

# Vazodilatacijski i antioksidacijski učinci fenolnih kiselina iz vina, termički obrađenog te intaktnog vina u usporedbi s kupinovim vinom

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**SVEUČILIŠTE U SPLITU  
MEDICINSKI FAKULTET**

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**VAZODILATACIJSKI I ANTIOKSIDACIJSKI  
UČINCI FENOLNIH KISELINA IZ VINA,  
TERMIČKI OBRAĐENOG TE INTAKTNOG VINA  
U USPOREDBI S KUPINOVIM VINOM**

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## 1. POPIS OZNAKA I KRATICA



ABTS <sup>+</sup>	2,2'-azinodi (3-etilbenzotiazolin)-6-sulfonat
Ach	acetilkolin
ANOVA	analiza varijance
BV	bijelo vino iz grožđa
cGMP	ciklički gvanozin-monofosfat
CV	crno vino iz grožđa
ΔD	srednja vrijednost stupnja topološke udaljenosti vrhova grafa (engl. <i>Mean Distance Degree Deviation</i> )
EC <sub>50</sub>	koncentracija koja izaziva 50% maksimalnog vazodilatacijskog učinka
EDHF	čimbenik hiperpolarizacije endotelnog podrijetla (engl. <i>Endothelium - Derived Hyperpolarizing Factor</i> )
E <sub>max</sub>	maksimalni vazodilatacijski učinak
eNOS	endotelna NO sintetaza
ET-1	endotelin-1
FRAP	antioksidacijski kapacitet redukcije željeza (engl. <i>Ferric Reducing Antioxidant Power</i> )
GAE	ekvivalent galne kiseline (engl. <i>Gallic Acid Equivalent</i> )
GETAWAY	molekulski deskriptori koji se izvode iz geometrije, topologije i strukture molekule i skupa atomskih masa (engl. <i>Geometry, Topology and Atom Weights Assembly</i> )
HDL	lipoproteini velike gustoće (engl. <i>High Density Lipoprotein</i> )
HPLC	visoko djelotvorna tekućinska kromatografija (engl. <i>High Performance Liquid Chromatography</i> )
KV	kupinovo vino
LDL	lipoproteini male gustoće (engl. <i>Low Density Lipoprotein</i> )
M-3-GE	ekvivalent malvidin-3-glukozida (engl. <i>Malvidine-3-Glucoside Equivalent</i> )
NA	noradrenalin
NO	dušikov oksid (engl. <i>Nitric Oxide</i> )
PDA	fotodiodni detektor (engl. <i>Photodiode Array Detector</i> )
PGI <sub>2</sub>	prostaciklin
QSAR	analiza kvantitativne povezanosti strukture i aktivnosti (engl. <i>Quantitative Structure - Activity Relationship</i> )

RDF	deskriptori izvedeni iz funkcije radijalne raspodjele atoma (engl. <i>Radial Distribution Function</i> )
SAR	analiza povezanosti strukture i aktivnosti (engl. <i>Structure - Activity Relationship</i> )
TE	ekvivalent troloksa (engl. <i>Trolox Equivalent</i> )
TEAC	antioksidacijski kapacitet u ekvivalentima troloksa (engl. <i>Trolox Equivalent Antioxidant Capacity</i> )
TPSA	topološka ploština polarne površine molekule (engl. <i>Topological Polar Surface Area</i> )
TPTZ	2,4,6-tri-(2-piridil)-2-triazin
VS	nasuprot (engl., lat. <i>Versus</i> )
3D-MoRSE	trodimenzijska molekulska reprezentacija struktura utemeljena na elektronskoj difrakciji (engl. <i>Molecule Representation of Structure based on Electron diffraction</i> )

## 2. PREGLED OBJEDINJENIH RADOVA

Doktorska disertacija nastala je objedinjenjem triju znanstvenih članaka:

1. Mudnic I, Modun D, Rastija V, Vukovic J, Brizic I, Katalinic V, Kozina B, Medic-Sarić M, Boban M. Antioxidative and vasodilatory effects of phenolic acids in wine. *Food Chemistry* 2010;**119**:1205-1210.
2. Mudnic I, Budimir D, Modun D, Gunjaca G, Generalic I, Skroza D, Katalinic V, Ljubenkovic I, Boban M. Antioxidant and vasodilatory effects of blackberry and grape wines. *Journal of Medicinal Food* 2012;**15**(3):315-321.
3. Mudnic I, Budimir D, Jajic I, Boban N, Sutlovic D, Jeroncic A, Boban M. Thermally treated wine retains vasodilatory activity in rat and guinea pig aorta. *Journal of Cardiovascular Pharmacology* 2011;**57**:707-711.

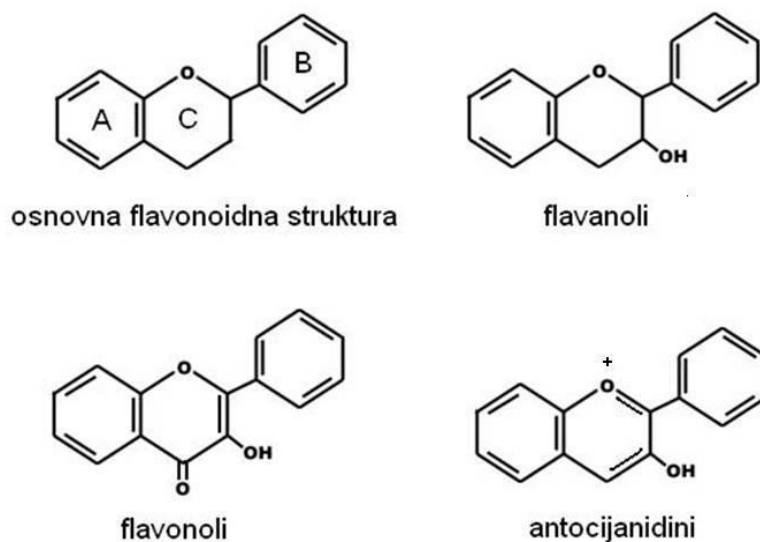
## 2.1. UVOD

Brojne epidemiološke studije pokazale su da umjerena konzumacija vina posreduje različite biološke učinke s povoljnim utjecajem na ljudsko zdravlje.<sup>1-4</sup> Jedan od takvih učinaka je snažna vazodilatacija, koja se povezuje sa sadržajem polifenola u vinu i u eksperimentalnim uvjetima *in vitro* nije ovisna o sadržaju etanola.<sup>5-7</sup> Osim tonusa krvnih žila, vino i vinski fenoli moduliraju krvožilnu funkciju tako što smanjuju ekspresiju adhezijskih molekula i faktora rasta, inhibiraju migraciju i proliferaciju stanica glatkih mišića i inhibiraju agregaciju trombocita.<sup>8,9</sup>

Vazodilacijski učinak vina je izravan, ovisan o endotelu te različitim mehanizmima povezan s dušikovim oksidom (NO). U kratkoročne mehanizme spada povećana proizvodnja i biodostupnosti NO-a,<sup>10-13</sup> a u dugoročne povećanje ekspresije endotelne NO sintetaze (eNOS) i smanjenje sinteze endotelina-1.<sup>14,15</sup> Učinci vina i vinskih polifenola na endotel su od potencijalno velikog značaja s obzirom na važnost disfunkcije endotela u patogenezi vaskularnih bolesti, uključujući i ishemijsku bolest srca.

Prvu studiju o vazodilacijskim učincima vina i vinskih fenola proveli su Fitzpatrick i suradnici 1993. godine na izoliranoj štakorskoj aorti.<sup>5</sup> Studija opisuje vazodilacijske učinke nekoliko sorti crnog i bijelog vina, soka iz grožđa i pojedinih polifenolnih spojeva iz vina i pokazuje da je vazodilacijski učinak vina posredovan proizvodnjom NO-a i cikličkog gvanozin-monofosfata (cGMP-a), kao drugog glasnika. Slijedile su studije o učincima vinskih polifenola i vina na različitim humanim i životinjskim izoliranim krvnim žilama.<sup>7,10,16-18</sup> Tako su Flesch i suradnici pokazali da vina iz Italije i Francuske dilatiraju humane koronarne žile, za razliku od njemačkih vina koja nemaju takav učinak,<sup>7</sup> a u Rendigovoj studiji crno vino (Merlot i Cabernet Sauvignon) nije pokazalo vazodilacijski učinak na koronarnim žilama kunića.<sup>19</sup> Istraživanja o mehanizmima vazodilacijskog učinka koja su slijedila, osim NO-a i cGMP-a, opisuju uključenost prostaciklina (PGI<sub>2</sub>),<sup>20</sup> čimbenika hiperpolarizacije endotelnog podrijetla (eng. *Endothelium - Derived Hyperpolarizing Factor* (EDHF))<sup>13,21</sup> te purinergičkog sustava<sup>22</sup> u vazodilacijskom odgovoru na vino i vinske polifenole. Vazodilacijski učinak vina *in vitro* ovisi ne samo o korištenoj sorti vina i vrsti krvne žile, nego i o životinjskoj vrsti. U nedavnoj studiji Brizića i suradnika pokazano je da isto vino, u istim eksperimentalnim uvjetima, ostvaruje različite vazodilacijske učinke na aorti štakora u odnosu na aortu zamorčića.<sup>23</sup>

Uz vazodilatacijski učinak pokazano je da vino i vinski polifenoli mogu djelovati pozitivno protiv oksidativnog stresa, kao važnog patofiziološkog mehanizma vaskularnih i drugih bolesti. Vinski fenoli primijenjeni *in vitro* inhibiraju oksidaciju lipoproteina male gustoće (LDL),<sup>24,25</sup> a nakon konzumacije vina povećava se razina lipoproteina velike gustoće (HDL)<sup>26,27</sup> i antioksidacijski kapacitet plazme u ljudi.<sup>28-30</sup> Fenolni spojevi u vinu podrazumijevaju veliku skupinu od više stotina različitih molekula. Jednostavni fenoli su spojevi s jednim aromatskim prstenom koji imaju jednu ili više hidroksilnih skupina i osnovna su građevna jedinica svih polifenola. Polifenoli su spojevi koji imaju više fenolnih prstenova unutar svoje strukture. Vinski polifenoli dijele se na dvije osnovne skupine, flavonoide i ne-flavonoide. Flavonoidi su polifenolni spojevi koji imaju karakterističnu jezgru od tri aromatska prstena. Na benzenski prsten A izravno je vezan heterociklički prsten C koji na drugom ugljikovom atomu ima spojen prsten benzena B (Slika 1). Pojedine skupine flavonoida imaju drugačiji prsten C. Prsten C može biti heterociklički piran kod skupine flavanola (tipični predstavnik je katehin) i antocijanidina (tipični predstavnik je cijanidin) ili piron kod skupine flavonola (tipični predstavnik je kvercetin).<sup>31,32</sup>



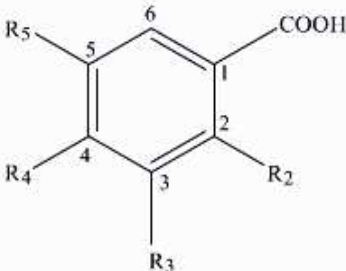
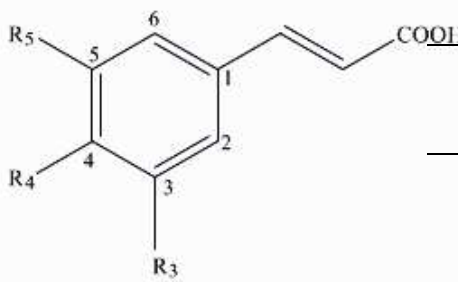
**Slika 1.** Glavne skupine vinskih flavonoida

Flavanoli su prevladavajući flavonoidi, a u vinu i grožđu se nalaze u slobodnom obliku, za razliku od ostalih pripadnika flavonoida koji su vezani za šećere. Osim monomera katehina i epikatehina, prisutni su i kao polimeri (procijanidini). Antocijanidini su

pigmenti vina i grožđa, a njihov specifičan konjugirani C prsten zaslužan je za plavo ili crveno obojenje. Najvažniji predstavnici su cijanidin i malvidin. Antocijanidini su vrlo nestabilni i zapravo ne postoje u slobodnom obliku u vinu, već se nalaze kao glikozidi i tada se nazivaju antocijani.<sup>32</sup>

Upravo se procijanidini i antocijani izdvajaju kao flavonoidni spojevi posebno značajni za vazodilatacijsku aktivnost vina.<sup>33,34</sup>

Važna frakcija vinskih fenola su fenolne kiseline. Fenolne kiseline su neflavonoidne tvari prisutne u slobodnoj formi ili kao glikozilirani derivati i esteri u crnom i bijelom vinu.<sup>32</sup> Osim u grožđu i vinu, fenolne su kiseline prisutne i u voću, povrću, žitaricama te u čaju.<sup>35</sup> Fenolne kiseline se dijele na derivate hidroksibenzojeve kiseline (*p*-hidroksibenzojeva, protokatehinska, vanilinska, galna, siringinska, gentisinska, elaginska kiselina) i derivate hidroksicimetne kiseline (*p*-kumarinska, kafeinska, ferulinska, sinapinska kiselina) (Slika 2).

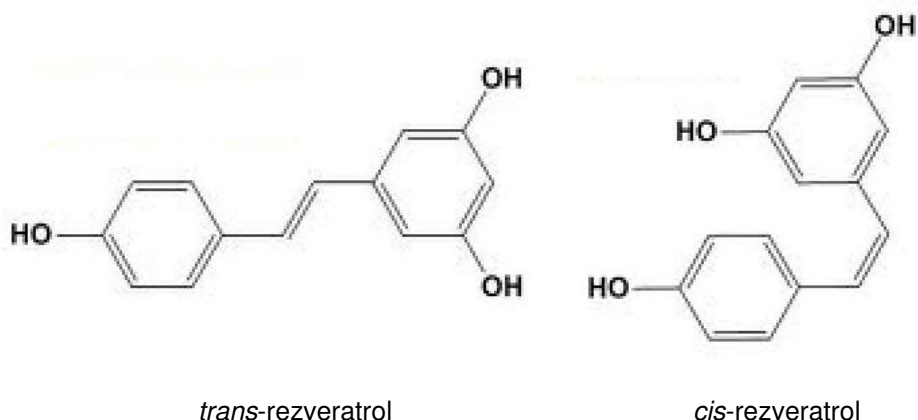
	<b>Hidroksibenzojeva kiselina</b>					
	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	R <sub>5</sub>		
	<i>p</i> -hidroksibenzojeva	H	H	OH	H	
	protokatehinska	H	OH	OH	H	
	vanilinska	H	OCH <sub>3</sub>	OH	H	
	galna	H	OH	OH	OH	
siringinska	H	OCH <sub>3</sub>	OH	OCH <sub>3</sub>		
	<b>Hidroksicimetna kiselina</b>					
			R <sub>3</sub>	R <sub>4</sub>	R <sub>5</sub>	
	<i>p</i> -kumarinska			H	OH	H
	kafeinska			OH	OH	H
	ferulinska			OCH <sub>3</sub>	OH	H
sinapinska			OCH <sub>3</sub>	OH	OCH <sub>3</sub>	

**Slika 2.** Fenolne kiseline

Derivati hidroksicimetine kiseline su glavni fenolni spojevi bijelog vina, a koncentracija galne kiseline, kao glavnog predstavnika derivata hidroksibenzojeve kiseline, povećava se starenjem vina.<sup>32</sup>

Fenolne kiseline su pokazale antioksidacijsku aktivnost u različitim eksperimentalnim uvjetima,<sup>36-39</sup> te je za nekoliko fenolnih kiselina utvrđena povezanost strukture i antioksidacijskog učinka *in vitro*.<sup>40</sup> Također je pokazana vazodilatacijska aktivnost nekih fenolnih kiselina.<sup>16</sup> Tako ferulinska kiselina snižava krvni tlak spontano-hipertenzivnim štakorima<sup>41</sup> i obnavlja vazodilataciju ovisnu o endotelu u aortama spontano-hipertenzivnih štakora, povećavajući biodostupnost NO.<sup>42</sup> Unatoč velike zastupljenosti fenolnih kiselina u biljnom svijetu i različitim pićima, biološki učinci većine fenolnih kiselina su slabo istraženi u odnosu na ostale fenolne spojeve iz vina.

Nasuprot fenolnim kiselinama, veliku pozornost znanstvenika privukao je rezveratrol, specifičan neflavonoidni polifenolni spoj koji se ubraja u skupinu stilbena (Slika 3). Stilbeni su prirodni spojevi vinove loze nastali kao obrambeni odgovor na UV zračenje, infekcije različitim mikroorganizmima i druge oblike stresa.<sup>32</sup> Rezveratrol se u grožđu nalazi primarno u kožici. Količina rezveratrola je različita u različitim sortama grožđa, a ovisi o geografskom podrijetlu i izloženosti stresu iz okoliša. Sadržaj rezveratrola u vinu ovisi i o duljini fermentacijskog vremena tijekom kojeg je vino bilo u dodiru s kožicama grožđa. Prosječno crno vino sadrži između 0,2 i 5,8 mg/L rezveratrola dok je u bijelih vina ta razina višestruko niža. Razlog tome je fermentacija crnog vina u dodiru s pokožicama grožđa što omogućava prijelaz rezveratrola u vino, dok kod bijelog vina fermentira samo sok odvojen od sjemenki i pokožica.<sup>32</sup>



**Slika 3.** Izomeri rezveratrola



Brojne studije su opisale vazodilatacijsku aktivnost *trans*-rezveratrola u različitim eksperimentalnim uvjetima.<sup>43,44</sup> Štoviše, u studiji Oralla i suradnika *trans*-rezveratrol je istaknut kao ključni sastojak odgovoran za kardioprotektivni učinak vina.<sup>45</sup>

Osim vina iz grožđa vinove loze (*Vitis vinifera*), postoje i brojna druga vina čija je konzumacija sve popularnija. Nekoliko studija pokazalo je da su voćna vina također potencijalno bogati izvor polifenola i da pokazuju značajan antioksidacijski učinak *in vitro*.<sup>46-48</sup> Među voćnim vinima posebno se izdvaja kupinovo vino (*Rubus fruticosus*), za kojega je karakterističan sadržaj ukupnih polifenola sličan crnom vinu<sup>47</sup> i antioksidacijski učinak čak i veći od crnog vina.<sup>49</sup> Kupinova vina su, kao i crna vina od grožđa, bogata antocijanima, a slično bijelim vinima bogata ne flavonoidnim spojevima, osobito galnom kiselinom. Učinci kupinovitih vina u biološkim sustavima još nisu istraživani.

Uz uobičajeni način konzumacije, vino se često pije i kao topli napitak, tzv. kuhano vino. Također se vrlo često osobito u mediteranskoj kuhinji koristi kao dodatak u pripremi hrane. U oba je slučaja vino izloženo zagrijavanju koje može značajno promijeniti izvorna fizikalno-kemijska svojstva te posljedično biološki učinak. Primjerice, alkohol prilikom kuhanja brzo isparava ovisno o intenzitetu i vremenu zagrijavanja.<sup>50</sup> Polifenolni sastojci su također podložni termičkoj razgradnji s posljedičnim dinamičkim promjenama u antioksidacijskoj aktivnosti.<sup>51,52</sup> Biološki učinci termički tretiranih vina praktički su neistraženi.

### 2.1.1. Ciljevi istraživanja

Unatoč brojnim istraživanjima vazodilatacijskog učinka pojedinih polifenolnih spojeva prisutnih u vinu, vazodilatacijski učinak fenolnih kiselina u koncentracijskom rasponu, koji je usporediv s izmjerenim koncentracijama fenolnih kiselina u plazmi ljudi nakon konzumacije vina, do sada nije bio određen. Stoga je **prvi cilj** istraživanja utvrditi vazodilatacijski i antioksidacijski učinak 9 fenolnih kiselina iz vina. Dodatni je cilj ovog dijela istraživanja utvrditi međusobnu povezanost bioloških učinaka sa strukturnim karakteristikama molekula ispitivanih fenolnih kiselina (analiza kvantitativne povezanosti strukture i aktivnosti, engl. *Quantitative Structure - Activity Relationship* (QSAR)).

Vazodilatacijski učinak crnog vina je snažan i usporediv s učinkom vazodilatacijskih lijekova te osim s grožđanim sokom i ekstraktima obogaćenima polifenolima nije uspoređivan sa sličnim alkoholnim napitcima. S druge strane bijelo je vino slabi vazodilatator, bez ili s vrlo niskim sadržajem antocijana. Uz vino iz grožđa, popularna je konzumacija voćnih vina, među kojima je kupinovo vino posebno bogato polifenolima te jači antioksidans od crnog vina *in vitro*. U biološkim sustavima nije do sada opisan učinak kupinovog vina. Stoga je **drugi cilj** istraživanja usporediti vazodilatacijski i antioksidacijski učinak crnog i bijelog vina s kupinovim vinom. Dodatni je cilj ovog dijela istraživanja utvrditi međusobnu povezanost ispitivanih bioloških učinaka te doprinos pojedine polifenolne frakcije i/ili pojedinačnog polifenolnog spoja (posebice rezveratrola) vazodilatacijskom učinku ispitivanih vina.

Uz standardni način, vino se često konzumira i kao topli napitak, kuhano vino i koristi kao dodatak u pripremi hrane. Stoga je **treći cilj** istraživanja ispitati vazodilatacijski učinak vina koje je tijekom 45 minuta zagrijavano na 75 i 125 °C. Vazodilatacijski učinci termički obrađenih vina uspoređeni su s onima intaktnog vina i vina dealkoholiziranog bez termičkog stresa. Dodatni je cilj ovog dijela istraživanja usporediti vazodilatacijske učinke intaktnog, dealkoholiziranog i termički obrađenog vina na aorti štakora u odnosu na aortu zamorčića.

## 2.2. MATERIJALI I METODE

### 2.2.1. Ispitivana vina i derivati

U istraživanju su korištena crna vina: Refošk 2007 i Vinagra 2005 i 2006, vinarija Brič iz Slovenije; bijela vina Pošip 2007, Čara Korčula i Zlatna žlahtina 2007, Vrbnik Krk, iz Hrvatske te kupinova vina KupiFe 2007, Split, Kupinovo vino Šuler 2007, Kutina, BIO&BIO 2008, Orlov put, Baranjsko kupinovo vino 2006, Cerine iz Hrvatske.

Za pripremu dealkoholiziranog uzorka korišteno je crno vino Vinagra 2006, vinarija Brič, Slovenija. Priprava dealkoholiziranog vina izvodila se pomoću rotacijskog vakuum uparivača (Laborota 4000, Heidolph Instruments Inc., Schwabach, Njemačka). Kako bi se izbjegao mehanički i termički stres uzorka, vakuum se primjenjivao postupno i progresivno do očitavanja od  $-130$  kPa, pri temperaturi od  $30$  °C, tijekom 45 minuta i rotaciji posude s uzorkom od 150 okretaja u minuti.

