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VERSITA

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eNOS PROMOTER ACTIVATION BY RED WINE POLYPHENOLS: AN INTERACTION STUDY

AKTIVÁCIA eNOS PROMÓTERA POLYFENOLMI Z ČERVENÉHO VÍNA: INTERAKČNÁ ŠTÚDIA

Original research article

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Abstract The beneficial effects of red wine polyphenols on cardiovascular health are well known. The aim of our research was an interaction study of four red wine polyphenols – resveratrol (R), quercetin (Q), kaempferol (KF) and isorhamnetin (IR) of their ability to activate endothelial NO synthase (eNOS) promoter when used alone and in equimolar mixtures. To determine their activity, we performed a luciferase reporter gene assay on EA.hy926 cells stably transfected with a luciferase reporter gene construct containing eNOS promoter. The Bradford assay was also performed to account the cytotoxicity and/or the cell number differences. The median effect equation, as an interaction analysis evaluating synergy or antagonism of the combinations was done according to mass–action law principle. Isobolographic method was performed on selected double mixtures and dose reduction index was calculated for all mixtures. All single polyphenols activated eNOS promoter. The EC_{50} values were in micromolar concentrations ranging from 3.44 μM (R^2 = 0.96) for kaempferol to 9.89 μM for isorhamnetin (R^2 = 0.94). All mixtures activated eNOS promoter, but their interactions varied from synergy (Q+R, Q+IR+KF, Q+R+KF and Q+R+IR+KF), through additive (R+IR+KF) to antagonistic interaction (R+IR, R+KF, Q+IR, Q+KF, IR+KF and R+Q+IR). In this study, we show for the first time that red wine polyphenols activated eNOS promoter when used alone and in mixtures with different types of interactions.

Slovak abstract Zdraviu prospešné účinky polyfenolov z červeného vína na srdcovocievny systém sú dobre známe. Cieľom našej práce bola interakčná štúdia štyroch polyfenolov z červeného vína – resveratrolu (R), kvercetínu (Q), kempferolu (KF) a izoramnetínu (IR) ich schopnosti aktivovať promóter endotelovej NO syntázy (eNOS) samostatne a v ekvimolárnych zmesiach. Na určenie ich aktivity sme použili metódu luciferázového reporterového génu na transfektovaných endotelových EA.hy926 bunkách, pričom meranie bolo normalizované na obsah bielkovín v bunkových lyzátoch pomocou metódy podľa Bradforda. Interakčnú analýzu synergie či antagonizmu zmesí sme vyhodnotili podľa rovnice stredového účinku na podklade princípov zákona o účinkoch hmoty. Izobolografická metóda bola použitá na vybrané dvojkombinácie a index zníženia dávky bol vypočítaný pre všetky zmesi. Všetky polyfenoly samostatne aktivovali eNOS promóter. Hodnoty EC_{50} dosahovali mikromolárne koncentrácie v rozsahu od 3,44 μM (R^2 = 0,96) pre kempferol do 9,89 μM pre izoramnetín (R^2 = 0,94). Všetky zmesi aktivovali eNOS promóter, ale ich interakcie sa líšili od synergických (Q+R, Q+IR+KF, Q+R+KF a Q+R+IR+KF) cez aditívne (R+IR+KF) až po antagonistické (R+IR, R+KF, Q+IR, Q+KF, IR+KF and R+Q+IR). Štúdiou sme po prvý krát preukázali, že polyfenoly z červeného vína *in vitro* aktivujú eNOS promóter samostatne aj v zmesiach s rozdielnym typom interakcie.

Keywords *eNOS promoter – luciferase reporter gene assay – resveratrol – quercetin – kaempferol – isorhamnetin – interaction study*

Kľúčové slová: *eNOS promóter – metóda luciferázového reporterového génu – resveratrol – kvercetín – kaempferol – izoramnetín – interakčná štúdia*

1. Introduction

A significantly reduced incidence of ischaemic heart-disease deaths despite a high saturated and monounsaturated fat intake in certain areas of France has led to the concept of the "French paradox". This phenomenon was attributed to a higher intake of alcohol and, in particular, of wine in France (St Leger *et al.*, 1979). Moderate ethanol intake from any type of beverage improves lipoprotein metabolism and, lowers cardiovascular mortality risk, but wine, particularly red wine with its abundant content of phenolic acids, polyphenols, and flavonoids seems to confer additional health benefits. These include increase in high-density lipoprotein cholesterol levels and decreased oxidation of low-density lipoprotein (LDL) cholesterol, antioxidant activity, decreased platelet aggregation and adhesion, as well as improved endothelium-dependent vasodilatation. Many of these effects are compatible with the action of endothelium-derived nitric oxide (NO) (Wallerath *et al.*, 2003).

