50 in the high dose group. There was no effect by quercetin on the kidneys of female rats across all dose groups. Based on these results, the conclusion of this study determined that there was some evidence of carcinogenic activity by quercetin in male F344/N rats.

This is relevant insofar as cited in Table 3.1 and Table 3.2 of this document; quercetin is a minor component of both the MegaNatural™ Gold GSE and GSKE products. Chemical analysis of six GSE lots determined the mean value of 0.0045 g of quercetin per 100 g of GSE. The mean of six lots of GSKE was determined contain 0.23 g of

quercetin per 100 g of GSKE. As this relates to the consumption of one beverage serving of 8 ounces, containing 50 mg of GSKE, quercetin intake would be approximately 0.115 mg. A heavy user of GSKE, in the 90th percentile of intake, would consume 200 mg of GSKE. This would equate to a 90th percentile intake of 0.46 mg of quercetin. If GSE were substituted for GSKE, quercetin intake would be approximately 0.002 mg for one serving and 0.009 mg at the 90th percentile of intake. These amounts are only a fraction of the 1,900 mg/kg (40,000 ppm) dose of dietary quercetin associated with an increase in the occurrence of tumors in the kidney of male F344 rats in the 2-year NTP dietary bioassay [NTP, 1992].

Importantly, the Expert Panel noted that the increase in tumors was small, confined to male rats in the highest treatment group, not observed in the females, and not reproduced in a second comparable and contemporary 2-year study employing an even higher 5% dietary treatment (50,000 ppm) [Ito, N *et al.*, 1989]. Based on these considerations, the Expert Panel concluded that GSE and GSKE are not carcinogenic when consumed in food under the proposed conditions of use.

8.4 Reproductive Toxicity

No formal reproductive toxicological work has been conducted with polyphenolic PAC compounds. However, no basis for concern has been established. These compounds are a naturally-occurring part of the everyday diet and have provided researchers no reason to believe that they possess estrogenic activity. Furthermore, in the 90-day rat study detailed in section 8.2.1, no histopathological findings were reported in the reproductive organs of both male and female rats receiving either 2.50% GSE or GSKE in the diet [Bentivegna and Whitney, 2002]. Nor were histopathological findings noted in the reproductive organs of rats evaluated using the comparable grape seed extract products Activin® and Gravinol-Super™ [Wren *et al.*, 2002; Yamakoshi *et al.*, 2002].

9.0 CLINICAL SAFETY

Research by Malaveille *et al.*, [1996] indicated that phenolic compounds inhibited the mutagenicity of 2-amino-1methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). The anti-mutagenic effects of 15 polyphenolic compounds, including catechin, were evaluated by comparing the levels of tobacco-related DNA adducts in exfoliated urothelial cells from 10 smoking volunteers with the anti-mutagenic activity in corresponding 24-hour urine samples. At concentrations ranging from 0.2 to 6μg per assay, the highest inhibitory effects were obtained with flavonols, flavones, flavanones, and one anthocyanidin. An inverse relationship was found between PhIP mutagenicity by urine extracts *in vitro* and two DNA adduct measurements: the level of tentatively identified N-(deoxyguanosine-8-yl)-4-amino-biphenyl adduct and the total level of all tobacco smoke related carcinogen adducts, including those probably derived from PhIP. Although results regarding catechin were inconclusive in this assay, overall the investigators suggest that study results indicated that smokers ingesting dietary phenolic compounds such as those found in onions, lettuce, apples, and red wine, may be partially protected against the harmful effects of tobacco carcinogens in the bladder and other sites.

The pharmacological treatment of non-complicated chronic venous insufficiency is a current and well-debated topic. The introduction of new products with action on the venous system, improved knowledge on the physiopathology of venous insufficiency, and the possibility provided by new analytical instruments, have given new impetus to the consolidation of the clinical value of phlebotonics in this indication. In light of this, Costantini *et al.*, [1999] examined 24 patients with non-complicated chronic venous insufficiency treated with a daily oral administration of 100 mg/day of oligomeric PACs (Pycnogenols-OPC). To evaluate the therapeutic efficacy of the treatment, instrumental evaluation by optical probe capillaroscope was performed in addition to the traditional subjective clinical parameters: swelling, itching, heaviness and pain. The videocapillaroscope examination was performed at the lower third of the leg and the first toe. Edema in the capillaroscopic field, the number of observable capillaries, and capillary dilation were the parameters chosen to evaluate the efficacy of treatment. All

