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Enzymatically-Modified Isoquercitrin Improves Endothelial Function in Volunteers at Risk of Cardiovascular Disease

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1 ABSTRACT

2 A higher intake of foods rich in flavonoids such as quercetin can reduce the risk of
3 cardiovascular disease. Enzymatically modified isoquercitrin (EMIQ[®]) has a bioavailability
4 17-fold higher than quercetin aglycone and has shown potential cardiovascular disease
5 moderating effects in animal studies. The present study aimed to determine if acute ingestion
6 of EMIQ[®] improves endothelial function, blood pressure, and cognitive function in human
7 volunteers at risk of cardiovascular disease. Twenty-five participants (12 males, 13 females)
8 with at least one cardiovascular disease risk factor completed this randomized, controlled,
9 crossover study. In a random order, participants were given EMIQ[®] (2 mg aglycone
10 equivalent)/kg body weight or placebo alongside a standard breakfast meal. Endothelial
11 function, assessed by flow mediated dilatation (FMD) of the brachial artery was measured
12 before and 1.5 hrs after intervention. Blood pressure (BP), arterial stiffness, cognitive
13 function, BP during cognitive stress and measures of quercetin metabolites, oxidative stress
14 and markers of nitric oxide (NO) production were assessed post-intervention. After
15 adjustment for pre-treatment measurements and treatment order, EMIQ[®] treatment resulted in
16 a significantly higher FMD response compared to the placebo [0.60%, 95% CI: 0.03, 1.17
17 (p=0.04)]. Plasma concentrations of quercetin metabolites were significantly higher
18 (p<0.001) after EMIQ[®] treatment compared to the placebo. No changes in blood pressure,
19 arterial stiffness, cognitive function, or biochemical parameters were observed. In this
20 human intervention study, the acute administration of EMIQ[®] significantly increased
21 circulating quercetin metabolites and improved endothelial function. Further clinical trials are
22 required to assess whether health benefits are associated with long-term EMIQ[®]
23 consumption.

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30 INTRODUCTION:

31 There is mounting evidence from both cohort studies and clinical trials that an increased
32 intake of plant foods, specifically those rich in flavonoids, can reduce the risk of
33 cardiovascular disease ⁽¹⁾. Quercetin (in its glycosylated form) is the most commonly
34 consumed flavonoid compound within the flavonol subclass ⁽²⁾. The main dietary contributors
35 to quercetin intake include tea, apples, onions, and broccoli ⁽³⁾.

36 Evidence for the health promoting effects of quercetin comes primarily from animal and *in*
37 *vitro* studies ⁽⁴⁾. In a randomized-controlled cross-over study of men and women, apples with
38 skin significantly reduced systolic blood pressure, improved endothelial function and
39 increased plasma nitric oxide (NO) ⁽⁵⁾. These apples with skin provided 184 mg of quercetin
40 and 180 mg of (-)-epicatechin while the apple flesh only (control) provided less than 5 mg of
41 quercetin and (-)-epicatechin. Previous studies using both pure quercetin aglycone (1095 mg)
42 ⁽⁶⁾ and quercetin glucoside supplementation (50 – 400 mg) ^(7; 8) have shown no effect on
43 endothelial function. This may be due to in part to the bioavailability of the quercetin
44 interventions.

45 Quercetin in foods is usually found in its glycosylated form, with the presence and position of
46 the sugar moiety influencing the site and extent to which it is absorbed ⁽⁹⁾. The bioavailability
47 of quercetin glucosides can be enhanced by enzymatic α -oligoglucosylation of this sugar
48 moiety ⁽¹⁰⁾. Enzymatically modified isoquercitrin (EMIQ[®]) is a water-soluble glucoside of
49 quercetin produced by the deglycosylation and subsequent α -oligoglucosylation of rutin ⁽¹⁰⁾.
50 Plasma concentrations of quercetin metabolites have been shown to be approximately 2-3
51 times higher after the consumption of EMIQ[®] in comparison to isoquercitrin (quercetin-3-*O*-
52 glucoside) and its bioavailability is 17-fold higher than that of quercetin ^(10; 11).

53 EMIQ[®] has been shown to suppress the increase in systolic BP observed in spontaneously
54 hypertensive rats, ⁽¹²⁾ and decrease oxidative stress in a mouse model of atherosclerosis ⁽¹³⁾.
55 EMIQ[®] is approved in Japan as a food additive and is safe for human consumption. Human
56 trials carried out over a 12-week period indicate a significant reduction in body fat ⁽¹⁴⁾. The
57 effects of EMIQ[®] on vascular and cognitive function have yet to be investigated.

58 Therefore, the aim of the present study was to determine if acute ingestion of EMIQ[®]
59 improves endothelial function, blood pressure, and cognitive function in human volunteers at
60 risk of cardiovascular disease.