In the development of atherosclerosis, reduced bioavailability of NO, formed by endothelial nitric oxide synthase (eNOS)

precedes the appearance of visible vessel alterations. Thus, improved NO bioavailability would be a promising step in the therapy and prevention of cardiovascular disorders (Räthel *et al.*, 2007), implying that NO may be a mediator of the cardiovascular protection provided by red wine. As the long-term treatment of cultured endothelial cells with red wine or red wine polyphenols induces eNOS expression and causes a sustained increase in endothelial NO production, upregulation of eNOS is probably based on synergistic mechanisms between the different polyphenolic components (Schmitt & Dirsch, 2009). The concentration of active ingredients in some herbs or dietary source is lower than therapeutic dosages, which has led to scepticism and suggestion that herbal therapeutic efficacies are because of placebo effects. By contrast, there have been reports of the total contents of herbal product showing a significantly better effect than an equivalent dose of a single isolated active ingredient (Ma *et al.*, 2009). There is an increasing awareness that analyses of single components are not always adequate to clearly assess the health benefits of natural product mixtures from dietary sources, since they involve interaction effects (Kurin *et al.*, 2012). Interactions are generally described as being synergistic or antagonistic. Synergy means, broadly, "working together" and antagonism means "working against each other", and these terms imply the existence of some intermediate, zero-interactive state in which agents do neither of the above (e.g. additivity) (Berenbaum, 1989).

The aim of our study was an interaction study of four red wine polyphenols (resveratrol – R, quercetin – Q, kaempferol – KF, isorhamnetin – IR; Fig. 1) on eNOS promoter activation in endothelial EA.hy926 cells using median effect equations, where the effects of single compounds and their equimolar mixtures were determined and the interactions of combinations were evaluated according to Chou (2006).

2. Materials and methods

Cell Culture

The human endothelial cell line EA.hy926 (Edgell *et al.*, 1983), stably transfected with the plasmid p-eNOS-3500-Hu-Luc-neo (Li *et al.*, 1998) containing 3600 base pairs of the human eNOS promoter driving a luciferase reporter gene (EA.hy926-heNOS-Luc) were used for measuring the eNOS promoter activity.

Luciferase Reporter Gene Assay

Stably transfected EA.hy926-heNOS-Luc cells were grown in Dulbecco's modified Eagle's medium without phenol red supplemented with 584 mg/ml glutamine, 100 U/ml benzylpenicillin, 100 mg/ml, streptomycin (Lonza, Belgium), HAT supplement (100 µM hypoxanthine, 0.4 µM aminopterin, 16 µM thymidine) (Biochrom, Germany) and 10% heat-inactivated foetal bovine serum (Gibco via Invitrogen, UK) until passage 15. For the experiments, the cells were seeded for 24 hours in 96-well plates at a density of 4×10^5 cells/well and were stimulated with polyphenols – resveratrol (≥99% purity), quercetin (≥98% purity) (Sigma-Aldrich, USA), kaempferol (≥99% purity) and isorhamnetin (≥ 99% purity) (Carl Roth, Germany) dissolved in dimethyl sulfoxide (DMSO). Phorbol-12-myristate-13-acetate (Alexis Biochemicals, Austria) was used as a reference (positive control) and the final DMSO concentrations in all treatment did not exceed 0.1%. Control cells were always treated with an equal volume of solvent. The concentration of single polyphenols used was 3–100 µM and their equimolar combinations final mixture concentration were 1–30 µM, (e.g., the 30 µM final equimolar combination Q+R was composed of 15 µM of R and 15µM of Q). After 18 hours incubation with the respective compounds, the cells were washed with PBS and lysed with lysis buffer (Promega, Germany). To determine eNOS promoter activity, the luminescence generated from the luciferase activity was measured using Tecan Genios Pro (Tecan, Austria) plate reader. The values were then normalised to the protein level determined by the Bradford assay as described by Bradford with slight modifications (Bradford, 1976).

Statistical and Interaction Analysis

All data were obtained in three independent experiments performed in quadruplets. Data are expressed as mean ±SD. Differences between groups for statistical significance were evaluated by ANOVA with Bonferroni *post hoc* test using GraphPad Prism software (version 5.01, GraphPad Software, Inc., La Jolla, CA, USA). *P*-values < 0.05 were considered significant.

The concentration of sample leading to 50% effect (EC_{50}) was calculated from the dose–effect relationship of polyphenols effect on eNOS promoter activation using GraphPad Prism software. The interaction analysis evaluating synergy or antagonism of the combinations was done according to mass–action law principle (Chou, 2006), described by Equation (1) for *n*-drug combination at *x*% inhibition, using combination index (CI) for interaction interpretation

$$^{n}(\mathrm{CI})_x = \sum_{j=1}^{n} (D)_j / (D_x)_j \qquad \text{Equation 1}$$

$^{n}(\mathrm{CI})_x$ is the sum of the dose of *n* drugs that exerts *x*% inhibition in a combination. In the denominator (D_x) is for D "alone" that inhibits a system *x*%. If CI value is =, > or < 1, an additive, synergistic or antagonistic effect is indicated.