Za pripremu termički obrađenih uzoraka korišteno je crno vino Vinagra 2006, vinarija Brič, Slovenija. Termička obrada vina izvodila se u termostatski kontroliranoj uljnoj kupelji (Heidolph, HB Digital, Schwabach, Njemačka) pri temperaturama od  $75$  i  $125$  °C tijekom 45 minuta.

### 2.2.2. Pokusne životinje

Za potrebe cjelokupnog istraživanja korišteno je 126 štakora (mužjaka sojeva Wistar i Sprague-Dawley) i 44 zamorčica (mase  $330 \pm 20$  g i  $290 \pm 20$  g; 3-4 mjeseca starosti) iz Sveučilišne nastambe za pokusne životinje u Splitu. Svi postupci provedeni u opisanim istraživanjima odobreni su od Etičkog povjerenstva za biomedicinska istraživanja Medicinskog fakulteta Sveučilišta u Splitu.

Nakon anestezije životinje (i.p. 1 g/kg t.t. uretana), učinila se torakotomija, preparirala torakalna aorta, disecirala te prenijela u preparativni bazenčić sa hladnom i oksigeniranom Krebs–Henseleitovom otopinom.<sup>53</sup> Nakon pažljivog čišćenja masnog i vezivnog tkiva, aorta se izrezala na prstenove širine 3-4 mm. Prstenove se zatim namjestilo u organske bazenčiće (20 mL zapremine) s Krebs – Henseleitovom otopinom koja se kontinuirano zagrijavala i oksigenirala ( $t = 37$  °C,  $\text{pH} = 7,4$ ). Dvije paralelne čelične žice provukle su se kroz lumen prstena, od kojih se jedna fiksirala za dno organskog bazenčića, a druga koncem povezala na pretvarač vlačne sile

(FORT 10, World Precision Instruments, Berlin, Njemačka), radi mjerenja tonusa žilnog prstena. Pretvarač sile se spajao na pojačalo (QUAD Bridge, ADInstruments, Castle Hill, Australija) i na analogno/digitalni pretvarač (MacLab/8e, ADInstruments), što je primjenom računalnog programa (CHART za Windows, verzija 4.2.4, ADInstruments) omogućilo kontinuirani grafički prikaz tijeka pokusa na ekranu računala.

Nakon ispiranja prstenovi su se prekontrahirali kontrolnom dozom noradrenalina (NA, prstenovi štakora  $10^{-7}$  mol/L, prstenovi zamorčića  $10^{-6}$  mol/L). Kada se postigao stabilni plato kontrakcije, očuvanost endotela se testirala acetilkolinom (Ack, prstenovi štakora  $10^{-6}$  mol/L, prstenovi zamorčića  $10^{-5}$  mol/L). Relaksacija se izražavala kao postotak smanjenja vazokonstrukcije uzrokovane noradrenalinom. Isključivo prstenovi s očuvanim endotelom (acetilkolinska relaksacija veća od 70%), prekontrahirani noradrenalinom, bili su podvrgnuti jednom od sljedećih protokola:

1. radi određivanja vazodilatacijskog učinka fenolnih kiselina, prstenovi su bili izloženi kumulativnoj dozi ( $10^{-6}$ - $10^{-3}$  g/L) ispitivane pojedinačne fenolne kiseline (*p*-hidroksibenzojeva, *p*-kumarinska, vanilinska, ferulinska, protokatehinska, kafeinska, siringinska, sinapinska i galna kiselina), (n = 12 aortnih prstenova štakora po ispitivanoj fenolnoj kiselini)
2. radi određivanja vazodilatacijskog učinka termički obrađenog vina, prstenovi su bili izloženi kumulativnoj dozi (0,1-8%) ispitivanog pojedinačnog uzorka intaktnog vina, dealkoholiziranog vina, vina zagrijavanog na 75 °C, te vina zagrijavanog na 125 °C (n = 21 aortni prsten štakora i n = 23 aortna prstena zamorčića po uzorku)
3. radi usporedbe vazodilatacijskih učinaka crnog i bijelog vina iz grožđa s kupinovima vinom, prstenovi su bili izloženi kumulativnoj dozi (1-8%) ispitivanog pojedinačnog uzorka od po dva crna i bijela vina od grožđa, te četiri kupinova vina (n = 15 aortnih prstenova štakora). Ispitivani uzorci prikazani su u Tablici 1.

**Tablica 1.** Ispitivana vina iz grožđa i kupine (izvor, berba i sadržaj etanola).

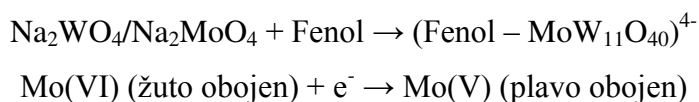
	Uzorak vina (kratica)	Naziv, proizvođač, porijeklo	Godina	Sadržaj etanola (% volumena)
1	Crno (CV1)	Refošk, Brič, Slovenija	2007	13,5
2	Crno (CV2)	Vinagra, Brič, Slovenija	2005	13,0
3	Bijelo (BV1)	Pošip, Čara Korčula, Hrvatska	2007	14,0
4	Bijelo (BV2)	Zlatna žlahtina, Vrbnik Krk, Hrvatska	2007	11,5
5	Kupinovo (KV1)	KupiFe, Split, Hrvatska	2007	9,9
6	Kupinovo (KV2)	Kupinovo vino Šuler, Kutina, Hrvatska	2007	13,7
7	Kupinovo (KV3)	BIO&BIO, Orlov put, Hrvatska	2008	10,6
8	Kupinovo (KV4)	Baranjsko kupinovo vino, Cerine, Hrvatska	2006	13,0

CV, crno vino iz grožđa; BV, bijelo vino iz grožđa; KV, kupinovo vino

### 2.2.3. Metode analize polifenolnog sastava i antioksidacijskog kapaciteta

Koncentracije ukupnih fenola, flavonoida, ne-flavonoida i antocijana, kao i antioksidacijski kapacitet svih ispitivanih vina i derivata određeni su spektrofotometrijskim metodama. Spektrofotometrijska mjerenja provedena su na UV – VIS spektrofotometru „Specord 200” Analytik Jena GmbH, Jena, Njemačka.

Koncentracija ukupnih fenola određena je primjenom Folin-Ciocalteu metode.<sup>56</sup> Metoda se temelji na oksidaciji fenolnih spojeva dodatkom Folin-Ciocalteu reagensa uz nastajanje obojenog produkta te mjerenjem nastalog intenziteta obojenja određivanjem apsorbancije pri valnoj duljini 725 nm. Folin-Ciocalteu reagens je smjesa volframofosfatnih i molibdofosfatnih aniona koji se reduciraju i daju plavo obojenje. Intenzitet obojenja proporcionalan je koncentraciji fenolnih spojeva.



Ista se metoda koristila za određivanje koncentracije flavonoida i ne-flavonoida, nakon precipitacije s formaldehidom. Mjerenja su vršena u triplikatu, a rezultati izražavani kao mg/L ekvivalentata galne kiseline (GAE).

Za određivanje koncentracije antocijana primijenjena je metoda izbjeljivanja bisulfitom.<sup>57</sup> Metoda se temelji na vezivanju  $\text{HSO}_3^-$  iona na antocijane u položaju 2' te se time obojeni kation oblik antocijana prevodi u bezbojni leuko oblik. Mjerenja su vršena u triplikatu, a rezultati izražavani kao mg/L ekvivalentata malvidin-3-glukozida (M-3-GE).

Antioksidacijski kapaciteti fenolnih kiselina, intaktnog, dealkoholiziranog i termički obrađenog vina određeni su primjenom FRAP (engl. *Ferric Reducing Antioxidant Power*) metode.<sup>54</sup> FRAP se temelji na sposobnosti uzorka (antioksidansa) da reducira  $\text{Fe}^{3+}$  u  $\text{Fe}^{2+}$ . Nastali  $\text{Fe}^{2+}$  oblik u reakciji s 2,4,6-tri-(2-piridil)-2-triazinom (TPTZ) daje spoj koji apsorbira svjetlo pri valnoj duljini 593 nm, a intenzitet plavog obojenja ovisi o antioksidacijskoj sposobnosti prisutnog antioksidansa.



Mjerenja su vršena u triplikatu. Rezultati su uspoređivani sa standardnim krivuljama s različitim koncentracijama troloksa (vodotopljivog analoga vitamina E) i izražavani kao mikromolarni ekvivalenti troloksa (TE).

Antioksidacijski kapacitet fenolnih kiselina je, osim FRAP metodom, određen i primjenom TEAC (engl. *Trolox Equivalent Antioxidant Capacity*) metode. TEAC se temelji na sposobnosti uzorka (antioksidansa) da reducira radikal kation 2,2'-azinodi (3-etilbenzotiazolin)-6-sulfonat ( $\text{ABTS}^{+\cdot}$ ), koji je plavo-zelene boje. TEAC metoda mjeri promjene apsorbancije kod 734 nm zbog nastajanja bezbojnog produkta. Mjerenja su vršena u triplikatu, a rezultati izražavani kao milimolarni ekvivalenti troloksa (TE).<sup>55</sup>

Sadržaj etanola određen je plinskim kromatografom (Shimadzu, model 2010, Kyoto, Japan) s analizatorom vršnih para i plameno - ionizacijskim detektorom.

Koncentracija pojedinačnih polifenolnih spojeva, uključujući i rezveratrol, određena je primjenom tekućinske kromatografije visoke djelotvornosti, HPLC. Korišten je HPLC, Varian (Palo Alto, Kalifornija, SAD) opremljen fotodiodnim detektorom PDA 330 (engl. *Photodiode Array Detector*), ternarnom gradijentnom tekućinskom pumpom, Pro Star 230, grijačem kolone model 500 i radnom stanicom „Star chromatography”, verzija 6,0. Polifenoli su odvajani na oktadecilnoj koloni (Zorbax Eclipse XDB-C18; unutarnjeg promjera 4,6 mm, duljine 250 mm; debljina filma 5  $\mu\text{m}$ ; Agilent, Palo Alto, SAD) pri 30 °C. Prije analize uzorci vina su filtrirani (veličina pora filtera 0,45  $\mu\text{m}$ ) i izravno injektirani u kromatografski sustav. Gradijentna elucija uključuje primjenu

dvaju otapala: otapalo A (voda/octena kiselina, 98:2 *vol/vol*) i otapalo B (acetonitril/octena kiselina, 99:1 *vol/vol*) uz brzinu protoka od 1mL/min. Detekcija se vršila pri valnoj duljini od 280 nm. Pojedinačni fenolni spojevi identificirani su prema retencijskom vremenu i apsorpcijskom spektru, a kvantificirani usporedbom s kalibracijskim krivuljama vanjskih standarda. Svaki je uzorak dva puta injektiran u kromatografski sustav.

Sve kemikalije i reagensi su bili analitičkog stupnja čistoće i kupljeni od Sigma Chemical Co (Steineheim, Njemačka) i Merck (Darmstadt, Njemačka). Deionizirana (Milli Q) voda korištena je za pripremu svih otopina i reagensa.

#### 2.2.4. Studija kvantitativne povezanosti strukture i aktivnosti (*Quantitative Structure - Activity Relationship* (QSAR))

QSAR je skup metoda koje se temelje na ideji da postoji veza između kemijske strukture molekula i njihove biološke aktivnosti i da je tu vezu moguće kvantitativno opisati. Najčešće korištena matematička tehnika u QSAR analizi je višestruka linearna regresija (pretpostavlja da postoji linearna veza između skupa nezavisnih varijabli koje opisuju strukturu molekule i zavisnih varijabli koje opisuju njihovu aktivnost). Kemijski deskriptor je konačni rezultat matematičkog i logičkog postupka kojim se kemijske informacije pretvaraju u broj. Danas je poznato više od 3000 molekularnih deskriptora; mogu biti izračunati ili izmjereni eksperimentalno, dobiveni od 1D, 2D ili 3D reprezentacije molekule.

5 grupa 3D deskriptora korišteno je u tvorbi QSAR modela o povezanosti strukture i biološke aktivnosti fenolnih kiselina: geometrijski deskriptori, trodimenzijska molekulska reprezentacija struktura utemeljena na elektronskoj difrakciji (3D-MoRSE, engl. *Molecule Representation of Structure based on Electron diffraction*), Randić molekularni profili, molekularni deskriptori koji se izvede iz geometrijske, topološke strukture molekule i skupa atomskih masa (GETAWAY, engl. *Geometry, Topology and Atom Weights Assembly*), i deskriptori izvedeni iz funkcije radijalne raspodjele atoma (RDF, engl. *Radial Distribution Function*).<sup>58</sup> Dodatni deskriptori uključeni u QSAR studija fenolnih kiselina bili su: ukupan broj hidroksilnih i metoksi grupa na fenilnom prstenu ( $n_{OH}$ ,  $n_{OCH_3}$ ) i indikatorske varijable. Indikatorske su varijable definirane kao prisustvo ( $I_{3,4-OH} = 1$ ) ili odsustvo 3,4-OH grupa ( $I_{3,4-OH} = 0$ ), temeljem prethodnih studija o odnosu strukture i aktivnosti (engl. *Structure - Activity relationship* (SAR)).<sup>59,60</sup>

### 2.2.5. Statistička obrada

Rezultati su izraženi kao srednje vrijednosti  $\pm$  standardna pogreška sredine ili s 95 % intervalima pouzdanosti. Vrijednost  $p < 0,05$  predstavlja granicu statističke značajnosti. Za statističku analizu vazodilatacijskih učinaka koristila se jednosmjerna (usporedba maksimalnih učinaka ( $E_{\max}$ )) i dvosmjerna (usporedba učinaka uz varijable doza i vrsta uzorka) analiza varijance (ANOVA) sa Bonferronijevim *post hoc* testom. Obzirom da su se ispitivani uzorci vina i derivata razlikovali prema polifenolnom i etanolnom sadržaju, koristili smo logaritam razrjeđenja, umjesto koncentracije, za izračunavanje  $EC_{50}$ , koncentracije koja izaziva 50% maksimalnog vazodilatacijskog učinka. Pri tome je korištena nelinearna regresijska analiza krivulja doza-učinak. Analize povezanosti učinaka i sastava ispitivanih uzoraka izvršene su linearnom regresijom, a analiza odnosa bioloških učinaka i strukture ispitivanih spojeva (fenolnih kiselina) provedena je jednostavnom i višestrukom linearnom regresijom. Statističke analize fizikalno-kemijskih svojstava i sastava uzoraka vina u studiji s termički obrađenim vinom, učinjene su primjenom Mann-Whitney  $U$  testa. Rezultati su analizirani primjenom programa GraphPad Prism (verzija 4.03; GraphPad Software, San Diego, SAD) i STATISTICA (verzija 6.0; Statsoft. Inc., Tulsa, SAD).



## 2.3. REZULTATI

### 2.3.1. Sažetak objedinjenih rezultata

Vazodilatacijski i antioksidacijski učinci pojedinačnih fenolnih kiselina međusobno su negativno povezani. Najbolji primjer ove negativne korelacije je galna kiselina, s najmanjim maksimalnim vazodilatacijskim učinkom i s najvećom antioksidacijskom aktivnosti. Najvažnije strukturne karakteristike vezane za vazodilatacijski učinak fenolnih kiselina su lipofilnost molekula, dok su za antioksidacijsku aktivnost najvažniji broj i raspored hidroksilnih skupina te stupanj grananja i zbijenosti molekule.

S druge strane, razmatramo li učinke vina kao složene otopine smjese polifenola, povezanost je vazodilatacijskog i antioksidacijskog učinka pozitivna i nadalje povezana sa sadržajem ukupnih polifenola. Tako su ispitivana bijela vina najsiromašnija polifenolima, najslabiji antioksidansi i najslabiji vazodilatatori. Fenolne kiseline su najzastupljeniji polifenolni spojevi bijelih vina. Vazodilatacijski i antioksidacijski učinci bijelih vina i pojedinih fenolnih kiselina su slični.

Crno je vino potentniji vazodilatator u usporedbi s kupinovim vinom. S druge strane, kupinovo je vino, unatoč manjem sadržaju ukupnih polifenola, bolji antioksidans *in vitro* od crnog vina. Moguće objašnjenje ovog fenomena je viša razina neflavonoidnih polifenola, primjerice fenolnih kiselina, u kupinovom vinu u odnosu na crno vino.

Važan rezultat opisanog istraživanja je povezanost vazodilatacijskog učinka s antocijanima, ali i taj da vazodilatacijska aktivnost nije povezana s koncentracijom pojedinih polifenolnih tvari, izmjerenih tijekom istraživanja (galna kiselina, katehin, epikatehin, epigalokatehin-galat, procijanidin B<sub>2</sub>, kvercetin-4-glukozid, rezveratrol, piceid, astringin).

Termički obrađeno vino pod temperaturnim uvjetima koji su primjenjivi u svakodnevnom životu prilikom pripremanja hrane, zadržava vazodilatacijsku učinkovitost unatoč značajnim promjenama u fizikalno-kemijskim svojstvima izazvanim zagrijavanjem. Vina zagrijavana na 75 °C i 125 °C, intaktno vino i dealkoholizirano vino pokazali su sličan vazodilatacijski o dozi ovisan učinak, i njihovi se maksimalni učinci nisu međusobno razlikovali. Ipak, u najnižim koncentracijama, do 1%, dealkoholizirano vino bez termičkog stresa koje je i najbogatije polifenolima, uzrokovalo je statistički značajno snažniju vazodilataciju, u usporedbi s intaktnim vinom i vinom zagrijavanjem na 75 i 125 °C. Ovaj rezultat se može objasniti

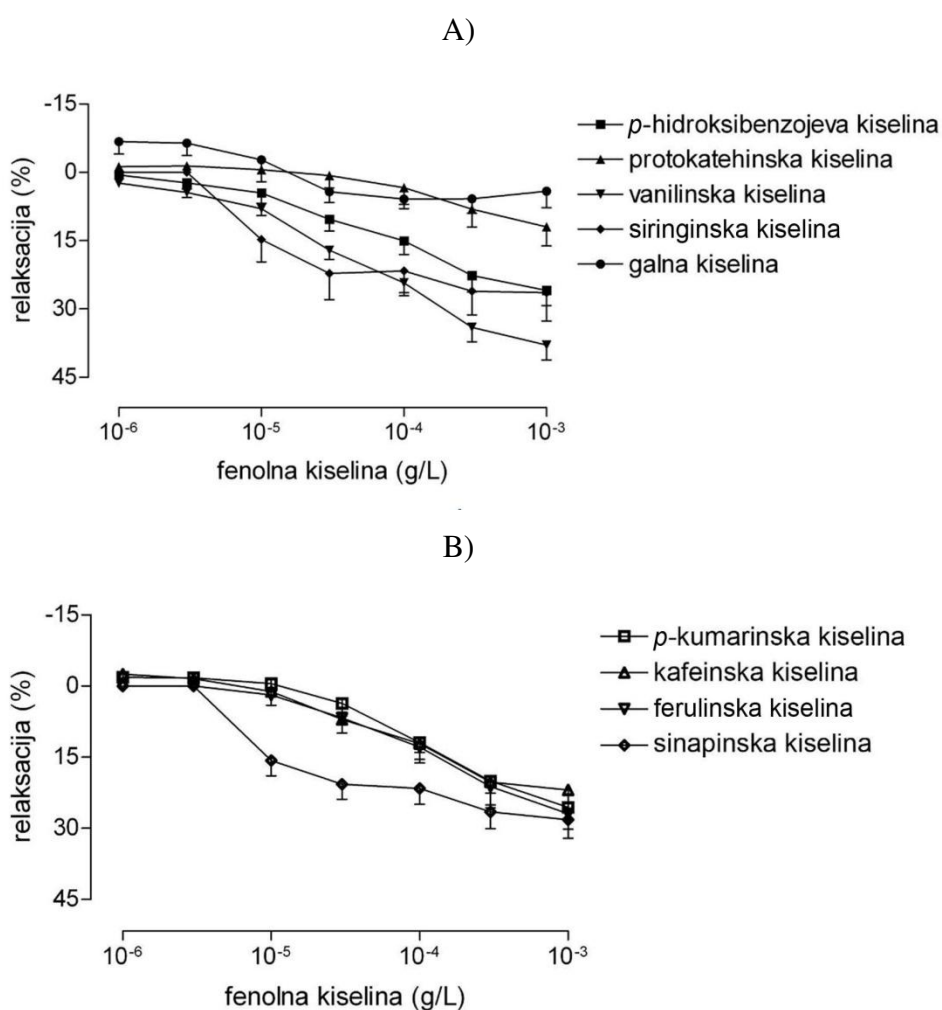
djelomičnom razgradnjom izazvanom zagrijavanjem i promjenama polifenola koji su, unatoč približno 10%-tnom smanjenju u ukupnoj koncentraciji i u koncentracijama pojedinačnih frakcija, ostvarili tek nešto slabiji vazodilatacijski učinak. Povećanjem doze vina, razlika u vazodilatacijskom odgovoru se izgubila. Sličnost intaktnog vina i vina zagrijavanog na 75 °C, s druge strane, ukazuje da termički stres pri ovoj temperaturi nije dovoljno snažan da bi kompromitirao vazodilatacijsku potentnost i učinkovitost vina, unatoč diskretnim promjenama fizikalno-kemijskih svojstava.

Konačno, u opisanom istraživanju nije se mogla nedvojbeno odrediti uloga rezveratrola u vazodilatacijskom odgovoru. Koncentracija je rezveratrola u ispitivanim intaktnim vinima, dealkoholiziranom i termički obrađenim vinima bila prilično niska, te je nakon razrjeđenja u organskim bazećima bila oko 10 puta manja od koncentracija koje su se koristile u studijama koje opisuju vazodilatacijski učinak rezveratrola.

### 2.3.2. Rezultati rada „Antioxidative and vasodilatory effects of phenolic acids in wine”

Ispitivane fenolne kiseline pokazale su različit vazodilatacijski učinak ovisan o dozi (Slika 4).

Najbolji QSAR model koji opisuje njihovu vazodilatacijsku aktivnost uključuje parametre topološka ploština polarne površine molekule (TPSA) i GETAWAY deskriptor  $R^+_3(u)$ . Nadalje, postoji značajna negativna korelacija između maksimalnog vazodilatacijskog učinka i broja hidroksilnih skupina u strukturi fenolnih kiselina ( $r = -0,893, p = 0,0011$ ).