61 **SUBJECTS AND METHODS:**

62 Participants

63 Twenty-five eligible men and women, aged between 50 and 70 years, were recruited by
64 newspaper advertisement from the general population of Perth, Western Australia (**Figure 1**).
65 These volunteers attended a screening appointment within the University of Western
66 Australia, School of Biomedical Sciences, located in the Medical Research Foundation
67 building at Royal Perth Hospital. The screening appointment consisted of an
68 electrocardiograph, a standard medical history questionnaire, and the measurement of height,
69 weight, body mass index (BMI) and BP. Finally, a blood sample was taken for measurement
70 of fasting serum total cholesterol, HDL-cholesterol (HDL-C), triglycerides (TG), LDL-
71 cholesterol (LDL-C), and glucose. Volunteers were included in the study if they had at least
72 one of the following risk factors for CVD: systolic BP between 120 mmHg and 160 mmHg,
73 fasting plasma glucose between 5.6 mM and 6.5 mM, total cholesterol between 5 mM and 8
74 mM or a waist circumference >94 cm for men or >80 cm for women. Exclusion criteria
75 included a BMI <18 or >35 kg/m²; a systolic BP ≤100 or ≥160 mmHg; a diastolic BP ≤50 or
76 ≥90 mmHg; diagnosed diabetes; fasting plasma glucose concentrations ≥6.5 mmol/L; use of
77 BP lowering or cholesterol lowering medication; alcohol intake >210 g per week for women
78 and >280 g per week for men; current or recent (within previous 6 months) significant weight
79 loss or gain (>6% of body weight); actively trying to lose weight; current or recent (<12
80 months) smoking; history of cardiovascular or peripheral vascular disease; psychiatric or any
81 other major illnesses; and women were lactating, pregnant or wishing to become pregnant
82 during the study.

83 The study was carried out in accordance with the Declaration of Helsinki and was approved
84 by the University of Western Australia Human Research Ethics Committee (Approval
85 number RA/4/1/9260). All participants provided written informed consent before inclusion in
86 the study. The trial was registered with the Australian New Zealand Clinical Trials Registry
87 (ACTRN12617001202358).

88 Study design

89 This acute, randomized, controlled crossover trial was conducted between September 2017
90 and June 2018. Participants were required to visit the department three times (one practice
91 visit and two study visits). The practice visit involved the completion of a selection of

92 cognitive function tests designed to induce cognitive stress (see description below) three
93 times, in order to control for practice effects and to allow familiarization with procedure. The
94 practice day data were not included in any analyses. Participants were required to fast for 12
95 hrs (water allowed *ad libitum*) prior to each subsequent study visit. These two visits were
96 separated by at least 7 days to ensure that there were no carry-over treatment effects.
97 Participants were instructed to have the same meal the night before, not to consume any
98 alcohol for 24 hrs prior to the visit and not to take any medication or undergo any exercise on
99 the morning of their visit. Endothelial function was assessed before and 1.5 hrs post-
100 intervention. A blood pressure measurement was taken prior to and every 10 min for 80 min
101 post treatment. Measurements of arterial stiffness were taken 2 hrs post-intervention.
102 Participants completed the selection of cognitive function tests 2.5 hrs post-intervention,
103 during which blood pressure was measured every 2 min. Finally, a blood sample was taken
104 by venipuncture 3.5 hrs post-intervention.

105 Intervention

106 Participants were randomly assigned to one of two treatment orders generated using a
107 pseudorandom number generator (<https://www.randomizer.org/>). The EMIQ[®] was prepared
108 by San-Ei Gen F.F.I., Incorporated (Osaka, Japan). The active treatment consisted of 4.89 mg
109 EMIQ[®] (2 mg aglycone equivalent)/kg body weight ⁽¹⁰⁾ plus ½ teaspoon of maltodextrin and
110 1½ tablespoons of Cottee's Raspberry flavoured cordial mixed with 250 mL water. The
111 placebo treatment was ½ teaspoon of maltodextrin and 1½ tablespoons of cordial mixed with
112 250 mL water. The cordial was used to mask the taste and the colour of the EMIQ[®]. Both
113 treatments were given with a standardised breakfast of 2 pieces of white bread and cheese.
114 Participants and all researchers performing the tests and analysing the results were blinded to
115 the treatment.

116 Assessment of endothelial function

117 In order to assess endothelial function, flow-mediated dilatation (FMD) of the brachial artery
118 was calculated by a trained ultrasonographer, dedicated to the research protocol and blinded
119 to the interventions used. Participants were studied in a quiet, temperature-controlled room
120 (21 to 25 °C). Participants rested in a supine position for 20 min prior to the initiation of the
121 FMD measurement. The right arm was extended and supported comfortably on a foam mat.
122 For the ultrasound, a 12-MHz transducer connected to a Philips CX50 Ultrasound Machine

123 was clamped in position over the brachial artery, 5 to 10 cm proximal to the antecubital
124 crease. After a baseline artery diameter recording of 1 min, a blood pressure cuff placed
125 around the left forearm was inflated to 200 mmHg. After 5 min the cuff was released,
126 inducing reactive hyperaemia. The brachial artery image was recorded for 4 min (240
127 seconds) post-cuff deflation to assess FMD. Images were downloaded for retrospective
128 analysis. Analysis of FMD was performed using a semi-automated edge detection software
129 (Vascular Research Tools, Medical Imaging Applications LLC, Coralville IA), which
130 automatically calculates the brachial artery diameter, corresponding to the internal diameter.
131 Responses were calculated as the percentage change in brachial artery diameter from
132 baseline, at 10 second intervals, for 240 seconds after cuff deflation.

