EVALUATION OF MEGANATURAL™ WHOLE GRAPE EXTRACT, MEGANATURAL™ GRAPE SEED EXTRACT, AND MEGANATURAL™ GRAPE SKIN EXTRACT

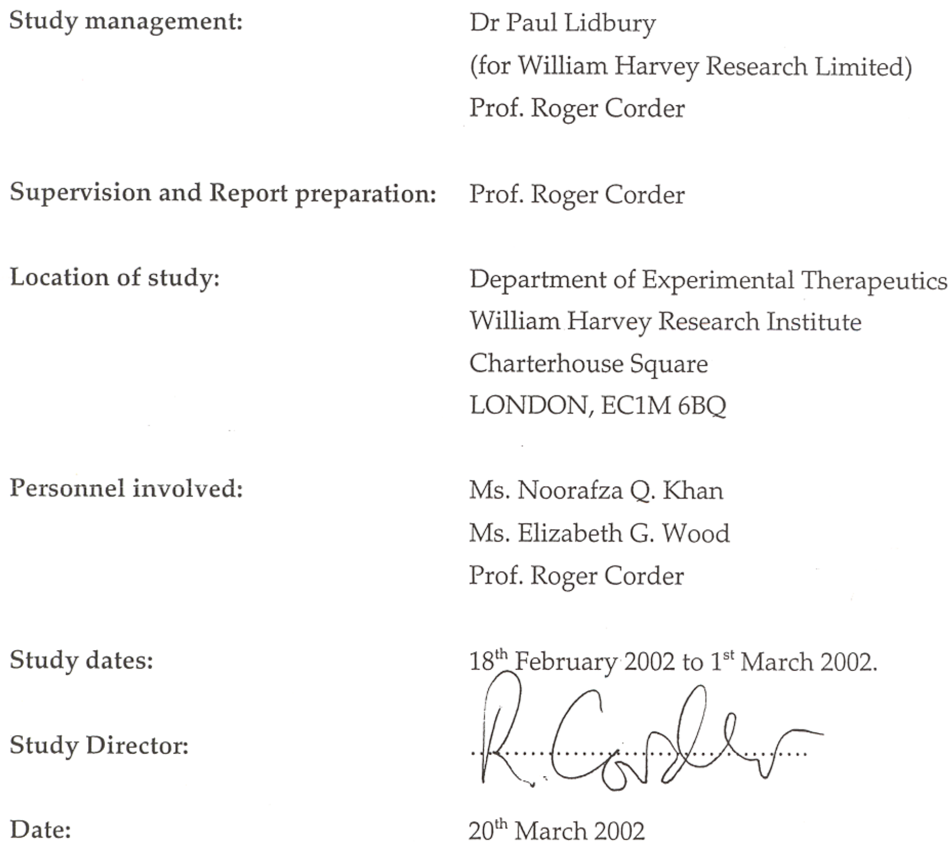
WILLIAM HARVEY RESEARCH INSTITUTE
CHARTERHOUSE SQUARE
LONDON, EC1M 6BQ, UK

March 2002

Evaluation of MegaNatural™ Whole Grape Extract, MegaNatural™ Grape Seed Extract, and MegaNatural™ Grape Skin Extract

AIM OF STUDY

The aim of these investigations was to compare the effects of **MegaNatural™ Whole Grape Extract**, **MegaNatural™ Grape Seed Extract**, and **MegaNatural™ Grape Skin Extract** on endothelin-1 synthesis by bovine aortic endothelial cells.