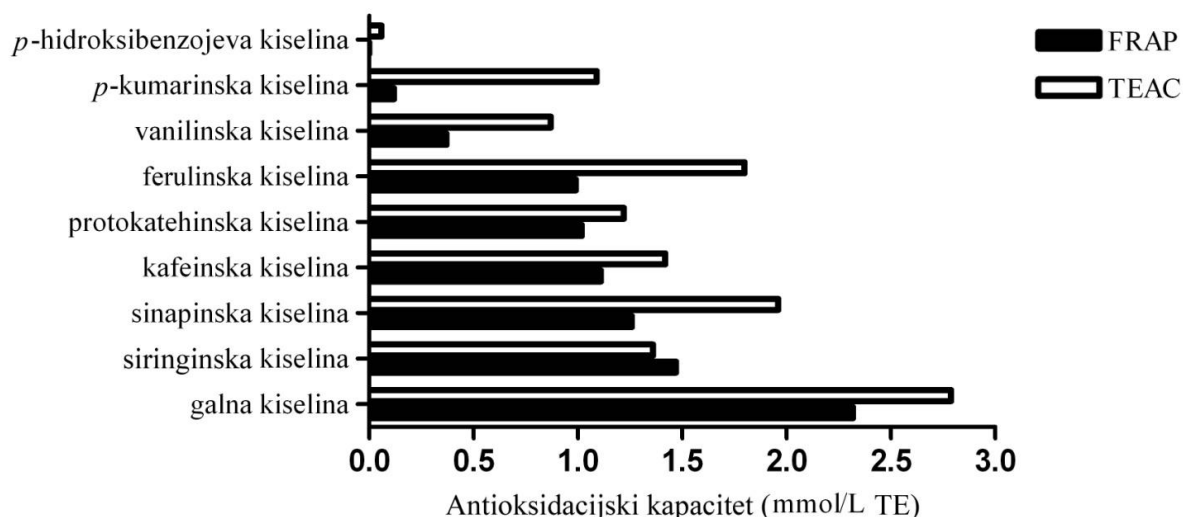


**Slika 4.** Krivulje doza-učinak (koncentracija-relaksacija) fenolnih kiselina na prstenovima aorte štakora ( $NA 10^{-7}$  mol/L), ( $n=12$  prstenova po kiselini)

A) derivati hidroksibenzojeve kiseline

B) derivati hidroksicimetne kiseline

Najbolji model za opisivanje antioksidacijske aktivnosti izmjerene FRAP metodom dobiven je primjenom dvaju deskriptora: topološke ploštine polarne površine molekule (TPSA) i srednje vrijednosti stupnja topološke udaljenosti vrhova grafa ( $\Delta D$ ). Najbolja povezanost antioksidacijske aktivnosti izmjerene TEAC metodom i strukture molekule je pokazana QSAR modelom koji uključuje dva GETAWAY deskriptora,  $R7p$  i  $HATS 3e$ , a koji su vezani uz polarizabilnost i elektronegativnost atoma.

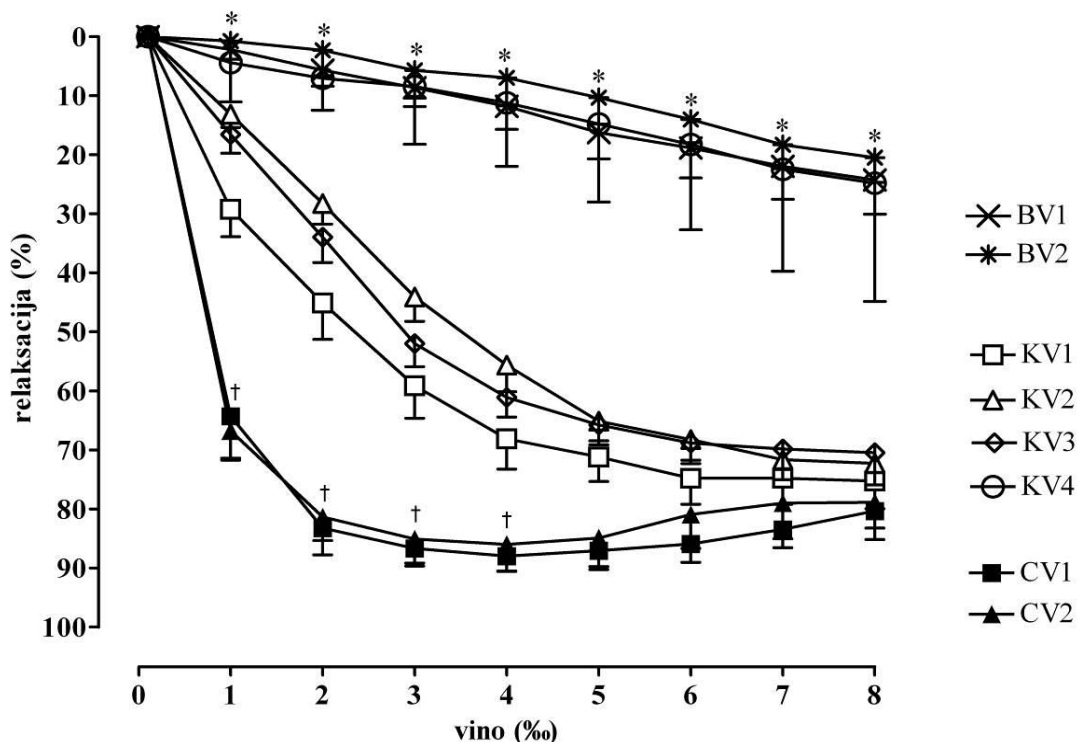


**Slika 5.** Antioksidacijska aktivnost fenolnih kiselina izmjerena FRAP i TEAC metodama

FRAP i TEAC metode za mjerenje antioksidacijske aktivnosti fenolnih kiselina međusobno su pozitivno korelirale ( $r = 0,8859$ ,  $p = 0,0015$ ). Suprotno tome, vazodilatacijski i antioksidacijski učinci fenolnih kiselina međusobno su negativno povezani ( $r = -0,7348$ ,  $p = 0,0241$ ).

### 2.3.3. Rezultati rada „*Antioxidant and vasodilatory effects of blackberry and grape wines*”

Iako su sva ispitivana vina (Tablica 1) izazvala relaksaciju štakorske aorte prekontrahirane noradrenalinom, njihove krivulje doza-učinak (razrjeđenje-relaksacija) su se međusobno razlikovale (Slika 6).



**Slika 6.** Krivulje doza-učinak (razrjeđenje-relaksacija) ispitivanih vina na prstenovima aorte štakora ( $NA 10^{-7}$  mol/L)

\* $p < 0,05$  vs. crnih vina (CV1 i CV2) i kupinovitih vina (KV1, KV2 i KV3); †  $p < 0,05$  vs. kupinovitih vina (KV1, KV2, KV3)

Općenito, crna vina su bila najpotentniji vazodilatatori ( $EC_{50} = -3,17$  i  $-3,24$  za crno vino 1 i crno vino 2). Kupinova vina 1, 2 i 3 pokazala su srednju vazodilatacijsku potentnost s  $EC_{50}$  od  $-2,85$ ,  $-2,65$  i  $-2,72$ , dok je kupinovo vino 4 bilo značajno manje potentan vazodilatator s  $EC_{50}$  od  $-2,31$ . Ipak, maksimalni vazodilatacijski učinak ( $E_{max}$ ) kupinovitih vina 1, 2 i 3 bio je sličan crnim vinima ( $E_{max} = 75,27 \pm 4,67\%$ ,  $72,27 \pm 3,65\%$  i  $70,48 \pm 3,40\%$  za kupinovo vino 1, 2 i 3 nasuprot  $87,99 \pm 2,50\%$  za crno vino 1 i  $86,00 \pm 4,56\%$  za crno vino 2).  $E_{max}$  bijelih vina i kupinovog vina 4 bio je značajno manji ( $24,28 \pm 5,79\%$ ,  $20,53 \pm 4,17\%$  i  $24,27 \pm 3,86\%$  za bijelo vino 1, 2 i kupinovo vino 4, ( $p < 0,05$ ). (Tablica 2)

**Tablica 2.** Vazodilatacijska aktivnost vina izražena maksimalnim vazodilatacijskim učinkom ( $E_{max}$ ) i koncentracijom 50% maksimalnog učinka ( $EC_{50}$ ).

Vino	$E_{max}$ (%)	$EC_{50}$ (CI)
Crno (CV1)	87,99 ± 2,50*	-3,17 (od -3,18 do -3,03) <sup>†</sup>
Crno (CV2)	86,00 ± 4,56*	-3,24 (od -3,47 do -3,01) <sup>†</sup>
Bijelo (BV1)	24,28 ± 5,79	-2,44 (od -2,55 do -2,32)
Bijelo (BV2)	20,53 ± 4,17	-2,34 (od -2,44 do -2,24)
Kupinovo (KV1)	75,27 ± 4,67*	-2,85 (od -2,93 do -2,77)*
Kupinovo (KV2)	72,27 ± 3,65*	-2,63 (od -2,64 do -2,59)*
Kupinovo (KV3)	70,48 ± 3,40*	-2,72 (od -2,77 do -2,67)*
Kupinovo (KV4)	24,27 ± 3,86	-2,31 (od -2,38 do -2,25)

$E_{max}$ , maksimalni vazodilatacijski učinak;  $EC_{50}$ , koncentracija 50% maksimalnog učinka (izračunata primjenom nelinearne regresijske analize).  $EC_{50}$  vrijednosti su logaritamske vrijednosti koncentracija (razrjeđenja) koje ostvaruju 50% maksimalnog vazodilatacijskog učinka, (1‰ = 0,001 => log 0,001 = -3, 2‰ = 0,002 => log 0,002 = -2,70);

CI, 95%-tni interval pouzdanosti.

\* $p < 0,05$  vs. bijelih vina (1 i 2) i kupinovog vina 4;

<sup>†</sup> $p < 0,05$  vs. kupinovitih vina (1, 2, 3 i 4) i bijelih vina (1 i 2)

Kao što je pokazano u Tablici 3, ukupni fenolni sadržaj bio je najveći u crnim vinima, slijede kupinova vina, dok je najniži bio u bijelim vinima.

U odnosu na crna vina, kupinova vina su bila siromašnija u flavonoidnom sadržaju, ali nekoliko puta bogatija u neflavonoidnim polifenolima. Antocijani su bili prisutni samo u crnim i kupinovim vinima. Njihova je koncentracija bila viša u crnim vinima (212±9 i 287±3 mg/L ekvivalenata malvidin-3-glukozida) u odnosu na kupinova vina, gdje se koncentracija antocijana kretala u rasponu od 13 do 164 mg/L ekvivalenata malvidin-3-glukozida (Tablica 3).

**Tablica 3.** Ukupni sadržaj fenola, glavne fenolne frakcije i antioksidacijska aktivnost vina.

Vino	Ukupni fenoli mg GAE/L	Flavonoidi mg GAE/L	Ne-flavonoidi mg GAE/L	Antocijani mg M-3-GE/L	FRAP mmol TE/L
CV1	3313±27	3002±22	311±4	212±9	12,6±0,6
CV2	3225±26	2902±21	323±7	287±3	12,7±0,2
BV1	482±3	103±2	379±5	0	1,9±0,1
BV2	379±3	58±3	321±5	0	1,2±0,1
KV1	2628±29	1417±14	1210±11	135±3	13,9±0,7
KV2	2025±23	951±11	1074±10	148±2	10,8±0,4
KV3	2789±27	1303±14	1486±12	164±3	15,8±0,6
KV4	1697±20	924±11	773±8	13±1	7,8±0,4

GAE (engl. *gallic acid equivalent*) – ekvivalent galne kiseline; M-3-GE (engl. *malvidine-3-glucoside equivalent*) – ekvivalent malvidin-3-glukozida; FRAP (engl. *Ferric reducing antioxidant capacity*) – antioksidacijski kapacitet redukcije željeza; TE (engl. *Trolox equivalent*) – ekvivalent Troloksa; CV – crno vino iz grožđa; BV – bijelo vino iz grožđa; KV – kupinovo vino. Podaci su dobiveni iz najmanje triju nezavisnih uzoraka i prikazani su kao srednja vrijednost ± SEM.

Najzastupljeniji monomer flavanol u vinima iz grožđa je bio katehin. U kupinovim vinima sadržaj flavanola je varirao više nego u vinima iz grožđa. Najbolji primjer je epigalokatehin galat koji se kretao u rasponu od 3,5 do 148,8 mg/L u kupinovim vinima. Procijanidin B<sub>2</sub>, dimer epikatehina također se kretao u širokom koncentracijskom rasponu (od 6,1 do 77,1 mg/L) u kupinovim vinima; tako su kupinova vina 1 i 2 bila bogatija procijanidinom B<sub>2</sub> u odnosu na crna vina.

Među ne-flavonoidima, stilbeni su bili izmjereni u svim vinima. Monomeri rezveratrola su bili zastupljeni u malim koncentracijama (od 0 do 2,2 mg/L), ali njihovi derivativi, (*E*)-piceid i astringin su pronađeni u višim koncentracijama, bez obzira na vrstu vina. Ne-flavonoid galna kiselina također je bila prisutna u svim vinima, ali je njena koncentracija bila dva do tri puta viša u kupinovim vinima u odnosu na vina iz grožđa. Identificirani polifenolni spojevi i njihove koncentracije navedeni su u Tablici 4.

**Tablica 4.** Koncentracije (mg/L) analiziranih fenolnih spojeva.

Vino	Galna kiselina	(+)-Katehin	(-)-Epikatehin	Epigalo-katehin galat	Procijanidin B2	Kvercetin-4-glukozid	<i>cis</i> -Rezveratrol	<i>trans</i> -Rezveratrol	( <i>E</i> )-Piceid	Astringin
CV1	26,4±0,1	13,5±0,4	7,8±1,4	12,7±0,5	12,3±1,6	1,4±0,1	0,6±0,1	1,1±0,1	1,5±0,2	5,2±0,1
CV2	14,9±0,1	15,8±0,9	4,9±0,3	1,6±0,3	10,0±1,3	0,4±0,1	0,6±0,1	1±0,1	1,7±0,1	n.p.
BV1	21,8±0,1	21,7±0,2	1,5±0,3	9,8±0,1	4,4±0,3	n.p.	0,5±0,1	1,1±0,1	2,3±0,1	2,4±0,2
BV2	3,3±0,2	18,4±0,9	0,4±0,01	17,8±0,6	n.p.	n.p.	0,1±0	0,2±0	0,8±0,1	2,3±0,2
KV1	59,0±1,4	18,7±1,5	34,7±3,7	148,8±0,8	77,1±3,3	2,6±0,1	1,5±0,1	0,7±0,1	5,2±0,5	7,9±0,8
KV2	45,4±0,3	16,8±1,4	7,9±1,7	16,8±2,2	34,5±4,3	2,4±0,2	n.p.	0,5±0,1	2,6±0,1	n.p.
KV3	50,1±0,3	45,2±2,9	n.p.	n.p.	6,1±0,2	2,1±0,1	0,2±0,1	0,7±0,1	6,5±0,4	12,1±0,8
KV4	53,1±3,2	9,1±0,9	12,2±1,7	3,5±0	8,8±0,4	n.p.	0,1±0	0,9±0,1	2,0±0,4	n.p.

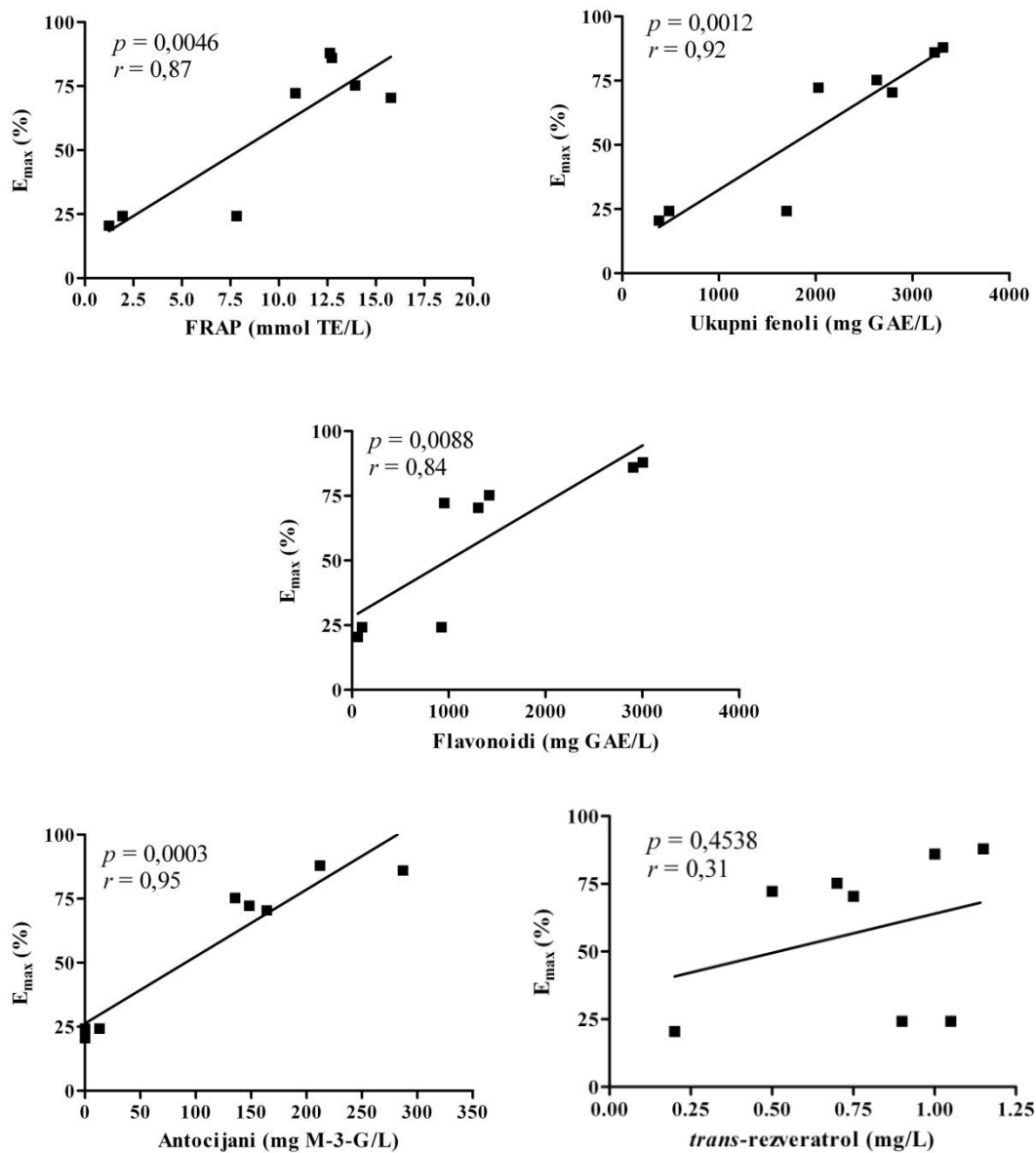
CV – crno vino iz grožđa; BV – bijelo vino iz grožđa; KV – kupinovo vino.

n.p. = nije potvrđen

Podaci su dobiveni iz najmanje dvaju nezavisnih uzoraka i prikazani su kao srednja vrijednost ± SEM.

Vazodilatacijska aktivnost je snažno korelirala s ukupnim fenolnim sadržajem vina ( $r = 0,92$ ,  $p = 0,0012$ ). Među fenolnim frakcijama snažna pozitivna korelacija pronađena je za sadržaj antocijana ( $r = 0,95$ ,  $p = 0,0003$ ), i flavonoidni sadržaj ( $r = 0,84$ ,  $p = 0,0088$ ). Nije pronađena korelacija između koncentracije pojedinačnih polifenolnih spojeva i vazodilatacijske aktivnosti. Korelacija između  $E_{\max}$  i antioksidacijske aktivnosti te sadržaja ukupnih fenola, flavonoida, antocijana i *trans*-rezveratrola prikazana je na Slici 7.





**Slika 7.** Povezanost vazodilatacijske aktivnosti i antioksidacijske aktivnosti te sadržaja ukupnih fenola, flavonoida, antocijana i *trans*-rezveratrola ispitivanih uzoraka vina.

$E_{max}$  je maksimalni vazodilatacijski učinak. Antioksidacijska aktivnost mjerena je FRAP (engl. *Ferric reducing antioxidant power*) metodom i izražena u mmol/L ekvivalenata troloksa (TE). Sadržaj ukupnih fenola i flavonoida izražen je u mg/L ekvivalenata galne kiseline (GAE, engl. *gallic acid equivalent*), a antocijana u mg/L ekvivalenata malvidin-3-glukozida (M-3-GE)

#### 2.3.4. Rezultati rada „Thermally treated wine retains vasodilatory activity in rat and guinea pig aorta”

Zagrijavanje na 125 °C i vakuum-dealkoholizacija vina rezultirala je vrlo sličnim smanjenjem volumena i sadržaja etanola. Koncentracija ukupnih fenola očekivano se povećala sa smanjenjem volumena uzorka u oba slučaja. Ipak, u vinu termički obrađenom na 125 °C, došlo je do prosječno 10% smanjenja koncentracije polifenola u pojedinačnim fenolnim frakcijama u odnosu na dealkoholizirano vino, što upućuje na zagrijavanjem uzrokovanu degradaciju polifenola u odgovarajućim frakcijama. Opisane promjene nisu opažene u vinu zagrijavanom na 75 °C. Antioksidacijski kapacitet uzoraka povećao se u termički obrađenim uzorcima i u dealkoholiziranom vinu sukladno promjenama u koncentraciji ukupnih fenola (Tablica 5). Koncentracija *trans*-rezveratrola bila je najviša u dealkoholiziranom vinu, a najniža u vinu termički obrađenom na 125 °C, što upućuje na termičku osjetljivost rezveratrola.

**Tablica 5.** Učinci termičke obrade i dealkoholizacije na volumen, sadržaj etanola, ukupnih fenola, pojedinačne fenolne frakcije, sadržaj *trans*-rezveratrola i antioksidacijski kapacitet uzoraka vina

	Intaktno vino	Vino zagrijavano na 75 °C	Vino zagrijavano na 125 °C	Dealkoholizirano vino
Volumen nakon 45 min.	/	140±1*	88±1*	85±3*
Etanol (vol %)	12,9±0,1	10,4±0,4*	0,7±0,3*	0,6±0,2*
Ukupni fenoli (mg GAE/L)	3200±38	3390±42	5530±61*	5690±51*
Flavonoidi (mg GAE/L)	2940±32	3110±24	5200±29*	5280±42*
Ne-flavonoidi (mg GAE/L)	263±3	278±3	334±3*	413±4*
Antocijani (mg M-3-GE/L)	240±9	247±7	338±9*	398±7*
<i>Trans</i> -rezveratrol (mg/L)	2,0±0,1	1,9±0,2	1,0±0,1*	2,9±0,1*
FRAP (mmol TE/L)	17±1	18±1	24±1*	27±1*

Prije obrade početni volumen svih uzoraka bio je 150 mL.

Podaci su dobiveni iz najmanje triju nezavisnih uzoraka i prikazani su kao srednja vrijednost ± SEM.