The dose-reduction index (DRI) means how many-fold the dose of each drug in a synergic combination could be reduced at a given effect level compared with the doses of each drug alone. The DRI value for each corresponding drug was given for n-drug combinations, Equation (2)

$$(\mathrm{DRI})_1 = \frac{(D_x)_1}{(D)_1};\ (\mathrm{DRI})_2 = \frac{(D_x)_2}{(D)_2} \ldots\text{etc.}$$

Equation 2

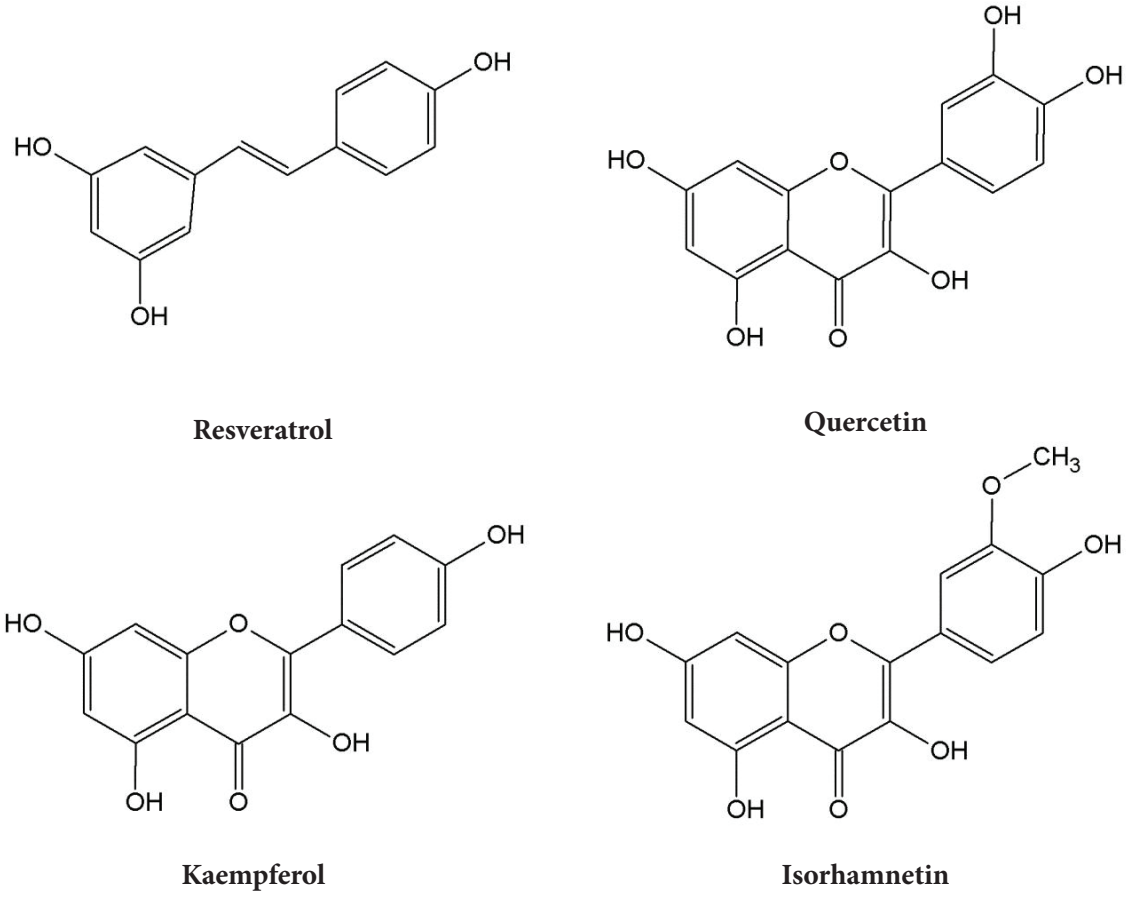

Figure 1. Structures of resveratrol, quercetin, kaempferol and isorhamnetin.

Value of DRI > 1 indicates a favourable dose reduction, and the higher DRI value indicates the higher dose reduction for a given therapeutic effect, but does not necessarily always indicate synergism. Both CI and DRI were calculated using a median-effect analysis by CompuSyn software (version 1.0.1, ComboSyn, Inc., Paramus, NJ, USA).