Study management:	Dr Paul Lidbury (for William Harvey Research Limited) Prof. Roger Corder
Supervision and Report preparation:	Prof. Roger Corder
Location of study:	Department of Experimental Therapeutics William Harvey Research Institute Charterhouse Square LONDON, EC1M 6BQ
Personnel involved:	Ms. Noorafza Q. Khan Ms. Elizabeth G. Wood Prof. Roger Corder
Study dates:	18th February 2002 to 1st March 2002.
Study Director:	 ....
Date:	20th March 2002

Background

Epidemiological studies of mortality from coronary heart disease have shown an inverse relationship with the level of wine consumption [1,2]. Nevertheless, the question as to whether red wine possesses a specific property that enables it to prevent atherosclerosis and reduce the incidence of coronary artery disease has been the subject of debate for many years [3,4]. Generally it has been concluded that moderate alcohol consumption reduces the incidence of myocardial infarction [3,4]. However, it is far more controversial whether red wine confers additional benefit over and above that of other alcoholic beverages [3,4]. Similarly, experimental studies in animal models of atherosclerosis have often provided support for actions of red wine that lead to a reduction in lesion area [5,6], but other investigations have failed to endorse these observations [7]. In part this may be due to the different preparations being used in these investigations having variable levels of the relevant active principle for prevention of atherosclerosis. Indeed, the precise identity of the active components of red wine that inhibit atherosclerosis and their mechanisms of action remain to be identified. In recent years most attention has been given to the antioxidant properties of wines [8-10], however there is no proof that antioxidants can prevent heart disease [11]. In fact the majority of large-scale clinical trials of antioxidants have failed to show any benefit in terms of reductions in cardiac events such as myocardial infarction [11].

In 1993 wine and grape extracts were shown to cause endothelium-dependent vasorelaxing effects [12]. This effect has now been confirmed by several research groups [13-17] and linked to the polyphenolic components of grapes, for which red wine is an abundant source. Recent human volunteer studies have also shown that red wine components can increase blood flow through the forearm, implying that sufficient red wine polyphenols are absorbed from the gastrointestinal tract to modify vascular function [18].

Perhaps the most important aspect of the work by Fitzpatrick and colleagues was the observation that red wine and grape components could modify endothelial cell function [12]. The importance of this finding cannot be overstated because the first event in the process of atherosclerotic lesion formation in coronary arteries and other vessels is widely thought to be endothelial dysfunction, which results in decreased endothelium dependent vasodilatation and increased production of pro-atherosclerotic mediators such as endothelin-1 (ET-1) and monocyte chemotactic protein-1 (MCP-1) [19]. Hence, if red wine polyphenols can prevent endothelial dysfunction then they have very real potential to inhibit the development of coronary artery disease. In recent years, a number of studies have shown the pivotal role ET-1 plays in atherosclerotic lesion formation [20-22]. Therefore, we investigated the action of red wine extracts on ET-1 synthesis by cultured bovine aortic endothelial cells [23]. The relevance of these studies in bovine aortic endothelial cells to human ET-1 synthesis was demonstrated by investigating the regulation of a human ET-1 gene construct that was

incorporated into these bovine cells using molecular techniques [23]. These studies showed very clearly that red wine potently inhibits ET-1 synthesis to an extent that could account for how red wine reduces heart disease [23]. Inhibition of ET-1 synthesis appears to be unrelated to the antioxidant properties of red wine because antioxidant concentrations of polyphenols such as quercetin had no effect on ET-1 production. The magnitude of the effect of red wine on ET-1 synthesis correlated to the polyphenol content of the wines tested [23]. This demonstrates that wines or other grape products with very high polyphenol content are likely to have the greatest potential for preventing heart disease. The specific wine polyphenols that exert an effect on endothelial function have yet to be identified. In the meantime there is an opportunity to develop products that are enriched in the key polyphenols that suppress ET-1 (irrespective of any antioxidant properties) so that individuals can have the choice of taking a daily polyphenol supplement to maintain healthy coronary arteries rather than consuming wine.

Previous research has shown that grape seeds contain catechin oligomers and polymers collectively known as procyanidins [24,25]. In view of the fact that red wine, and not white wine phenolics elicited strong inhibition of ET-1 synthesis, it may be inferred that the winemaking process itself, and specifically, the alcohol generated in the fermentation, extracts the procyanidins from the skin and seed components of the grape. Consistent with this concept, a progressive increase in phenolics during vinification of red wine has been described [26]. Hence, because red wines are fermented on the skins and seeds whereas white (and rosé) wines are fermented after crushing and separation of those fractions. It is logical, therefore, to assume that extracts prepared from fresh grape seeds and skins will isolate those same bioactive phenolics as found in red wines.