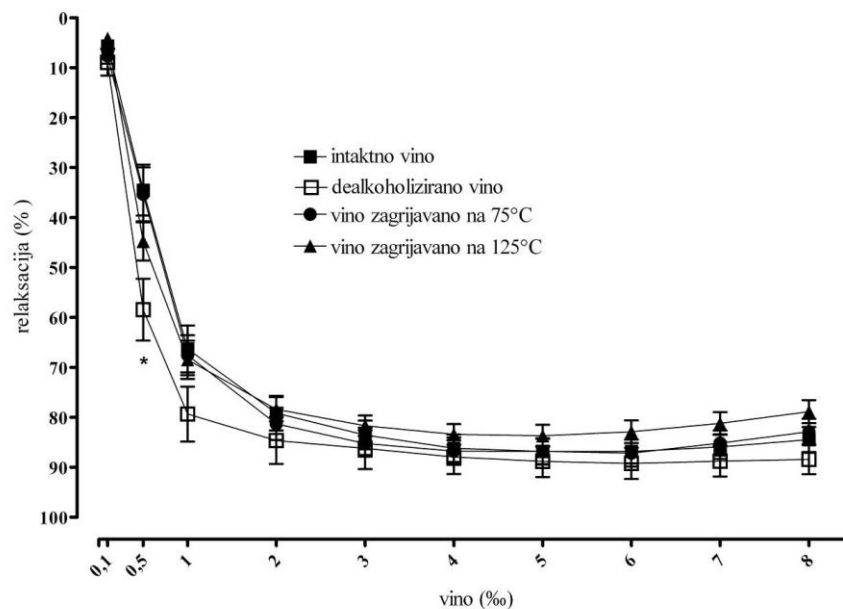
\* $p < 0,05$  vs. intaktno vino.

GAE (engl. *gallic acid equivalent*) - ekvivalent galne kiseline; M-3-GE (engl. *malvidin-3-glucoside equivalent*) – ekvivalent malvidin-3-glukozida; FRAP (engl. *Ferric reducing antioxidant power*) – antioksidacijski kapacitet redukcije željeza; TE (engl. *trolox equivalent*) - ekvivalent troloksa.

Kontraktilni odgovor izazvan noradrenalinom (NA) izoliranih aortnih prstenova štakora i zamorčića nije se značajno razlikovao ( $16,71 \pm 0,85$  mN za aortu štakora i  $16,65 \pm 0,76$  mN za aortu zamorčića). Suprotno tome, kontrolna vazodilatacija prekontrahiranih prstenova izazvana acetilkolinom (Ach) bila je značajno veća kod prstenova izoliranih iz štakora ( $77,73 \pm 3,22\%$ ) u odnosu na zamorčića ( $41,81 \pm 2,53\%$ ).

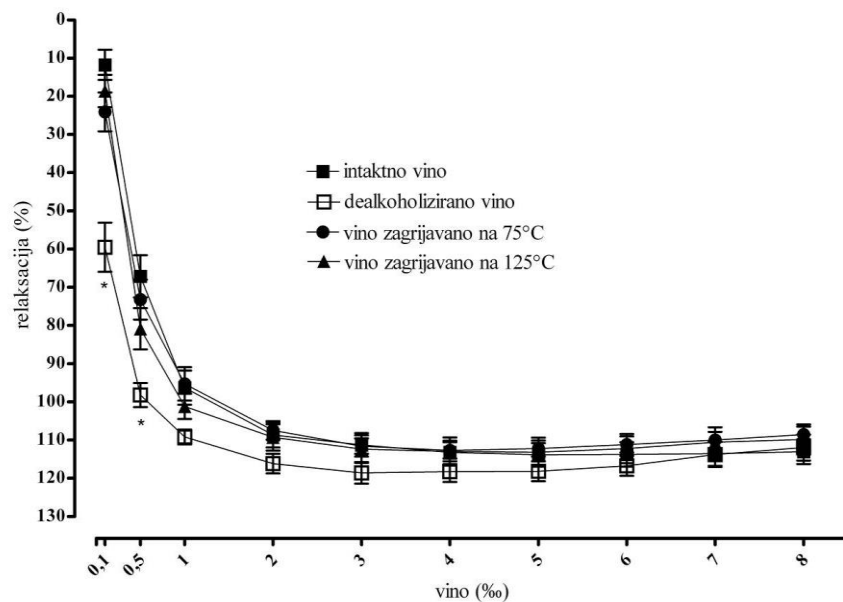
Vazodilatacijska aktivnost intaktnog vina bila je snažnija kod prstenova aorte zamorčića u odnosu na aortu štakora. Maksimalni vazodilatacijski učinak ( $E_{\max}$ ) aorte štakora i zamorčića bili su  $86,84 \pm 2,53\%$  i  $113,87 \pm 3,06\%$  (Slike 8 i 9). Općenito, termička obrada intaktnog vina nije značajno utjecala na ukupnu izravnu vazodilatacijsku aktivnost niti u aorti zamorčića niti štakora. Vino zagrijavano na  $75$  °C uzrokovalo je  $E_{\max}$  od  $87,16 \pm 2,70\%$  u aorti štakora i  $112,72 \pm 2,14\%$  u aorti zamorčića, dok je vino zagrijavano na  $125$  °C uzrokovalo  $E_{\max}$  od  $83,70 \pm 2,19\%$  u aorti štakora i  $113,17 \pm 3,78$  u aorti zamorčića.

Konačno, dealkoholizirano vino ostvarilo je najsnažniji, iako statistički neznačajan, maksimalni vazodilatacijski odgovor u usporedbi s intaktnim i termički obrađenim vinima postižući  $E_{\max}$  od  $89,22 \pm 3,11\%$  i  $118,58 \pm 2,83\%$  u aorti štakora i zamorčića. Ipak, u najnižim koncentracijama (do  $1\%$ ), dealkoholizirano vino je izazvalo statistički značajno veću relaksaciju u aortama obje životinjske vrste kao što je prikazano na Slikama 8 i 9.



**Slika 8.** Relaksacija aortnih prstenova štakora prekontrahiranih noradrenalinom nakon izlaganja intaktnom, dealkoholiziranom vinu te vinima zagrijavanim na 75 i 125 °C

Rezultati su prikazani kao srednje vrijednosti ± SEM. \* $p < 0,05$  za dealkoholizirano vino vs. intaktno vino i vina zagrijavana na 75 i 125 °C (n = 21 prsten po uzorku).



**Slika 9.** Relaksacija aortnih prstenova zamorčića prekontrahiranih noradrenalinom nakon izlaganja intaktnom, dealkoholiziranom vinu te vinima zagrijavanim na 75 i 125 °C.

Rezultati su prikazani kao srednje vrijednosti ± SEM. \* $p < 0,05$  za dealkoholizirano vino vs. intaktno vino i vina zagrijavana na 75 i 125 °C (n = 23 prstena po uzorku)

Sličnosti u ukupnoj vazodilatacijskoj aktivnosti svih ispitivanih vina i vinskih derivata također je potvrđena njihovom sličnom vazodilatacijskom potentnosti, što je očito iz pripadajućih EC<sub>50</sub> vrijednosti (Tablica 6).

**Tablica 6.** Učinci termičke obrade i dealkoholizacije na EC<sub>50</sub> ispitivanih vina.

	EC <sub>50</sub> (CI)	
	Aorta štakora	Aorta zamorčića
Intaktno vino	-3,19 (od -3,24 do -3,14)	-3.33 (od -3,38 do -3,27)
Dealkoholizirano vino	-3,38 (od -3,47 do -3,30)	-3.41 (od -3,51 do -3,30)
Vino zagrijavano na 75°C	-3,19 (od -3,24 do -3,14)	-3.33 (od -3,39 do -3,27)
Vino zagrijavano na 125°C	-3,30 (od -3,35 do -3,25)	-3.41 (od -3,49 do -3,33)

EC<sub>50</sub>, koncentracija 50% maksimalnog učinka (izračunata primjenom nelinearne regresijske analize). EC<sub>50</sub> vrijednosti su logaritamske vrijednosti koncentracija (razrjeđenja) 50% maksimalnog vazodilatacijskog učinka, (1 ‰ = 0,001 => log 0,001 = -3,2‰ = 0,002 => log 0,002 = -2,70); n = 21 prsten za uzorak vina na aorti štakora; n = 23 prstena za uzorak vina na aorti zamorčića. CI, 95%-tni interval pouzdanosti.

Poredak vazodilatacijske potentnosti vina uzimajući u obzir EC<sub>50</sub> za štakorsku aortu je bio: dealkoholizirano vino > vino zagrijavano na 125 °C > intaktno vino = vino zagrijavano na 75 °C. Na aortnim prstenovima zamorčića, oba termički obrađena vina bila su jednako potentni vazodilatatori kao njihove kontrole bez termičkoga stresa: dealkoholizirano vino = vino zagrijavano na 125 °C > intaktno vino = vino zagrijavano na 75 °C.

## 2.4. RASPRAVA

### 2.4.1. Vazodilatacijski i antioksidacijski učinci fenolnih kiselina

Ključni rezultat ovog dijela studije je negativna korelacija *in vitro* antioksidacijske i vazodilatacijske aktivnosti ispitivanih fenolnih kiselina. Ovo je najbolje prikazano slabom vazodilatacijskom aktivnosti galne kiseline, najjačeg antioksidansa među ispitivanim fenolnim kiselinama. Općenito, fenolne su kiseline slabiji vazodilatatori nego antioksidansi. QSAR modeli za predviđanje antioksidacijske aktivnosti (TEAC i FRAP) ukazuju da fenolne kiseline s više hidroksilnih skupina imaju veću antioksidacijsku aktivnost. Sukladno tome, galna je kiselina s tri hidroksilne skupine imala najveći FRAP kapacitet (2,32 mmol/L TE), protokatehinska kiselina s dvije hidroksilne skupine imala je FRAP kapacitet 1,02 mmol/L TE, dok *p*-hidroksibenzojeva kiselina, sa samo jednom hidroksilnom skupinom, uopće nije pokazala aktivnost. Ovi rezultati su konzistentni s rezultatima Sroke i Cisowskog,<sup>61</sup> koji su pokazali pozitivnu korelaciju između broja hidroksilnih skupina vezanih za aromatski prsten fenolnih kiselina i sposobnosti „gašenja” slobodnih radikala.

Stupanj grananja i oblik molekule također prilično utječu na antioksidacijsku aktivnost. Kompaktnije i manje razgranate molekule imaju niži antioksidacijski kapacitet.<sup>62</sup> Ovo je potvrđeno i dobivenim rezultatom da su derivati hidroksicimetne kiseline, čije su molekule dulje i razgranatije, ujedno i učinkovitiji antioksidansi u odnosu na male i manje razgranate derivate hidroksibenzojeve kiseline. Primjerice, unatoč istim supstitucijskim skupinama na aromatskom prstenu, sinapinska (3,5-dimetoksi-4-hidroksicimetna) kiselina ima veći TEAC kapacitet (1,96 mmol/L TE) nego siringinska (3,5-dimetoksi-4-hidroksibenzojeva), kiselina (TEAC = 1,36 mmol/L TE).

Trodimenzijska raspodjela polarizabilnosti atoma (deskriptor *R7p*) također ima utjecaj na antioksidacijsku aktivnost fenolnih kiselina. Ovim se objašnjava zašto zamjena 3- i 5-hidroksi skupina s metoksi skupinama u siringinskoj kiselini rezultira smanjenom antioksidacijskom aktivnosti (FRAP 1,47 i TEAC 1,36 mmol/L TE) u usporedbi s galnom kiselinom s tri hidroksilne skupine (FRAP 2,32 i TEAC 2,79 mmol/L TE).

Opisana studija po prvi put prikazuje QSAR analizu vazodilatacijske aktivnosti fenolnih kiselina. Osim negativne povezanosti maksimalnog vazodilatacijskog učinka i broja hidroksilnih skupina,

najvažnije varijable u modelima za predviđanje vazodilatacijske aktivnosti fenolnih kiselina vezane su za lipofilnost i distribuciju lokalnog atoma.

Rezultati studije o vazodilatacijskim učincima fenolnih kiselina *in vitro* ne dozvoljavaju zaključke o njihovim mogućim učincima *in vivo*. Najveći vazodilatacijski učinak *in vitro* uvijek je postignut pri njihovim najvišim koncentracijama. Te koncentracije nisu ostvarive u uvjetima *in vivo* nakon konzumacije vina ili neke druge hrane bogate polifenolima.

Kako bi ovu studiju učinili usporedivom s rezultatima sličnih studija *in vitro*, a ujedno koristili koncentracije spojive s uvjetima *in vivo*, koncentracije ispitivanih fenolnih kiselina u ovoj studiji odabrane su sukladno studijama Andriambelosona<sup>16</sup> i Caccette.<sup>63</sup> Andriambelosen je koristio galnu, vanilinsku, kumarinsku i kafeinsku kiselinu u koncentracijskom rasponu od  $10^{-5}$  do  $3 \times 10^{-1}$  g/L, što približno odgovara 50 nmol/L do 2 mmol/L.<sup>16</sup> Primjenom 10 puta nižih koncentracija, ostvarili smo koncentracije koje se postižu u ljudskoj plazmi nakon konzumacije vina (176 nmol/L za 4-O-metilirani oblik galne kiseline i 84 nmol/L za kafeinsku kiselinu).<sup>63</sup>

U ovoj su studiji samo siringinska i sinapinska kiselina, pri usporedivim koncentracijama pokazale vazodilatacijski učinak. Ipak, zanemarivi vazodilatacijski učinak *in vitro* ostalih fenolnih kiselina pri ovim koncentracijama nužno ne odražava njihovu neučinkovitost *in vivo*. Naime, u biološkim učincima vina pojedinačni polifenolni spoj nikako ne može imati prediktivnu ili najvažniju ulogu. Primjerice, primjena katehina *in vitro* nije izazvala vazodilataciju štakorske aorte.<sup>10</sup> Također se promjena endotelne funkcije kod ljudi nakon konzumacije vina nije mogla predvidjeti na temelju plazmatske koncentracije katehina.<sup>6</sup> Suprotno tome, maksimalna indukcija endotelne NO sintetaze (eNOS) na ljudskim endotelnim stanicama postignuta je sinergističkim učinkom niza različitih fenola iz vina, uključujući i neke fenolne kiseline.<sup>64</sup> Sličan sinergistički učinak potvrđen je također i za antioksidacijsku aktivnost fenola iz vina.<sup>65</sup>

Nakon konzumacije vina ili druge hrane bogate fenolima, teško je predvidjeti koncentracije fenolnih kiselina u plazmi. Prisutne su u vinu u različitim koncentracijama i različitim konjugiranim oblicima. Pojavljuju se u esterificiranim oblicima s organskim kiselinama, šećerima ili lipidima, što može utjecati na njihov metabolizam i bioraspoloživost. *In vivo* fenolne kiseline podliježu konjugacijskim reakcijama sa sulfatom, glukuronatom, S-adenozil-metioninom ili njihovom kombinacijom, a stvaranje konjugiranih oblika može značajno utjecati na biološku aktivnost početnih tvari.<sup>35</sup> Osim toga, konačna je koncentracija fenolnih kiselina u plazmi rezultat složenih metaboličkih puteva ne samo fenolnih kiselina, nego i drugih polifenola (katehin,

kvercetin, miricetin, procijanidini, antocijani).<sup>66-68</sup> Naime, polifenoli koji se ne apsorbiraju u tankom crijevu dolaze do debeloga crijeva gdje crijevna mikroflora hidrolizira glikozide i oslobađa aglikone te metabolizira oslobođene aglikone u različite aromatske kiseline. One se dalje metaboliziraju u derivate benzojeve kiseline, koji se mogu apsorbirati u krv.<sup>35</sup>

#### 2.4.2. Vazodilatacijski i antioksidacijski učinci kupinovog vina u usporedbi s bijelim i crnim vinima iz grožđa

Rezultati ove studije pokazuju da su kupinova vina u uvjetima *in vitro* relativno učinkoviti izravni vazodilatatori i još bolji antioksidansi. Rezultat da su neka kupinova vina (kupinovo vino 1 i 3) učinkovitiji antioksidansi nego crna vina iz grožđa potvrda je prethodno objavljenih rezultata istraživanja Pinhera i Paliyatha.<sup>49</sup> Međutim, ovo je prilično neočekivani rezultat ako se uzme u obzir niži sadržaj ukupnih fenola kupinovitih vina u usporedbi s crnim vinima. Antioksidacijski kapacitet različitih napitaka *in vitro*, određivan FRAP metodom, snažno je povezan sa sadržajem ukupnih fenola.<sup>69-71</sup> Moguće objašnjenje ovog neočekivanog rezultata može biti povezano s visokim sadržajem ne-flavonoida (osobito galne kiseline) u kupinovitim vinima u odnosu na crna vina. U studiji o antioksidacijskim i vazodilatacijskim učincima fenolnih kiselina pokazano je da je galna kiselina najpotentniji antioksidans *in vitro*, ali najslabije učinkovit izravni vazodilatator.

Drugi je važan rezultat ove studije povezanost vazodilatacijske aktivnosti ispitivanih vina sa sadržajem flavonoida i ukupnih fenola, a osobito, najznačajnija povezanost sa sadržajem antocijana. Međutim, vazodilatacijska aktivnost nije povezana s koncentracijom niti jedne od pojedinih polifenolnih tvari izmjerenih tijekom istraživanja (galna kiselina, katehin, epikatehin, epigalokatehin galat, procijanidin B<sub>2</sub>, kvercetin-4-glukozid, *trans*-rezveratrol, (*E*)-piceid, astringin).

Povezanost vazodilatacijske aktivnosti i sadržaja antocijana crnih vina već su pokazali Burns i suradnici, koji su ispitali 16 različitih crnih vina na aortnim vaskularnim prstenovima kunića.<sup>34</sup> Ipak, ostalo je nerazjašnjeno zašto su upravo antocijani tako snažno povezani s vaskularnim odgovorom. Rezultati studija *in vitro* s pojedinačnim antocijanidinskim spojevima su bili kontradiktorni, jer je samo delfinidin (ali ni malvidin niti cijanidin) uzrokovao relaksaciju štakorskih aortnih prstenova ovisnu o endotelu.<sup>16</sup> Studija Nakamure i suradnika pokazala je da



četiri pojedinačne antocijanske tvari, primijenjene same ili u kombinaciji, ne ostvaruju vazodilatacijski učinak na štakorskoj aorti.<sup>72</sup> Slično je i Serraino pokazala da cijanidin 3-O-glukozid nije utjecao na kontraktilni niti na vazodilatacijski odgovor aortnih prstenova ovisan o endotelu pod bazalnim uvjetima, iako je osigurao zaštitu protiv vaskularne disfunkcije posredovane peroksinitritom.<sup>73</sup> Izostanak izravnog vazodilatacijskog učinka antocijana ili antocijanidina *in vitro* ne odražavaju nužno njihovu neučinkovitost u uvjetima *in vivo* gdje se oni konzumiraju kao dio složenih mješanih otopina, poput vina. Koncentracije antocijana u našoj studiji su bile nanomolarne (primjerice 1‰ kupinovog vina 3 odgovara koncentraciji od 319 nmol/L ekvivalenta malvidin-3-glukozida u organskom bazenčiću), što se podudara s gornjim granicama njihove koncentracije u plazmi nakon konzumacije antocijanima bogate hrane ili pića.<sup>74</sup> Osim toga, nedavne publikacije pokazuju da antocijani podliježu opsežnom matabolizmu nakon što se unesu u tijelo i da njihovi metaboliti iz tankog crijeva i jetrenih stanica, kao i niskomolekularni katabolički produkti mikroflore kolona (među njima su i fenolne kiseline) putuju tijelom u cirkulacijskom sustavu i mogu biti odgovorni za određene biološke učinke koji se pripisuju antocijanima.<sup>75,66</sup> Ovi bi metaboliti mogli biti most između opaženih bioloških učinaka antocijana *in vitro* i *in vivo*.

Važnost antocijana u vazodilatacijskoj aktivnosti kupinovog vina najbolje je pokazana na primjeru kupinovog vina 4. Djelovalo je kao slabi izravni vazodilatator slično kao i bijela vina s  $E_{max}$  od  $24,27 \pm 3,86\%$  i  $24,28 \pm 5,79\%$  za kupinovo vino 4 i bijelo vino 2, iako je fenolni sadržaj bio 3,5 puta viši od bijelih vina (Tablica 3), ali je antocijanski sadržaj bio 10 puta niži od ostalih kupinovitih vina (Tablica 3). Brojne su studije nastojale identificirati polifenolne spojeve odgovorne za vazodilatacijski učinak crnog vina. Među vinskim fenolima posebno je istaknut *trans*-rezveratrol, kao tvar s brojnim korisnim kardiovaskularnim učincima, uključujući i vazodilatacijsku aktivnost.<sup>76</sup> Vazodilatacijski učinci u ovoj studiji ne mogu se povezati sa sadržajem rezveratrola u vinskim uzorcima koji su ispitivani. Međutim, treba naglasiti da je sadržaj rezveratrola u ispitivanim vinima bio prilično nizak (do 2,2 mg/l), i da je nakon razrjeđenja u organskim bazenčićima njegova koncentracija bila tisuću puta niža nego u studijama koje opisuju dilatacijske učinke rezveratrola *in vitro*.<sup>43</sup>

Procijanidini su također potentni *in vitro* vazodilatatori<sup>77</sup> te istaknuti kao tvari odgovorne za vazodilatacijski učinak vina.<sup>33</sup> Iako je koncentracija procijanidina B<sub>2</sub> u uzorcima i nakon razrjeđenja bila unutar raspona praga vazodilatacijskog odgovora (od 0,4-5µg/L)<sup>8</sup>, nije pronađena

značajna korelacija između koncentracije procijanidina B<sub>2</sub> i E<sub>max</sub> ( $r = 0.41$ ,  $p = 0.31$ ). Uzevši sve u obzir, dobiveni rezultati potvrđuju da se vazodilatacijski učinak složenih otopina, kao što je vino, ne može isključivo pripisati pojedinačnom polifenolnom spoju. Dapače, biološki je učinak rezultat sinergističkog djelovanja različitih polifenola, kao što je pokazano i za druge biološke učinke u različitim eksperimentalnim uvjetima.<sup>78,64</sup>

### 2.4.3. Vazodilatacijski učinci termički obrađenog vina u usporedbi s intaktnim i dealkoholiziranim vinom

Najvažniji rezultat ove studije je da termički obrađeno vino, pod temperaturnim uvjetima koji su primjenjivi u svakodnevnom životu prilikom pripremanja hrane, zadržava vazodilatacijsku učinkovitost. Ovo je prva studija koja ispituje biološke učinke termički obrađenog vina na kardiovaskularni sustav i, osim nedavne studije o antibakterijskim učincima termički obrađenih vina,<sup>79</sup> nudi novi pristup istraživanjima bioloških učinaka vina.

Donekle je iznenađujuće da termički obrađena vina i njihovi potencijalni biološki učinci nisu privukli veću znanstvenu pažnju unatoč uobičajenoj praksi u svakodnevnom životu gdje se termički obrađena vina konzumiraju kao topli napitak (kuhano vino) ili koriste prilikom pripremanja jela.

Fizikalno-kemijski sastav se, ovisno o uvjetima zagrijavanja vina, značajno mijenja. Najočitiije su i najlakše mjerljive promjene bile smanjenje volumena i sadržaja etanola. Kako su vinski fenoli glavni sastojci vina odgovorni za njegovu *in vitro* antioksidacijsku aktivnost,<sup>34,80,81</sup> porast u koncentraciji ukupnih fenola, nastao kao posljedica smanjenja volumena, pratio je odgovarajući porast antioksidacijske aktivnosti uzoraka (Tablica 5).

