1 **Anti-Atherogenic Properties of *Allium Ursinum* Liophylizate: Impact on Lipoprotein**
2 **Homeostasis and Cardiac Biomarkers in Hypercholesterolemic Rabbits**

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20 **Abstract:** The present investigation evaluates the capacity of *Allium ursinum* (Wild garlic)
21 leaf lyophilisate (WGLL; alliin content: 0.261%) to mitigate cardiovascular damage in
22 hypercholesterolemic rabbits. New Zealand rabbits were divided into three groups: (i)
23 cholesterol-free rabbit chow (control); (ii) rabbit chow, containing 2% cholesterol (HC);
24 (iii) rabbit chow containing 2% cholesterol + 2% WGLL (HCT); for 8 weeks. At the 0- and
25 8-week timepoints, echocardiographic measurements were made, along with determination
26 of basic serum parameters. Following the treatment period, after ischemia-reperfusion
27 injury, hemodynamic parameters were measured using an isolated working heart model.
28 Western blot analyses of heart tissue followed for evaluating protein expression and
29 histochemical study for the atheroma status determination. WGLL treatment mediated
30 increases in fractional shortening; right ventricular function; peak systolic velocity;
31 tricuspidal annular systolic velocity in live animals; along with improved aortic and
32 coronary flow. Western blot analysis revealed WGLL-associated increases in HO-1 protein
33 and decreases in SOD-1 protein production. WGLL-associated decreases were observed in
34 aortic atherosclerotic plaque coverage; plasma ApoB and activity of LDH and CK in
35 plasma. Plasma LDL was also significantly reduced. The results clearly demonstrate that
36 WGLL has complex cardioprotective effects, suggesting future strategies for its use in
37 prevention and therapy for atherosclerotic disorders.

38 **Keywords:** *Allium* species; atherosclerosis; lipoprotein; cardiovascular homeostasis;
39 echocardiography.

40 **1. Introduction**

41 Wild garlic (*Allium ursinum* L.) is a wild plant belonging to the Amaryllidaceae family.
42 It is distributed widely in Asia and Europe, and known variously as bear's garlic, buckrams,
43 bear's leek, wood garlic, and ransoms [1]. The intense flavor of the plant makes it a popular
44 flavoring and regular dietary component for people and animals living in regions where it
45 grows [2]. The mild garlic-like scent of the plant is attributable to its content of
46 sulfur-containing compounds. These include, prominently, sulfoxides and glutamyl peptides.
47 The species also contains high levels of odorless, non-volatile metabolites:
48 S-alk(en)yl-l-cysteine-sulfoxides, which hydrolyze under physiological conditions to

volatile (poly)sulfides and thiosulphinates, imparting the characteristic odor and flavor of the plant [3]. Wild garlic also contains high levels of polyphenolic compounds, particularly in leaves and bulbs – which accounts substantially for antioxidant and therapeutic properties of these sections of the plant [4-6]. It also combines two additional health-enhancing properties: the plant has approximately 20 times the level of adenosine as common garlic (*Allium sativum*), plus it has significantly higher levels of ajoene, both of which combine to stabilize blood pressure and cholesterol levels, reduce excessive thrombocyte aggregation, and improve physiological control of cholesterol metabolism [7]. Indeed, the cardiovascular benefits of using this plant were observed to be so substantial that the Association for the Protection and Research on European Medicinal Plants designated it "Plant of the Year" for 1992 [7]. However, contrary to common garlic, wild garlic has not been studied in clinical trials, and although its cardiovascular effect may be hypothesized based on its chemical constituents, the preclinical confirmation is rather incomplete. *A. ursinum* was also selected as the subject of the present investigation based on outcomes of previous work by this laboratory demonstrating cardioprotective properties of other plant extracts derived from traditional medicines [8,9].

Hypercholesterolemia, a syndrome characterized by abnormally elevated levels of blood cholesterol and lipoproteins[10,11], was chosen as a model disease for the present study due to its association with a wide range of pathologies, particularly atherosclerosis [12], with associated thrombosis, stroke, and heart failure [13]. Although *A. ursinum* is not typically used as a stand-alone medication, its anti-atherogenic properties are well known, to the extent it is used as a dietary treatment for these disorders at Bucharest University Hospital in Romania [14]. *In vitro* evaluation for effects of several *A ursinum* fresh leaf extract preparations on aggregation of human platelets, revealed that ADP-induced aggregation was significantly suppressed by ethanolic extracts. The observed data suggested similarity of pharmacological action to Clopidogrel a thienopyridine clot formation inhibitor that is a potent antiplatelet drug [15].   A likely explanation for this outcome is the known antiaggregatory properties of the β-sitosterol 3-O-β-D-glucopyranoside and 1,2-di-O-α-linolenoyl-3-O-β-D-galactopyranosyl-sn-glycerol (DLGG) components of *A ursinum* [16].   It was further noted that 45-day administration of feed supplemented with 1% w/w wild garlic *Allium ursinum* (wild garlic), or alternatively with 1% w/w *Allium sativum* (cultivated garlic) to spontaneously hypertensive rats (SHR), in groups of 10 animals per experiment mediated significant reduction in final mean systolic blood pressure (SBP) [17].

 The possible underlying mechanisms include the ability of Ramson to inhibit the activity of angiotensin-converting enzyme (ACE). *In vitro* tests on the water extract from the leaves (at the concentration of 0.300 mg/ml) showed significantly increased activity on enzyme inhibition when compared to leaves with extract of garlic (58 vs. 30%) [7]. Moreover, significantly lower levels of ACE activity were noted in blood of animals fed for 8 weeks with a standard rodent chow containing 2% pulverized whole leaf *A. ursinum*, versus untreated control rats [18].

    The physiologic significance of hypercholesterolemia induced by elevated cholesterol in feed administered to animals is particularly well illustrated by consideration of how such diets affect inflammatory processes, dysregulation of which, imposes increased oxidative stress on a wide range of tissues, and to which cells of the cardiovascular system are

96 particularly sensitive[19]. For example, pigs maintained on diets supplemented with 2%
97 cholesterol, exhibited impairment of coronary endothelial function associated with decreased
98 capacity to neutralize free radicals, decreased expression of nitric oxide synthetase and
99 elevate activation of nuclear factor-kappa beta, a pro-inflammatory transcription factor[20].
100 Outcomes of these investigations underscore the particular significance of
101 hypercholesterolemia for investigation of cardiac function – as was demonstrated in by gene
102 transfer studies conducted [21,22].
103   Previous work by the authors shows that methods of extraction used to recover, purify,
104 and concentrate plant products may cause some degradation in the bioactivity of component
105 molecules [23]. For this reason, lyophilization was used to process the *A. ursinum*
106 administered to animals in the present study. This method is easily accomplished, and
107 optimally preserves the native properties of extracted biological molecules [24].