133 Measurement of blood pressure and arterial stiffness

134 Blood pressure measurements were taken using a Dinamap 1846SX/P oscillometric recorder
135 (Critikon, Tampa, FL, USA) attached to the non-dominant arm with participants in a seated
136 position. Following a 10 min rest, one BP measurement was taken immediately prior to
137 treatment consumption. BP was then measured every 10 min post-treatment consumption for
138 80 min (9 measurements in total).

139 Office BP, central diastolic BP, and arterial stiffness were assessed using the SphygmoCor
140 Xcel (AtCor Medical, Sydney, Australia). Following a 5 min rest in a supine position, BP
141 measurements were taken on three occasions at 1 min intervals, the first measurement was
142 discarded and the second and third measurements were averaged. Arterial stiffness was
143 determined by measuring augmentation Index (AIx) using the Sphygmocor Xcel as described
144 previously⁽¹⁵⁾ and was standardised to a heart rate of 75 bpm (AIx75).

145 Cognitive stress test and blood pressure

146 A selection of cognitive function tests designed to induce cognitive stress using the
147 Computerised Mental Performance Assessment System (COMPASS, BPNRC, Newcastle
148 Upon Tyne, UK) was used. This series of tests, described in detail previously⁽¹⁶⁾, comprised
149 of two computerized serial subtraction tasks (Serial Threes and Serial Sevens) and a Bakan
150 Rapid Visual Information Processing task (RVIP), each repeated three times. Briefly, for the
151 Serial Three and Serial Seven subtraction tasks, participants were asked to continuously
152 subtract three or seven from a random starting number between 800 and 999. This task lasted
153 for two min and both the number of correct responses and the total number of subtractions

154 completed were recorded. For the RVIP task, participants were asked to identify when three
155 odd or three even digits appeared consecutively in series of single digit numbers presented at
156 a rate of 100 per min. This task lasted for five min and both the number of correct responses
157 and the average response time were recorded. Participants rested for 20 min prior to and 15
158 min after the cognitive testing, which took approximately 30 min. For the duration of this
159 task (65 min total), blood pressure was measured every 2 min with a Dinamap 1846SX/P
160 oscillometric recorder (Critikon, Tampa, FL, USA) attached to the non-dominant arm of
161 participants in a seated position.

162 Plasma analyses

163 Plasma samples, obtained 3.5 hrs post-intervention by venepuncture, were collected into
164 EDTA tubes with added butylated hydroxytoluene and immediately centrifuged at 5000 x g,
165 at 4°C for 5 min. One 2 mL aliquot of plasma was kept on liquid nitrogen for immediate
166 analysis of nitrogen oxides (NO_x) and the remaining plasma was stored at -80°C until
167 analysis.

168 *Quercetin metabolites*

169 Plasma quercetin metabolites were measured by liquid chromatography/tandem mass
170 spectrometry (LCMSMS) as described previously ⁽⁷⁾. Briefly, plasma (250 µL) collected in
171 EDTA was incubated for 2 hrs with β-glucuronidase enzyme. As β-glucuronidase has both
172 glucuronidase and sulfatase activity, but is unable to enzymatically cleave methyl conjugates,
173 isorhamnetin (O-methylated quercetin) was measured. Following solid phase extraction,
174 quercetin aglycone and isorhamnetin were measured on a Thermo Scientific TSQ Quantum
175 Ultra Triple Quadrupole LCMS System (ThermoFisher Scientific, Waltham, MA, USA).
176 Calibration curves for quercetin and isorhamnetin with the fisetin as the internal standard
177 were used for quantification.

178 *Measurement of plasma total nitrogen oxides (NO_x)*

179 The concentrations of total NO_x (nitric oxide, nitros(yl)ated species and nitrite) in plasma
180 were determined using a previously described gas phase chemiluminescence assay ⁽¹⁷⁾. Blood
181 was collected into N-ethylmaleimide (10 mmol/L) and EDTA (2 mM), mixed and centrifuged
182 at 3000 x g (5 min, 4°C). Fresh plasma was kept on ice in the dark and analysed within 1 hr.
183 Antifoam (200 µL) was added prior to injection into the radical purger containing potassium

184 iodide (0.125 g) and iodine (0.05 g) in water (2.5 mL) / glacial acetic acid (7.5 mL) at room
185 temperature. Plasma NO_x was quantified by the NO signal peak area of samples against a
186 nitrite standard (300 µL, 0.5 µM NaNO₂⁻). Quantification of NO released by the redox
187 reactions occurred by its chemiluminescence reaction with ozone using the Nitric Oxide
188 Analyzer (CLD66, Eco Physics, Sweden).