Mnogo suptilnije i složenije promjene uzrokovane toplinom mogu se pojaviti unutar samih vinskih polifenola, što je pokazano promjenama koncentracija pojedinačnih fenolnih frakcija i rezveratrola termički obrađenih uzoraka (Tablica 5). Međutim, studije o kinetici termičke degradacije različitih fenolnih spojeva s odgovarajućim dinamičkim promjenama u njihovoj antioksidacijskoj aktivnosti ukazuju da su toplinom uzrokovane interakcije polifenola i putevi njihove degradacije slabo objašnjeni i teško predvidivi, čak i u jednostavnim smjesama fenola.<sup>51,52,82,83</sup> Također, važno je razlikovati akutne od dugoročnih učinaka zagrijavanja vina. Ako je vino iznenada slučajno izloženo visokim temperaturama tijekom nekoliko sati (obično

tijekom distribucije, negdje unutar distribucijskog lanca), može postati pokvareno ili „skuhano”. Kao posljedica oksidacije i ostalih neželjenih reakcija koje dramatično rastu pri višim temperaturama dolazi do preuranjenog tamnjenja i stvaranja neželjenih okusa. S druge strane, kontrolirano zagrijavanje je standardna enološka metoda kojom se tretiraju vina radi uništenja mikroorganizama, za stabilizaciju bijelih vina ili za prevenciju određenih vrsta koloidalne precipitacije.

Bez obzira na sve promjene u vinu uzrokovane akutnim termičkim stresom, njegova je vazodilatacijska aktivnost bila u velikoj mjeri očuvana i to je najvažniji rezultat ove studije.

Termički obrađeni uzorci vina i njihove kontrole bez termičkog stresa pokazale su sličan vazodilatacijski obrazac i njihovi se maksimalni učinci nisu razlikovali. Ipak, aorta zamorčića pokazala je veću reaktivnost na vino u odnosu na aortu štakora (Slike 8 i 9) što potvrđuje prijašnje rezultate da su različiti mehanizmi uključeni u relaksaciju aorte ovih dviju vrsta te da ekstrapolacija rezultata s jedne životinjske vrste na drugu može biti pogrešna.<sup>23</sup> Razlike u osjetljivosti na vino nadalje su izražene nižim  $EC_{50}$  vrijednostima na aorti zamorčića u odnosu na odgovarajuće  $EC_{50}$  vrijednosti kod štakora (Tablica 6).

Ipak je dealkoholizirano vino (bez termičkog stresa) i na aorti štakora i na aorti zamorčića uzrokovalo značajno snažniju vazodilataciju pri najnižim koncentracijama (do 1‰), u usporedbi s intaktnim vinom i vinima zagrijavanima na 75 i 125 °C (Slike 8 i 9). Ovaj se rezultat može objasniti s toplinom uzrokovanim djelomičnim gubitkom unutar fenolnih frakcija kod vina zagrijavanog na 125 °C. S druge strane sličnost intaktnog vina i vina zagrijavanog na 75 °C ukazuje da termički stres pri ovoj temperaturi nije dovoljan da kompromitira vazodilatacijsku potentnost i učinkovitost unatoč diskretnim promjenama u fizikalno-kemijskim svojstvima vina.

## 2.5. ZAKLJUČCI

1. Fenolne su kiseline slabiji vazodilatatori nego antioksidansi *in vitro*. Postoji negativna povezanost između antioksidacijskog i vazodilatacijskog učinka fenolnih kiselina *in vitro* što je potvrđeno i analizom kvantitativne povezanosti strukture i aktivnosti (Quantitative Structure-Activity Relationship (QSAR)). Određivanje antioksidacijske aktivnosti fenolnih tvari *in vitro* nije osnova za predviđanje njihovih drugih bioloških učinaka.
2. QSAR modeli ukazuju na to da se povećan broj hidroksilnih grupa u aromatskom prstenu povezuje s dobrom antioksidacijskom, a slabijom vazodilatacijskom aktivnosti. Važne strukturalne osobine koje doprinose antioksidacijskoj aktivnosti fenolnih kiselina su stupanj razgranatosti i zbijenosti molekula te trodimenzijska raspodjela polarizabilnosti atoma.
3. QSAR modeli, primjenjeni u ovoj studiji, mogu biti pogodni za istraživanje ostalih bioloških učinaka fenolnih kiselina i/ili drugih vinskih fenola.
4. Kupinovo vino je relativno učinkovit, izravni vazodilatator *in vitro*. Crno je vino, u usporedbi s kupinovima vinom potentniji vazodilatator.
5. Kupinovo vino je, unatoč manjem sadržaju ukupnih polifenola, bolji antioksidans *in vitro* od crnog vina. Moguće objašnjenje ovog fenomena je viša razina neflavonoidnih polifenola u kupinovom vinu u odnosu na crno vino.
6. Bijelo je vino najslabiji vazodilatator i antioksidans *in vitro*. Vazodilatacijski i antioksidacijski učinci bijelog vina i fenolnih kiselina su slični.
7. Postoji pozitivna povezanost između vazodilatacijskog i antioksidacijskog učinka vina.
8. Vazodilatacijska aktivnost vina povezana je sa sadržajem ukupnih fenola, flavonoida i antocijana. Vazodilatacijska aktivnost vina nije povezana s koncentracijama pojedinačnih polifenolnih spojeva (*trans*-rezveratrol, *cis*-rezveratrol, galna kiselina, katehin, epikatehin, epigalokatehin galat, procijanidin B<sub>2</sub>, kvercetin-4-glukozid, (*E*)-piceid, astringin). To ukazuje da se vazodilatacijska aktivnost složenih otopina kao što je vino ne temelji, niti se može predvidjeti na temelju koncentracije pojedinačnog sastojka.
9. Termički obrađeno vino, pod temperaturnim uvjetima primjenjivim u svakodnevnom životu prilikom pripreme hrane, u velikoj mjeri zadržava vazodilatacijsku aktivnost *in vitro* unatoč značajnim promjenama u fizikalno – kemijskim svojstvima vina.

10. Aortni prstenovi zamorčiča pokazuju snažniji vazodilatacijski odgovor na intaktno i dealkoholizirano te termički obrađeno vino u odnosu na aortne prstenove štakora.

## 2.6. SAŽETAK

Za razliku od ostalih polifenolnih spojeva iz vina i unatoč njihovoj velikoj zastupljenosti u drugim napitcima i hrani, biološki su učinci fenolnih kiselina slabije istraživani. Kardiovaskularni učinci termički obrađenog vina također do sada nisu istraživani, iako je primjena vina u kuhanju i konzumacija vina kao toplog napitka (kuhano vino) široko rasprostranjena. Osim vina iz grožđa, postoje brojna voćna vina čija je konzumacija sve popularnija, a njihovi su biološki učinci također slabije istraživani. Stoga je cilj ovog istraživanja bio utvrditi vazodilatacijske i antioksidacijske učinke fenolnih kiselina iz vina, termički obrađenog vina i kupinovog vina.

Antioksidacijski učinci mjereni su FRAP (Ferric reducing antioxidant power) i TEAC (Trolox equivalent antioxidant capacity) metodama, dok je vazodilatacijska aktivnost određena na prekontrahiranim vaskularnim prstenovima aorte štakora i zamorčića. Fenolne kiseline bile su bolji antioksidansi nego vazodilatatori. Njihovi su antioksidacijski kapaciteti i maksimalni vazodilatacijski učinci pokazali negativnu povezanost.

Kupinova su vina manje potentni vazodilatatori, a unatoč nižem sadržaju ukupnih fenola učinkovitiji antioksidansi u odnosu na crna vina. Vazodilatacijska aktivnost vina iz grožđa te kupinovih vina, osim s flavonoidnim i ukupnim fenolnim sadržajem, najznačajnije je povezana sa sadržajem antocijana, ali ne i sa sadržajem rezveratrola.

Vazodilatacijski učinci crnog vina zagrijavanog pri 75 i 125 °C uspoređivani su s učincima intaktnog vina i vina dealkoholiziranog bez termičkog stresa. Iako je pri najnižim koncentracijama dealkoholizirano vino ostvarilo jaču vazodilataciju, svi su uzorci vina postigli sličan maksimalni vazodilatacijski učinak, veći na aorti zamorčića.

## 2.7. SUMMARY

In contrast to other wine polyphenolic compounds, and despite their great abundance in other drinks and foods, biological effects of phenolic acids have been scarcely investigated. Also cardiovascular effects of thermally treated wine have not been investigated, although cooking with wine and consumption of mulled wine is common practice throughout the world. Besides grape wines, consumption of other fruit wines has become increasingly popular, but their biological effects have received little scientific attention. Therefore the aim of this study was to determine vasodilatory and antioxidant effects of wine phenolic acids, thermally treated and blackberry wine.

Antioxidant effects were measured by FRAP (Ferric reducing antioxidant power) and TEAC (Trolox equivalent antioxidant capacity) methods. Vasodilatory effects were measured in the isolated rat and guinea pig aortic rings.

Phenolic acids were better antioxidants than vasodilators. There was negative correlation between their antioxidant capacities and maximal vasodilatory effects.

Blackberry wines were less potent vasodilators, and despite their lower total phenolic content, more effective antioxidants in comparison to red wines. Vasodilatory activity of grape and blackberry wines, in addition to their flavonoid and total phenolic content, was most significantly associated with their anthocyanins content. No association of vasodilation with the resveratrol content was found.

Vasodilatory effects of red wine heated at 75 and 125 °C were compared with the effects of the intact and wine dealcoholized without thermal stress. Although at the lowest concentrations dealcoholized wine produced greater vasodilation, all the tested wine samples produced similar maximal vasodilatory effect, more pronounced in the guinea pig aorta.

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### 3. PRESLIK RADOVA



### 3.1. Antioxidative and vasodilatory effects of phenolic acids in wine



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## Antioxidative and vasodilatory effects of phenolic acids in wine

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## ABSTRACT

Phenolic acids represent important fraction of wine phenolics, but their biological effects have been scarcely investigated. We examined the interrelationship between antioxidative capacity and vasodilatory activity, two potentially beneficial biological effects, of nine phenolic acids from wine. The observed antioxidative and vasodilatory effects of the tested phenolic acids were further evaluated through quantitative structure–activity relationship (QSAR) analysis, by using molecular properties, “two-dimensional” (2D) and “three-dimensional” (3D) molecular descriptors. The antioxidative capacity of phenolic acids was measured by ferric reducing antioxidant power (FRAP) and Trolox equivalent antioxidant capacity (TEAC) methods, whereas their vasodilatory activity was determined in the precontracted rat aortic rings.

FRAP and TEAC values for antioxidative capacity positively correlated, but antioxidative capacity and maximal vasodilatory effect of the acids showed a negative correlation. This was best illustrated by poor vasodilatory activity of gallic acid, which is the strongest antioxidant among the tested phenolic acids. QSAR study described how antioxidative and vasodilatory effects of phenolic acids relate to the number of hydroxyl groups in the phenyl ring, degree of compactness and branching of molecules, and three-dimensional distributions of atomic polarisability of the tested molecules.

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## 1. Introduction

Epidemiological studies have shown that moderate wine intake may be beneficial for human health (Renaud & De Lorgeril, 1992). Among many beneficial effects, wine inhibits low density lipoprotein (LDL) oxidation (Frankel, Kanner, German, Parks, & Kinsella, 1993), increases antioxidative capacity in humans (Maxwell, Cruickshank, & Thorpe, 1994; Modun et al., 2008) and modulates vascular function by inducing vasodilation through increased production of nitric oxide (NO) (Fitzpatrick, Hirschfeld, & Coffey, 1993; Flesch, Schwarz, & Bohm, 1998). These effects are mainly attributed to the wine phenolics, especially flavonoids, as their intake is also inversely associated with the incidence of many diseases, including coronary heart disease (CHD) (Stoclet et al., 2004).

An important fraction of wine phenolics are phenolic acids (German & Walzem, 2000). Phenolic acids and stilbenes are important non-flavonoid compounds present in grapes and wine. Phenolic acids are present in their free form or as glycosylated derivatives and esters of tartaric, quinic and shikimic acid in both red and

white wines (Monagas, Bartolome, & Gomez-Cordoves, 2005). As well as grapes and wine, phenolic acids are also present in other fruits and vegetables, and in tea (Manach, Scalbert, Morand, Remesy, & Jimenez, 2004). In spite of that, phenolic acids have been insufficiently investigated, especially for a possible interrelationship between their different biological effects, like antioxidative and vasodilatory activity. The structure–antioxidative activity relationship *in vitro* has been determined for several phenolic acids (Kim & Lee, 2004). Also, some wine phenolic acids have been shown *in vitro* to have vasodilatory activity (Andriambeloson et al., 1998). Ferulic acid lowers blood pressure in spontaneously hypertensive rats (Suzuki et al., 2002), and restores endothelium-dependent vasodilatation in aortas from spontaneously hypertensive rats, by increasing NO bioavailability due to its antioxidative activity (Suzuki et al., 2007).

The aim of this study was to determine and correlate antioxidative and vasodilatory activities of nine phenolic acids from wine: *p*-hydroxybenzoic, protocatechuic, vanillic, gallic, and syringic acids, as derivatives of hydroxybenzoic acid, and *p*-coumaric, caffeic, ferulic, and sinapic acids, as derivatives of hydroxycinnamic acid (Fig. 1). The observed antioxidative capacities and vasodilatory activities of the tested phenolic acids were further evaluated

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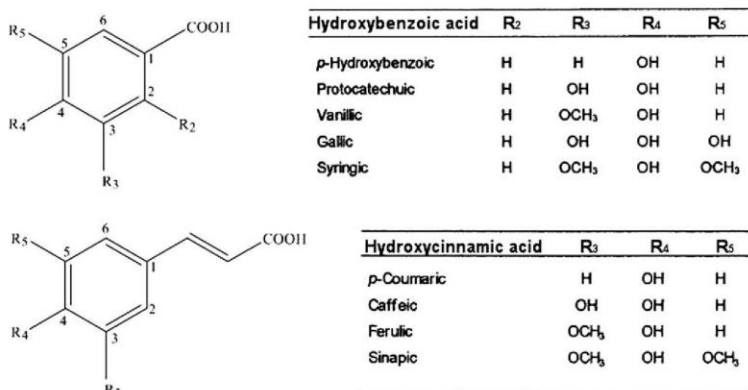


Fig. 1. Structures and classification of the tested phenolic acids.

through quantitative structure–activity relationship (QSAR) analysis, by using molecular properties, “two-dimensional” (2D) and “three-dimensional” (3D) molecular descriptors.

## 2. Materials and methods

### 2.1. Chemicals

Phenolic acids (*p*-hydroxybenzoic, *p*-coumaric, vanillic, ferulic, protocatechuic, caffeic, sinapic, syringic, and gallic acid) were purchased as pure compounds from Sigma–Aldrich Chemie (Steinheim, Germany). Ferric chloride (FeCl<sub>3</sub>), 2,4,6-tri-(2-pyridyl)-*s*-triazine (TPTZ), 2,2'-azinobis (3-ethylbenzothiazoline 6-sulfonate) (ABTS), Trolox, potassium persulfate, noradrenaline (NA) and acetylcholine (Ach) were also obtained from Sigma–Aldrich Chemie. All solvents used were of HPLC grade or the highest purity available. All solutions and reagents were made with deionised (Milli Q) water.

### 2.2. Antioxidative capacity of phenolic acids

The antioxidative capacity of phenolic acids (1 mM) was measured by ferric reducing antioxidant power (FRAP) and Trolox equivalent antioxidant capacity (TEAC) methods.

In the FRAP assay, antioxidants are evaluated as reductants of Fe<sup>3+</sup> to Fe<sup>2+</sup>, which is chelated by TPTZ to form a Fe<sup>2+</sup>–TPTZ complex absorbing at 593 nm (Benzie & Strain, 1996). TEAC assay is based on the inhibition of the absorbance of the radical cation of 2,2'-azinobis (3-ethylbenzothiazoline 6-sulfonate) (ABTS<sup>•+</sup>), which has a characteristic long-wavelength absorption spectrum showing a maximum at 734 nm (Re et al., 1999), with the tested antioxidant. Absorbance was monitored by a UV–Vis spectrophotometer (Specord 200, Analytik Jena Inc., Jena, Germany), equipped with a six-cell holder and a thermostatically controlled bath. All measurements were done in triplicate. Results were compared with a standard curve prepared daily with different concentrations of Trolox, a water-soluble analogue of vitamin E, and were expressed as millimolar Trolox equivalents.

### 2.3. Vasodilatory properties of phenolic acids

All animal experiments were conducted in accordance with international ethical guidelines. The study was approved by the Ethics Committee of the University of Split School of Medicine. Wistar rats, 3 months old and 330 ± 20 g of body weight were used

for this study. The animals received an intraperitoneal injection of urethane (1.2 g/kg). After becoming unresponsive to noxious stimuli, they were decapitated. The descending thoracic aorta was dissected free from the connective tissue and placed in modified Krebs–Henseleit solution. The aorta was carefully cleaned of the adhering fat and cut into four rings of 3–4 mm in length. After wash-out and stabilisation in modified Krebs–Henseleit solution, each ring was precontracted with the test dose of noradrenaline (NA, 10<sup>−7</sup> M). When the contraction reached the plateau phase, endothelium-dependent relaxation was induced by acetylcholine (Ach, 10<sup>−6</sup> M). The functionality of endothelium was confirmed if 10<sup>−6</sup> M Ach induced more than 70% relaxation of precontracted rings. The relaxation was expressed as the percent decrease of vasoconstriction induced by NA. The rings that relaxed less than 70% were excluded from the study. After triple wash-out and tension stabilisation, the aortic rings were again precontracted with NA (10<sup>−7</sup> M). After the stable plateau was reached, the rings (*n* = 12 per acid) were randomly exposed to cumulative concentrations of the tested phenolic acid (10<sup>−6</sup>–10<sup>−3</sup> g/l) corresponding to 7.25 × 10<sup>−9</sup> to 7.25 × 10<sup>−6</sup> M of *p*-hydroxybenzoic acid, to 6.09 × 10<sup>−9</sup> to 6.09 × 10<sup>−6</sup> M of *p*-coumaric acid, to 5.95 × 10<sup>−9</sup> to 5.95 × 10<sup>−6</sup> M vanillic acid, to 5.15 × 10<sup>−9</sup> to 5.15 × 10<sup>−6</sup> M ferulic acid, to 6.49 × 10<sup>−9</sup> to 6.49 × 10<sup>−6</sup> M protocatechuic acid, to 5.55 × 10<sup>−9</sup> to 5.55 × 10<sup>−6</sup> M caffeic acid, to 5.05 × 10<sup>−9</sup> to 5.05 × 10<sup>−6</sup> M syringic acid, to 4.46 × 10<sup>−9</sup> to 4.46 × 10<sup>−6</sup> M sinapic acid, and to 5.88 × 10<sup>−9</sup> to 5.88 × 10<sup>−6</sup> M gallic acid.

### 2.4. QSAR study

#### 2.4.1. Generation of physicochemical properties and molecular descriptors

The geometry optimisation of the nine phenolic acids was performed using the Austin Model 1 (AM1) semi-empirical method (Dewar, Zoebisch, Healy, & Stewart, 1985) applying the HyperChem 8.0 Evaluation software package (Hypercube Inc., Gainesville, USA). Some of the molecular properties (e.g., surface area, volume of the molecule, hydration energy, refractivity, polarisability) were calculated by HyperChem. 2D topological indices (e.g., Wiener index (*W*), mean distance degree deviation (*ΔD*)), 3D molecular descriptors, and some molecular properties (lipophilicity, Topological Polar Surface Area) were calculated applying the online software Parameter Client (PCLIENT, an online version of the Dragon software, Milano, Italy). Five groups of 3D descriptors were used to generate QSAR models: geometrical, Molecule

Representation of Structures based on Electron diffraction (3D-MORSE), Randic molecular profiles, GETAWAY (Geometry, Topology, and Atom Weights Assembly) and RDF (Radial Distribution Function) (Tetko et al., 2005).

QSAR study also includes the number of hydroxyl and methoxy groups on the phenyl ring ( $n_{OH}$ ,  $n_{OCH_3}$ ) and indicator variables as descriptors. Indicator variables were defined on the basis of former SAR studies (Nenadis & Tsimidou, 2002; Rice-Evans, Miller, & Panganga, 1996) as the presence of 3,4-OH groups ( $I_{3,4-OH} = 1$ ) or their absence ( $I = 0$ ).

#### 2.4.2. Selection of descriptors and statistical analysis

The statistical analysis was performed using STATISTICA 6.0 (StatSoft, Inc., Tulsa, OK). The relationship between 2D, 3D descriptors, physicochemical properties, indicator variables and antioxidative/vasodilatory activities of phenolic acids were determined by simple linear and multiple regression analyses. The selection of predictor variables for regression was performed by best-subset method in which a regression equation was fitted to every subset of independent variables. The criterion used to determine "best" is based on the  $r^2$  values of analysed models. To test the quality and accuracy of derived models, the following statistical parameters were used: squared correlation coefficient ( $r^2$ ), standard deviation of regression ( $s$ ) and Fisher ration values ( $F$ ).

The best possible QSAR models, which are presented in this report, were selected on the basis of the highest correlation coefficients and  $F$ -test, and the lowest standard deviations. The selected models were additionally validated by the calculation of quality factor ( $Q$ ) and significance level of the model  $p$ . Quality factor  $Q$  is defined as a ratio of correlation coefficient ( $r$ ) and standard deviation of regression ( $Q = r/s$ ) and is used for accounting predictive power of the model (Pogliani, 1996).

The number of descriptors in multiple regression analysis was limited to two, in accordance with the rule that the number of compounds in the data set should be three to six times greater than the number of parameters in the equation (Agrawal, Srivastava, & Khadikar, 2004). Terminal selection of the models was based on an inter-correlation study between variables included in the equation. Two-parameter models with highly collinear descriptors ( $|r| \geq 0.7$ ) were not considered.

### 3. Results

#### 3.1. Antioxidative capacity

Antioxidative capacity, measured by FRAP method, was 0, 0.12, 0.37, 0.99, 1.02, 1.11, 1.26, 1.47, and 2.32 mM Trolox equivalents and the values obtained by TEAC method were 0.06, 1.09, 0.87, 1.80, 1.22, 1.42, 1.96, 1.36, and 2.79 mM Trolox equivalents, for

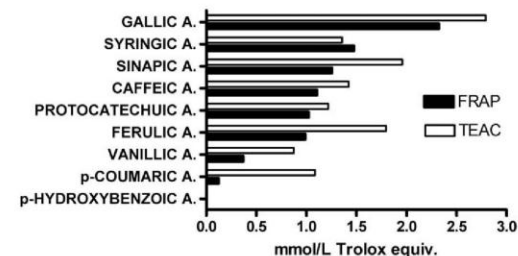


Fig. 2. Antioxidative capacity of nine phenolic acids from wine, determined by FRAP and TEAC methods, and expressed as mmol/L of Trolox equivalents.