2. Results

2.1. Bioanalytical Analysis of Wild Garlic Leaf Lyophilisate.

110 Alliin (*S*-Allyl-L-cysteine sulfoxide) is a nonprotein amino acid abundant in most of the
111 *Allium* species. It is the natural substrate of alliinase. Therefore, its content in the pure form is
112 commonly analyzed by HPLC. The percentage of total alliin was analyzed by HPLC.
113 Analysis of a representative lyophilized sample revealed the leaf to contain 0.261% alliin by
114 weight (RSD% = 0.45%). The major peak at 3.8 min (detection at 204 nm) is identical with
115 alliin based on its identical UV spectrum and detection time with those of a reference
116 standard.
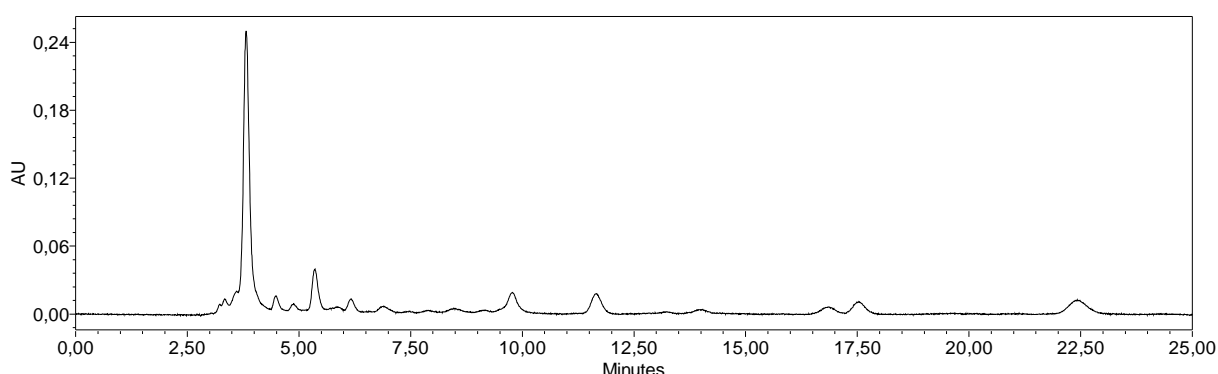
118   Figure 1. High-performance liquid chromatography (HPLC) spectrum analysis: HPLC
119 chromatogram of the WGLL reporting one major peak at 3.8 min.

2.2. Echocardiographic Analyses.

122   All echocardiographic examinations were completed within a 20-minute time interval
123 with outcomes shown in Table 1. End-systolic diameter (ESD) of the left ventricle measured
124 in M-mode, exhibited significant increases in HC animals (1.242±0.045 cm for HC, versus
125 1.016±0.091 cm noted in the Control group). Nevertheless, no change in this outcome was
126 observed in HCT animals (1.184±0.020 cm) in comparison with this parameter in the Control
127 group.
128   Fractional shortening (FS) and ejection fraction (EF) data correlated strongly with
129 measurements of both the parasternal long and short axis views. FS and EF of HC animals
130 were significantly decreased in comparison with this outcome evaluated in animals in the
131 control group (FS$_{HC}$: 29.010±1.056 versus FS$_{Control}$: 32.310±0.718 and EF$_{HC}$: 49.810±1.140

132   versus EF$_{Control}$: 56.910±1.294, respectively). Additionally, significant increases in fractional
133   shortening were observed in in the WGLL-treated (HCT) group in comparison with the HC
134   group (FS$_{HCT}$: 32.970±1.131, and EF$_{HCT}$: 55.990±1.756). Diastolic function of the left
135   ventricle was evaluated by E/A ratios measured in Doppler (PW) mode. E/A ratios were
136   significantly lower in the HC group in comparison to the Control animals (HC:
137   1.207±0.037versus the Control: 1.376±0.045). These results notwithstanding, no significant
138   changes were observed in the E/A ratios of treated animals (HCT: 1.344±0.076) in
139   comparison to Controls. Deceleration time of the E wave (DecT) exhibited significant
140   lengthening in the HC animals (HC: 87.440±3.534 ms versus the Control: 71.250±4.101 ms).
141   However, DecT values of WGLL-treated animals were significantly lower compared to the
142   HC rabbits (HCT: 69.540±4.787 ms). Tissue velocity imaging (TDI) revealed a
143   non-significant trend toward decreased lateral E'/A' ratios in WGLL-treated animals.
144   Surprisingly, right ventricle function characterized by measuring peak systolic velocity (S')
145   waves and tricuspidal annular plane systolic excursion (TAPSE) exhibited significant
146   improvement in WGLL-treated animals. Amplitudes of S' waves were significantly
147   increased in WGLL-treated animals, compared to the HC group (HCT: 9.156±0.210 cm/s
148   versus HC: 8.103±0.216 cm/s), and TAPSE values were also significantly elevated in the
149   WGLL-treated HCT animals compared to the HC rabbits (HCT: 0.646±0.020 cm versus.
150   HC: 0.5762±0.015 cm). Additionally, right ventricle E'/A' ratios of WGLL-treated animals
151   were slightly decreased.
152

Table 1. Echocardiographic outcomes

mean±SEM	HR (bpm)	Ao (cm)	LV ESD (cm)	LV EDD (cm)	FS PLAX (%)
Control	180,8±4,145	0,946±0,024	1,016±0,091	1,655±0,050	39,370±5,021
HC	150,2±4,303*	0,919±0,025	1,242±0,045*	1,756±0,063	29,220±0,803*
HCT	185,0±7,053**	0,898±0,012	1,184±0,020	1,793±0,031	33,820±1,312**

	EF PLAX (%)	LV mass PLAX (g)	FS SAX (%)	EF SAX (%)	LV mass SAX (g)
Control	56,910±1,294	6,632±0,478	32,310±0,717	54,130±0,961	6,573±0,351
HC	49,810±1,140*	8,218±0,628	29,010±1,056*	49,430±1,517*	8,315±0,792*
HCT	55,990±1,756**	8,769±0,169*	32,970±1,131**	54,930±1,522**	8,195±0,226*