TEST MATERIALS

MegaNatural™ Whole Grape Extract (**WGE**) Pin: GK2001; Lot: 138-64
MegaNatural™ Grape Seed Extract (**GSE**) Pin: VW7000; Lot: 2501-120319
MegaNatural™ Grape Skin Extract (**GSKE**) Pin: GK2000; Lot: 2511-040060
(All extracts were from Polyphenolics, 12667 Rd. 24, Madera, CA 93637, USA).

METHODS

1. Preparation of polyphenol extracts for cell studies

Samples of each polyphenol extract (i.e. **WGE**, **GSE** and **GSKE**) were weighed into sterile tubes and then reconstituted at a concentration of 40 mg/ml in 10 mM citric acid containing 20% ethanol.

2. Cell studies

Confluent cultures of bovine aortic endothelial cells were grown in 24-well plates

with Dulbecco's modified Eagle medium (DMEM) containing 10% foetal calf serum as previously described [23,27]. Immediately prior to each experiment dilutions of the reconstituted polyphenol extracts were prepared in serum free DMEM. Six dilutions were made for each sample: 40, 20, 10, 5, 2.5 and 1.25 μg/ml. Confluent cultures were rinsed once with warmed DMEM (0.5 ml/well). Then the media in each well was replace by the dilutions of the polyphenol extracts (0.3 ml/well). Each dilution was tested in triplicate. Once all the dilutions had been transferred onto the cells, the cells were incubated for 6 h at 37°C in a humidified incubator with an atmosphere of 5%CO_2 in air. Each plate also included three control wells for measuring basal ET-1 release. Dilutions of a representative wine extract were also included in each experiment for comparison. At the end of the incubation period the conditioned media were collected for immunoassay. Media samples were frozen at –20°C and stored for subsequent immunoassay. ET-1 synthesis was measured using a well-characterised, sensitive and specific double-recognition site sandwich immunoassay [23,28]

To demonstrate that the effects of the polyphenol extracts were not due to non-specific cytotoxicity an MTT (methylthiazoletetrazolium) test was performed immediately after collection of the media samples for ET-1 immunoassay. The MTT test measures mitochondrial dehydrogenase activity by intracellular conversion of MTT to insoluble formazan (for more information see the description in the Sigma Biochemical and Reagents catalogue or www.sigma-aldrich.com). MTT solution diluted in DMEM was added to each well (0.4 mg/ml; 250μl/well), and the cell culture plates were then incubated for a further 1 h at 37°C in 5% CO_2 incubator. At the end of the incubation, medium was aspirated and the plates left to dry at room temperature. Insoluble formazan in each well was dissolved in DMSO and quantified by measuring absorbance spectrophotometrically at 550nm using a 96-well plate reader.

Each of the polyphenol extracts was evaluated in two separate experiments. Results expressed as means ± sem analysed using Graphpad Prism (GraphPad Software, San Diego, CA, USA) and Statview (SAS Institute Inc., Cary, NC, USA).

Reproducibility of this method for measuring the effect of red wine polyphenols on ET-1 synthesis by bovine aortic endothelial cells has been established in previous evaluations. Determination of the IC_{50} value ten times for the same wine has shown this cell-based method to have an intra-assay coefficient of variation of 5.7%. This variability is influenced by the efficiency of the extraction procedure which itself has a coefficient of variation of 4.3% when determined as total polyphenols.

3. Total polyphenol content

The total polyphenol content of each extract was measured with Folin and Ciocalteu's reagent using gallic acid as a reference.

RESULTS

Both **GSE** and **GSKE** caused a concentration-dependent inhibition of ET-1 synthesis over the concentration range 40 – 1.25 $\mu g.ml^{-1}$, with IC_{50} values of 5.6 and 5.8 $\mu g.ml^{-1}$ respectively (Figure 1, and Appendix 1). Although **GSE** tended to have a greater effect than **GSKE** this did not reach statistical significance. **WGE** did not produce a concentration-dependent reduction in ET-1 production over the concentration range tested.