*p*-hydroxybenzoic, *p*-coumaric, vanillic, ferulic, protocatechuic, caffeic, sinapic, syringic, and gallic acid, respectively (Fig. 2). There was a positive correlation between FRAP and TEAC values for antioxidative capacity of phenolic acids ( $r = 0.8859$ ,  $p = 0.0015$ ).

The best di-parametric model for expression of FRAP values was obtained using topological polar surface area with polar contributions of nitrogen and oxygen atoms ( $TPSA_{No}$ ) and topological descriptor, mean distance degree deviation ( $\Delta D$ ):

$$FRAP = -2.973(\pm 0.38) + 0.032(\pm 0.001)\Delta D + 0.051(\pm 0.005)TPSA_{No} \quad (1)$$

$$n = 9, r^2 = 0.950, s = 0.148, F = 60.93, Q = 6.59, p = 0.0001.$$

The best relation between antioxidative capacity expressed by TEAC values and structure of molecules was demonstrated by QSAR model that included two GETAWAY descriptors,  $R7p$  and  $HATS\ 3e$ , which were related to atomic polarisabilities and electro-negativities, respectively:

$$TEAC = -17.558(\pm 2.857) - 0.011(\pm 0.001)R7p + 0.162(\pm 0.024)HATS\ 3e \quad (2)$$

$$n = 9, r^2 = 0.955, s = 0.151, F = 64.00, Q = 6.47, p = 0.00009.$$

A scatter plot of the observed ( $FRAP_{obs.}$ ) versus the calculated (using Eq. (1)) FRAP values ( $FRAP_{calc.}$ ), together with the observed ( $TEAC_{obs.}$ ) versus the calculated (using Eq. (2)) TEAC values ( $TEAC_{calc.}$ ) is shown in Fig. 3.

#### 3.2. Vasodilatory activity

Basal tension of the rat aortic rings ( $n = 108$ ) after exposure to NA was  $14.4 \pm 0.1$  mN. The tested phenolic acids caused different concentration-dependent vasodilatory response in the NA-precontracted vascular rings (Fig. 4).

Maximal vasodilation induced by derivatives of hydroxybenzoic acid was  $26.0 \pm 3.3$ ,  $12.0 \pm 4.1$ ,  $38.0 \pm 3.3$ ,  $21.7 \pm 3.3$ , and  $5.9 \pm 2.1$  % for *p*-hydroxybenzoic, protocatechuic, vanillic, syringic and gallic acid, respectively. Maximal vasodilation induced by derivatives of hydroxycinnamic acid was  $25.6 \pm 4.6$ ,  $27.0 \pm 1.4$ ,  $22.0 \pm 6.1$ , and  $21.7 \pm 5.4$ %, for *p*-coumaric, caffeic, ferulic, and sinapic acid, respectively.

There was a significant negative correlation between antioxidative capacity (measured by FRAP method) and maximal vasodilatory effect of the phenolic acids ( $r = -0.7348$ ,  $p = 0.0241$ ).

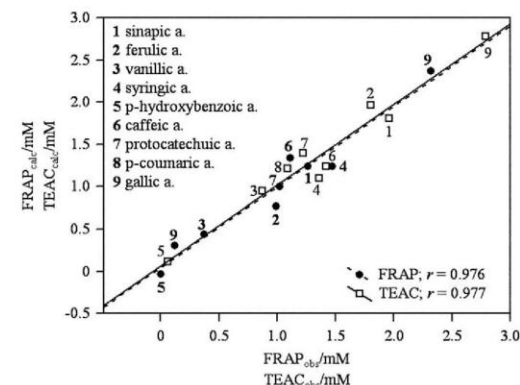
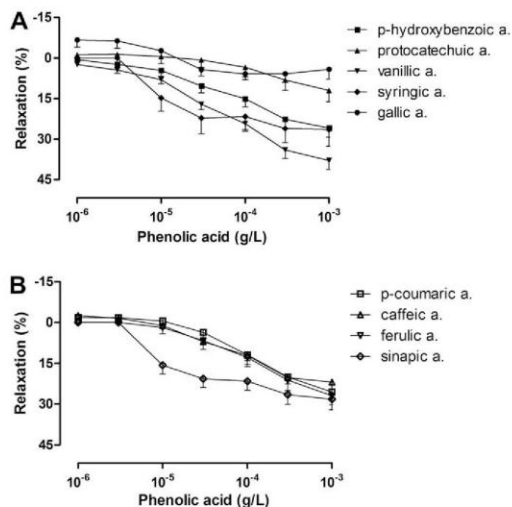


Fig. 3. Relationship between observed ( $FRAP_{obs.}$ ) and calculated FRAP values ( $FRAP_{calc.}$ ), with relationship between observed ( $TEAC_{obs.}$ ) and calculated TEAC values ( $TEAC_{calc.}$ ), for nine phenolic acids from wine.



**Fig. 4.** Dose–response vasodilatory effect of nine phenolic acids (derivatives of hydroxybenzoic acid in panel A; derivatives of hydroxycinnamic acid in panel B) in NA ( $10^{-7}$  M)-precontracted rat aortic rings ( $n = 12$  per acid). Data are expressed as mean  $\pm$  SEM (standard error of the mean). Phenolic acids doses are expressed in g/L.

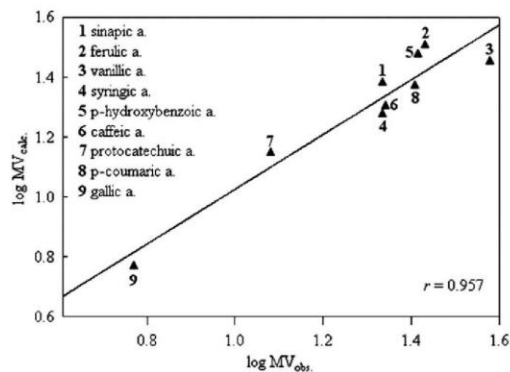
The best QSAR model for vasodilatory activity of phenolic acids (expressed as log of % maximal vasodilation,  $\log MV$ ) was obtained by multiple regression with Topological Polar Surface Area ( $TPSA_{No}$ ) and GETAWAY descriptor  $R^*_3(u)$ :

$$\log MV = 7.685(\pm 1.345) - 0.052(\pm 0.013)R^*_3(u) - 0.014(\pm 0.003)TPSA_{No} \quad (3)$$

$$n = 9, r^2 = 0.916, s = 0.065, F = 32.763, Q = 14.72, p = 0.00059.$$

A scatter plot of the experimental data ( $\log MV_{obs.}$ ) versus the calculated (using Eq. (3))  $\log MV$  values ( $\log MV_{calc.}$ ) is shown in Fig. 5.

Significant correlation was also obtained by simple linear regression between the maximal vasodilatory activity and the number of hydroxyl groups ( $n_{OH}$ ):



**Fig. 5.** Relationship between observed maximal vasodilation ( $MV$ ) (expressed as log of %  $MV_{obs.}$ ) and calculated  $\log MV$  values ( $\log MV_{calc.}$ ) for nine phenolic acids from wine.

$$\log MV = 1.722(\pm 0.889) - 0.293(\pm 0.056)n_{OH} \quad (4)$$

$$n = 9, r^2 = 0.798, s = 0.100, F = 27.69, Q = 8.93, p = 0.0011.$$

#### 4. Discussion

In this study, we examined interrelationship between antioxidative capacity and vasodilatory activity, two potentially beneficial biological effects, of nine phenolic acids from wine. The key finding of this study is the negative correlation between *in vitro* antioxidative and vasodilatory activity of the tested phenolic acids. This is best illustrated by poor vasodilatory activity of gallic acid, the strongest antioxidant among the tested phenolic acids. Generally, phenolic acids appear to be weaker vasodilators than antioxidants.

Considering that antioxidative action of phenolic compounds arises from scavenging the free radicals by the donation of a hydrogen atom to radicals, antioxidative activities of these compounds greatly depend on the number and position of hydroxyl groups in the aromatic ring (Rice-Evans et al., 1996). In the present study, QSAR models for the prediction of antioxidative activities (TEAC and FRAP) of phenolic acids also demonstrated the relevance of electronegative atoms, precisely, oxygen atoms from hydroxyl groups. Namely, positive coefficients of  $TPSA_{No}$  (an area of surface that arises from oxygen or nitrogen atoms or hydrogen atoms attached to oxygen atom) in Eq. (1), and  $HATS\ 3e$  in Eq. (2), indicate that molecules with more hydroxyl groups in the phenyl ring have greater antioxidative activity.

Accordingly, gallic acid had highest FRAP value (2.32 mM Trolox equiv.) due to three hydroxyl groups attached to the phenyl ring, hydroxybenzoic acid with two hydroxyl groups (protocatechuic acid) had FRAP of 1.02 mM Trolox equiv., while *p*-hydroxybenzoic acid, as the representative of hydroxybenzoic acids with only one hydroxyl group, demonstrated no activity. These results are consistent with data reported by Sroka and Cisowski (2003) who also showed a positive correlation between the number of hydroxyl groups bonded to the aromatic ring and the ability to scavenge free radicals by phenolic acids.

The degree of branching and shape of molecules also considerably affect FRAP values. Namely, positive coefficient of mean distance degree deviation ( $\Delta D$ ) in Eq. (1) implies that more compact and less branched molecules have a lower antioxidative capacity (Konstantinova, 1996). This is in agreement with the fact that hydroxycinnamic acids, which are larger more branched molecules, are generally more effective than smaller and less branched hydroxybenzoic acids. For example, in spite of the identical substitutions in the aromatic ring, sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) has a higher TEAC value (1.96 mM Trolox equiv.) than syringic acid (3,5-dimethoxy-4-hydroxybenzoic acid) (1.36 mM Trolox equiv.).

Presence of descriptor  $R7p$  in Eq. (2) indicates that the three-dimensional distribution of atomic polarisability also plays a role in the antioxidative activity of the phenolic acids. Because of a negative coefficient of that descriptor in Eq. (2), it would be expected that phenolic acids with more pairs of distant atoms with elevated polarisabilities have a less efficient antioxidative activity. That explains why the substitution of the 3- and 5-hydroxy groups with methoxy groups in syringic acid result in decreased antioxidative activity (FRAP 1.47 and TEAC 1.36 mM Trolox equiv.) in comparison to the trihydroxybenzoic acid, gallic acid (FRAP 2.32 and TEAC 2.79 mM Trolox equiv.).

The most important variables in models for prediction of vasodilatory activity are related to the lipophilicity and local atom distribution. Previous QSAR studies have shown that beside lipophilicity and molar refractivity, the polar surface area also correlated well with the drug transport properties, such as intestinal and oral

absorption, as well as with blood–brain barrier penetration (Clark, 1999; Kelder, Grootenhuys, Bayada, Delbressine, & Ploemen, 1999).

The negative correlation between antioxidative activity and vasodilatory effect of the phenolic acids are related to negative coefficients of variables  $TPSA_{No}$  in Eq. (3), and  $n_{OH}$  in Eq. (4), indicating that the increase in the number of hydroxyl groups in the phenyl ring is unfavourable for vasodilatory effect. Hence, mono-hydroxybenzoic acids, vanillic, p-hydroxybenzoic, and syringic acid, with low corresponding  $TPSA_{No}$  values ( $37.97 \text{ \AA}^2$ ;  $57.53 \text{ \AA}^2$ ;  $75.99 \text{ \AA}^2$ , respectively), induce greater vasodilation than the dihydroxybenzoic acid, protocatechuic acid, with  $TPSA_{No}$  value of  $77.76 \text{ \AA}^2$ . Accordingly, trihydroxybenzoic acid, gallic acid, with the largest  $TPSA_{No}$  value of  $97.99 \text{ \AA}^2$  showed the smallest vasodilatory potential. Considering that  $n_{OH}$  and  $TPSA_{No}$  have negative influence on vasodilatory activity, we may conclude that polar atoms cause steric hindrance over the process of vasodilation.

Taken together, QSAR models presented in this study, revealed that the number of hydroxyl groups in the phenyl ring, degree of compactness and branching, and the three-dimensional distributions of atomic polarisability are important molecular properties of the phenolic acids, which contribute to their antioxidative activity. The increased number of hydroxyl groups in the aromatic ring is related to good antioxidative but poor vasodilatory activity of phenolic acids. Rare QSAR studies of other workers on activities of phenolic acids (Cheng, Ren, Li, Chang, & Chen, 2002), cannot be compared with our study, as they include different descriptors. Because GETAWAY, RDF, 3D-MORSE and Randic molecular profile descriptors were developed recently, QSAR studies of antioxidative activity of phenolic acids using these descriptors are lacking. Moreover, there is no literature evidence about QSAR study for vasodilatory activity.

Although, the results of the present study do not allow conclusions on possible *in vivo* vasodilatory effects of the tested phenolic acids, it should be noticed that maximal vasodilation was always achieved at the highest concentrations, which are not expected to be reached under normal *in vivo* conditions following intake of wine or other foods rich in phenolics.

In order to make our study comparable with the results of similar *in vitro* studies, and at the same time, to roughly “cover” concentrations that are feasible with the *in vivo* conditions, concentrations of the tested phenolic acids used in our study were chosen according to the studies by Andriambelosen et al. (1998) and Caccetta, Croft, Beilin, and Puddey (2000).

Andriambelosen et al. used vanillic, gallic, caffeic and coumaric acids over the concentration range of  $10^{-5}$  to  $3 \times 10^{-1} \text{ g/l}$ , which approximately corresponds to 50 nM to 2 mM. By using 10 times lower concentrations than the Andriambelosen's group, we were able to reasonably cover concentrations attainable in human plasma after wine consumption, as shown for 4-O-methylated form of gallic acid (176 nM) and caffeic acid (84 nM) by Caccetta et al. (2000).

At comparable concentrations, only syringic and sinapic acids showed vasodilatory effect in our study. However, negligible vasodilatory effect of other phenolic acids at these concentrations does not necessarily reflect their ineffectiveness under *in vivo* conditions. Namely, there is not a single phenolic compound from wine, which would be of predictive, or principal importance for its biological effects. For example, *in vitro* application of catechin failed to induce vasodilation in rat aorta (Andriambelosen et al., 1997) and modulation of endothelial function in humans after wine consumption could not be predicted on the basis of plasma catechin concentration (Boban et al., 2006). In contrast, a maximal induction of endothelial NO synthase in human endothelial cells was achieved by synergistic effect of a blend of different wine phenolics, including some phenolic acids (Wallerath, Li, Godtel-Ambrust, Schwarz, & Forstermann, 2005). Similar synergistic effect was also found for the antioxidative activity of wine phenolics (Pignatelli et al., 2006). Therefore, determining levels and related

biological effects of a single phenolic compound *in vitro* could be misleading in terms of its expected biological effects *in vivo*.

In addition, the concentrations of different phenolic acids in human plasma after consumption of wine, or other foods rich in phenolics, are mostly unknown and are difficult to predict. The tested acids are present in wine in different concentrations and in different conjugated forms. Phenolic acids occur in foods mainly in esterified forms with organic acids, sugars and lipids, which may affect their bioavailability and metabolism. Phenolic acids undergo conjugation reactions *in vivo* with sulfate, glucuronate, S-adenosylmethionine or their combination (Manach et al., 2004). Formation of the conjugated forms can significantly influence biological properties of the parent compounds.

Polyphenols that are not absorbed in the small intestine reach the colon where colonic microflora hydrolyse glycosides into aglycones and metabolise aglycones into various aromatic acids, which are further metabolised to derivatives of benzoic acid that also may be absorbed in blood. Hence, final plasma concentration of phenolic acids is the result of complex metabolic pathways followed by not only phenolic acids, but also other polyphenols (Manach et al., 2004).

Future studies are needed to determine possible additive or synergistic effects of phenolic acids and/or their metabolites.

Taken together, results of the present QSAR study show that recently developed molecular profile descriptors, GETAWAY, RDF, 3D-MORSE and Randic can be successfully used to model possible vasodilatory activity of phenolic acids. QSAR techniques used in our study could also be suitable for investigation of other biological activities of phenolic acids and/or other wine phenolics.

Furthermore, our results imply that determining antioxidative capacity of phenolic compounds *in vitro* has no relevance for prediction of their other biological effects.

In conclusion, our results point out the relevance of wine phenolic acids as potent biologically active compounds that deserve more thorough research effort.

#### Acknowledgments

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### 3.2. Antioxidant and vasodilatory effects of blackberry wine and grape wines



## Antioxidant and Vasodilatory Effects of Blackberry and Grape Wines

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**ABSTRACT** In contrast to the well-described various biological effects of grape wines, the potential effects of commonly consumed blackberry wine have not been studied. We examined *in vitro* antioxidant and vasodilatory effects of four blackberry wines and compared them with the effects of two red and two white grape wines. Although some blackberry wines had lower total phenolic content relative to the red grape wines, their antioxidant capacity was stronger, which may be related to a higher content of non-flavonoid compounds (most notably gallic acid) in blackberry wines. Although maximal vasodilation induced by blackberry wines was generally similar to that of red wines, blackberry wines were less potent vasodilators. Vasodilatory activity of all wines, in addition to their flavonoid and total phenolic content, was most significantly associated with their content of anthocyanins. No association of vasodilation with any individual polyphenolic compound was found. Our results indicate the biological potential of blackberry wines, which deserves deeper scientific attention.

**KEY WORDS:** • anthocyanins • antioxidant effects • blackberry • vasodilation • wine

### INTRODUCTION

**D**IFFERENT FRUITS and their processed products, such as wine, are rich sources of polyphenolic compounds. Epidemiological studies have demonstrated a significant negative correlation between polyphenol consumption and cardiovascular risk.<sup>1,2</sup> It has been indicated that polyphenols may act beneficially against oxidative stress, as the main pathophysiological mechanism, and the endothelium, as the main target organ, in the development of various cardiovascular diseases. In addition to, and independently from, their antioxidant effects,<sup>3</sup> polyphenols enhance the production of vasodilating agents (nitric oxide, endothelium-derived hyperpolarizing factor) and inhibit the production of vasoconstrictor (endothelin-1) factors in endothelial cells.<sup>4</sup> Among polyphenol-rich food products, the phenolic composition and biological effects of red wine produced from grapes (*Vitis vinifera*) have received particular attention.<sup>5,6</sup> An important and well-documented biological effect of red wine is the direct, endothelium-dependent vasodilatory activity that is mostly related to the polyphenols.<sup>7–9</sup> In contrast to red wine, the polyphenolic content of white wine is low, and related direct vasodilatory activity is poor.<sup>8,10</sup>

Besides grape wines, there are several other commonly consumed fruit wines. Although their consumption has be-

come increasingly popular, they have received little scientific attention. Several studies showed that fruit wines are also a potentially rich source of polyphenols, exhibiting noticeable antioxidant activity *in vitro*.<sup>11–13</sup> Among them, blackberry (*Rubus fruticosus*) wine was indicated as a rich source of phenolics,<sup>12</sup> with even higher *in vitro* antioxidant activity than grape wine.<sup>14</sup> However, no effects of blackberry wines in biological systems have been studied so far.

In this study, we examined antioxidant and vasodilatory effects of four blackberry wines in the isolated rat aorta and compared them with the effects of two red and two white grape wines. The interrelationship between the vasodilatory activity of the tested wines with their phenolic content and composition was also examined. All tested samples were biochemically characterized with respect to their total phenolic content, high-performance liquid chromatography (HPLC) “phenolic fingerprint,” antioxidant capacity, and ethanol content.

### MATERIALS AND METHODS

All wines were commercially available and purchased from local pharmacy and grocery stores. Information about the source, vintage, and ethanol content of the tested grape (red and white) and blackberry wine samples are shown in Table 1.

Animal experiments were conducted in accordance with international ethical guidelines. The study was approved by the Ethics Committee of the University of Split School of Medicine, Split, Croatia.

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TABLE 1. GRAPE AND BLACKBERRY WINE SOURCE, VINTAGE, AND ETHANOL CONTENT USED IN THE STUDY

Wine number	Wine sample (abbreviation) <sup>a</sup>	Name, producer, region	Year	Ethanol content (volume %)
1	Red (RW1)	Refosk, Bric, Slovenia	2007	13.5
2	Red (RW2)	Vinagra, Bric, Slovenia	2005	13.0
3	White (WW1)	Posip, Cara Korcula, Croatia	2007	14.0
4	White (WW2)	Zlatna zlahtina, Vrbnik Krk, Croatia	2007	11.5
5	Blackberry (BW1)	KupiFe, Split, Croatia	2007	9.9
6	Blackberry (BW2)	Kupinovo vino Suler, Kutina, Croatia	2007	13.7
7	Blackberry (BW3)	BIO&BIO, Orlov put, Croatia	2008	10.6
8	Blackberry (BW4)	Baranjsko kupinovo vino, Cerine, Croatia	2006	13.0

<sup>a</sup>BW, blackberry wine; RW, grape red wine; WW, grape white wine.

#### Preparation of aortic rings

Male Sprague–Dawley rats ( $n=48$ ) weighing  $330 \pm 20$  g were used for this study. The animals received an intraperitoneal injection of urethane (1.2 g/kg). After becoming unresponsive to noxious stimuli, they were decapitated. The descending thoracic aorta was dissected free from the connective tissue and placed in the modified Krebs–Henseleit solution. The aorta was carefully cleaned of the adhering fat and cut into rings as previously described.<sup>15</sup> After a washing-out and stabilization period, the rings were precontracted with a test dose of noradrenaline ( $10^{-7}$  M). When the contraction reached the plateau phase, functionality of endothelium was confirmed by acetylcholine ( $10^{-6}$  M)-induced relaxation. The relaxation was expressed as the percentage decrease of the noradrenaline-induced vasoconstriction. After triple washout and tension stabilization, the precontracted rings were randomly exposed to cumulative concentrations (0.1‰ to 8‰ final dilutions in organ baths) of each of the tested wine samples ( $n=15$  per wine sample).

#### Biochemical analysis of the wine samples

**Phenolic content and composition.** The contents of total phenolics and their subgroup (flavonoid, non-flavonoid, and anthocyanin) were measured spectrophotometrically, whereas the individual phenolic compounds were determined by HPLC.

The total phenolic content of the samples was determined by the Folin–Ciocalteu method, and the results are expressed as gallic acid equivalents per liter. Non-flavonoid compounds were determined by the Folin–Ciocalteu method after precipitation of flavonoids with formaldehyde, and the flavonoid content was calculated as the difference between total phenolic and non-flavonoid contents.

Total anthocyanin content was determined using the bisulfite bleaching method, and the results are expressed as milligrams of malvidin-3-glucoside per liter.

Absorbances were monitored by an ultraviolet-visible spectrophotometer (Specord 200, Analytik Jena Inc., Jena, Germany), equipped with a six-cell holder and a thermostatically controlled bath. The data presented are the averages of three measurements. A more detailed description of

the above-mentioned methods has been previously published.<sup>16</sup>

Individual polyphenols were identified and quantified by HPLC. The HPLC system was composed of a Varian (Palo Alto, CA, USA) ultraviolet-visible PDA 330 detector, a ternary gradient liquid Pro Star 230 pump, model 500 column heater, and Star chromatography workstation version 6.0. The polyphenolic compounds were separated on an octadecyl column (Zorbax Eclipse XDB-C18; 4.6 mm × 250 mm; film thickness, 5 μm; Agilent, Palo Alto) maintained at 30°C. Wine samples were filtered through a membrane filter (pore size, 0.45 μm) and directly injected through a 20-μL fixed loop into a C18 guard column. Wine samples were diluted three times with eluent prior to injection.