	E/A	DecT (ms)	E/E'	LVOTVmax (cm/s)	LVOTVTI (cm)
Control	1,376±0,045	71,250±4,101	1,417±0,058	84,280±2,131	0,071±0,002
HC	1,207±0,037*	87,440±3,534*	1,775±0,101	87,940±5,719	0,080±0,005
HCT	1,344±0,076	69,540±4,787**	1,718±0,155	77,150±2,157	0,0685±0,002

	E'/A' (lateral)	MAPSE (cm)	RV S' (cm/s)	RV E'/A'	TAPSE (cm)
Control	1,303±0,058	0,527±0,018	8,935±0,273	1,336±0,051	0,576±0,012
HC	1,109±0,071	0,571±0,025	8,103±0,215*	1,233±0,092	0,576±0,015
HCT	1,065±0,117	0,592±0,030*	9,156±0,210**	1,055±0,077*	0,644±0,020**

153

154   **Table 1. Echocardiographic outcomes.** Outcomes of echocardiographic evaluations on
155   animals fed normal cholesterol-free rabbit chow (Control); normal rabbit chow, containing
156   2 % cholesterol (HC); rabbit chow containing 2% cholesterol + 2% WGLL (HCT).
157   Outcomes evaluated included the following: heart rate (HR); beats per minute (bpm); aortic
158   diameter (Ao); left ventricle (LV); right ventricle (RV); end-systolic diameter (ESD);
159   end-diastolic diameter (EDD); parasternal long axis view (PLAX); short axis view (SAX);
160   fractional shortening (FS) of the left ventricle; ejection fraction of the left ventricle (EF);
161   calculated weight of the left ventricle (LV mass); peak mitral early diastolic inflow
162   velocity/peak atrial diastolic inflow velocity (E/A); decelaration time of the E wave from
163   maximum to baseline (DecT); peak mitral inflow velocity/avarage of spectral tissue
164   Doppler peak early diastolic velocities at the septal and lateral corner of mitral annulus
165   (E/E'); maximal velocity of left ventricle outflow (LVOTVmax); left ventricle outflow tract
166   velocity time integral (LVOTVTI); peak early diastolic velocity of the lateral wall, spectral
167   tissue Doppler/peak atrial diastolic velocity of the lateral wall, spectral tissue Doppler
168   (E'/A'); peak systolic velocity (S); mitral annular plane systolic excursion (MAPSE); and
169   tricuspidal annular plane systolic excursion (TAPSE).
170   * $P < 0.05$ in comparison to mean values of Control group.
171   ** $P < 0.05$ in comparison to mean values of HC group.

172   *2.3. Cardiac Function in Isolated Working Hearts*

173   Figure 2 shows the effect on cardiac functional parameters of elevated dietary
174   cholesterol-induced hypercholesterolaemia and WGLL treatment. Cardiac functions
175   evaluated included: aortic flow (AF, 2A), coronary flow (CF, 2B), aortic pressure (Aop, 2C),
176   heart rate (HR, 2D), cardiac output (CO, 2E) and stroke volume (SV, 2F). Measurement of
177   these functions in animals in the HC and HCT groups revealed decreases in AF, HR, and CO
178   for basal functions of the perfused hearts, compared to Controls ($P < 0.05$). There were
179   significant increase under preischemia AoP, both in hypercholesterolemic and WGLL
180   treated hypercholesterolemic groups, compared to the Control group ($P < 0.05$). After 60
181   minutes of reperfusion, animals in all groups showed decreases in AF, CF, HR, and CO
182   compared with preischemic values. Significant increases in recovery of AF and HR were
183   observed in the WGLL-treated group, compared with the other groups ($P < 0.05$). Subsequent
184   correlation with results of echocardiographic measurements (Table 1) further supported the
185   cardioprotective capacity of WGLL.
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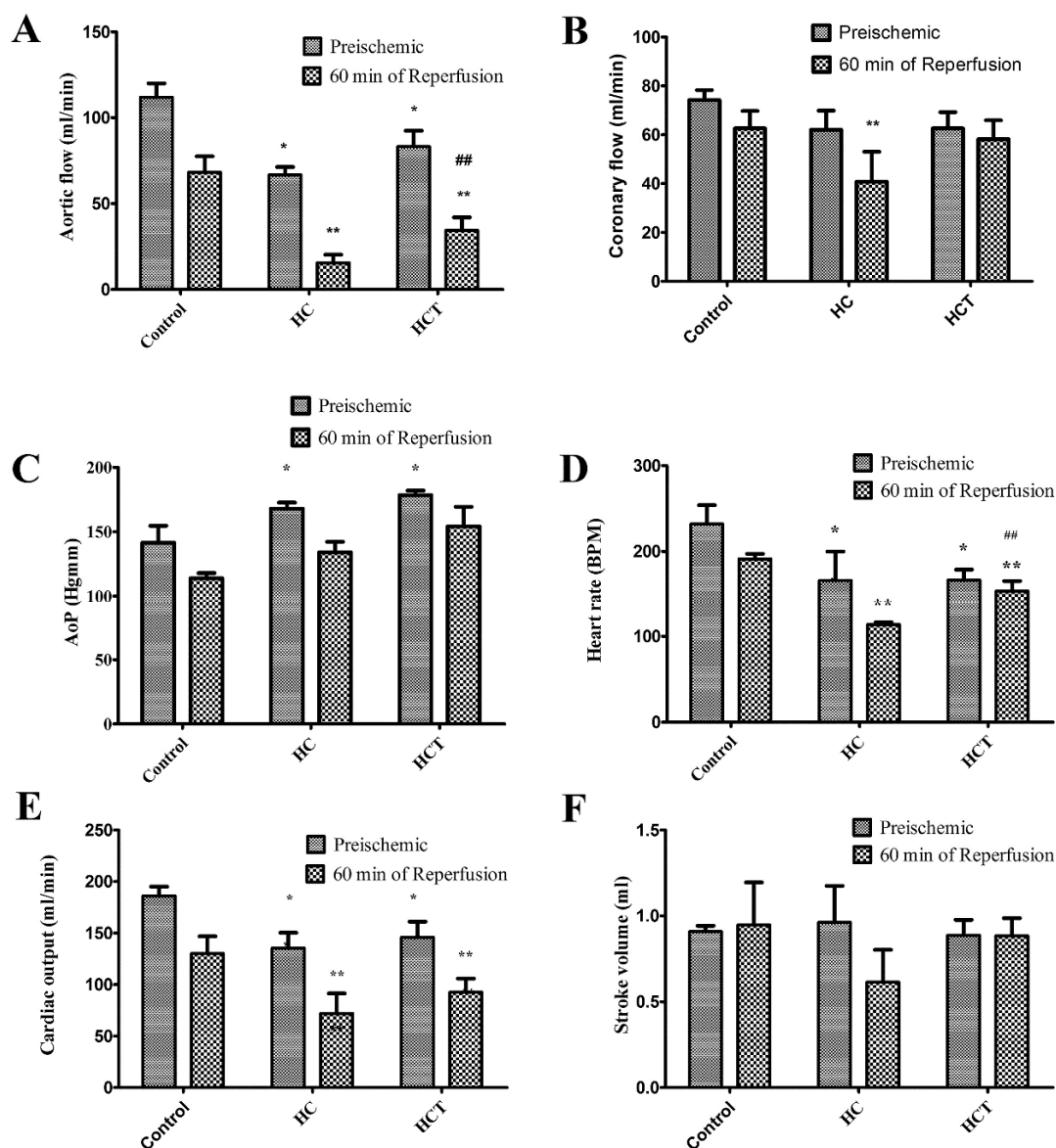
Figure 2. Effect of high cholesterol and WGLL on cardiac function. Hearts were isolated from 3 groups of animals (n=6), defined as follows: non-hypercholesterolemic animals fed with normal chow (Control); hypercholesterolemic group fed with 2 % cholesterol-supplemented chow (HC); and a group of hypercholesterolemic animals treated with WGLL (HCT). Isolated working hearts harvested from each animal in each group were subjected to global ischemia followed by 120 minutes of reperfusion (I/R). Cardiac functions were registered before ischemia (Preischemic) and 60 es after global ischemia (60 minutes of Reperfusion). Results are shown as average values from each group of rabbits ±SEM of aortic flow (AF, ml/min, 2A); coronary flow (CF, ml/min, 2B); Aortic pressure (AoP, Hgmm, 2C); Heart rate (HR, beat/min, 2D); Cardiac output (CO, ml/min, 2E); Stroke volume (SV, ml, 2F). *P <0.05 compared with Preischemic Control. **P<0.05 compared with 60 minutes of Reperfusion Control. ##P<0.05 compared with 60 minutes of Reperfusion HC.