patients completed the study with no reports of adverse events during the period of observation. The results obtained showed a positive clinical response of improved or absent symptoms in over 80% of patients, with significant improvement of symptoms already evident after the first 10 days of treatment; rapid reduction in the swelling of the lower limbs was observed. Correlated with this were other evaluable symptoms: heaviness and itching. Particularly striking results were observed for itching and pain, which completely disappeared during the course of therapy in 80% and 53% of the patients respectively. Noteworthy is the good correlation between the clinical and instrumental data, with improvement in a total of 70% of patients. The results obtained in the course of this clinical experience indicated that treatment with oligomeric proanthocyanidins was associated with improvement during the first weeks of treatment and the absence of adverse events.

The literature provides some examples of clinical studies that attempt to establish the pharmacological efficacy of grape seed / grape skin extract and PACs in humans. However, in light of the centuries of safe use and numerous supportive animal studies, very few clinical studies have been conducted to assess the safety of a given PAC product as a specific clinical endpoint. We have therefore reviewed those clinical pharmacological studies available to us as evidence of safety. The results of our evaluation, summarized in Table 9.1, confirmed the general safety of PACs for clinical use.

Table 9.1 Summary Evaluation of Clinical Safety

Reference	No. of Subjects	Dose	Length of Exposure	Findings	Indication of Toxicity
Maxwell, 1997	6	250 ml of port wine	Single dose w/ a blood draw every 30 minutes for 2 hours	Increased antioxidant activity in the serum after consumption of port wine.	No
Maxwell, 1997	10	5.7 ml / kg of red wine	Single dose w/ a 240 minute follow up time	Increased antioxidant activity in human extracellular fluid following red wine consumption.	No
Nigdikar *et al.*, 1998	9	375 ml / day of red wine, white wine red wine polyphenols (RWPS) or RWPS dissolved in white wine	2 weeks	Decreased thiobarbituric acid-reactive substances, lipid peroxides and conjugated dienes and increased plasma and LDL polyphenols.	No
Nutall *et al.*, 1997.	20	300 mg of procyanidin extract twice daily	5 days followed by a 2 week washout period followed by another 5 day regimen	Increased serum antioxidant activity	No
Maalej *et al.*, 1997	1600	Red wine	NA	Reduced platelet response to ADP and collagen but not to thrombin.	No
Maalej *et al.*, 1997	-	Red wine	15 days	Increased HDL levels and decreased ADP induced platelet aggregation	No
Maalej *et al.*, 1997	20	Red wine	3 months	Reduced thrombin-initiated platelet aggregation and LDL in hypercholesterolemics.	No
Whitehead *et al.*, 1995	9	300 ml of red wine	Consumed over 30 minutes blood drawn at 1 hr and 2 hr	A rise in serum antioxidant levels after ingestion of red wine.	No

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Appendix A **Expert Panel Statement: Determination of the GRAS Status of MegaNatural™ Gold Grape Seed Extract (GSE) and MegaNatural™ Gold Grape Skin Extract (GSKE) for Use as an Antioxidant Ingredient in Beverage Products**

Expert Panel Statement

Determination of the GRAS Status of Meganatural™ Gold Grape Seed Extract (GSE) and Meganatural™ Gold Grape Skin Extract (GSKE) for Use as an Antioxidant Ingredient in Beverage Products.

The undersigned, an independent panel of recognized experts (hereinafter referred to as Expert Panel), qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients, was requested by Polyphenolics Incorporated, a subsidiary of Canandaigua Wine Company, to determine the Generally Recognized as Safe (GRAS) status of two natural products from grape seed and skin. These products, which shall be referred to in this document as grape seed extract (GSE) and grape skin extract (GSKE), contain primarily oligomeric and polymeric proanthocyanidins and other natural phenolic compounds. GSE and GSKE are intended for interchangeable addition to fruit juice and fruit flavored beverages at composite total concentrations up to 210 ppm as antioxidants to retard deterioration. Their use may be alone or in combination with other safe and appropriate antioxidant substances. A comprehensive search of the scientific literature for safety and toxicity information on GSE, GSKE and constituent proanthocyanidins and related polyphenols was conducted through August 2001 and made available to the Expert Panel. A report based on this comprehensive literature review as well as results and analyses of safety studies on the two specific GSE and GSKE ingredients aided and facilitated the work of the Expert Panel. The Expert Panel independently evaluated the material submitted, as well as other materials deemed appropriate or necessary. Following independent, critical evaluation, the Expert Panel conferred and unanimously agreed to the decisions described herein.