A gradient consisting of solvent A (water/acetic acid, 98:2, vol/vol) and solvent B (acetonitrile/acetic acid, 99:1, vol/vol) was applied at a flow rate of 1.0 mL/minute as follows: 0 minute, 93% A/7% B; 18 minutes, 80% A/20% B; 25 minutes, 60% A/40% B; 30 minutes, 40% A/60% A; 40 minutes, 40% A/60% B; 43 minutes, 93% A/7% B; and 45 minutes, 93% A/7% B. The signal was monitored at 280 nm. Phenolic compounds were identified by their retention times and absorption spectra. Quantification was carried out by comparison with external standard calibration curves: 20–80 mg/L for gallic acid and (–)-epicatechin; 10–60 mg/L for epigallocatechin gallate, quercetin-4-glucoside, *trans*-resveratrol, (*E*)-piceid, and astrigin; and 30–170 mg/L for (+)-catechin and procyanidin B2. The stock solution of *cis*-resveratrol isomer was prepared by ultraviolet irradiation at 254 nm of an alcoholic solution of *trans*-resveratrol according to Romero-Pérez *et al.*<sup>17</sup> Each sample was injected twice into the chromatographic system.

**Antioxidant capacity.** Total antioxidant capacity of the samples was determined using the ferric reducing antioxidant power (FRAP) assay.<sup>18</sup> In this assay, antioxidants are evaluated as reductants of  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ , which is chelated by 2,4,6-tripyridyl-*s*-triazine to form a  $\text{Fe}^{2+}$ -2,4,6-tripyridyl-*s*-triazine complex absorbing at 593 nm. Measurements were done in triplicate. Results were compared with a standard curve prepared with different concentrations (0.5–2 mM) of Trolox, a water-soluble analog of vitamin E, and expressed as Trolox equivalents.

**Ethanol content.** Ethanol concentration in the samples was measured by a Shimadzu (Kyoto, Japan) model 2010 gas chromatograph with a headspace and flame ionization detector. Ultrapure-grade helium was used as the carrier gas at a flow rate of 11.70 mL/minute. An RTX-BAC2 chromatographic column was used (fused silica; 30 m long and 0.53 mm i.d.; film thickness, 0.20  $\mu$ m). The injection temperature was 200°C, and the column conditions were 3 minutes at 60°C with the flame ionization detector at 200°C.

**Chemicals.** All analytical-grade chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA), Aldrich Chemical Co. (Steinheim, Germany), and Merck (Darmstadt, Germany). The resveratrol derivatives (*E*)-piceid (*trans*-3,5,4'-trihydroxystilbene-3-*O*- $\beta$ -D-glucopyranoside), isorhapontin (*trans*-3,4',5-trihydroxy-3'-methoxystilbene-3-*O*- $\beta$ -D-glucopyranoside), and astringin (*trans*-3,4,3',5'-tetrahydroxystilbene-3'-*O*- $\beta$ -D-glucopyranoside) were obtained from Polyphenols Laboratories (Sandnes, Norway). Deionized (Milli Q<sup>®</sup>, Waters Corp., Milford, MA, USA) water was used for the preparation of all solutions and reagents.

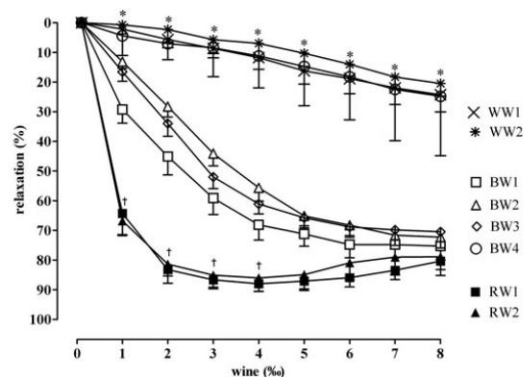
#### Statistical analysis

Data were analyzed using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA, USA). For statistical analysis of vasodilatation responses, one- and two-way analysis of variance followed by Bonferroni's *post hoc* tests was used. All data are expressed as mean  $\pm$  SEM values.  $P < .05$  was considered statistically significant. Because the wine samples differed significantly in their total phenolic and ethanol contents, we used dilution and logarithm of dilution, instead of concentration, to express 50% effective concentration ( $EC_{50}$ ) values. Nonlinear regression analysis was used to calculate  $EC_{50}$ .

## RESULTS

#### Vasodilatory effects of the wines

Basal tension of the rat aortic rings ( $n=120$ ) following exposure to noradrenaline was  $18.31 \pm 4.27$  mN. Although all wines showed vasodilatory activity, they induced different concentration-dependent vasodilatory responses in the noradrenaline-precontracted rat aortic rings (Fig. 1). Generally, red grape wines were the most potent vasodilators ( $EC_{50} = -3.17$  and  $-3.24$  for RW1 and RW2). Blackberry wines BW1, BW2, and BW3 showed intermediate vasodilatory potency with  $EC_{50}$  values of  $-2.85$ ,  $-2.63$ , and  $-2.72$ , respectively, whereas blackberry wine BW4 was a significantly less potent vasodilator with an  $EC_{50}$  of  $-2.31$ . However, maximum vasodilation ( $E_{max}$ ) induced by blackberry wines BW1, BW2, and BW3 was generally similar to that of red grape wines ( $E_{max} = 75.27 \pm 4.67\%$ ,  $72.27 \pm 3.65\%$ , and  $70.48 \pm 3.40\%$  for BW1, BW2, and BW3, respectively, vs.  $87.99 \pm 2.50\%$  for RW1 and  $86.00 \pm 4.56\%$  for RW2). Maximal relaxation produced by white grape wines and blackberry wine BW4 was signifi-



**FIG. 1.** Relaxation in noradrenaline-precontracted rat aortic rings following exposure to grape red (RW1 and RW2), grape white (WW1 and WW2) and blackberry (BW1, BW2, BW3, and BW4) wines. Results are shown as mean  $\pm$  SEM values ( $n=15$  per wine sample). By two-way analysis of variance, Bonferroni's *post hoc* test: \* $P < .05$  versus red grape (RW1 and RW2) and blackberry (BW1, BW2, and BW3) wines; † $P < .05$  versus blackberry wines (BW1, BW2, and BW3).

cantly smaller ( $24.28 \pm 5.79\%$ ,  $20.53 \pm 4.17\%$ , and  $24.27 \pm 3.86\%$  for WW1, WW2, and BW4, respectively) ( $P < .05$ ) (Table 2).

#### Biochemical analysis of the wine samples

**Phenolic content and antioxidant capacity.** As shown in Table 3, total phenolic content was highest in the red grape wines, followed by blackberry wines, whereas the lowest was found in the white grape wines.

**TABLE 2.** VASODILATING ACTIVITY OF THE GRAPE AND BLACKBERRY WINES EXPRESSED BY MAXIMUM VASODILATION AND 50% EFFECTIVE CONCENTRATION

Wine sample	$E_{max}$ (%)	$EC_{50}$ (CI)
RW1	$87.99 \pm 2.50^*$	$-3.17$ ( $-3.18$ to $-3.03$ ) <sup>†</sup>
RW2	$86.00 \pm 4.56^*$	$-3.24$ ( $-3.47$ to $-3.01$ ) <sup>†</sup>
WW1	$24.28 \pm 5.79$	$-2.44$ ( $-2.55$ to $-2.32$ )
WW2	$20.53 \pm 4.17$	$-2.34$ ( $-2.44$ to $-2.24$ )
BW1	$75.27 \pm 4.67^*$	$-2.85$ ( $-2.93$ to $-2.77$ ) <sup>*</sup>
BW2	$72.27 \pm 3.65^*$	$-2.63$ ( $-2.64$ to $-2.59$ ) <sup>*</sup>
BW3	$70.48 \pm 3.40^*$	$-2.72$ ( $-2.77$ to $-2.67$ ) <sup>*</sup>
BW4	$24.27 \pm 3.86$	$-2.31$ ( $-2.38$ to $-2.25$ )

Maximum vasodilation ( $E_{max}$ ) values are mean  $\pm$  SEM values, and the 50% effective concentration ( $EC_{50}$ ) was calculated using nonlinear regression analysis.  $EC_{50}$  values are a log of dilution giving 50% of relaxation relative to the sample's own maximal relaxation ( $1\%_{00} = 0.001$ ;  $\log 0.001 = -3$  and  $2\%_{00} = 0.002$ ;  $\log 0.002 = -2.70$ ), with the 95% confidence interval (CI) in parentheses. In all measurements  $n=15$  per wine sample.

By one-way analysis of variance, Bonferroni's *post hoc* multiple comparison test: \* $P < .05$  versus white grape wines (WW1 and WW2) and blackberry wine (BW4); † $P < .05$  versus blackberry wines (BW1, BW2, BW3, and BW4) and white grape wines (WW1 and WW2).

TABLE 3. CONTENT OF TOTAL PHENOLICS, MAIN PHENOLIC FRACTIONS, AND ANTIOXIDANT ACTIVITY OF THE GRAPE AND BLACKBERRY WINES

	Total phenolics (mg of GAE/L)	Flavonoids (mg of GAE/L)	Non-flavonoids (mg of GAE/L)	Anthocyanins (mg of M-3-G/L)	FRAP (mmol of TE/L)
RW1	3313±27	3002±22	311±4	212±9	12.6±0.6
RW2	3225±26	2902±21	323±7	287±3	12.7±0.2
WW1	482±3	103±2	379±5	ND	1.9±0.1
WW2	379±3	58±3	321±5	ND	1.2±0.1
BW1	2628±29	1417±14	1210±11	135±3	13.9±0.7
BW2	2025±23	951±11	1074±10	148±2	10.8±0.4
BW3	2789±27	1303±14	1486±12	164±3	15.8±0.6
BW4	1697±20	924±11	773±8	13±1	7.8±0.4

Data are averages of at least three independent samples and are shown as mean±SEM values.

FRAP, ferric reducing antioxidant capacity; GAE gallic acid equivalents; M-3-G, malvidin-3-glucoside; ND, not detected; TE, Trolox equivalents.

Relative to the red grape wines, blackberry wines were lower in flavonoid content but were several times richer in non-flavonoid phenolic compounds. Anthocyanins were present only in the red grape and blackberry wines. Their concentrations were higher in the red grape wines (212±9 and 287±3 mg/L malvidin-3-glucoside), relative to the blackberry wines, in which anthocyanins ranged from 13 to 164 mg/L malvidin-3-glucoside (Table 3).

The wine with the highest antioxidant capacity was blackberry wine BW3. The order of the antioxidant capacity considering FRAP was BW3>BW1>RW2>RW1>BW2>BW4>WW1>WW2. The antioxidant capacity of the white wines was fourfold lower relative to the blackberry wine with the lowest FRAP (Table 3).

*Analysis of phenolic compounds by HPLC.* The most abundant flavanol monomer in grape wines was catechin. In blackberry wines the flavanol content varied more than in grape wines. The best example of that is epigallocatechin gallate, which ranged in concentration from 3.5 to 148.8 mg/L in the blackberry wines. Similarly, procyanidin B2, the dimer of epicatechin, ranged in concentration from 6.1 to 77.1 mg/L in blackberry wines; thus BW1 and BW2 were richer in procyanidin B2 content than red wines.

Among non-flavonoids, stilbenes were detected in all wines. Resveratrol monomers were found in small amounts (0–2.2 mg/L), but their derivatives, (*E*)-piceid and astringin, were found in higher concentrations, regardless of wine type. The non-flavonoid gallic acid was also present in all wines, but its concentrations were two to three times higher in blackberry than in grape wines. Compounds identified and their concentrations are listed in Table 4.

#### *Relationship between vasodilatory activity and phenolic content of the tested wines*

Vasodilatory activity correlated strongly with total phenolic content of the wines ( $r=0.92$ ). Among phenolic fractions a strong positive correlation was found for anthocyanin content ( $r=0.95$ ), followed by flavonoid content ( $r=0.84$ ). No correlation was found between the levels of individual phenolic compounds and vasodilatory activity.

The relationship between  $E_{max}$  and total phenolic, flavonoid, and anthocyanin content is shown in Figure 2.

## DISCUSSION

Results of this study indicate that blackberry wines are relatively effective direct vasodilators and even better antioxidants *in vitro*. The finding that some blackberry wines (BW1 and BW3) were more effective antioxidants than red grape wines is supportive of the previously published results by Pinhero and Paliyath.<sup>14</sup> Nonetheless, this is a rather unexpected finding taking into account the lower total phenolic content of blackberry wines in comparison with red grape wines, as it has been repeatedly documented that *in vitro* antioxidant capacity of different beverages, as determined by FRAP, is highly related to their total phenolic content.<sup>16,19,20</sup> A possible explanation for this discrepancy may be attributed to the higher content of non-flavonoids (most notably gallic acid) in blackberry wines relative to the red grape wines. In our previous study on antioxidant and vasodilatory effects of phenolic acids we showed that gallic acid was the most potent *in vitro* antioxidant but the least effective direct vasodilator.<sup>21</sup>

Another important finding of this study is that vasodilatory activity of all tested wines, in addition to their flavonoid and total phenolic contents, was most significantly associated with their anthocyanin content. In contrast to that, we found no association of vasodilation with any individual polyphenolic compound, at least not with ones that we analyzed.

The correlation between vasodilatory activity and anthocyanin level of red wines was already demonstrated by Burns *et al.*,<sup>7</sup> who tested 16 different red wine samples on rabbit aortic vascular rings. However, it remains unclear as to why the anthocyanins are so strongly associated with the vascular response to wines. Results of *in vitro* studies with individual anthocyanidin compounds (sugar free molecules, anthocyanin aglycones) were contradictory, as only delphinidin, but not malvidin or cyanidin, caused endothelium-dependent relaxation of rat aortic rings.<sup>22</sup> The study by Nakamura *et al.*<sup>23</sup> showed that four individual anthocyanin compounds, applied either alone or in combination, caused

TABLE 4. CONCENTRATIONS OF SELECTED PHENOLIC COMPOUNDS IN THE GRAPE AND BLACKBERRY WINES

Wine	Concentration (mg/L)										
	Galic acid	(+)-Catechin	(-)-Epicatechin	Epigallocatechin gallate	Procyanidin B2	Quercetin-4-glucoside	cis-Resveratrol	trans-Resveratrol	(E)-Piceid	Astringin	
RW1	26.4±0.1	13.5±0.4	7.8±1.4	12.7±0.5	12.3±1.6	1.4±0.1	0.6±0.1	1.1±0.1	1.5±0.2	5.2±0.1	
RW2	14.9±0.1	15.8±0.9	4.9±0.3	1.6±0.3	10.0±1.3	0.4±0.1	0.6±0.1	1±0.1	1.7±0.1	ND	
WW1	21.8±0.1	21.7±0.2	1.5±0.3	9.8±0.1	4.4±0.3	ND	0.5±0.1	1.1±0.1	2.3±0.1	2.4±0.2	
WW2	3.3±0.2	18.4±0.9	0.4±0.01	17.8±0.6	ND	ND	0.1±0	0.2±0	0.8±0.1	2.3±0.2	
BW1	59.0±1.4	18.7±1.5	34.7±3.7	148.8±0.8	77.1±3.3	2.6±0.1	1.5±0.1	0.7±0.1	5.2±0.5	7.9±0.8	
BW2	45.4±0.3	16.8±1.4	7.9±1.7	16.8±2.2	34.5±4.3	2.4±0.2	ND	0.5±0.1	2.6±0.1	ND	
BW3	50.1±0.3	45.2±2.9	ND	ND	6.1±0.2	2.1±0.1	0.2±0.1	0.7±0.1	6.5±0.4	12.1±0.8	
BW4	53.1±3.2	9.1±0.9	12.2±1.7	3.5±0	8.8±0.4	ND	0.1±0	0.9±0.1	2.0±0.4	ND	

Data are averages of two independent samples and are shown as mean±SEM values.

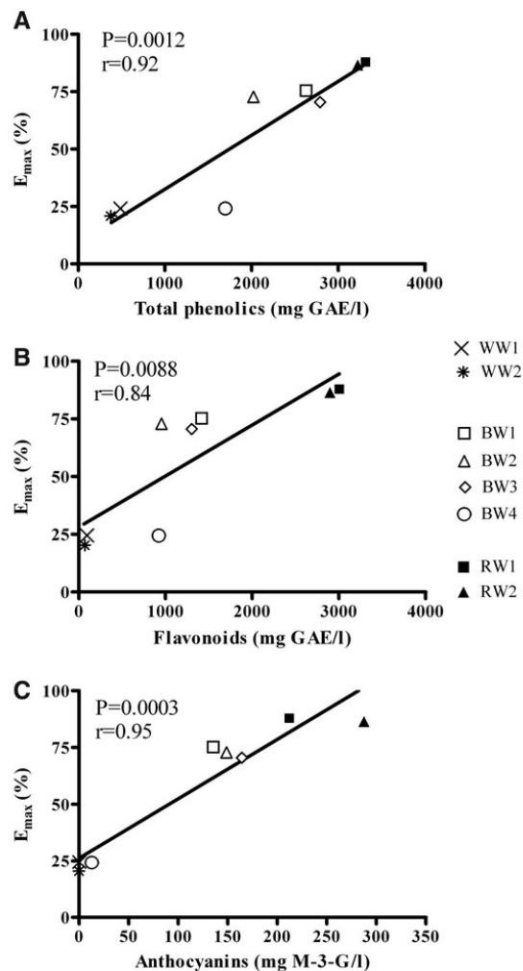


FIG. 2. Relationships between vasodilatory activity ( $E_{max}$ ) and (A) total phenolic, (B) flavonoid, and (C) anthocyanin content of the tested wine samples. Total phenolic and flavonoid contents were expressed in milligrams of GAE per liter, and anthocyanin content was expressed in milligrams of M-3-G per liter.

no vasodilatory effect in the rat aorta. Similarly, Serraino et al.<sup>24</sup> showed that cyanidine-3-*O*-glucoside had no effect in the contractile or in the endothelium-dependent vasodilating response of the aortic rings under basal conditions, although it provided protection against peroxynitrite-mediated vascular dysfunction. Negligible direct vasodilatory effects of anthocyanin and anthocyanidin compounds *in vitro* do not necessarily reflect their effectiveness under *in vivo* conditions when they are consumed as a part of a complex mixed solution, such as wine. The concentrations of anthocyanins

in our study were in the nanomolar range (for example, 1‰ of BW3 roughly equals 310 nM malvidin-3-glucoside in the organ bath), which corresponds with the upper limits of their plasma concentrations following consumption of anthocyanin-rich foods or beverages.<sup>25</sup> In addition, the most recent reports indicate that anthocyanins undergo substantial metabolism after being ingested and that their metabolites, formed in the small intestine and hepatic cells, as well as low-molecular-weight catabolic products of the colonic microflora, such as phenolic acids, travel around the human body in the circulatory system and may be responsible for the distinctive biological effects of “anthocyanins.”<sup>26,27</sup> These metabolites might be the key to filling the gap between *in vitro* and *in vivo* observed biological effects of the anthocyanins.

The importance of anthocyanins in the vasodilatory activity of blackberry wine is best illustrated with the BW4 sample. It acted as a poor direct vasodilator similar to the white grape wines with  $E_{max}$  of  $24.27 \pm 3.86\%$  and  $24.28 \pm 5.79\%$  for BW4 and WW1, respectively, although its phenolic content is 3.5-fold higher than that of white grape wine (WW1), but its anthocyanin content was about 10-fold lower than that of other blackberry wines. Numerous studies were undertaken in order to identify polyphenolic compounds responsible for the vasodilatory effect of the red grape wine. Among wine phenolics, a significant role has been attributed to resveratrol in wine-mediated cardiovascular protection, including vasodilatory activity.<sup>28</sup> Vasodilatory response in this study could not be associated with the resveratrol content of the wine samples examined. However, it should be noted that the resveratrol content in the wines examined was rather low (up to 2.2 mg/L), and after dilution in the organ bath its concentration was in the low nanomolar range, which is an order of magnitude lower than in the studies examining the dilatory effects of resveratrol *in vitro*.<sup>29</sup> Procyanidins were also suggested as the principal vasoactive polyphenols in red grape wine.<sup>30</sup> Although procyanidin B2 concentrations in our samples were within the range of the vasodilation threshold (from 0.5 to 4 µg/L),<sup>31</sup> we found no significant correlation between procyanidin B2 concentration and  $E_{max}$  ( $r=0.41$ ,  $P=.31$ ). Taken together, these results support the notion that the biological effect of a complex solution, such as wine, cannot be exclusively attributed to a single phenolic compound. Rather, it is a result of the synergistic effect of different polyphenolics, as shown for various biological effects under different experimental conditions.<sup>9,32,33</sup>

In conclusion, we have confirmed potent *in vitro* antioxidant and vasodilatory effects of blackberry wines that are roughly comparable to those of red grape wines.

These results justify the need for examining cardiovascular effects of blackberry wines in human subjects.

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#### AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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### 3.3. Thermally treated red wine retains vasodilatory activity in rat and guinea pig aorta



## Thermally Treated Wine Retains Vasodilatory Activity in Rat and Guinea Pig Aorta

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**Abstract:** In contrast to the intact wine, cardiovascular effects of the thermally treated wine have not been studied, despite widespread habits of cooking with wine and consumption of mulled wine. Vasodilatory effects of the red wine heated at 75 and 125°C were examined in the isolated rat and guinea pig aorta and compared with the intact and wine dealcoholized without thermal stress. Samples were analyzed for their phenolic content, antioxidant capacity, resveratrol and ethanol contents. Heating-induced degradation of individual phenolic fraction was observed only in the samples treated at 125°C, although total phenolic concentration and related antioxidant activity increased in the thermally treated samples due to the reduction in their volume. All wine samples regardless of treatment caused similar maximal relaxation in both species, but the response was stronger in aortas from guinea pigs. At the lowest concentrations up to 1‰, dealcoholized wine produced vasodilation greater than that produced by intact wine and wines treated at 75 and 125°C, which showed similar vasodilating activity at all concentrations. Our results indicate that wine thermally treated under heating conditions applicable to the preparation of a mulled wine and cooking with wine largely retains vasodilatory activity in vitro despite significant heat-induced changes in its composition.

**Key Words:** red wine, thermally treated wine, vasodilation, isolated aorta

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### INTRODUCTION

Thermal treatment of wine is quite usual in everyday life as it is commonly used in the kitchen in the processes of food preparation or consumed as a warm beverage known as “mulled wine.” In contrast to intact wine, the biological effects

of thermally treated wine have practically not been studied. An important and well-documented biological effect of wine is the direct, endothelium-dependent vasodilatory activity that is mainly related to the phenolic compounds in wine.<sup>1–3</sup>

The process of heating may significantly change the physical–chemical properties and the composition of wine. For example, alcohol readily evaporates,<sup>4</sup> and polyphenolic compounds are subject to thermal degradation,<sup>5,6</sup> which in turn may result in altered or lost biological activity.