203 *2.4. Western Blot Analysis for Biomarkers of Cardiac Tissue Function*

204 Myocardial tissue levels of four major mediators of cardiac homeostasis, measured by
205 Western blot analysis, is shown in Figure 3. The outcomes of treatments administered to
206 rabbits in these experiments revealed that expression of HO-1 protein was significantly
207 greater in tissue harvested from HCT animals, compared to the levels observed in the HC
208 group (3A, $P<0.05$). Tissue expression of SOD-1 in the HC group was observed to be
209 significantly higher compared to Control and HCT animals (3C, $P <0.05$). COXIII and
210 VEGF proteins were expressed at lower levels both in HC and HCT groups versus quantities
211 of these proteins found in hearts harvested from the Control animals (3B, 3D, $P <0.05$).
212
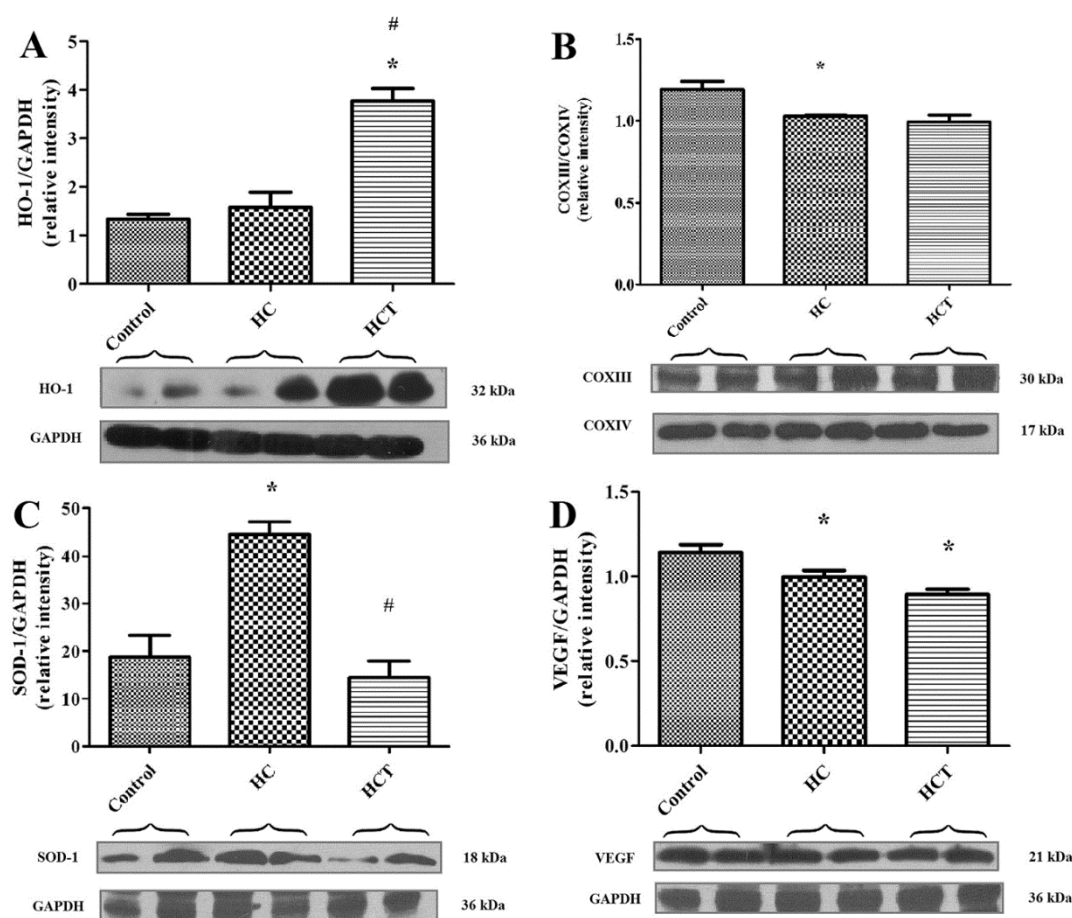
213