3. Results and discussion

NO is one of the main mediators of vasodilatation, and decreased NO levels play a central role in endothelial dysfunction. In mammalians, endothelial NO is produced by the enzyme eNOS, which converts L-arginine in the presence of O_2 and NADPH into L-citrulline and NO (Appeldoorn *et al.*, 2009). The generation of NO plays a major role in maintaining cardiovascular homeostasis by governing blood pressure, improving endothelial function, suppressing vascular smooth muscle mitogenesis, inhibiting leukocyte adhesion and platelet aggregation. Dietary polyphenols are widely distributed in vegetables, fruits and beverages such as tea and wine. Several recent studies have demonstrated that polyphenols such as resveratrol, quercetin, epigallocatechin-3-gallate and delphinidin enhance NO output to improve endothelium-dependent vascular relaxation (Xu *et al.*, 2004).

As the moderate regular red wine consumption are associated with a reduced risk of cardiovascular diseases and are related with activation of eNOS system at different levels (Wallerath *et al.*, 2005), in this study, we investigated the influence of resveratrol, quercetin, kaempferol and isorhamnetin, the polyphenols that are present in red wines, on the eNOS promoter activity. Both individual substance or in their equimolar mixtures were investigated. Further, we evaluated their interactions when used in combinations. As it was described in Materials and Methods, first of all we explored activity of single polyphenols on eNOS promoter activation in four different concentrations of polyphenols (3–10–30–100 μM) and from the dose–effect relationship the EC_{50} values using GraphPad Prism software were determined. PMA as a positive control activated eNOS promoter (Fig. 2). As it is seen in Table 1 and in supplementary information Table S1 (where the statistic evaluation of each sample is done), all polyphenols activated eNOS promoter, where the EC_{50} values were in micromolar concentration ranging from 3.44 μM (R^2 = 0.96) for kaempferol to 9.89 μM for isorhamnetin (R^2 = 0.94).

Table 1. Activation of eNOS promotor with single wine polyphenols

Compound	EC_{50} (μM)	R^2
Resveratrol	4.07	0.93
Quercetin	6.92	0.93
Isorhamnetin	9.89	0.94
Kaempferol	3.44	0.96

EC_{50} (effective concentration) means the concentration (in μM) of the compound leading to the half maximal effect. EC_{50} and R^2 (value quantifying the goodness of fit) were calculated using GraphPad Prism (version 5.01, USA).

Wallerath *et al.* (2005) demonstrated that quercetin has no effect on eNOS promoter activity up to 33 μM. However, in our experiment, we found out that quercetin activated eNOS promoter (EC_{50} 6.92 μM; R^2 = 0.93). We also demonstrated that quercetin activates eNOS promoter not only alone but also in mixtures with other red wine polyphenols (Tables 1 and 2; Tables S2 and S3). It is known that kaempferol significantly induces NO production in endothelial cells (Chen *et al.*, 2010) and isorhamnetin has shown inhibitory effect on ox-LDL induced eNOS downregulation (Bao & Lou, 2006), but for the first time we described that kaempferol and isorhamnetin activate eNOS promoter (Table 1). Resveratrol has been shown to enhance the expression of eNOS modulate the deacetylation of eNOS and increase the plasma NO levels (Wang *et al.*, 2012). The ability of resveratrol to activate eNOS promoter was described in the two previous studies (Wallerath *et al.*, 2002; 2005) and our results (Table 1) are in accordance with them.

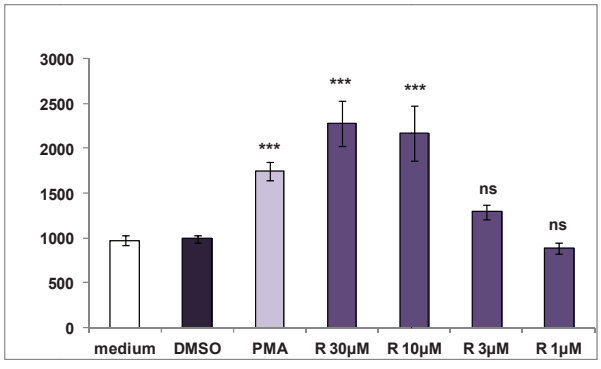

Figure 2. PMA vs. resveratrol in eNOS promoter activation.