Published values indicate estimated dietary intake of flavanoids, catechins and proanthocyanidins by the average American consumer is in the range of 460-1,000 mg/day. Intake arises from the common occurrence of these substances in fruits, juices, tea, chocolate, vegetables, and in many other foods and beverages. The intake of additional polyphenolic substances arising from the combined proposed beverage uses of GSE and GSKE was estimated using data contained in the USDA CSFII (1994-1996, 1998). Additional intake was calculated to be approximately 70 mg/day and 130 mg/day for the mean and 90th percentile beverage user, respectively. The highest 90th percentile intake among population age sub-groups was projected to occur in teenage males who may consume 200 mg GSE/GSKE daily. The Expert Panel concluded that such intake is within the population variability in background consumption.

The composition of Meganatural™ Gold GSE and GSKE is comprised of approximately 90% gallic acid equivalents by weight of flavan-3-ol based monomeric, oligomeric and polymeric phenolic compounds, 60-80% of which are oligomers while polymer content is 25% or less and monomer content approximately 10 - 15%. Regarding individual components, the Expert Panel found the composition to represent a typical spectrum of natural positional and structural isomers consistent with that reported in the literature. The diversity of polymeric forms was observed to be somewhat greater in material derived from grape seed than that from grape skin. Review of minor components indicated no unexpected substances. The presence of 0.1 - 1% combined quercetin and quercetin glycosides were noted. Results of analysis of multiple production batches demonstrated compliance with compositional specifications as well as compliance with typical food quality standards regarding microbiologic activity, heavy metal content and pesticide residues permitted in grapes. The Expert Panel concluded that Meganatural™ Gold GSE and GSKE products were representative of naturally occurring grape phenolics and that the products conformed to applicable standards regarding microbiologic activity and residue limits.

The Expert Panal examined a significant body of information regarding the antioxidant activity of phenolic compounds, polyphenols in particular and those in GSE and GSKE specifically. Ample evidence, including mechanistic investigations, demonstrating both *ex-vivo* and *in-vivo* antioxidant activity was found. Evidence from *in-vitro* investigation of Activin, a similar commercial grape seed proanthocyanidin extract product demonstrated antioxidant activity of this substance toward superoxide anion and hydroxyl radical which was equal to or exceeded that of vitamins E and C at concentrations examined in the range of 50-100 mg/L (ppm). The Expert Panel was not presented with specific data regarding the antioxidant activity of the Meganatural™ products when added to fruit juice and fruit flavored beverages at concentrations up to 210 ppm. However, the Expert Panel noted that these same substances occur naturally at concentrations up to several hundred ppm in grape wines and juice as well as in the fruit and juice of apples, cranberries, and in numerous other foods wherein their antioxidant activity is well recognized. The Expert Panel, considering the well characterized mechanism of action and diversity of evidence, concluded that Meganatural™ Gold GSE and GSKE exhibit antioxidant activity and would do so when added to fruit juice and fruit flavored beverage at a concentration up to 210 ppm as specified.

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The Expert Panel also considered the likelihood for GSE and GSKE to exhibit pro-oxidant activity under the conditions of intended use. The Expert Panel limited its

consideration to possible implications for consumer safety. Evidence was assessed which demonstrated that under certain experimental *in-vitro* conditions, polyphenolic substances present in GSE and GSKE can, like many other antioxidants, exhibit pro-oxidant activity. These conditions generally require the presence of polyphenols in high concentration combined with the presence of transition or other metal ions or certain compounds such as bleomycin capable of sustained oxidation-reduction reaction cycling. The Expert Panel concluded that such conditions were not relevant to prospective consumers of fruit juice and fruit flavored beverage products containing 210 GSE and/or GSKE. This conclusion was based in part on consideration that the polyphenolic dose to a high-end (90th %) consumer from the proposed uses would be on the order of only 200 mg/day which, due to their limited bioavailability, would not be expected to produce highly elevated polyphenol concentrations in body fluids and tissues. Importantly, administration to clinical trial subjects of 300 mg of procyanidin extract twice daily for 5 days induced increased serum antioxidant activity with no reported pro-oxidant effects. Similarly, administering red wine polyphenols for 2 weeks resulted in decreased thiobarbituric acid reactive substances, lipid peroxides and conjugated dienes.