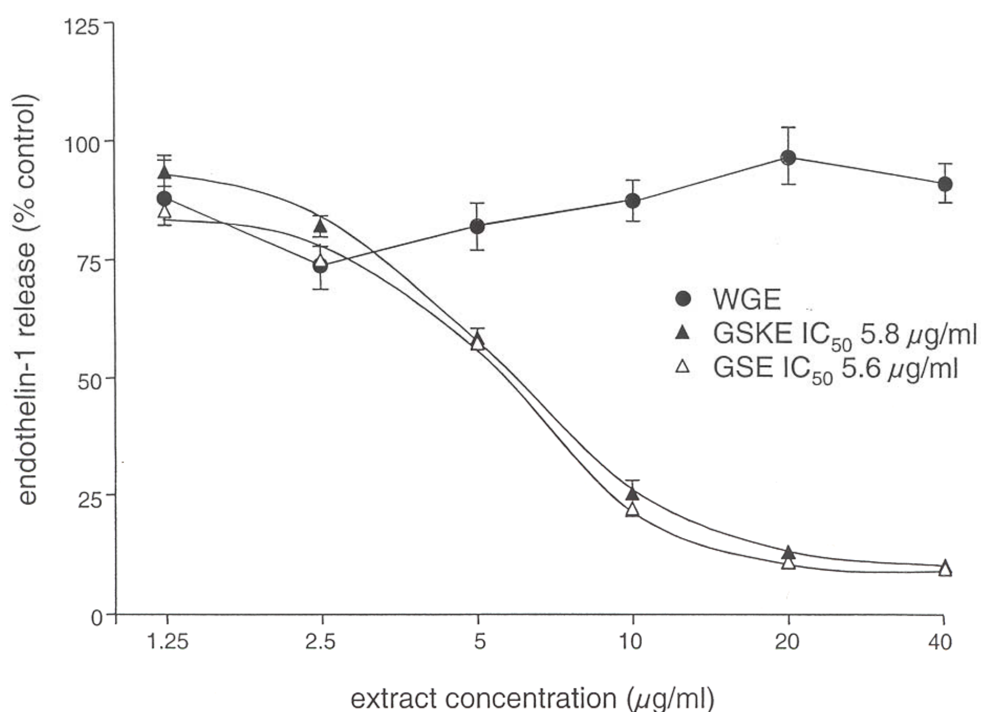


Figure 1. Comparison of the effect of WGE, GSKE and GSE on ET-1 synthesis by bovine aortic endothelial cells.

Analysis of the polyphenol content of the three extracts using Folin and Ciocalteu's reagent revealed the following values:

WGE 39.96 ± 1.33 g GAE/100g
GSKE 72.61 ± 0.59 g GAE/100g
GSE 71.61 ± 0.53 g GAE/100g

These values are 84.6%, 82.9% and 77.5% of the values on the data sheet provided. Although we cannot exclude the possibility that these differences may result from the analysis procedure employed at the William Harvey Research Institute, the most likely explanation for these lower values is a change in moisture content since initial quantification.