*Positive control – phorbol-12-myristate-13-acetate (2 μM; PMA) vs. resveratrol activity in eNOS promoter activation. The bars represent mean ±SD, n=3; *P<0.05, **P<0.01, ***P<0.001 against DMSO treatment alone (ANOVA/Bonferroni).*

It is known that resveratrol has protective effects on multitargets related to cardiovascular diseases. It seems that a drug targeting multiple points may exhibit better therapeutic efficacy than one target blocking or activating in complex conditions. Common disorders such as cardiovascular diseases tend to result from multiple molecular abnormalities (Wang *et al.*, 2012), thus multi-targeting drugs or combinations of drugs seem to bring much more efficiency into therapy or prevention. We performed an interaction study of polyphenols combinations in the second step of our work. We prepared binary, tertiary and quaternary mixtures of tested polyphenols in four concentrations (1–3–10–30 μM), where the contribution of each part was always equimolar (e.g. the 30 μM final equimolar combination Q+R was composed of 15 μM of R and 15 μM of Q) and gave the same final molar concentration of the mixture as the single compound samples. As it is shown in Table 2, EC_{50} values of polyphenols mixtures ranged from 3.31 μM (R^2 = 0.97) for R+Q to 11.09 μM (R^2 = 0.95) for IR+KF.

For Q+R and IR+KF mixture isobolographic analysis was performed as seen in Figures 3 and 4. The solid line in the isobologram is based on the individual median effects (e.g. EC_{50}) of both compounds and represents the collection of concentration pairs which have zero interaction (e.g. they display additivity). From the definition, points under this solid line represent synergic composition (as they give the same effect with a smaller dose in total), whereas points above this solid line describe antagonistic ones (as they give the same effect with a higher dose). The point in the middle of the curved dashed line shows the experimentally determined position of the EC_{50} value of the Q+R mixture (Fig. 3) or IR+KF mixture (Fig. 4) and its position manifests the synergic or antagonistic interaction of the mixture.

When we used combination index analysis based on the mass–action law for quantifying drug interactions, we were able to determine not only binary mixtures interactions (as with isobologram) but also perform n>2-drug combinations interactions analysis. The results of Q+R and IR+KF isobolograms analysis were in concordance with the CI analysis where the CI for Q+R was 0.65 (from the definition CI < 1 indicates synergy) and CI for IR+KF was 2.17 (from the definition CI > 1 indicates antagonism). One can see in Table 2, that four mixtures acted synergic (Q+R, Q+IR+KF, R+Q+KF and Q+R+IR+KF) where the CI vary from 0.65 to 0.87, one mixture effect was nearly additive R+IR+KF (CI = 1.08) and other six mixtures were antagonistic with CI from 1.16 to 2.01.

Interaction studies, which determine synergy or antagonism of substances, are relatively well known for at least three decades amongst antioxidants. Examples are synergic effects between vitamins E and C (Scarpa *et al.*, 1984; Han *et al.*, 1991), vitamin E and β-carotene (Palozza & Krinsky, 1992) or vitamin E with flavan-3-ols (Zhou *et al.,* 2005), with rootlets extracts (Peyrat-Maillard *et al.*, 2001) or with tea polyphenols (Zhou *et al.*, 2000). Nevertheless, there is no information about wine polyphenols interactions related to eNOS path-

Table 2. EC_{50}, CI, and DRI values of polyphenol mixtures at 50% effect dose level

Polyphenol mixture	EC_{50} (μM)	R^2	CI	Interaction	DRI
R+Q	*3.31* (1.655 : 1.655)	0.97	0.65	*Synergy*	2.5 : 4.2
R+IR	*6.76* (3.38 : 3.38)	0.99	1.16	*Slight antagonism*	1.2 : 3.0
R+KF	*4.54* (2.27 : 2.27)	0.95	1.22	*Moderate antagonism*	1.8 : 1.5
Q+IR	*11.48* (5.74 : 5.74)	0.96	1.41	*Moderate antagonism*	1.2 : 1.7
Q+KF	*5.43* (2.715 : 2.715)	0.89	1.18	*Slight antagonism*	2.6 : 1.3
IR+KF	*11.09* (5.545 : 5.545)	0.95	2.17	*Antagonsim*	1.8 : 0.6
R+Q+IR	*7.31* (2.436 : 2.436 : 2.436)	0.99	1.20	*Slight antagonism*	1.7 : 2.8 : 4.1
Q+IR+KF	*4.89* (1.63 : 1.63 : 1.63)	0.95	0.87	*Slight synergy*	4.2 : 6.1 : 2.1
R+Q+KF	*3.50* (1.16 : 1.16 : 1.16)	0.97	0.79	*Moderate synergy*	3.5 : 5.9 : 2.9
R+IR+KF	*5.06* (1.686 : 1.686 : 1.686)	0.97	1.08	*Additivity*	2.4 : 5.9 : 2.0
Q+R+IR+KF	*3.65* (0.9125 : 0.9125 : 0.9125 : 0.9125)	0.97	0.71	*Moderate synergy*	7.6 : 4.5 : 10.8 : 3.8