Therefore, the aim of this study was to examine the direct vasodilatory effects of the thermally treated red wine in comparison with those of the intact wine and the wine dealcoholized without thermal stress. Because of the differences between guinea pigs (GPs) and rats in their vasodilatory response to wine,<sup>7</sup> the study was conducted on the isolated aorta from both species. The applied temperatures corresponded to the heating conditions when preparing a mulled wine or cooking with wine. All tested samples were biochemically characterized with respect to their total phenolic content and related antioxidant capacity. Besides the effects of the thermal treatments on major phenolic fractions, special attention was given to the effects on a specific wine phenolic, resveratrol, as it has been attributed an important role in wine-mediated vasodilation.<sup>8–10</sup>

### METHODS

#### Aortic Ring Preparation

All animal experiments were conducted in accordance with international ethic guidelines. The study was approved by the Ethics Committee of the University of Split School of Medicine. Forty-eight male Sprague–Dawley rats, weighting 330 ± 20 g and 44 GPs, weighting 290 ± 20 g were used for this study. The animals received an intraperitoneal injection of urethane (1.2 g/kg). After becoming unresponsive to noxious stimuli, they were decapitated. The descending thoracic aorta was dissected free from the connective tissue and placed in modified Krebs–Henseleit solution. The aorta was carefully cleaned of the adhering fat and cut into rings as previously described.<sup>11</sup> After washing out and stabilization in modified Krebs–Henseleit solution, rings were precontracted with the test dose of norepinephrine (NE 10<sup>-7</sup> M for rat aortic rings and 10<sup>-6</sup> M for GP aortic rings). When the contraction reached the plateau phase, the functionality of the endothelium was confirmed by acetylcholine-induced relaxation (10<sup>-6</sup> M for rat aortic rings and 10<sup>-5</sup> M for GP aortic rings). The relaxation was expressed as the percent decrease of the NE-induced

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vasoconstriction. After triple washout and tension stabilization, the precontracted rings were randomly exposed to cumulative concentrations (0.1‰–8‰ final dilutions in organ baths) of one of the tested wine samples (rat aortic rings,  $n = 21$  per wine sample and for the GP aortic rings,  $n = 23$  per sample).

### Preparation of Wine Samples

All tested samples were obtained from the original red wine Vinagra, vintage 2006, Bric winery, Slovenia. Thermally treated wine samples were obtained by heating for 45 minutes in a thermally controlled oil bath, at 75 and 125°C, respectively. Heating at 75°C corresponds to the thermal conditions in preparation of a mulled wine (avoidance of boiling), whereas heating at 125°C is feasible with the thermal conditions of cooking with wine. Dealcoholized wine was used as a thermal stress-free control to the wine samples heated at 125°C, as their volume and ethanol content were similarly reduced during dealcoholization and heating, respectively. Dealcoholized wine was prepared in vacuo in a rotary evaporator (Laborota 4000, Heidolph Instruments Inc, Schwabach, Germany). To avoid mechanical and thermal stress, a vacuum was applied progressively and gradually up to a reading of  $-130$  kPa, at 30°C for 45 minutes.

### Biochemical Analysis of the Wine Samples

#### Phenolic Compounds and Antioxidant Capacity

The total phenolic content of the samples was determined by the Folin–Ciocalteu method, and the results are expressed as gallic acid equivalents (GAE) per liter. Flavonoid and nonflavonoid contents were determined by the Folin–Ciocalteu method after precipitation of flavonoids with formaldehyde. The anthocyanin content in wine was determined using bisulfite bleaching method, and the results are expressed as milligrams per liter of malvidin-3-glucoside. Catechin content was determined using vanillin assay, and (+)-catechin was used as a standard.

The antioxidant capacity of the samples was measured as ferric reducing antioxidant power. Results are expressed as millimoles of Trolox equivalents per liter.

All measurements were performed spectrophotometrically. Absorbances were monitored by a UV–VIS spectrophotometer (Specord 200, Analytik Jena Inc., Jena, Germany), equipped with a 6-cell holder and a thermostatically controlled bath. The presented data are the averages of 3 measurements. A more detailed description of the above-mentioned methods has been previously published.<sup>12</sup>

#### Trans-resveratrol Content

The content of *trans*-resveratrol was determined using a high-performance liquid chromatography system that consisted of LC-10AT pump and SPD-10 A VP ultraviolet detector, CTO-10 ACVP column oven (Shimadzu, Kyoto, Japan) with a C18 column from Shimadzu ( $150 \times 4.6$ -mm, with 5- $\mu$ M particles), as previously described.<sup>12</sup>

Standards solutions of *trans*-resveratrol were prepared weekly and were kept protected from air and light exposure. The presented data are the average of 2 parallel measurements.

### Ethanol Content

Ethanol concentration in the samples was measured by Shimadzu 2010 gas chromatography with a headspace and flame ionization detector, as previously described.<sup>12</sup>

### Chemicals

All chemicals and reagents were of analytical grade and were obtained from Sigma Chemical Co (St Louis, MO), Aldrich Chemical Co (Steinheim, Germany), and Merck (Darmstadt, Germany). Deionized (Milli Q) water was used for the preparation of all solutions and reagents.

### Statistical Analyses

Data were analyzed using GraphPad Prism, version 4.03 for Windows, GraphPad Software, San Diego, CA. Statistical analyses of the physical–chemical properties and the composition of wine samples were done using the Mann–Whitney *U* test. Statistical analysis of vasodilatation responses was done using 2-way analysis of variance, followed by the Bonferroni post hoc test. All data are expressed as mean  $\pm$  SEM.  $P < 0.05$  was considered statistically significant. Because the wine samples differed significantly in their total phenolic and ethanol content, we used dilution and logarithm of dilution, instead of concentration to express effective concentration 50 ( $EC_{50}$ ). Nonlinear regression analysis was used to calculate  $EC_{50}$ .

## RESULTS

Heating at 125°C and vacuum dealcoholization of the wine samples resulted in very similar decrease in their volume and ethanol content. Total phenolic concentration expectedly increased with the decrease in the sample volume, in both cases. Thermally treated samples at 125°C, however, reduced approximately 10% in the individual phenolic fraction content relative to the dealcoholized wine, indicating heating-induced degradation of polyphenols in the respective fractions. Heat-induced changes of the phenolic fraction in the thermally treated samples at 75°C were not observed. The antioxidant activity of the samples corresponded to their total phenolic concentration (Table 1). Resveratrol levels were highest in the dealcoholized and the lowest in the thermally treated samples at 125°C (Table 1).

After exposure to NE, the basal tension of the isolated rat and GP aortic rings did not significantly differ and were  $16.71 \pm 0.85$  and  $16.65 \pm 0.76$  mN, respectively. In contrast, control vasodilation to acetylcholine of the NE-precontracted rings significantly differed between rings isolated from rats ( $77.73\% \pm 3.22\%$ ) and GP ( $41.81\% \pm 2.53\%$ ).

Vasodilatory activity of the intact wine was dose dependent and stronger in the aortas from the GPs than in those from the rats. Maximal relaxation ( $E_{max}$ ) for the rat and GP aortic rings was  $86.84\% \pm 2.53\%$  and  $113.87\% \pm 3.06\%$ , respectively (Figs. 1, 2). In general, thermal treatment of the intact wine did not largely affect its overall direct vasodilatory activity in both rat and GP aortic rings. Wine heated at 75°C caused maximal relaxation  $E_{max}$  of  $87.16\% \pm 2.70\%$  in the rats and  $112.72\% \pm 2.14\%$  in the GP aorta, whereas wine heated at 125°C caused maximal relaxation  $E_{max}$  of  $83.70\% \pm 2.19\%$  in the rats and  $113.17\% \pm 3.78\%$  in the GP aorta.

**TABLE 1.** Effects of Thermal Treatment and Dealcoholization on Wine Sample Volume, Content of Ethanol, Total Phenolic Content, Individual Phenolic Fractions, Resveratrol Content, and Antioxidant Capacity

Measured Parameter	Intact Wine	Heating at 75°C	Heating at 125°C	Dealcoholized Wine
Volume after 45 min of treatment (mL)	/	140 ± 1*	88 ± 1*	85 ± 3*
Ethanol volume (%)	12.9 ± 0.1	10.4 ± 0.4*	0.7 ± 0.3*	0.6 ± 0.2*
Total phenolics (mg GAE/L)	3200 ± 38	3390 ± 42	5530 ± 61*	5690 ± 51*
Flavonoids (mg GAE/L)	2940 ± 32	3110 ± 24	5200 ± 29*	5280 ± 42*
Nonflavonoids (mg GAE/L)	263 ± 3	278 ± 3	334 ± 3*	413 ± 4*
Catechins (mM CE)	727 ± 10	746 ± 12	830 ± 9*	919 ± 17*
Anthocyanins (mg M-3-G/L)	240 ± 9	247 ± 7	338 ± 9*	398 ± 7*
Resveratrol (mg/L)	2.0 ± 0.1	1.9 ± 0.2	1.0 ± 0.1*	2.9 ± 0.1*
Ferric-reducing antioxidant power (mM TE)	17 ± 1	18 ± 1	24 ± 1*	27 ± 1*

Initial volume before treatment of all wine samples was 150 mL.  
 Data are averages of at least 3 independent samples and are shown as mean ± SEM.  
 Mann-Whitney U test.  
 \*P < 0.05 vs. intact wine.  
 CE, catechin equivalent; M-3-G, malvidin-3-glucoside; TE, trolox equivalent.

Finally, the dealcoholized wine induced the highest, although nonsignificant, maximal vasodilatory response in comparison with the intact and thermally treated wines, yielding  $E_{max}$  of  $89.22\% \pm 3.11\%$  and  $118.58\% \pm 2.83\%$  in the rat and the GP aortic rings, respectively. At the lowest concentrations (up to 1‰), however, dealcoholized wine produced significantly greater relaxation in both rat and GP aortic rings as shown in Figures 1 and 2.

Similarity in the overall vasodilating activity of all tested wine samples is also revealed by the similar vasodilating potency, as indicated by the respective  $EC_{50}$  values (Table 2). The order of the vasodilatory potency considering  $EC_{50}$  in rat aortic rings was as follows: dealcoholized wine > wine thermally treated at 125°C > intact wine = wine thermally treated at 75°C. In the GP aortic rings, both thermally treated samples were equally potent vasodilators as their heating-free controls: dealcoholized wine = wine thermally treated at 125°C > intact wine = wine thermally treated at 75°C.

**DISCUSSION**

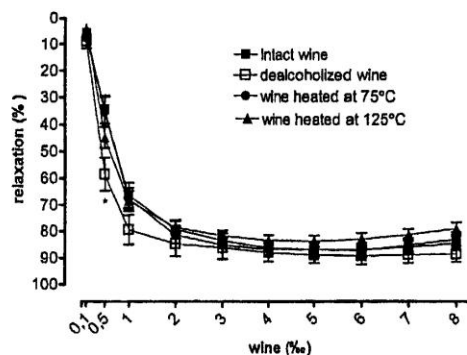
In this report, we demonstrate that thermal stress, applicable to the heating conditions in real life, does not largely interfere with the direct vasodilatory effects of the wine. This is the first study examining the biological effects of thermally treated wine in the cardiovascular system, and besides our recent study on antibacterial effects of thermally treated wine,<sup>12</sup> it offers a new approach to the investigation of the biological effects of wine.

It is somewhat surprising that investigation of the biological effects of thermally treated wine has not attracted scientific attention despite the common practice in everyday life where thermally treated wine is consumed as a warm beverage or used in the kitchen in food preparation.

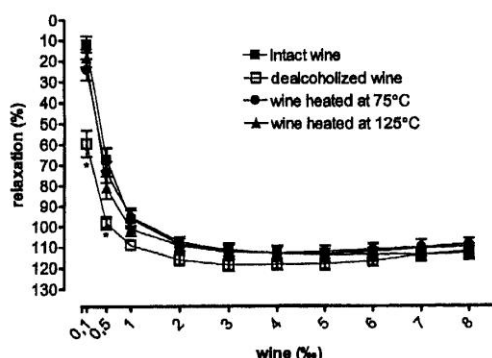
As described earlier, the physical-chemical composition of wine significantly changes, depending on the heating conditions. The most obvious and easily detectible heat-induced changes of the wine samples were reduction in their volume and ethanol content. This was associated with the

increase in the total phenolic concentration and corresponding increase in the antioxidant activity of the samples (Table 1), as wine phenolics are principal wine constituents responsible for its antioxidant activity in vitro.<sup>1,13,14</sup>

More subtle and complex heat-induced changes may occur with the wine phenolics itself, as indicated by changes in individual phenolic fraction and resveratrol content in the thermally treated samples (Table 1). However, the studies on kinetics of thermal degradation of different phenolic compounds with corresponding dynamic changes in their antioxidant activity indicate that heat-induced interactions of polyphenols and pathways of their degradation are poorly understood and hard to predict, even in simple phenolic mixtures.<sup>6,15-17</sup> Therefore, it would be very speculative and beyond the scope of this study to discuss the effects of thermal treatment on the phenolic compounds of a complex solution



**FIGURE 1.** Relaxation in the NE-precontracted rat aortic rings after exposure to the intact wine, dealcoholized wine, wine heated at 75°C and wine heated at 125°C (n = 21 per wine sample). Results are shown as mean ± SEM. \*P < 0.05 for dealcoholized wine versus intact wine, and wines heated at 75 and 125°C (2-way analysis of variance, Bonferroni post hoc test).



**FIGURE 2.** Relaxation in the NE-precontracted GP aortic rings after exposure to the intact wine, dealcoholized wine, wine heated at 75°C, and wine heated at 125°C ( $n = 23$  per wine sample). The results are shown as mean  $\pm$  SEM. \* $p < 0.05$  for dealcoholized wine versus intact wine, and wines heated at 75 and 125°C (2-way analysis of variance, Bonferroni post hoc test).

such as wine. Also, it is important to distinguish the acute from long-term effects of wine heating. Namely, if the wine is accidentally exposed to higher temperatures for several hours (usually somewhere along the distribution line), it may become spoiled or “cooked” with the production of off-flavors and premature browning resulting from oxidation and other undesirable reactions whose rates dramatically increase at the higher temperatures. On the other hand, controlled heating is a standard enological practice used to treat wines to destroy microorganisms, for stabilization of white wines or for the prevention of certain types of colloidal precipitation.<sup>18</sup>

Whatever acute thermal stress-related changes in the wine have occurred, its vasodilatory activity was highly preserved, and this is the key finding of this study.

Similar to the antioxidant activity, the direct vasodilatory effects of wines also highly correlate with their phenolic content.<sup>19,20</sup> Among different classes of wine phenolics, anthocyanins were shown to have a major influence on the vasodilating potential of wines.<sup>1</sup>

**TABLE 2.** Effects of Thermal Treatment and Dealcoholization on  $EC_{50}$  Values of the Tested Wine Samples

Wine Sample	$EC_{50}$ (CI)	
	RAT Aorta	GP Aorta
Intact wine	-3.19 (-3.24 to -3.14)	-3.33 (-3.38 to -3.27)
Dealcoholized wine	-3.38 (-3.47 to -3.30)	-3.41 (-3.51 to -3.30)
Heating at 75°C	-3.19 (-3.24 to -3.14)	-3.33 (-3.39 to -3.27)
Heating at 125°C	-3.30 (-3.35 to -3.25)	-3.41 (-3.49 to -3.33)

$EC_{50}$  was calculated using nonlinear regression analysis.  $EC_{50}$  values are log of dilution giving 50% relaxation relative to the sample's own maximal relaxation. 0.1% = 0.0001  $\approx$  log 0.0001 = -4; 0.5% = 0.0005  $\approx$  log 0.0005 = -3.30.  $n = 21$  per wine sample in rat aorta;  $n = 23$  per wine sample in GP aorta. CI, 95% confidence interval.

In vitro studies revealed several mechanisms of vascular function that are modulated by red wine and its polyphenols: vasodilatation of isolated vessels through increased production of nitric oxide (NO)<sup>2,21-23</sup>; enhancement of NO bioactivity by antioxidant action,<sup>24</sup> elevation of endothelial NO synthase protein expression,<sup>25</sup> and reduction of endothelin-1 synthesis.<sup>26</sup>

Thermally treated wine samples and their thermal stress-free controls showed a similar vasodilating pattern, and their maximal effects did not differ. However, the GP aorta showed higher reactivity to the wine than the rat aorta did (Figs. 1, 2), confirming our previous results that different pathways are involved in the wine-induced vasorelaxation between the 2 species and that extrapolation of the results from one species to another can be misleading.<sup>7</sup> The differences in the sensitivity to the wine are further indicated by lower  $EC_{50}$  values in the GP aorta relative to the corresponding  $EC_{50}$  in the rats (Table 2).

In both species, however, at the lowest concentrations up to 1‰ dealcoholized wine without thermal stress caused significantly stronger vasodilation in comparison with that of the intact wine and wines heated at 75 and 125°C (Figs. 1, 2). This finding can be explained with the heat-induced partial loss in phenolic fractions in the wine treated at 125°C. On the other hand, similarity of the intact wine and wine treated at 75°C indicates that thermal stress at this temperature is not sufficient to compromise vasodilating potency and effectiveness of the wine despite distinct changes in its physical-chemical properties.

The role of resveratrol could not be clearly determined, but it is not likely to play a significant role in the vasodilatory effects observed. The concentration of resveratrol in the intact wine we used is rather low (2 mg/L or  $9 \times 10^{-6}$  M) yielding after dilution in the organ baths concentrations in the range of  $10^{-9}$  M, which is an order of magnitude lower than in the studies examining dilatory effects of resveratrol in vitro.<sup>8-10</sup> Nonetheless, based on our previous findings and findings of other authors, the complex biological effect of mixed solutions, such as wine, cannot be attributed to a single phenolic compound.<sup>3,27</sup> In contrast, the application of a blend of polyphenolic compounds causes synergism in different biological systems, including maximal induction of endothelial NO synthase in human endothelial cells.<sup>28,29</sup>

In conclusion, this study indicates that wine thermally treated under heating conditions applicable to the preparation of a mulled wine and wine used for cooking, largely retains vasodilatory activity in vitro despite significant heat-induced changes in its composition. These results justify the need for examining cardiovascular effects of thermally treated wines in human subjects.

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#### 4. ZNANSTVENI DOPRINOS OBJEDINJENIH RADOVA

Znanstveni je doprinos opisanih istraživanja višestruk. Prvi se put istražio biološki učinak termički obrađenog vina na krvnim žilama. Utvrđeno je kako fizikalno-kemijske promjene vina uslijed zagrijavanja usporedivog s temperaturnim uvjetima pripremanja hrane u svakodnevnom životu utječe na vazodilatacijsku aktivnost. S obzirom da vazodilatacijski i antioksidacijski učinak doprinose prevenciji ishemijske bolesti srca, bolesti s najvećim morbiditetom i mortalitetom u svijetu, dobiveni rezultati mogu biti značajni s epidemiološkog aspekta, budući da je konzumacija intaktnog i kuhanog vina, te primjena vina u kuhanju, vrlo česta praksa u svijetu. Prvi su put također uspoređeni učinci crnog vina s kupinovima čija je proizvodnja i konzumacija u Hrvatskoj sve popularnija.

Dizajn istraživanja u kojem su određeni i uspoređivani biološki učinci crnog, bijelog i kupinovog vina, te fenolnih kiselina, omogućio je i odgovore na pitanja relativnog doprinosa pojedinih polifenolnih frakcija i spojeva vazodilatacijskom i antioksidacijskom učinku, s posebnim osvrtom na ulogu rezveratrola.

Sve više istraživanja pokazuje da je sinergističko djelovanje različitih polifenola ključno za ostvarivanje učinka složene otopine kao što je vino. Opisani rezultati o učincima fenolnih kiselina predstavljaju dodatni argument ove spoznaje.

Opisanim je istraživanjem utvrđeno na koji su način povezani vazodilatacijski i antioksidacijski učinak. Osim zdravstvenog značaja, ovo je važno i stoga što se u prehrambenoj tehnologiji daje veliki značaj upravo antioksidacijskoj aktivnosti namirnica, iako je često nejasno koji je značaj tog učinka nakon unosa namirnica u organizam.

Opisana su istraživanja u skladu sa svjetskim trendovima u smislu etnofarmakološkog pristupa istraživanjima zdrave hrane, a ostvareni rezultati predstavljaju poticaj kulturi zdrave prehrane te proizvodnji zdrave hrane.

## 5. ŽIVOTOPIS



Rođena sam 9. veljače 1976. godine. u Livnu, Bosna i Hercegovina. 1994. godine upisala sam studij medicine na Medicinskom fakultetu Sveučilišta u Zagrebu. 2000. godine sam diplomirala s prosječnom ocjenom 4.49.

Pripravnički staž odradila sam pri KBC Split te sam 2002. položila stručni ispit za doktora medicine.

Od 2001. radim na Katedri za farmakologiju Medicinskog fakulteta Sveučilišta u Splitu kao znanstveni novak, a potom kao asistent.

2001. upisala sam poslijediplomski znanstveni studij „*Temeljne i kliničke medicinske znanosti*” pri Medicinskom fakultetu u Splitu.

Tijekom 2002. i 2005. radi stručnog usavršavanja boravila sam na Institutu za farmakologiju i eksperimentalnu toksikologiju Medicinskog fakulteta Sveučilišta u Ljubljani.

Autor sam 13 znanstvenih članaka u časopisima koji su indeksirani u *Current Contentsu*, 2 članka u *Science Citation Index - Expandedu*, te 12 kongresnih priopćenja na domaćim i međunarodnim znanstvenim skupovima. Član sam Hrvatske liječničke komore i Hrvatskog društva farmakologa. Udana sam i majka troje djece.

#### Iskustvo rada u nastavi

-demonstrator na Zavodu za patofiziologiju Medicinskog fakulteta Sveučilišta u Zagrebu (1998.–1999.)

-Katedra za farmakologiju, studij medicine (2001.– danas)

-Katedra za farmakologiju, stručni studij sestrinstva (2005.– danas)

-Katedra za farmakologiju, studij dentalne medicine (2007.– danas)

#### Sudjelovanje na projektima

-Korisni učinci ekstrakta vina iz dalmatinskih autohtonih sorti grožđa na srce i krvne žile (TP-01/0216-05), suradnik na projektu (2001.– 2005.)

-Izvantjelesno očuvanje srca, znanstveno-istraživački projekt (MZOŠ 0216012), znanstveni novak (2002.– 2005.)

-Farmakologija kardiovaskularnog sustava, projekt u sklopu hrvatsko – slovenskog bilateralnog programa suradnje u području znanosti i tehnologije, istraživač (2003.– 2008.)

-Kardiovaskularni učinci vina i njegovih sastojaka (MZOŠ 216-2160547-0537), istraživač (2007.– danas)

-Prirodni izvori rezveratrola i njegov sinergijski učinak s drugim polifenolima (MZOŠ 011-2160547-2226), istraživač (2007.– danas)