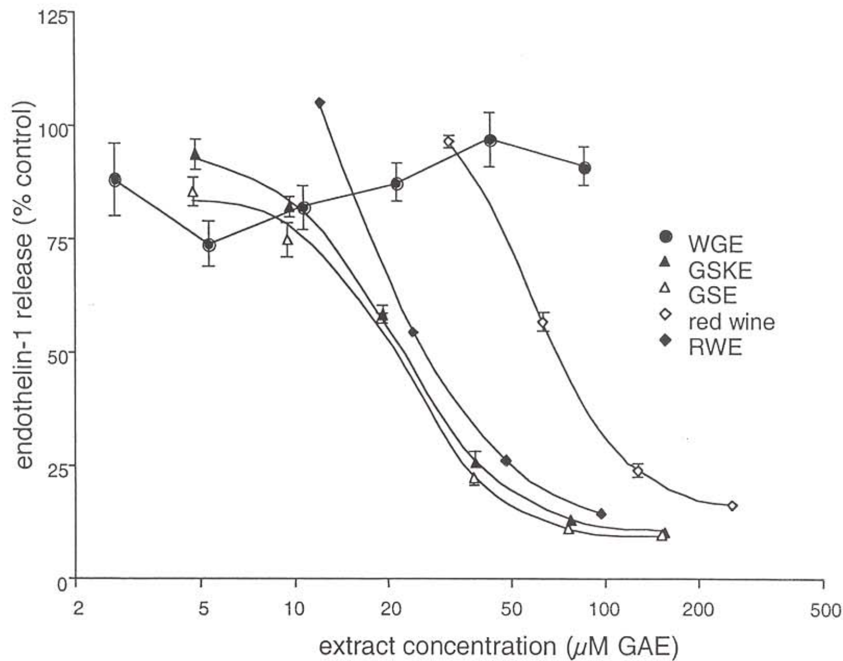


Figure 2. Comparison of the effect of WGE, GSKE and GSE expressed as GAE (μM) on ET-1 synthesis by bovine aortic endothelial cells. The red wine sample was included as a reference in these experiments to test the effects of WGE, GSKE and GSE. The red wine extract (RWE) from the Institut National de la Recherche Agronomique, France [see reference 23] was not tested in these experiments – the data are shown for comparison.

Figure 2 shows the relative effect of these extracts on ET-1 release expressed in gallic acid equivalents. This demonstrates that the **GSKE** and **GSE** are slightly more potent than the red wine extract used in previous studies (this RWE is a polyphenol enriched extract of Cabernet sauvignon wine, polyphenols ≈1.3 g per litre of wine, with an anthocyanin content of ≈5% [23]). Although not tested in these experiments the data is included here for comparison. The red wine sample was included as a comparison with the results described in earlier studies [23]. It was prepared from an Argentinian Cabernet Sauvignon (1997) as an alcohol free extract [23]. The results shown were obtained from the same experiments as the tests on **WGE**, **GSKE** and **GSE**. In terms of relative potency expressed as polyphenol content the red wine sample (IC_{50} 70.8μM GAE) was approximately three fold less active than the

GSKE and GSE (IC_{50}s 22.4μM GAE and 21.5μM GAE). In previous experiments the IC_{50} value for this red wine was shown to be 8.8 μl wine equivalents.ml^{-1} of culture medium, which is ≈5 fold less effective than the 2000 vintage from the same producer that was included in reference 23. In part this may be accounted for by a 50% lower total polyphenol content than the 2000 vintage, and the influence of aging or oxidation. Nevertheless, this shows that as inhibitors of ET-1 synthesis the **GSKE** and **GSE** are as effective as the more potent red wines studied in earlier work [23].

Evaluation of the three extracts in terms of cytotoxicity did not reveal any adverse effects (Figure 3). None of the extracts significantly suppressed MTT values (Figure 3, and Appendix 2). The highest concentrations of **GSKE** and **GSE** caused a significant increase in MTT value (mitochondrial dehydrogenase activity). The reason for this is unclear but it has been observed with the RWE and extracts of wines, and it way well reflect part of the mechanism of action of polyphenols on endothelial function. Whether it is related to the action on ET-1 synthesis awaits the isolation of specific polyphenol components to determine whether there is simultaneous inhibition of ET-1 synthesis and stimulation of MTT levels.