Polyphenols equimolar mixtures: R – resveratrol, Q – quercetin, IR – isorhamnetin, KF – kaempferol. EC_{50} (effective concentration) means the concentration (in μM) of the compound leading to the half maximal effect. EC_{50} and R^2 (value quantifying the goodness of fit) were calculated using GraphPad Prism (version 5.01., USA). CI – combination index, based on the mass–action law is quantifying drug interaction in terms of synergy, additivity or antagonism (CI <, = or >1). DRI represents the order of magnitude (fold) of dose reduction that is allowed in combination for a given degree of effect as compared with the dose of each drug alone. CI and DRI were calculated using CompuSyn software (version 1.0.1, USA). Interactions are determined according to Chou (2006).

way. Räthel *et al.* investigated apart from resveratrol also red wine polyphenol extracts (RWPE) from 180 wine types. Using luciferase reporter gene expression as an indicator for eNOS promoter activity they found out that all RWPE under investigation increased eNOS promoter activity, but the biological activity was dependent to an individual polyphenol pattern. When they compared the RWPE results with resveratrol, they discovered that resveratrol mimics the effects of RWPE at concentrations higher than that calculated to be present in analysed wines and thus, resveratrol alone does not account for the observed effects of RWPE. Thus, synergy with other compounds in red wine is suggested (Räthel *et al.*, 2007). Chan *et al.* have shown that the effects of ethanol on NO production and inducible nitric oxide synthase (iNOS) gene expression in murine macrophage cells (RAW 264.7) was synergistically increased when combined with quercetin and resveratrol in reducing NO production by both scavenging NO and reducing iNOS gene expression (Chan *et al.*, 2000). We found out that quercetin with resveratrol act synergistically in eNOS promoter activation (Table 2 and Fig. 3). This is in accordance with our previous results with resveratrol and quercetin, where they synergistically inhibited vascular smooth muscle cell proliferation when used in a mixture (Kurin *et al.*, 2012).

Besides CI we determined the DRI as well. DRI represents the order of magnitude (fold) of dose reduction that is allowed in combination for a given degree of effect as compared with the dose of each drug alone, or in other words it indicates to what extent the concentration of drug can be reduced in a mixture in order to achieve a given effect level compared with a single drug treatment. DRI values higher than 1 are desirable, but they do not necessarily indicate synergy. As seen in Table 2, in the Q+R mixture are DRI values 2.5 for quercetin and 4.2 for resveratrol, what means that in Q+R mixture we needed 2.5 times lower dose of quercetin and 4.2 times lower dose of resveratrol to achieve the same effect that would be reached by the single compound treatment.

Despite we are not able to explain the inner mechanism of interactions among tested red wine polyphenols in eNOS promoter activation, we take into account that as the eNOS promoter activation involves multiple processes, the interference with multiple different targets is needed. Herbal drugs as complexes of substances or prepared mixtures of natural compounds open the possibility of novel multicomponent treatment or prevention approach development through synergistic interactions, which could impact multiple targets simultaneously, thus being better suitable for controlling complex diseases or biochemical pathways such as eNOS (Zimmermann *et al.*, 2007). In a small experimental model, we have shown that red wine polyphenols when used in mixtures are needed in a smaller