214 **Figure 3. Cardiac tissue biomarker expression: Western blot outcomes.**
215 Expression of HO-1 (3A), COXIII (3B), SOD-1 (3C) and VEGF (3D) protein in
216 rabbit myocardial tissue, was measured in homogenized left ventricular cardiac
217 tissue samples drawn from 3 test groups (n=6), defined as follows: I/R injured
218 from non-hypercholesterolemic animals fed with normal, non-cholesterol
219 supplemented chow (Control); I/R injured hearts from hypercholesterolemic
220 animals fed with 2 % cholesterol-supplemented chow (HC); and I/R-injured hearts
221 harvested from hypercholesterolemic animals fed with 2 % cholesterol and 2 %
222 Wild garlic leaf lyophilisate-supplemented chow (HCT). GAPDH and COXIV
223 expression level was measured as reference protein. Western blot analysis were
224 conducted on each tissue homogenate in duplicate, and the signal intensity of
225 resulting bands corresponding to proteins of interest was measured using the Scion
226 for Densitometry Image program, Alpha 4.0.2.3. Tissue content of each protein is

227 shown in arbitrary units as the mean for each group of animal ±SEM. * *P*<0.05 for
228 comparison of average expression levels of HO-1, COXIII, SOD-1 and VEGF in
229 myocardium to non-hypercholesterolemic group (Control). **#** *P*<0.05 for
230 comparison to hypercholesterolemic group (HC).

231 *2.5. Rabbit Aortic Histology*

232 Histological sections of aortas stained with hematoxylin-oil red O from the 3 groups are
233 shown in Figure 4. No atherosclerotic lesions were observed in sections of these blood
234 vessels harvested from the Control animals fed normal rabbit chow (4A). At the end of the 8
235 week-treatment period, up to 35% of the total aortic area harvested from HC animals was Oil
236 red O positive (4B). The extent of atherosclerotic lesions observed in animals within the HC
237 group was significantly increased (4D, 38.610%±0.146) in comparison to lesional coverage
238 in aortic sections taken from animals in the Control group (*P*<0.05). Discrete lesion
239 formation was visualised by oil red O stain and consecutive quantitative analysis in aortas
240 form HCT group rabbits (4C). Aortas harvested from WGLL-treated animals in HCT group,
241 exhibited significantly reduced atherosclerotic lesional coverage in comparison with the HC
242 group (4D, 16.710%±3.421, *P*<0.05).

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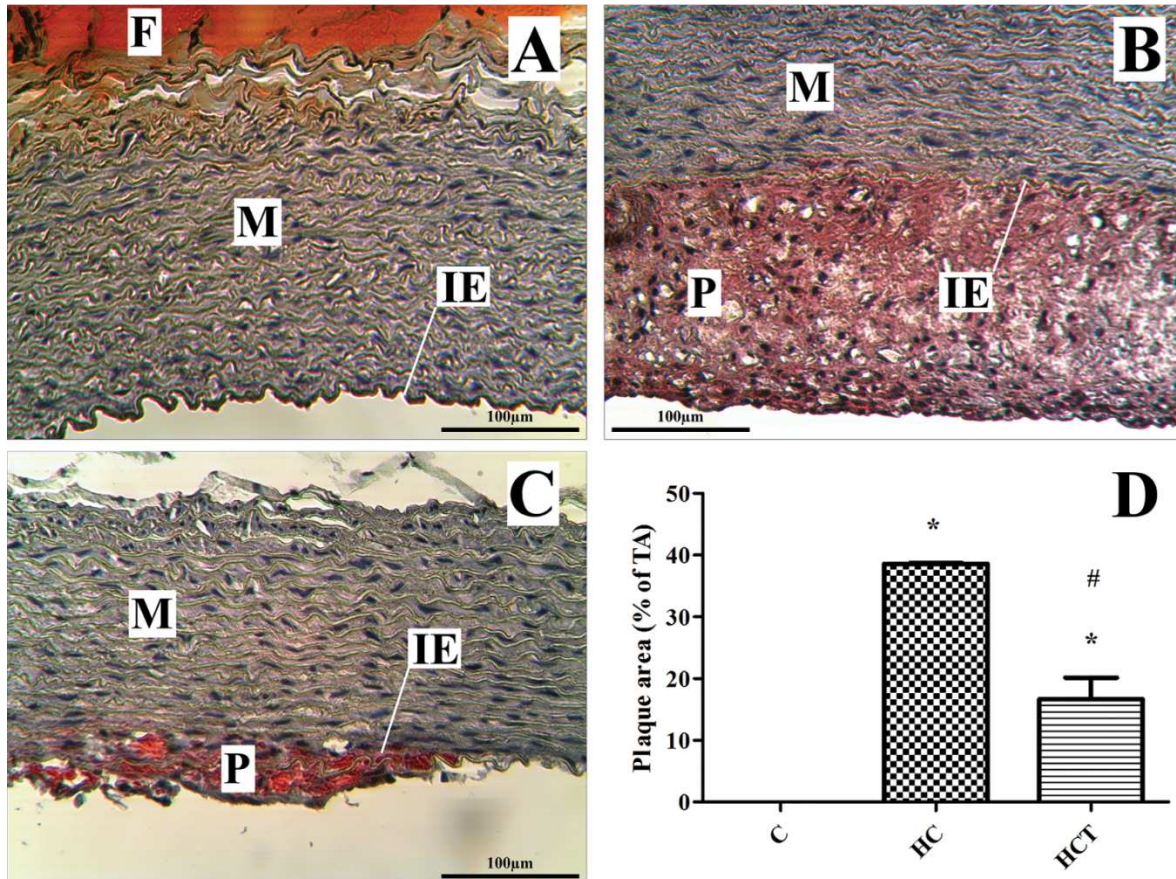
245 **Figure 4. Aortic histologic analysis.** Histological sections of aortas from 3 groups
246 of rabbits stained with hematoxilin-oil red O (A, B, C, magnification x25). Bright
247 red-stained lipid shows atherosclerotic plaques in the HC (4B) and HCT groups
248 (4C). Internal elastic lamina is shown in Figures 4 IE; M, media; P, atherosclerotic
249 plaques; and F, adventitial fatty tissue stained with oil red O. **P*<0.05 for
250 comparison with Control group outcomes. #*P*<0.05 for comparison with HC group
251 outcomes.