189 *NO₂ and NO₃*

190 Nitrate (NO₃) and nitrite (NO₂) concentrations were measured using gas
191 chromatography/mass spectrometry (GCMS) as described previously ⁽¹⁸⁾, with N¹⁵ labelled
192 NO₃ and NO₂ internal standards.

193 *Glucose*

194 Plasma glucose was measured using a fully automated analyser (Architect ci8200/c16000
195 Analyser).

196 *F₂-isoprostanes*

197 Systemic oxidative stress was determined by measuring plasma F₂-isoprostane concentrations
198 as described previously ⁽¹⁹⁾. Briefly, F₂-isoprostane concentrations were calculated in pmol/L
199 from ratio of the peak areas for the *m/z* 569 and *m/z* 573 ions, with a deuterium labelled
200 internal standard, 8-iso-Prostaglandin F_{2α}-d₄.

201 Statistics

202 Sample size was calculated with FMD as the primary endpoint. Assuming a within-subject
203 correlation of $r=0.25$, 24 pre-intervention measures, 24 post-intervention measures, and a
204 within group SD of 6%, a sample size of 25 subjects would provide more than 80% power at
205 $\alpha=0.05$ to detect a difference of 0.85% in FMD. Treatment effects for post-intervention
206 FMD were obtained using linear mixed models with adjustment for treatment order, the pre-
207 treatment FMD curve, and the time since cuff release with time included as a categorical
208 variable (0 to 240 seconds at 10 second intervals). In order to allow the treatment effect to
209 differ over time, we also included a treatment x time interaction term. The subject identifier
210 was included as a random intercept. Assessment of post-treatment BP was similar to that for
211 FMD, with time included as a categorical variable (0 to 80 minutes at 10-minute intervals).
212 When assessing the effect of EMIQ[®] on the BP during the cognitive stress test, we

213 additionally adjusted for the rest-period BP. For all other outcomes, treatment effects were
214 obtained using linear mixed models with adjustment for treatment order. Statistical analyses
215 were performed using STATA/IC 14.2 (StataCorp LLC).

216

217 **RESULTS:**

218 Baseline data

219 Recruitment began on 25th September 2017 and the study concluded on 12th June 2018.
220 Twenty-five participants (12 males, 13 females) completed the study (**Figure 1**). The baseline
221 characteristics of the study participants are shown in **Table 1**.

222 Endothelial function

223 Data from one participant was excluded prior to statistical analysis as the clarity of the FMD
224 image was poor. The observed mean percentages of FMD for 24 participants, between 0 and
225 240 seconds at 1.5 hrs post-intervention, are presented in **Figure 2**. Overall, FMD after the
226 EMIQ[®] treatment was significantly higher when compared to control [adjusted mean
227 difference=0.60%, 95% CI: 0.03, 1.17; p=0.040].

228 Blood pressure and arterial stiffness

229 Mean systolic BP and diastolic BP, measured immediately before treatment (time=0) and
230 then every 10 min for 80 min, are shown in **Figure 3**. There was no significant difference
231 between EMIQ[®] and placebo for post-treatment systolic BP (p=0.916) or diastolic BP
232 (p=0.073). Similarly, there was no difference for MAP (p=0.188) or heart rate (p=0.290).
233 There was no significant difference between treatments for mean systolic BP, diastolic BP,
234 central diastolic pressure, MAP, heart rate, or AIx75, measured 2 hrs post-treatment (**Table**
235 **2**).

236 Cognitive stress test and blood pressure

237 There were no significant effects associated with the EMIQ[®] treatment on any of the
238 cognitive stress test outcomes, nor on BP measurements taken during the cognitive stress
239 tests (**Tables 3 and 4**).

240 Plasma analyses

241 Plasma concentrations of quercetin aglycone and isorhamnetin were significantly higher
242 (p<0.001) after the ingestion of EMIQ[®] compared to the placebo (**Table 3**). There was no
243 significant difference in plasma glucose, NO_x, NO₂, NO₃, or F₂-isoprotanes between EMIQ[®]
244 and placebo treatments.

245

246 **DISCUSSION:**

247 This is the first study to investigate the acute effects of an enzymatically-modified
248 isoquercitrin compound on measures of vascular health, blood pressure and cognitive
249 function in humans. In this randomised controlled cross-over study, the acute administration
250 of EMIQ[®] significantly increased plasma quercetin metabolites and improved endothelial
251 function. No changes in blood pressure, arterial stiffness, cognitive function, blood pressure
252 during cognitive stress or biochemical parameters were observed.