With respect to GSE and GSKE safety, the Expert Panel carefully assessed evidence regarding potential mutagenic activity associated with these substances and their constituents. Evidence examined included results from several published studies as well as from a GLP-compliant assay conducted specifically on the Meganatural™ Gold products, which measured induction of micronuclei in polychromatic erythrocytes and in bone marrow cells of Crl:CD1 mice. The known presence of minor amounts of quercetin and its glycosides, substances with suspected mutagenic and carcinogenic activity, were of particular interest to the Expert Panel. Results available in the literature were inconsistent with respect to mutagenicity. Isolation and multiple fractionation of wine polyphenols was reported to yield a subfraction that exhibited mutagenic activity in the TA98 Salmonella strain when tested in the presence of human fecal glycosidase with or without S9 microsomal fraction. Rutin (3-rhamnoglucosyl quercetin) was identified as the probable active component. In contrast, several investigations demonstrated the antimutagenic, anticlastogenic, and antipromotional activity associated with (+) catechin and (-) epicatechin, as well as their oligomers and other related phenolic compounds. It was not clear that activity of the GSE and GSKE complex polyphenolic mixtures could be adequately assessed on the basis of the possible activity of certain constituents. The Expert Panel therefore assigned greatest importance to the mouse micronucleus assays conducted on the mixtures themselves the results of which demonstrated a complete lack of mutagenic activity when administered at oral doses up to 2,000 mg/kg. The Expert Panel also found it

reassuring that the greatest 90th percentile intake of GSE/GSKE (200 mg/day, in approximately 1 liter of beverage) among male teenagers (50 kg body weight) would result in only a 0.14 - 1.4 mg (0.003 - 0.028 mg/kg) intake of quercetin compared to the 25 mg estimated by NTP (NTP TR 409, 1992) as the average daily intake of quercetin by an individual in the United States. These amounts are also equal to only a fraction of the quercetin content of an equivalent amount of grape wine and comparable to the quercetin content of, for example, 0.2 - 2 fl. oz. of strong tea or 0.4 - 4 fl. oz. of tomato juice. They are also 633,000 – 68,000 times smaller than the 1,900 mg/kg dose (40,000 ppm in diet) associated with an increase in the occurrence of tumors in the kidney of male F344 rats in a modern 2-year NTP dietary bioassay (ibid.). Importantly, the Expert Panel noted that the increase in tumors was small, confined to male rats in the highest treatment group, not observed in the females and not reproduced in a second comparable and contemporary 2-year study employing an even higher dietary treatment (5%; 50,000 ppm) (Ito,N, et al.,JPN. J. CANCER RES. 80(4) :317-325, 1989). Based on these considerations, the Expert Panel concluded that GSE and GSKE are not carcinogenic when consumed in food under the proposed conditions of use.

The Expert Panel also assessed preclinical evidence regarding sub-chronic safety as well as possible adverse affects on nutrition. With respect to nutrition, one published study reported a possible impairment of growth and increased fecal nitrogen excretion in pair-fed rats given protein deficient or protein-free diets in combination with grape seed tannins incorporated in the diet at 2% by weight. This is believed to be the result of the interaction of the tannins to form less digestible complexes with dietary proteins as well as interaction with mucosal proteins, stimulating desquamation. These effects were not reported when dietary tannin content was 0.2% or in studies using protein-adequate diets. The Expert Panel concluded that the proposed GSE and GSKE use levels are sufficiently low and dietary protein intake sufficient high among consumers to assure an absence of these nutritional effects.