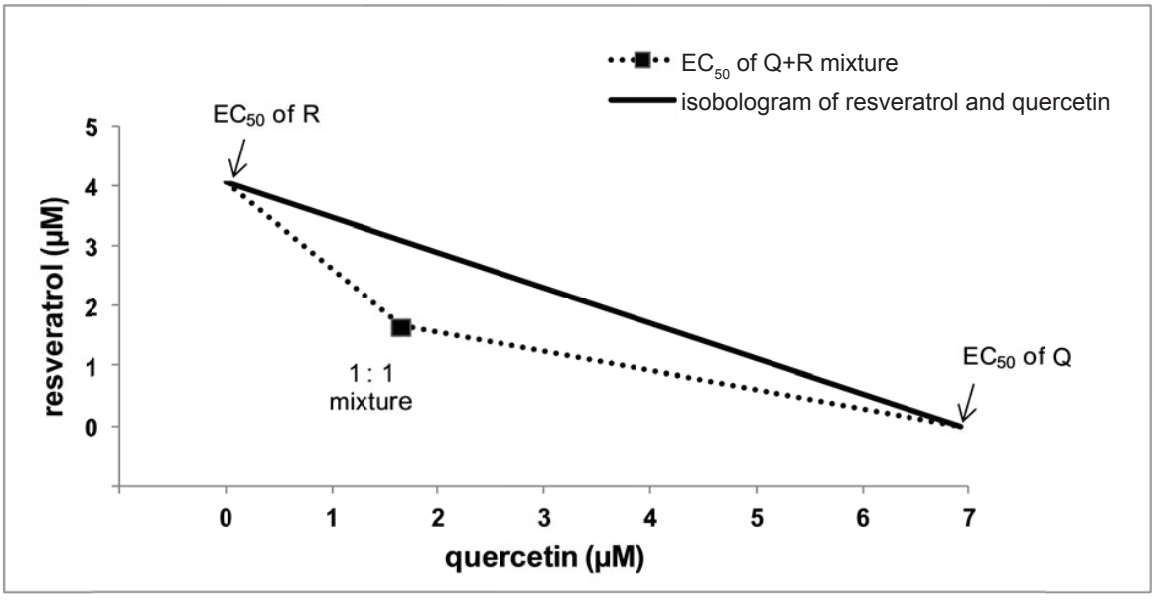

Figure 3. Isobologram of Q+R equimolar mixture.

amount and reach many times higher effects that is single molecule able to. "French paradox" until today has not been explained by a single effective molecule, our work suggests that the positive effects of red wine on cardiovascular system should be explained by the synergy of polyphenols mixtures present in red wine, thus despite their low concentration, their effects could be given by their cooperation in multiple system.

4. Conclusion

In conclusion, resveratrol, quercetin, kaempferol and isorhamnetin, the substances present in red wine, can activate eNOS promoter when used alone or in equimolar mixtures. The interaction study of red wine polyphenols indicated that in eNOS promoter activation, the final effects of mixtures vary from synergistic to antagonistic. Currently, the mechanism of their interaction is not known. However, when they are used together in a quaternary mixture, the final effect is synergic.

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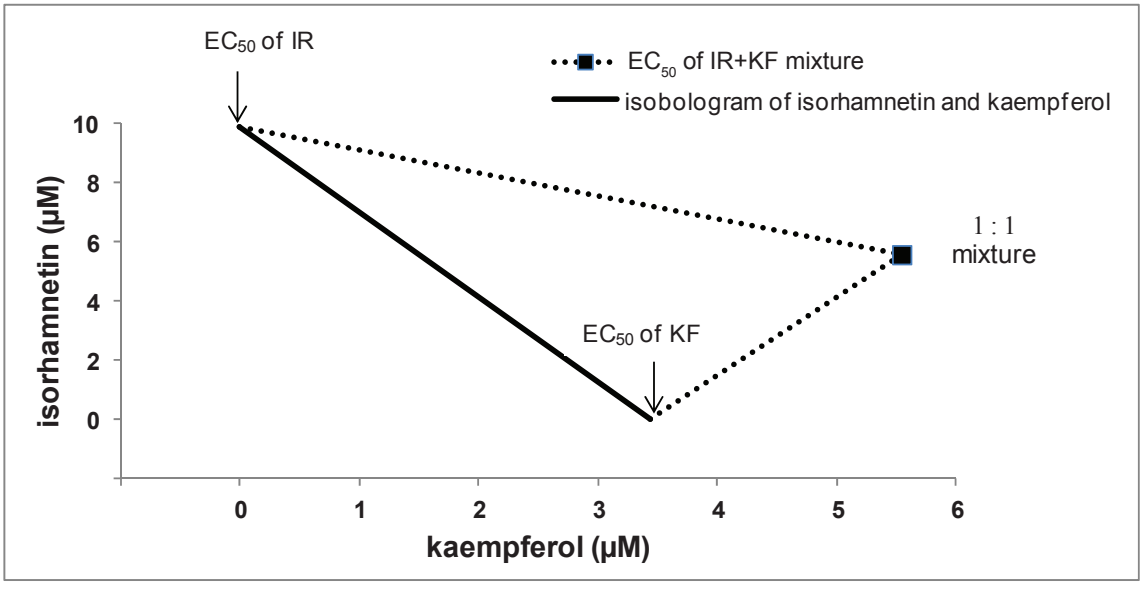

Figure 4. Isobologram of IR+KF equimolar mixture.

REFERENCES

[1] Appeldoorn MM, *et al.* Some phenolic compounds increase the nitric oxide level in endothelial cells *in vitro*. J Agric Food Chem 2009;57:7693–7699.

[2] Bao M, Lou Y. Flavonoids from seabuckthorn protect endothelial cells (EA.hy926) from oxidized low-density lipoprotein induced injuries via regulation of Lox-1 and eNOS expression. J Cardiovasc Pharmacol 2006;48:834–841.

[3] Berenbaum MC. What is synergy? Pharmacol Rev 1989; 41:93–141.

[4] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities utilizing the principle of protein dye binding. Anal Biochem 1976;72:248–254.

[5] Chan MM, Mattiacci JA, Hwang HS, Shah A, Fong D. Synergy between ethanol and grape polyphenols, quercetin, and resveratrol, in the inhibition of the inducible nitric oxide synthase pathway. Biochem Pharmacol 2000;60:1539–1548.