253 In the present study plasma concentrations of quercetin aglycone and isorhamnetin were
254 significantly higher after the ingestion of EMIQ[®] compared to the placebo. Previously, the
255 plasma concentration of conjugated quercetin metabolites has been shown to reach a maximal
256 level at approximately 1.5 h after intake of EMIQ[®] ⁽¹⁰⁾. For this reason, our primary outcome,
257 endothelial function, was assessed 1.5 hrs post-intervention.

258 Endothelial dysfunction, defined as an impairment in endothelium-dependent relaxation, is
259 implicated in prehypertension, hypertension, atherosclerosis, and stroke ^(20; 21), and is
260 associated with an increased risk of cardiovascular disease ^(22; 23). The results of the present
261 study suggest that EMIQ[®] improves postprandial endothelial function. In two previous
262 studies, acute quercetin administration (1095 mg quercetin aglycone ⁽⁶⁾; and doses of
263 quercetin-3-*O*-glucoside ranging from 50 - 400 mg ⁽⁷⁾) had no effect on FMD. In the study by
264 Larson *et al*, FMD was measured 10 hrs post-quercetin aglycone administration, the time at
265 which quercetin metabolites were shown to peak in the plasma ⁽⁶⁾. The pharmacokinetics of
266 quercetin aglycones and EMIQ are very different; in the present study FMD was measured
267 1.5 hrs post-intervention, at the time quercetin metabolites have been shown to peak in the
268 plasma following EMIQ administration ⁽¹⁰⁾. Quercetin-3-*O*-glucoside has been shown to peak
269 in the plasma between 1.5 and 3 hrs post-intervention; in our previous study ⁽⁷⁾, FMD was

270 measured 1 hr post-intervention. The differences in study design and the form in which the
271 quercetin was administered make the direct comparison to the present study difficult. Another
272 important discrepancy in study design is that EMIQ was administered alongside a meal in the
273 present study, likely influencing the effect of quercetin on endothelial function ^(24; 25). Overall,
274 FMD in the present study was relatively poor as we recruited participants with at least one
275 risk factor for cardiovascular disease. Although the improvement observed was subtle
276 (0.66%), if sustained it could have important clinical implications as a 1% increase in FMD is
277 associated with a 13% lower risk of cardiovascular events (RR: 0.87, 95% CI: 0.83- 0.91) ⁽²³⁾.

278 Evidence suggests that quercetin can improve endothelial health through NO-mediated
279 vasorelaxant activity as well as through the prevention of oxidant induced endothelial
280 dysfunction ⁽⁴⁾. An acute increase in plasma NO status has been observed after both a high-
281 flavonoid apple intervention ⁽¹⁷⁾ and quercetin (200 mg) intervention ⁽²⁶⁾. In the present study
282 we did not observe any increase in plasma NO_x, or the end products of NO metabolism, NO₃
283 and NO₂. This may have been due to the timing of our blood sample, which was 1.5 hrs after
284 the measurement of endothelial function, and 3.5 hrs post-intervention. Changes in NO are
285 likely to be transient, and because of the difficulty in detecting small changes in NO, the
286 timing of the measurement is likely to be critical.

287 In our previous randomized clinical trial with healthy men and women, improvements in BP
288 were also observed with the concomitant increase in nitric oxide status and FMD, after intake
289 of flavonoid-rich apples ⁽¹⁷⁾. In a meta-analysis of randomized controlled trials, significant
290 reductions in both systolic BP (-3.04 mm Hg, 95% CI: -5.75, -0.33, p=0.028) and diastolic
291 BP (-2.63 mm Hg, 95% CI: -3.26, -2.01, p<0.001) were seen following quercetin
292 supplementation ⁽²⁷⁾. Furthermore, in spontaneously hypertensive rats, EMIQ[®] administered
293 at a dose of 26 mg/kg/d suppressed the increase in systolic BP more effectively than
294 quercetin aglycone ⁽¹²⁾. In the present study we observed no effect of EMIQ[®] on post-
295 treatment BP nor on BP during the cognitive stress test. This lack of effect may be due to the
296 dose given; in the aforementioned meta-analysis there was only a significant effect on BP
297 when quercetin was administered at doses greater than 500 mg/day ⁽²⁷⁾. Additionally, the
298 health status of the participants may have played a role as a decrease in systolic BP following
299 quercetin supplementation is generally seen in hypertensive individuals ^(28; 29; 30), not in pre-
300 hypertensive or normotensive individuals ^(6; 30; 31; 32; 33; 34).

301 Many functions of the endothelium are affected under oxidative stress, including endothelial
302 cell apoptosis ⁽³⁵⁾ and adhesion of inflammatory cells ⁽³⁶⁾, initiating the development of
303 atherosclerosis. Quercetin may decrease oxidative stress through the stimulation of protective
304 defences and repair systems ⁽³⁷⁾. In apolipoprotein E (apoE)–deficient atherogenic mice,
305 EMIQ[®] supplementation for 14 weeks significantly suppressed aortic and aortic sinus
306 atherosclerotic lesion areas and decreased levels of 4-hydroxy-2-nonenal, a marker of
307 oxidative stress ⁽¹³⁾. In the present study we observed no acute changes in levels of plasma F₂-
308 isoprostanes, an *in vivo* biomarker of oxidative stress, following the EMIQ[®] intervention. It
309 may be that long-term interventions are required to observe changes in biomarkers of
310 oxidative stress. Evidence that quercetin may impede the development of CVD by
311 moderating oxidative stress is stronger in animal studies than human studies ⁽⁴⁾.