With respect to sub-chronic safety, the Expert Panel assigned primary importance to the results of a GLP-compliant 3-month study in which rats were administered the Meganatural™ Gold GSE and GSKE products in the diet at concentrations up to 2.5% by weight. This study included measurement of growth, serum chemistry, and hematology parameters, as well as comprehensive histopathologic examination of organs and tissues, including those related to reproduction and endocrine function. The only remarkable finding arising from this study related to an increase in occurrence of changes in the kidney of male rats administered a diet containing 2.5 % (w/w) GSKE. In this group, a significant ($p<0.05$) increase in the occurrence of a common renal lesion comprised predominately of lymphocytic interstitial infiltrates was reported in 11 of 20

animals compared to occurrence in 4 of 20 control animals. The severity in most cases was minimal and a similar increased frequency was not observed in female rats of the same treatment group or in male or female rats receiving dietary GSE. This lesion is commonly seen in male rats with increasing frequency and severity with aging and is considered a component of the entity of chronic nephropathy. Similar changes routinely observed in rats are considered background lesions. The Expert Panel examined histopathology findings for the individual animals and noted an absence of indications of a significant exacerbation of lesion severity. An acceleration in lesion severity would have been expected if administration of GSKE were acting to promote this chronic progressive spontaneously occurring condition. While the possibility of a renal effect by GSKE could not be entirely ruled out, particularly during the initial stage of the study when dose rates were highest, the Expert Panel concluded that a continuing significant affect is not supported by these findings. The Expert Panel also noted the species and sex specificity of this background renal syndrome and its unlikely relevance to human renal safety. Based on these considerations, the Expert Panel concluded that the reported renal changes do not constitute an adverse effect, are not relevant to humans, and may in fact represent normal fluctuation in the rate of appearance of this background lesion. The Expert Panel further concluded that the study results provided no evidence of toxicity or adverse effects including effects on endocrine and reproductive endpoints and supported a No-Observed-Effect Level (NOEL) of approximately 2,150 mg/kg/day for administration of GSE as well GSKE to female rats while in male rats 1,780 mg GSE/kg/day represented a NOEL and 1,780 mg GSKE/kg/day represented a No-Observed-Adverse-Effect Level (NOAEL). These values represent the time-weighed mean dose rates occurring in the high-treatment groups over the course of the study.

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Based on the critical evaluation discussed above, the Expert Panel has determined that, Meganatural™ Gold GSE and GSKE, meeting the specifications cited, is Generally Recognized As Safe (GRAS) by scientific procedures when used in fruit juice and fruit flavored beverages as an antioxidant to retard deterioration provided it is used in accordance with current Good Manufacturing Practice (21CFR§182.1(b)) in an amount not to exceed 210 ppm (w/v) in finished beverage product.

W. Gary Flamm, Ph.D., FACT
President, Flamm Associates

Walter H. Glinsmann, M.D.
Adjunct Professor, Georgetown University
President, Glinsmann, Inc.

Donald H. Hughes, Ph.D.

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Based on the critical evaluation discussed above, the Expert Panel has determined that, Meganatural™ Gold GSE and GSKE, meeting the specifications cited, is Generally Recognized As Safe (GRAS) by scientific procedures when used in fruit juice and fruit flavored beverages as an antioxidant to retard deterioration provided it is used in accordance with current Good Manufacturing Practice (21CFR§182.1(b)) in an amount not to exceed 210 ppm (w/v) in finished beverage product.

W. Gary Flamm, Ph.D., FACT
President, Flamm Associates

Walter H. Glinsmann, ~~M.D.~~
Fellow, Center for Food Safety and Nutrition, Virginia Tech
President, Glinsmann, Inc.

Donald H. Hughes, Ph.D.

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~~Based on the critical evaluation discussed above, the Expert Panel has determined that, Meganatural™ Gold GSE and GSKE, meeting the specifications cited, is Generally Recognized As Safe (GRAS) by scientific procedures when used in fruit juice and fruit flavored beverages as an antioxidant to retard deterioration provided it is used in accordance with current Good Manufacturing Practice (21CFR§182.1(b)) in an amount not to exceed 210 ppm (w/v) in finished beverage product.~~

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~~Adjunct Professor, Georgetown University~~
~~President, Glinsmann, Inc.~~

~~Donald H. Hughes, Ph.D.~~
President, Hughes Associates

Appendix B **Amendment to Expert Panel Statement: Confirmatory Review of Chemistry Data and Published Critical Safety Data of Similar Grape Seed Derived Products**

Addendum to the Expert Panel Statement

Determination of the GRAS Status of Meganatural™ Gold Grape Seed Extract (GSE) and MegaNatural™ Gold Grape Skin Extract (GSKE) for Use as an Antioxidant Ingredient in Beverage Products.