252 *2.6. Serological Correlates of Experimental Treatments*

253   The outcomes of analyses of peripheral blood serum from animals treated with selected
254 dietary regimens are shown in Table 2. Fasting plasma TC and LDL cholesterol were two
255 orders of magnitude higher, and HDL cholesterol concentration was 8-fold higher, in HC and
256 6-fold higher in HCT groups, compared to levels of these analytes in the serum of the Control
257 group animals ($P<0.05$). However, significantly lower plasma TC and LDL cholesterol
258 levels were observed in HCT, versus the HC groups ($P <0.05$), showing a possible protective
259 effect of the WGLL. Moreover, ApoA levels detected in blood of HC animals (0.022 ± 0.003)
260 were significantly lower, versus those of the Control rabbits fed diets with normal cholesterol
261 content (0.042 ± 0.005) and WGLL-treated rabbits in the HCT group (0.056 ± 0.008, $P <0.05$).
262 No significant differences were noted between serum ApoA content of serum from animals
263 in the Control group, versus HCT groups ($P> 0.05$). Serum levels of ApoB in rabbits from
264 both HCT and HC groups were significantly higher as compared to the Control group (P
265 <0.05). Moreover, ApoB levels detected in the serum of rabbits in the HCT group
266 (0.172 ± 0.019) were significantly lower compared with the content of this analyte in the HC
267 group (0.280 ± 0.063, $P <0.05$). Additionally, no significant differences were observed
268 between the serum TG content of these three groups. It was further noted that the serum
269 content of the pro-inflammatory biomarker c-reactive protein (CRP), was increased
270 significantly in blood from HC animals, compared to the Control group. GOT liver enzyme
271 levels were elevated in blood of HC group animals (48.8 ± 16.22), as compared to the Control
272 and HCT groups (29.670 ± 2.895, 24.910 ± 2.708; $P <0.05$). LDH (316.6 ± 37.17) and CK
273 (2851 ± 600.800) serum levels were significantly decreased in the HCT group compared to the
274 HC group (791.90 ± 325.4, 496 ± 135; $P <0.05$).
275

Table 2. Serum biomarkers of cardiac function.

Groups	TC	LDL-C	HDL-C	ApoA	ApoB
Control	0,793±0,067	0,230±0,023	0,523±0,045	0,042±0,005	0,015±0,003
HC	26,370±3,660[*,#]	23,550±3,032[*,#]	4,427±0,656[*]	0,022±0,003[*,#]	0,280±0,063[*,#]
HCT	20,030±1,947[*,**]	17±1,942[*,**]	3,314±0,369[*]	0,056±0,008[**]	0,172±0,019[*,**]

	TG	CRP	GOT	LDH	CK
Control	0,788±0,035	0,100±0,014	29,670±2,895	726,400±170,700	4213±623,200
HC	1,367±0,335	0,728±0,362[*]	48,800±16,220[*]	791,900±325,400	4955±1353
HCT	1,052±0,339	0,596±0,231	24,910±2,708[**]	316,600±37,170[**]	2851±600,800[**]

276 **Table 2. Serum biomarkers of cardiac function.**
277 Average serum TC, TG, LDL-C, HDL-C (mmol/L), ApoA and ApoB (g/L), sCRP (mg/L),
278 sGOT, sLDH and sCK (U/L) levels in 3 groups of rabbits (n=6), were analysed by

279　hematology analyser. Each analysis was conducted on peripheral blood serum harvested
280　from animals following the 8-week treatment periods, for: non-hypercholesterolemic
281　(Control), hypercholesterolemic rabbits (HC) and hypercholesterolemic animals receiving
282　WGLL-supplemented chow (HCT). Results are shown as average values from each group
283　animals ±SEM of serum total cholesterol (serum cholesterol, mmol/L) and low-density
284　lipoprotein cholesterol (LDL-C, mmol/L).
285　*p<0.05 compared with Control group values.
286　**p<0.05 compared with HC group values. #p<0.05 compared with HCT group values.

3. Discussion

288　　　As described in the Results section 3.1 of this report, bioanalytical analysis of a
289　representative WGLL sample revealed the leaf to contain 0.261% alliin by weight, a
290　property of this material that contributes to its ability to mitigate expression of other
291　biomolecules described here, which are involved in the atherosclerotic pathophysiologic
292　processes. This natural component of fresh garlic is a sulfoxide derived and forms from the
293　amino acid cysteine [25], and is a major contributor to the capacity of garlic extracts to
294　scavenge hydroxyl radicals, along with a wide variety of other antioxidant properties that
295　counteract oxidative tissue damage [26]. Alliin has also been demonstrated to potently
296　stabilize and strengthen immunoregulation, contributing to well-known curative properties
297　of garlic [27]. The outcomes of echocardiographic analyses conducted on live animals shown in
298　Table 1. reveal the effect of elevated dietary cholesterol and WGLL treatment. The
299　physiologic significance of these results may be stated according to 4 major interpretations
300　of the data shown. These may be summarized as follows:
301　　　(1) The observed stability throughout the evaluation period of heart rates, respiratory
302　frequencies, M-mode and Doppler measurements demonstrate that basal cardiopulmonary
303　activity was not substantially disrupted by hypercholesteremia, an outcome for which
304　preliminary evidence was provided by an earlier study conducted in the laboratory of the
305　authors [28].
306　　　(2) Moreover, in comparison to untreated control rabbits fed a normal diet, left
307　ventricular end-systolic diameter (ESD) measured in HC animals was significantly
308　increased, with or without WGLL treatment, along with decreased fractional shortening and
309　ejection fraction in HC (ii) animals – and a strong correlation was found between fractional
310　shortening (FS) and ejection fraction (EF) data measured on both the parasternal long and
311　short axis views. Also, the effects of WGLL treatment included observations that, relative to
312　HC animals not receiving the lyophilisate, HCT rabbits showed significant increases in
313　fractional shortening. Pathologically increased ESD is associated with greater risk of
314　mortality in heart disease [29], and decreased FS and ES have recently been implicated as
315　contributors a to fibrotic disease [30]. These results suggest that WGLL will contribute to
316　survival of cardiac patients and lower propensity for development of fibrosis.
317　　　(3) Table 1 diastolic function data, generated in Doppler (PW) mode also reveals that
318　elevated dietary cholesterol resulted in significantly lower left ventricular E/A ratios
319　relative to those observed in control animals. WGLL-treated animals showed values close
320　to controls. Moreover, significant lengthening was observed in deceleration time of the E
321　wave (DecT) in HC animals, showing an abnormal diastolic filling pattern of the ventricle,
322　which was counteracted by WGLL-supplemented diet, indicates slightly improved diastolic
323　function. (4) Finally, surprisingly significant increases were shown in Table 1, in right
324　function mediated by WGLL treatment of animals fed elevated cholesterol diets, which
325　obtained through evaluation of peak systolic velocity (S') waves and tricuspidal annular
326　plane systolic excursion (TAPSE). Reduction of peak systolic velocity identifies the
327　of RV dysfunction with high sensitivity. This reduction was significant in HC animals but