312 There is evidence that flavonols, the flavonoid subclass to which quercetin belongs, can
313 reduce the risk of type 2 diabetes (T2D). This comes from an observational study which
314 found that each 2.5- fold increase in flavonol intake was associated with a 26% lower
315 incidence of T2D ⁽³⁸⁾, and a meta-analysis demonstrating a significant reduction in fasting
316 plasma glucose (difference in mean = -0.18 mmol/L; 95% CI: -0.29, -0.08), following
317 flavanol intake ⁽³⁹⁾. However, fasting plasma glucose was not significantly lower in any of the
318 studies with a quercetin intervention. In the present study we saw no acute effect of EMIQ[®]
319 on plasma glucose. Given that the study population was relatively normoglycemic, this may
320 be better investigated in a hyperglycemic population.

321 Evidence that quercetin can improve cognitive function comes wholly from animal studies ^{(40;}
322 ^{41; 42)}. To our knowledge this is the first study to examine the acute effects of a quercetin
323 derivative on cognitive function in humans. We saw no significant effects of EMIQ[®] on any
324 measures of cognitive function in the present study. This warrants further investigation;
325 future studies could consider a long-term intervention and a broader range of cognitive
326 function measurements.

327 Strengths of the present study are that any inter-subject variability was accounted for by our
328 cross-over study design and adjustments for baseline measurements of FMD controlled for
329 day to day variability. There are, however, several limitations: firstly, only the primary
330 outcome was measured before and after the intervention. Secondly, the study was only
331 powered to detect a change in the primary outcome meaning that we may have been
332 underpowered to detect changes in secondary outcomes. So as not to affect the measurement

333 of any other outcomes, the blood sample was taken 1.5 hrs after the measurement of FMD
334 (3.5 hrs post-intervention). Additionally, this study only investigated one dose of EMIQ[®],
335 with the primary outcome measured at only one time-point.

336 In this human intervention study, the acute administration of EMIQ[®] significantly increased
337 circulating quercetin metabolites and improved postprandial endothelial function. The
338 addition of EMIQ[®] to commercially available foods and beverages may have positive effects
339 on vascular function. The potential health benefits associated with regular consumption
340 warrants investigation in longer term randomised controlled trials.

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346 Conflicts of Interest:

347 The authors declare no conflict of interest. The funding sponsors had no role in the design of
348 the study; in the collection, analyses, or interpretation of data; in the writing of the
349 manuscript, and in the decision to publish the results.

350 Authorship:

351 NPB, CPB, JMH, NCW, and KDC were responsible for the project conception; NPB and
352 CPB conducted the research; NPB and RJW analysed the data; NPB prepared the manuscript;
353 CPB, JMH, NCW, RJW and KDC critically reviewed the manuscript.

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TABLES

Table 1. Baseline characteristics of study participants

Characteristic	Mean \pm SD
Age (years)	64.1 \pm 6.3
Weight (kg)	77.6 \pm 14.9
BMI (kg/m ²)	27.0 \pm 3.7
Systolic BP (mmHg)	128.6 \pm 15.3
Diastolic BP (mmHg)	73.6 \pm 8.4
Total cholesterol (mM)	5.6 \pm 1.0
Triglyceride (mM)	1.0 \pm 0.3
Low- density lipoprotein cholesterol (mM)	3.7 \pm 0.8
High-density lipoprotein cholesterol (mM)	1.5 \pm 0.4
Fasting plasma glucose (mM)	5.2 \pm 0.5
Creatinine (mM)	67.9 \pm 10.3
eGFR (mL/min/1.73 m ²)	86 \pm 7.2

n=25; males=12, females=13
BP, blood pressure.

Table 2. Pulse wave analysis

	EMIQ [®]	Control	p-value
Systolic BP (mmHg)	129.3 \pm 3.2	130.9 \pm 3.2	0.489
Diastolic BP (mmHg)	73.8 \pm 1.8	74.1 \pm 1.8	0.702
Central DP (mmHg)	74.3 \pm 1.8	75.0 \pm 1.8	0.518
MAP (mmHg)	88.9 \pm 2.1	89.8 \pm 2.1	0.455
HR (bpm)	55.9 \pm 1.7	57.3 \pm 1.7	0.281
AIx75 (%)	13.9 \pm 2.0	13.2 \pm 2.0	0.635

Results are presented as means \pm sd (n=25). P-values for treatment effect were obtained using linear mixed models with adjustment for treatment order. BP, blood pressure; MAP, mean arterial pressure; PR, pulse rate.