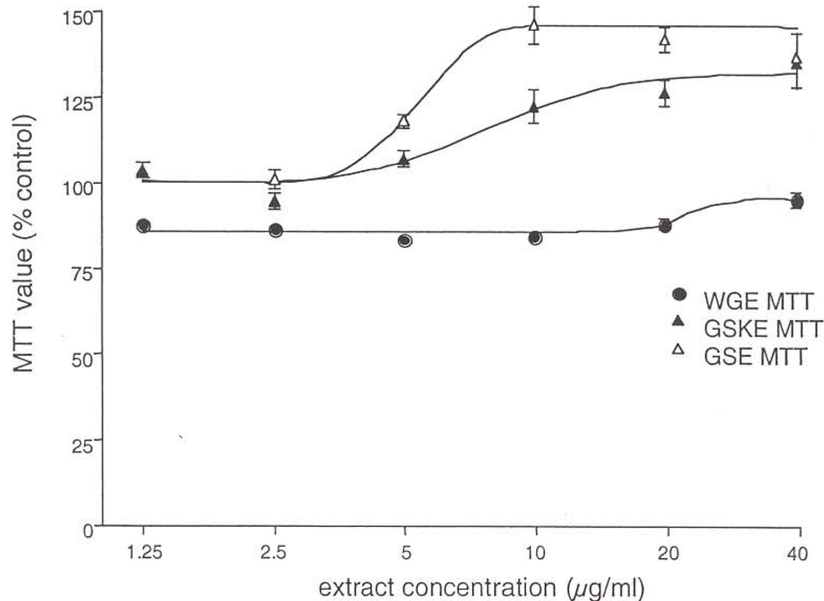


Figure 3. Comparison of the MTT values determined after 6 h incubation of bovine aortic endothelial cells with WGE, GSKE and GSE.

Conclusion

GSKE and **GSE** are potent inhibitors of ET-1 synthesis. Based on previous work they both represent a high quality preparation of grape polyphenols with the potential to prevent heart disease. In comparison, over the concentration range investigated the **WGE** lacks the ability to suppress ET-1 synthesis. The difference between these preparations needs to be compared in further systems that are relevant to the prevention of atherosclerosis and heart disease. This will help define the importance of specific polyphenols in preventing coronary heart disease as well as the relative contributions of antioxidant properties compared to their ability to modify endothelial function by inhibiting ET-1 synthesis

References

1. St. Leger, A.S., Cochrane, A.L. and Moore, F. (1979) Factors associated with cardiac mortality in developed countries with particular reference to the consumption of wine. Lancet i, 1017-1020.
2. Renaud, S. and De Lorgeril, M. (1992) Wine, alcohol, platelets, and the French paradox for coronary heart disease. Lancet i, 1523-1526.
3. Criqui, M.H. and Ringel, B.L. (1994) Does diet or alcohol explain the French paradox? Lancet 344, 1719-1723.
4. Law, M. and Wald, N. (2000) Why heart disease mortality is low in France: the time lag explanation. Brit. Medical J. 318, 1471-1476.
5. Hayek, T., Fuhrman, B., Vaya, J., Rosenblat, M., Belinky, P., Coleman, R., Elis, E. and Aviram M. (1997) Reduced progression of atherosclerosis in apolipoprotein E–deficient mice following consumption of red wine, or its polyphenols quercetin or catechin, is associated with reduced susceptibility of LDL to oxidation and aggregation. Arterioscler. Thromb. Vasc. Biol. 17, 2744-2752.
6. Vinson, J.A., Teufel, K. and Wu, N. (2001) Red wine, dealcoholized red wine, and especially grape juice inhibit atherosclerosis in a hamster model. Atherosclerosis 156, 67-72.
7. Bentzon, J.F., Skovenborg, E., Hansen, C., Møller, J., Saint-Cricq de Gaulejac, N., Proch, J. and Falk, E. (2001) Red wine does not reduce mature atherosclerosis in apolipoprotein E–deficient mice. Circulation 103, 1681-1687.
8. Frankel EN, Kanner J, German JB, Parks E and Kinsella JE. (1993) Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. Lancet. 341: 454-7.
9. Aviram M, Hayek T and Fuhrman B. (1997) Red wine consumption inhibits LDL oxidation and aggregation in humans and in atherosclerotic mice. Biofactors. 6: 415-9.
10. Kerry NL and Abbey M. (1997) Red wine and fractionated phenolic compounds prepared from red wine inhibit low density lipoprotein oxidation in vitro. Atherosclerosis. 135: 93-102.
11. Heinecke JW. (2001) Is the Emperor Wearing Clothes? Clinical Trials of Vitamin E and the LDL Oxidation Hypothesis. Arteriosclerosis, Thrombosis and Vascular Biology 21: 1261.
12. Fitzpatrick DF, Hirschfield SL and Coffey RG. (1993) Endothelium-dependent vasorelaxing activity of wine and other grape products. American Journal of Physiology. 265: H774-8.
13. Cishek MB. Galloway MT. Karim M. German JB. Kappagoda CT. (1997) Effect of red wine on endothelium-dependent relaxation in rabbits. Clinical Science. 93: 507-11.
14. Andriambeloson E. Kleschyov AL. Muller B. Beretz A. Stoclet JC. Andriantsitohaina R. (1997) Nitric oxide production and endothelium-dependent vasorelaxation induced by wine polyphenols in rat aorta. British Journal of Pharmacology. 120: 1053-8.
15. Flesch M. Schwarz A. Bohm M. (1998) Effects of red and white wine on endothelium-dependent vasorelaxation of rat aorta and human coronary arteries. American Journal of Physiology. 275:

H1183-90.

16. Andriambeloson E. Stoclet JC. Andriantsitohaina R. (1999) Mechanism of endothelial nitric oxide-dependent vasorelaxation induced by wine polyphenols in rat thoracic aorta. Journal of Cardiovascular Pharmacology 33:248-54.

17. Andriambeloson E, Magnier C, Haan-Archipoff G, Lobstein A, Anton R, Beretz A, Stoclet JC and Andriantsitohaina R. (1998) Natural dietary polyphenolic compounds cause endothelium-dependent vasorelaxation in rat thoracic aorta. Journal of Nutrition. 128:2324-33.

18. Hashimoto M, Kim S, Eto M, Iijima K, Ako J, Yoshizumi M, Akishita M, Kondo K, Itakura H, Hosoda K, Toba K and Ouchi Y. (2001) Effect of acute intake of red wine on flow-mediated vasodilatation of the brachial artery. Am J Cardiol 88: 1457-60.

19. Lüscher TF and Barton M (2000) Endothelins and Endothelin Receptor Antagonists: Therapeutic Considerations for a Novel Class of Cardiovascular Drugs. *Circulation* 102: 2434-40.

20. Barton, M., Haudenschild, C.C., d'Uscio, L.V., Shaw, S., Munter, K. and Luscher, T.F. (1998) Endothelin ET_A receptor blockade restores NO-mediated endothelial function and inhibits atherosclerosis in apolipoprotein E-deficient mice. Proc. Natl. Acad. Sci. USA 95, 14367-14372.

21. Corder, R. (2001) Identity of endothelin-converting enzyme and other targets for the therapeutic regulation of endothelin biosynthesis. Handbook of Experimental Pharmacology Vol. 152, Chapter 3 pp 35-67 "Endothelin and Its Inhibitors" Ed: Warner, T.D.; Springer-Verlag.

22. Kinlay, S., Behrendt, D., Wainstein, M., Beltrame, J., Fang, J.C., Creager, M.A., Selwyn, A.P. and Ganz, P. (2001) Role of endothelin-1 in the active constriction of human atherosclerotic coronary arteries. Circulation 104, 1114-1118.

23. Corder R, Douthwaite JA, Lees DM, Khan NQ, Viseu dos Santos AC, Wood EG & Carrier MJ (2001) Endothelin-1 synthesis reduced by red wine. *Nature* 414: 863-864.

24. Haslam E (1980) Oligomeric procyanidins and the aging of red wines. *Phytochemistry* 19: 2577-2582.

25. Prieur C, Rigaud J, Cheynier V & Moutounet M (1994) Oligomeric and polymeric procyanidins from grape seeds. *Phytochemistry* 36: 781-784.

26. Burns J, Gardner PT, Matthews D, Duthie GG, Lean MEJ & Crozier A (2001) Extraction of phenolics and changes in antioxidant activity of red wines during vinification. *J. Agric. Food Chem.* 49: 5797-5808.