The undersigned, an independent panel of recognized experts (hereinafter referred to as Expert Panel), qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients, was requested in 2001 by Polyphenolics Incorporated, a subsidiary of Canandaigua Wine Company, to determine the Generally Recognized a Safe (GRAS) status of two natural products from grape seed and skin. These products, which shall be referred to in this document as MegaNatural™ Gold grape seed extract (GSE) and grape skin extract (GSKE), contain primarily oligomeric and polymeric proanthocyanidins and other natural phenolic compounds. GSE and GSKE are intended for interchangeable addition to fruit juice and fruit flavored beverages at composite total concentrations up to 210 ppm as antioxidants to retard deterioration. Their use may be alone or in combination with other safe and appropriate antioxidant substances.

Based on its critical evaluation, in 2001 the Expert Panel determined that MegaNatural™ Gold GSE and GSKE, meeting the specifications cited at that time, are Generally Recognized As Safe (GRAS) by scientific procedures when used in fruit juice and fruit flavored beverages as an antioxidant to retard deterioration provided it is used in accordance with current Good Manufacturing Practice (21CFR§182.1(b)) in an amount not to exceed 210 ppm (w/v) in finished beverage product. Polyphenolics Incorporated then submitted to FDA a notification (GRN 00093) that it had determined that its GSE and GSKE products are GRAS for use in fruit juice and fruit flavored beverages, as specified. The FDA completed a review of the Polyphenolics notification and on June 5, 2002 replied that, in their view, the notification did not provide a sufficient basis for a determination that GSE and GSKE are GRAS under the conditions of intended use. In its response, FDA identified lack of publication of the primary GSE / GSKE safety studies as a principle deficiency and noted several areas requiring clarification, such as the flavonol and proanthocyanidin (PAC) content of GSE and GSKE for comparison with estimated background consumption of these classes of substances. Also noted was possible uncertainty created by NTP's nomination of grape seed extract for testing with respect to asserting an expert consensus regarding safety of these substances.

The Expert Panel has been recently provided with new data, information, and scientific argumentation compiled by Polyphenolics and intended to address concerns identified

by FDA. Polyphenolics plans to incorporate these into and submit a revised GRAS Notification. Accordingly, the Expert Panel has been requested to review and assess these new data, information, and scientific argumentation to determine whether they in any way alter the Panel's original opinion that use of GSE and GSKE, as specified, are GRAS.

With respect to public availability of pivotal safety information published in scientific journals, the Expert Panel acknowledges that at the time of its original determination, study results of GSKE and GSE, which it considered of primary importance, were then unpublished. It was believed that these results were "in press" and would imminently become available to the qualified scientific community. Unfortunately, publication was delayed, and the Expert Panel understands FDA's position regarding deficiency with respect to the "common knowledge" GRAS element. These studies are confirmed as currently in press with imminent publication expected[1,2].

Since the time of the Panel's original determination, data regarding the safety of grape seed extract products has appeared independently in the scientific literature. These data, contained in papers by Yamakoshi, *et al.* (2002) and Wren, *et al.* (2002) were provided to the Expert Panel. After careful examination, the Panel concluded that the results contained in these papers are directly applicable to evaluation of the safety of Polyphenolic's GSE and GSKE products and that these publications represent publicly available, pivotal scientific evidence.

Specifically, the Expert Panel found the composition of the test substance employed in the Yamakoshi studies to be substantially equivalent to GSE and GSKE in terms of total flavonoid and PAC content as well as monomer/polymer profile. The test substance employed by Wren was substantially similar, differing only in the occurrence of a higher proportion of dimers and trimers compared to the higher PAC polymeric content in MegaNatural™ Gold GSE and GSKE. It is the opinion of the Panel that the less polymerized PAC forms employed by Wren may be more subject to absorption and that the Wren study may be considered a "worst case" with respect to any systemic challenge following ingestion relative to an equivalent amount of GSE and GSKE wherein the PAC's are more highly polymerized. The results of these studies (Yamakoshi, *et al.,* 2002; Wren, *et al.,* 2002), which include a battery of mutagenesis assays, short-term toxicity, as well as 90-day subchronic tests, demonstrate an absence of adverse effects with respect to mutagenic activity. The also demonstrate an absence of toxicity following dietary administration for three months at daily doses equivalent to

[1] Erexson GL. 2003. Lack of in vivo clastogenic activity of grape seed and grape skin extracts in a mouse micronucleus assay. *Food and Chem. Toxicol.* 41(3):347-350.