328   was counteracted fully by WGLL treatment, showing that the aforementioned beneficial
329   effects of WGLL supplementation can be seen on right ventricle function as well. In heart
330   failure patients, the reduction of tricuspidal annular systolic velocity is associated with the
331   severity of RV dysfunction. Surprisingly, TAPSE values in WGLL-treated group were
332   significantly increased even compared to healthy animals. These findings indicates that diet
333   supplemented with WGLL could have positive effects on right ventricle systolic function,
334   but the relevance of this effects and the underlying mechanisms need further investigations.
335   Evaluation of cardiac functions in Langendorff-mounted isolated working hearts shown
336   in Figure 2 revealed significantly increased preischemic AoP values in both the
337   hypercholesterolemic and WGLL-treated hypercholesterolemic groups, relative to untreated
338   Control rabbits. Also, as shown in Figure 2, ischemic-reperfusion injury-associated
339   decreases in AF, CF, HR, and CO, versus preischemic values, along with significantly
340   increased recovery of AF and HR in animals fed the lyophilisate, further supported
341   anti-ischemic properties of WGLL. These effects are consistent with previous studies by the
342   authors, in which interventions that decrease oxidative stress on cardiac tissue, dramatically
343   improved recovery from ischemic events [31-33]. An interpretation of the significance of
344   these outcomes to the cardioprotective ability of WGLL should be considered in the context
345   of the fact that myocardial ischemic events typically reduce cardiac aortic blood pressure
346   (AoP), along with reduction in myocardial metabolic requirements, coronary blood flow, and
347   left ventricular tension. For these reasons, influences that decrease AoP may be either
348   detrimental or beneficial [34]. Thus, whereas increased preischemic AoP in HC animals
349   indicates that such an increase is pathological for animals maintained on a high cholesterol
350   diet, the failure of WGLL treatment to lower AoP suggests that the extract has negligible
351   effect on this aspect of cardiovascular function.
352   Data described in section 3.4 of the Results section of this article and shown in Figure 3,
353   provide ventricular tissue expression profiles of proteins implicated in pathogenesis and
354   adaptive response to atherosclerotic disease. Western blot analysis of myocardial tissue,
355   reveals significantly elevated content of HO-1 protein in tissue harvested from HCT
356   animals, versus that taken from rabbits in the HC group. The heat shock protein HO-1
357   (hsp-32) is a major antioxidant defense enzyme, which is increased in response to trauma
358   intrinsic to a wide range of diseases, including (and especially) atherosclerotic syndromes
359   [35]. Often, the effects of a disease process overwhelm the protective capacity afforded by
360   endogenous HO-1 expression[36-38]. However, its cardioprotective effects may be greatly
361   amplified by administration of pharmacological inducers, as demonstrated by the authors of
362   the present report [28]. The cytoprotective effects correlating with increased expression of
363   HO-1, are a likely effect of heme degradation by this enzyme to produce bilirubin and
364   carbon monoxide (CO), both of which enhance healthy function of cardiovascular tissue at
365   the concentration normally produced by HO-1 activity during normal heme clearance.
366   Therapeutic amplification of HO-1 in these studies was achieved using seed kernel
367   extracts of *Prunus cerasus* (sour cherry). The present investigation demonstrates that
368   lyophilisate of wild garlic leaf also mediates this effect. However, since this plant material
369   also stimulates other protective effects, based on the data shown here, it cannot be
370   determined to what extent WGLL-induced HO-1 expression contributes to the specific
371   cardioprotective outcomes revealed.
372   SOD1 levels measured by Western blot analysis in myocardial tissue of HC animals after
373   ischaemia/reperfusion injury were significantly elevated compared to the Controls, while
374   SOD1 expression in WGLL treated animals was maintained at the normal (Control) levels.
375   Both apoptotic signaling and adaptive responses to oxidative stress, involve processes for

376   which SOD1 activity is a critical component. This enzyme produces molecular oxygen and
377   hydrogen peroxide (H_2O_2) as an end metabolites of its main activity, which is to neutralize
378   superoxide [39]. H_2O_2 is itself a toxic reactive oxygen species (ROS) and may contribute to
379   ischemia-reperfusion injury of myocardial tissue, through abnormally high apoptotic
380   signaling and oxidative tissue damage in ischemic heart disease [40].
381   SOD1 is known to have a capacity to limit the detrimental effects of ROS by eliminate O_2^-
382   to produce H_2O_2 which is eliminated by Glutathione peroxidase or by Catalase to harmless
383   H_2O and O_2, but on the other hand, with free iron(II) H_2O_2 also can form free hydroxyl
384   radicals by Fenton's reaction (see graphical abstract). High SOD levels along with
385   considerable amounts of Fe^{2+} are associated with increased production of the highly toxic
386   hydroxyl radical, and may even enhance the extent of reperfusion injury [41]. An unbalance
387   between the production of prooxidant H_2O_2 (SOD1) and antioxidants, such as Glutathion
388   peroxidase and Catalase in the cell might lead to a strengthened production of free radicals
389   which could lead to serious cellular damage. This is supported by assessment of SOD
390   activity in blood of MI patients, which revealed that relative to healthy control individuals,
391   SOD levels in the patient group were significantly higher [42]. This difference likely reflects
392   a normal adaptive response to limit oxidative damage to the myocardium imposed by
393   ischemic (and other) tissue injury.
394   Western blot analyses conducted in this investigation, revealed that COXIII and VEGF,
395   which are both implicated in the pathophysiology of cardiovascular syndromes were
396   expressed at lower levels both in HC and HCT groups, versus quantities of these proteins
397   found in hearts harvested from the Control animals. In COXIII and VEGF protein levels,
398   there were no significant changes between WGLL-treated and hypercholesterolemic
399   groups. Our results suggest that wild garlic may develop its cardioprotective activity via
400   heme/HO system, and has no effect on COXIII and VEGF proteins.
401   The extent of atherosclerotic plaque coverage on the intimal surface of
402   hematoxylin-Oil red O-stained rabbit aortas reveals negligible plaque on sections harvested
403   from Control animals maintained on diets with normal cholesterol content, and lesional
404   extent of approximately 35% in sections from HC rabbits (Figure 4). The significantly
405   reduced lesional coverage observed in WGLL-treated rabbits fed high cholesterol chow
406   (HCT) is an effect also observed in previous work by these authors, in which HO-1
407   expression increased by adding sour cherry seed kernel extract to rabbit chow. This
408   protected against dietary cholesterol-induced arterial plaque formation [28].
409   The blood of animals utilized in the present study was evaluated for serum analytes
410   expressed at levels which may be used as diagnostic and therapy effect indicators for
411   cardiovascular disease severity along a wide range of other severe inflammatory
412   The outcomes of serum parameter measurements are shown in Table 2. They reveal
413   significantly elevated fasting plasma levels of TC and LDL cholesterol, which were two
414   orders of magnitude higher, and HDL cholesterol concentration, which was 8-fold higher,
415   HC and 6-fold higher HCT rabbits versus the Controls, effects that are an expected result of
416   high cholesterol diets [43]. However, elevated levels of HDL cholesterol in WGLL-treated
417   rabbits may indicate a cardioprotective property of the lyophilisate in the context of
418   beneficial effects of HDL. Significantly lower TC and LDL cholesterol levels were
419   in WGLL-treated (HCT) animals versus groups fed with high cholesterol chow but no
420   WGLL (HC), demonstrating that the lyophilisate is protective with respect to influence of
421   these analytes. ApoA levels in blood of HC animals were significantly lower versus rabbits
422   fed normal chow (Control). Thus, the lack of significant differences in ApoA content of
423   blood from animals fed normal diets (Control) versus content of this molecule in