27. Corder, R. and Barker, S. (1999) The expression of endothelin-1 and endothelin-converting enzyme-1 (ECE-1) are independently regulated in bovine aortic endothelial cells. J. Cardiovasc. Pharmacol. 33, 671-677.

28. Corder, R. (2002) Evaluation of endothelin-converting enzyme inhibitors using cultured cells. Methods in Molecular Medicine "Endothelin Protocols" Eds: Maguire, J.J. & Davenport, A,P. The Humana Press Inc.

Appendix 1

Means Table for ET-1 release (% control release)

Statistical significance is indicated compared to control values determined by using Scheffé's procedure for post hoc comparisons.

	n	Mean	Std. Dev.	Std. Err.	Stat. Significance
control	18	100.000	9.467	2.231	
1.25 μg/ml WGE	6	88.088	19.555	7.983	n.s.
2.5 μg/ml WGE	6	73.863	12.326	5.032	0.0112*
5 μg/ml WGE	6	82.000	12.088	4.935	n.s.
10 μg/ml WGE	6	87.462	10.182	4.157	n.s.
20 μg/ml WGE	6	97.040	14.990	6.120	n.s.
40 μg/ml WGE	6	91.282	10.111	4.128	n.s.
1.25 μg/ml GSKE	6	93.607	8.124	3.317	n.s.
2.5 μg/ml GSKE	6	82.023	5.661	2.311	n.s.
5 μg/ml GSKE	6	58.398	5.026	2.052	<0.0001*
10 μg/ml GSKE	6	25.470	7.029	2.870	<0.0001*
20 μg/ml GSKE	6	12.968	1.923	0.785	<0.0001*
40 μg/ml GSKE	6	10.213	1.132	0.462	<0.0001*
1.25 μg/ml GSE	6	85.287	7.599	3.102	n.s.
2.5 μg/ml GSE	6	74.838	8.967	3.661	0.0208*
5 μg/ml GSE	6	57.568	2.876	1.174	<0.0001*
10 μg/ml GSE	6	21.807	3.690	1.506	<0.0001*
20 μg/ml GSE	6	11.080	0.587	0.240	<0.0001*
40 μg/ml GSE	6	9.717	1.980	0.808	<0.0001*

Appendix 2

<u>Means Table for MTT values (% control value)</u>

Statistical significance is indicated compared to control values determined by using Scheffé's procedure for post hoc comparisons.

	n	Mean	Std. Dev.	Std. Err.	Stat. Significance
control	18	100.023	10.879	2.564	
1.25 μg/ml WGE	6	87.832	2.842	1.160	n.s.
2.5 μg/ml WGE	6	86.582	2.960	1.208	n.s.
5 μg/ml WGE	6	83.594	0.909	0.371	n.s.
10 μg/ml WGE	6	84.545	1.950	0.796	n.s.
20 μg/ml WGE	6	88.320	4.037	1.648	n.s.
40 μg/ml WGE	6	95.336	5.335	2.178	n.s.
1.25 μg/ml GSKE	6	103.959	5.931	2.421	n.s.
2.5 μg/ml GSKE	6	94.637	5.546	2.264	n.s.
5 μg/ml GSKE	6	107.156	6.139	2.506	n.s.
10 μg/ml GSKE	6	122.511	11.621	4.744	n.s.
20 μg/ml GSKE	6	126.636	9.709	3.964	0.0108*
40 μg/ml GSKE	6	135.481	18.890	7.712	<0.0001*
1.25 μg/ml GSE	6	103.456	3.439	1.404	n.s.
2.5 μg/ml GSE	6	101.300	6.770	2.764	n.s.
5 μg/ml GSE	6	118.355	5.365	2.190	n.s.
10 μg/ml GSE	6	146.483	13.923	5.684	<0.0001*
20 μg/ml GSE	6	142.260	8.824	3.602	<0.0001*
40 μg/ml GSE	6	137.118	17.246	7.040	<0.0001*