Table 3. Cognitive function measurements

	EMIQ [®]	Control	p-value
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Serial 3 subtractions total	25.1 ± 9.0	25.2 ± 9.7	0.710
Serial 3 subtractions correct	27.0 ± 8.7	26.8 ± 9.4	0.701
Serial 7 subtractions total	19.1 ± 7.1	18.6 ± 7.9	0.569
Serial 7 subtractions correct	16.3 ± 7.7	16.4 ± 7.7	0.595
RVIP correct	54.1 ± 22.5	53.5 ± 22.4	0.299
RVIP response time (msec)	559.8 ± 57.8	551.8 ± 56.9	0.210

Cognitive test measurements, 2.5 hours post consumption of EMIQ[®] or placebo treatment. Results are presented as means ± sd (n=23). P-values for treatment effect were obtained using linear mixed models with adjustment for treatment order and time.

Table 4. Blood pressure during cognitive stress testing

	EMIQ [®]	Control	p-value
Systolic BP (mmHg)	129.8 ± 18.0	125.2 ± 19.9	0.468
Diastolic BP (mmHg)	72.1 ± 10.1	73.3 ± 14.4	0.510
MAP (mmHg)	94.8 ± 12.4	93.4 ± 15.0	0.466
PR (bpm)	63.3 ± 10.1	65.8 ± 15.6	0.728

Results are presented as means ± sd (n=25). Blood pressure was measured every 2 minutes for 34 minutes (17 measures total). P-values for treatment effect were obtained using linear mixed models with adjustment for treatment order, time and blood pressure during pre-test rest period. BP, blood pressure; MAP, mean arterial pressure; PR, heart rate.

Table 5. Plasma analyses

	EMIQ [®]	Control	p-value
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Glucose (mmol/L)	4.7 ± 0.1	4.7 ± 0.1	0.994
NOx (nM NO ₂)	36.1 ± 6.8	36.3 ± 7.1	0.979
NO ₃ (μM)	29.9 ± 1.8	30.9 ± 1.8	0.587
NO ₂ (μM)	2.5 ± 0.2	2.8 ± 0.2	0.263
F ₂ -Isoprostanes (pmol/L)	540.7 ± 33.5	517.2 ± 33.5	0.510
Quercetin aglycone (nM)	144.9 ± 12.3	12.6 ± 12.3	<0.001
Isorhamnetin (nM)	245.5 ± 16.5	41.7 ± 16.5	<0.001

Measurements of plasma glucose, NOx, nitrate (NO₃), nitrite (NO₂), F₂-isoprostanes, and quercetin metabolites, 3 hours post consumption of EMIQ[®] or placebo treatment. Results are presented as means ± sd (n=25). P-values for treatment effect were obtained using linear mixed models with adjustment for treatment order.

FIGURES

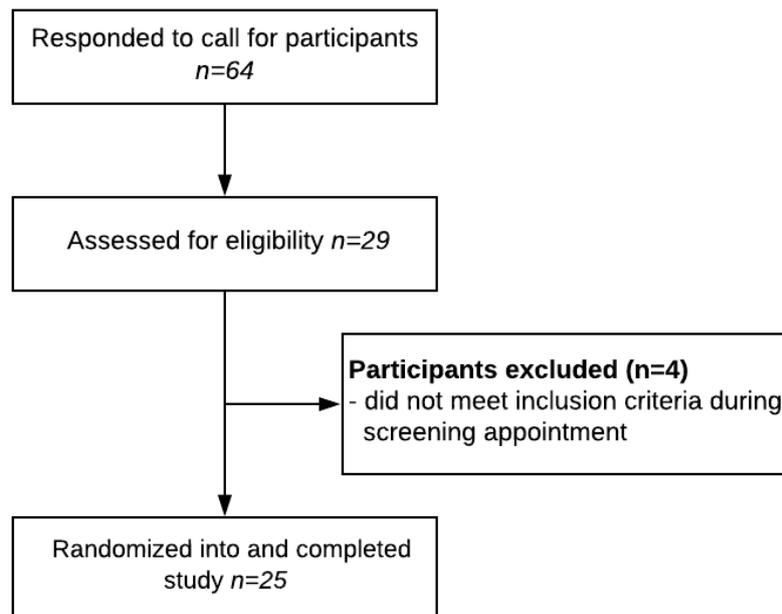


Figure 1. Participant flow diagram.