[2] Bentivegna SS, Whitney KM. 2002. Subchronic 3-month oral toxicity study of grape seed and grape skin extracts. *Food and Chem. Toxicol.* 40:1731-1743.

approximately 1400 mg/kgbw/day (males) and 1500 mg/kgbw/day (females) in the Yamakoshi study and approximately 1600 mg/kgbw/day (males) and 1900 mg/kgbw/day (females) in the Wren study. The Expert Panel finds these published results to be consistent with and strongly supported by the results of the micronucleus (Erexson, 2003) and 90-day safety (Bentivegna and Whitney, 2002) studies of GSE and GSKE examined by the Panel during its initial GRAS evaluation and confirms the Panel's initial view that use of GSE and GSKE in beverages, as specified, is GRAS.

With respect to the position taken by NTP in its nomination of a grape seed extract product for mutagenesis, 90-day, and reproductive safety testing, the Expert Panel examined NTP's "Summary of Data for Chemical Selection", prepared in July 2000 for "Oligomeric Proanthocyanidins from Grape Seeds and Pine Bark", and finds no basis to conclude that NTP has acted on the basis of identified potential safety concerns regarding grape seed extract or that current use in dietary supplement form is seen as unsafe. Rather, the discussion contained in the NTP document appears to indicate that nomination was based on recognition of increasing consumption and potential healthful importance of these substances and a desire to mitigate a lack of formal safety information, at the time of the recommendation in 2000, in the public peer-reviewed scientific literature. While the Expert Panel is not able to predict future NTP action, recent and pending publication of safety studies conducted on grape seed extract products has placed in the peer-reviewed literature the data recommended by NTP, including a demonstrated absence of any effect on reproductively sensitive structures or systems in the 90-day studies. The Expert Panel is not persuaded that the NTP nomination for testing of grape seed extract in any way impairs development of a clear consensus that the intended beverage uses of GSE and GSKE is GRAS by scientific principles.

Lastly, the Expert Panel examined new and revised presentation of existing analytical data detailing the compositional characteristics of GSE and GSKE. The Panel found the data to be consistent with its initial assessment, indicating the products to consist by weight, of approximately 80% PAC's as the major part of the 90% total flavonoids, which in turn represent the majority of the 95% total phenolic substances present in the MegaNatural™ Gold products. Graphic presentation of these data were effective in conveying compositional characteristics and reaffirmed the Panel's initial assessment that the intended beverage uses of GSE and GSKE would result in intake by consumers of approximately 70 mg/day (mean) to 130 mg/day (90th percentile user) of additional polyphenolic substances composed primarily of PAC and other flavonoids as described above. Further, this additional intake is well within the reported range of 460-1,000 mg/day for intake from background dietary sources.

Based on the critical evaluation discussed above, the Expert Panel reaffirms its earlier Opinion that Meganatural™ Gold GSE and GSKE, meeting the specifications cited, is Generally Recognized As Safe (GRAS) by scientific procedures when used in fruit juice and fruit flavored beverages as an antioxidant to retard deterioration provided it is used in accordance with current Good Manufacturing Practice (21CFR§182.1(b)) in an amount not to exceed 210 ppm (w/v) in finished beverage product.

Sept 20, 2002

W. Gary Flamm, Ph.D., FACT
President, Flamm Associates

Walter H. Glinsmann, M.D.
President, Glinsmann Inc.

Donald H. Hughes, Ph.D.

Based on the critical evaluation discussed above, the Expert Panel reaffirms its earlier Opinion that Meganatural™ Gold GSE and GSKE, meeting the specifications cited, is Generally Recognized As Safe (GRAS) by scientific procedures when used in fruit juice and fruit flavored beverages as an antioxidant to retard deterioration provided it is used in accordance with current Good Manufacturing Practice (21CFR§182.1(b)) in an amount not to exceed 210 ppm (w/v) in finished beverage product.