[6] Chen CC, Ke WH, Ceng LH, Hsieh CW, Wung BS. Calcium- and phosphatidylinositol 3-kinase/Akt-dependent activation of endothelial nitric oxide synthase by apigenin. Life Sci. 2010;87:743–749.

[7] Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev 2006; 58:621–681.

[8] Edgell CJ, McDonald CC, Graham JB. Permanent cell line expressing human factor VIII-related antigen established by hybridization. Proc Natl Acad Sci USA 1983;80:3734–3737.

[9] Han D, Yi OS, Shin HK. Solubilization of vitamin C in fish oil and synergistic effect with vitamin E in retarding oxidation. J Am Oil Chem Soc 1991;68:740–743.

[10] Kurin E, Atanasov AG, Donath O, Heiss EH, Dirsch VM, Nagy M. Synergy study of the inhibitory potential of red wine polyphenols on vascular smooth muscle cell proliferation. Planta Med 2012;78:772–778.

[11] Leikert JF, Räthel TR, Wohlfart P, Cheynier V, Vollmar AM, Dirsch VM. Red wine polyphenols enhance endothelial nitric oxide synthase expression and subsequent nitric oxide release from endothelial cells. Circulation 2002;106:1614–1617.

[12] Li H, *et al.* Activation of protein kinase C alpha and/or epsilon enhances transcription of the human endothelial nitric oxide synthase gene. Mol Pharmacol 1998;53:630–637.

[13] Ma XH, *et al.* Synergistic therapeutic actions of herbal ingredients and their mechanisms from molecular interaction and network perspectives. Drug Discov Today. 2009;14:579–588.

[14] Palozza P, Krinsky NI. Beta-carotene and alpha-tocopherol are synergistic antioxidants. Arch Biochem Biophys. 1992;297:184–187.

[15] Peyrat-Maillard MN, Bonnely S, Rondini L, Berset C. Effect of vitamin E and vitamin C on the antioxidant activity of malt rootlets extracts. Lebensm Wiss Technol 2001;34:176–182.

[16] Räthel TR, Samtleben R, Vollmar AM, Dirsch VM. Activation of endothelial nitric oxide synthase by red wine polyphenols: impact of grape cultivars, growing area and the vinification process. J Hypertens 2007;25:541–549.

[17] Scarpa M, Rigo A, Maiorino M, Ursini F, Gregolin C. Formation of alpha-tocopherol radical and recycling of alpha-tocopherol by ascorbate during peroxidation of phosphatidylcholine liposomes: an electron paramagnetic resonance study. Biochim Biophys Acta 1984;801:215–219.

[18] Schmitt CA, Dirsch VM. Modulation of endothelial nitric oxide by plant-derived products. Nitric Oxide 2009;21:77–91.

[19] St Leger AS, Cochrane AL, Moore F. Factors associated with cardiac mortality in developed countries with particular reference to the consumption of wine. Lancet 1979;313:1017–1020.

[20] Wallerath T, *et al.* Resveratrol, a polyphenolic phytoalexin present in red wine, enhances expression and activity of endothelial nitric oxide synthase. Circulation 2002;106:1652–1658.

[21] Wallerath T, Poleo D, Li H, Förstermann U. Red wine increases the expression of human endothelial nitric oxide synthase a mechanism that maycontribute to its beneficial cardiovascular effects. J Am Coll Cardiol 2003;41:471–478.

[22] Wallerath T, Li H, Gödtel-Ambrust U, Schwarz PM, Förstermann U. A blend of polyphenolic compounds explains the stimulatory effect of red wine on human endothelial NO synthase. Nitric Oxide 2005;12:97–104.

[23] Wang H, Yang YJ, Qian HY, Zhang Q, Xu H, Li JJ. Resveratrol in cardiovascular disease: what is known from current research? Heart Fail Rev 2012;17:437–448.

[24] Xu JW, Ikeda K, Yamori Y. Cyanidin-3-glucoside regulates phosphorylation of endothelial nitric oxide synthase. FEBS Lett 2004;574:176–180.

[25] Zhou B, Jia ZS, Chen ZH, Yang L, Wu LM., Liu ZL. Synergistic antioxidant effect of green tea polyphenols with alpha-tocopherol on free radical initiated peroxidation of linoleic acid in micelles. J Chem Soc Perkin Trans 2000;2:785–791.

[26] Zhou B, Wu LM, Yang L, Liu ZL. Evidence for alfa-tocopherol regeneration reaction of green tea polyphenols in SDS micelles. Free Radic Biol Med 2005;38:78–84.

[27] Zimmermann GR, Lehár J, Keith CT. Multi-target therapeutics: when the whole is greater than the sum of the parts. Drug Discov Today 2007;12:34–42.

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