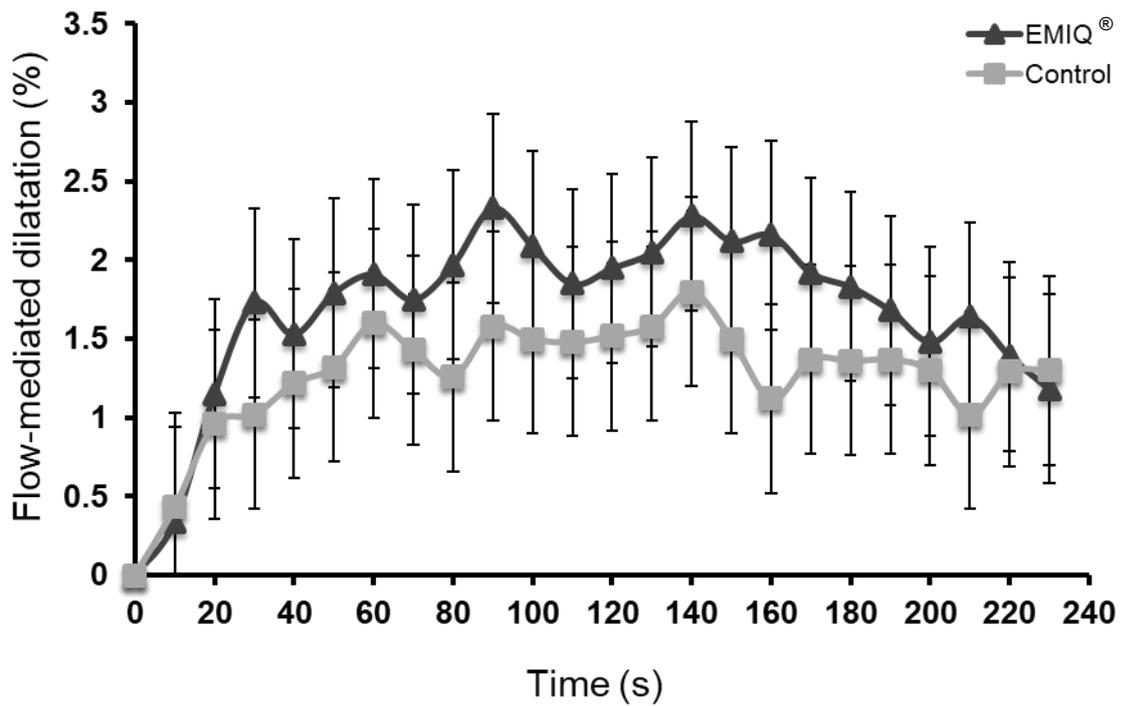


Figure 2. Acute changes in flow-mediated dilatation (FMD) over 240 s measured 1.5 h post-intervention. Results are presented as means (n=24). P-values for treatment effect were obtained using linear mixed models with adjustment for treatment order, time, and baseline (pre-treatment) FMD. Over the time course there was a significant difference between interventions ($p=0.040$). EMIQ®, Enzymatically-Modified Isoquercitrin.

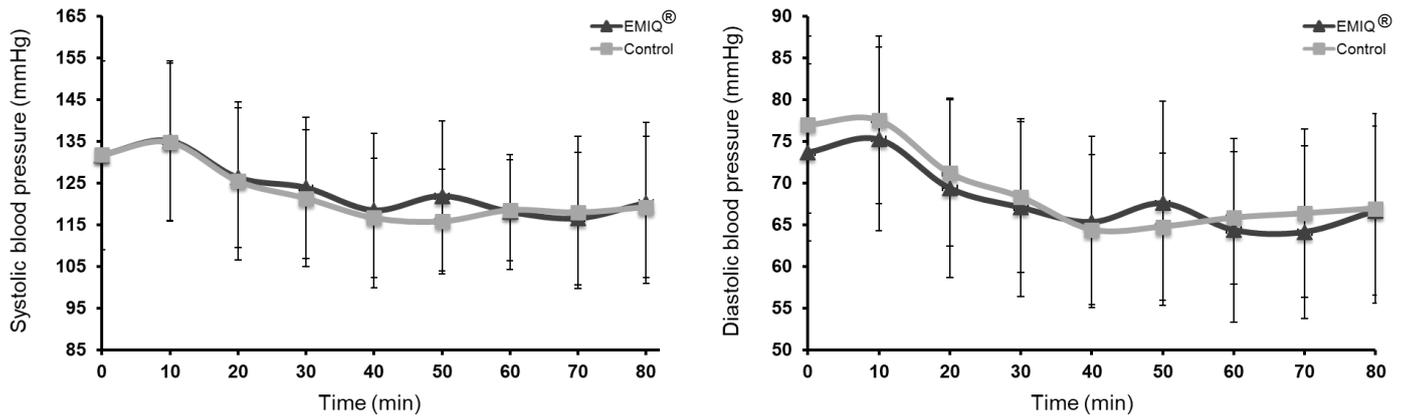


Figure 3. Acute changes in systolic and diastolic blood pressure over 80 minutes immediately post-intervention. Treatment was given immediately after the first measurement at time=0. Results are presented as means \pm sd error bars (n=25). P-values for treatment effect were obtained using linear mixed models with adjustment for treatment order and time. Over the time course there was no significant difference between interventions (systolic, $p=0.916$; diastolic, $p=0.073$). EMIQ[®], Enzymatically Modified Isoquercitrin.