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W. Gary Flamm, Ph.D., FACT
President, Flamm Associates

Walter H. Glinsmann, M.D.
President, Glinsmann Inc.

Donald H. Hughes, Ph.D.

End Submission

FAX COVER SHEET

M

Polyphenolics

Canandaigua Wine Company
12667 Road 24
Madera, CA 93637
Fax Number (559) 661-3430

To: Lisa Lubin
Fax No: 202 418 3131
Location: FDA
From: Anil J. Shrikhande

Number of Pages (Including Cover Page): 2

Date April 11, 2003

Message

Please see the attached document.

Thank you.

Anil Shrikhande

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If you do not receive a complete transmission, please call Beverly Ortegon (559) 661-5544.

A CONSTELLATION COMPANY

CW

Canandaigua Wine

April 11, 2003

Ms. Lisa F. Lubin, M.S., R.D.
Division of Biotechnology & GRAS Notice Review
Center for Food Safety and Applied Nutrition
Food & Drug Administration
College Park, MD 20740
Fax: 202 418 3131

Reg: GRAS Notice No. GRN000125

Dear Ms. Lubin:

We received your letter of March 5, 2003, informing us that FDA has received our notification of February 6, 2003 in accordance with the agency's proposed regulation 21CFR 170.36 and assigned a GRN. No. 000125.

Pursuant to the submission of this notice, Mr. Edward Race of Polyphenolics, Inc. has left the company and, therefore, all correspondence on this notice should be sent to:

Anil J. Shrikhande Ph.D.
Vice President Research & Development
Polyphenolics, Inc.
12667 Road 24
Madera, CA 93637
Phone: 559 661 5671
Fax: 559 661 3430

Sincerely yours,

Anil J. Shrikhande

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Polyphenolics

Polyphenolics • 12667 Road 24, Madera, CA 93639 • (559) 661 5500 • FAX (559) 661 3400 • www.cwino.com

CanandaiguaWine

April 24, 2003

Ms. Lisa F. Lubin, M.S., R.D.
Division of Biotechnology & GRAS Notice Review
Center for Food Safety and Applied Nutrition
Food & Drug Administration
College Park, MD 20740
Fax: 202 418 3131

Reg: GRAS Notice No. GRN000125

Dear Ms. Lubin:

This is in reference to your telephone call of April 11, 2003 informing us that our terminology of Grape Skin Extract (GSKE) for GRAS Exemption Claim, may be in conflict with Grape Skin Extract, an exempt color already published under 21 CFR.73.170. You also advanced a suggestion by ONPLDS for seed/skin extract and Grape Pomace Extract as a suitable alternative to Grape Skin Extract (GSKE).

After conferring within the Polyphenolics Business Unit, we have reached a conclusion to adopt the terminology (product) name to be Grape Pomace Extract (GPE) for our GRAS Notice No: GRN 000125. Please substitute this Grape Pomace Extract (GPT) as synonym for Grape Skin Extract (GSKE) used in our notification.

Sincerely yours,

Anil J. Shrikhande Ph.D.
Vice President Research & Development
Polyphenolics, Inc.
12667 Road 24
Madera, CA 93637
Phone: 559 661 5671
Fax: 559 661 3430

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A CONSTELLATION COMPANY

CW
CanandaiguaWine

M

April 30, 2003

Ms. Lisa F. Lubin, M.S., R.D.
Division of Biotechnology & GRAS Notice Review
Center for Food Safety and Applied Nutrition
Food & Drug Administration
College Park, MD 20740
Fax: 202 418 3131

Reg: GRAS Notice No. GRN000125

Dear Ms. Lubin:

Please refer to my letter of April 24, 2003 requesting change in terminology from Grape Skin Extract (GSKE) to Grape Pomace Extract (GPE)

We have found a typing error in the second paragraph and in the last sentence. The corrected sentence should read "Please substitute this Grape Pomace Extract (GPE) as synonym for Grape Skin Extract (GSKE) used in our notification.

Sincerely yours,

Anil J. Shrikhande Ph.D.
Vice President Research & Development
Polyphenolics, Inc.
12667 Road 24
Madera, CA 93637
Phone: 559 661 5671
Fax: 559 661 3430

Polyphenolics

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