424 WGLL-treated rabbits maintained on high cholesterol (HCT) indicated a normalizing effect
425 of the lyophilisate on this outcome. The significance of this result is that ApoA-I deficiency
426 causes both hypertriglyceridemia and increased atherosclerosis in animal models [44],
427 can be counteracted by WGLL-supported diet.
428 Analysis of ApoB revealed that systemic levels of this analyte in rabbits from both
429 HCT and HC groups were significantly higher versus its levels in blood of animals fed
430 chow with normal cholesterol content (Controls). Moreover, ApoB levels detected in blood
431 taken from rabbits in the HCT group were significantly lower in comparison to content of
432 this analyte in the HC group. This result is well correlated with LDL levels measured in the
433 two groups. This finding was expected since ApoB is the primary apolipoprotein of
434 chylomicrons, VLDL, IDL, and LDL particles.
435 Elevation in serum TG of the HC group was tendentious but not significant compared to
436 that of the Controls. One interpretation is that triglyceride metabolism is unaffected by
437 either influence within the constraints of the present study. Further analysis of blood from
438 each of the three test groups revealed significant elevation of c-reactive protein (CRP) in
439 HC animals versus the Controls. Since CRP is a reliable systemic indicator of a wide range
440 of inflammatory pathologies, this result implies that levels of dietary cholesterol
441 administered to animals in this study managed to induce onset of inflammatory processes.
442 Analysis for serum liver enzyme activities demonstrated significantly elevated GOT in
443 blood of HC group animals versus the Control and HCT groups, suggesting a hepatotoxic
444 effect of elevated dietary cholesterol intake, an effect noted by previous investigators [45].
445 Finally, the significantly lower levels of LDH and CK observed in HCT animals, versus
446 group rabbits maintained on high cholesterol (HC), indicated that effects of dietary
447 supplementation with WGLL may have beneficial effects on impaired liver function caused
448 by hypercholesteroleamic state. TNFα-induced ICAM-1 mRNA transcription, which has
449 been demonstrated by *in vitro* studies to suppress adhesion of monocytes to porcine arterial
450 tissues and HUVECs, was significantly inhibited by treatment with Alliin
451 (S-Allyl-L-Cysteine Sulfoxide). Moreover, Alliin is also protected against depolarization of
452 mitochondrial membrane potential and overproduction of the superoxide anion, which occur
453 as a downstream effect of TNFα – and may correlate with suppression of NOX4 mRNA
454 transcription by HUVECs. Additionally, treatment of HUVECs with Alliin was also
455 observed to reduce TNF-alpha-mediated ERK1/2 IjB, and IjB (but not p38) phosphorylation.
456 These results provide improved insight into the mechanisms by which Alliin acts as a
457 countermeasure to atherosclerotic pathomechanisms [46] and are consistent with the
458 protective effects of the plant extract reported here.

459 4. **Experimental Section (methodology)**.

460 *4.1. Sample lyophilization and Bioanalytics.*

461 Deep-frozen *Allium ursinum* leaves (Toltelekgyar Ltd., Zalakomar, Hungary) were
462 lyophilized for 24 hours in a Martin-Christ ALPHA 1-4 freeze dryer (Martin Christ
463 Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) at an ambient pressure of
464 0.120 millibars (mb), with a condensor temperature of -50°C and shelf temperature of 35°C.
465 The ratio of the frozen, leaf to fresh and desiccated plant material was 5:6:1. HPLC analysis
466 was accomplished using a Waters 600 system (Waters Corporation, Milford, CT, USA),
467 equipped with a 2998 photodiode array detector, on-line degasser, and auto sampler, using a
468 reversed phase Phenomenex Synergi 4 µm Hydro-RP 80Å (250 mm x 4.6 mm) column

469   (Phenomenex, Torrance, CA, USA). With a column temperature of 25°C, a gradient elution
470   was applied as follows: 0-15 minutes: 100% of mobile phase A (water + 0.1% phosphoric
471   acid); 15-20 minutes: the ratio of mobile phase B (acetonitrile) increased to 100%; 20-25
472   minutes: 100% B; 25-27 minutes: A increased to 100%; 27-45 minutes: 100% A. The flow
473   rate was 0.75 ml/min, and alliin was monitored at 204 nm. Alliin was detected at 4.6 minutes.
474   Data acquisition and evaluation were performed using Empower Pro software.
475   Alliin was purchased from LGC Standards. Acetonitrile used for chromatographic
476   analysis (LiChrosolv® HPLC grade) was obtained from Merck (Darmstadt, Germany).
477   Millipore Direct-Q UV3 clarifier (Molsheim, France) was used to produce purified water for
478   HPLC measurements. Stock standard solutions of alliin were prepared with methanol and
479   stored at 4°C. The calibration range was 0.5–5 µg alliin/injection. The calibration standard
480   was injected in triplicate at six volume levels. Extraction alliin from the lyophilized plant
481   material was carried out with 10 ml MeOH at room temperature for 3 minutes from 1 g
482   sample in an ultrasonic bath. After filtering through a filter membrane (Acrodisc® GHP 13
483   mm, 0.45 µm, Waters Corporation, Milford, CT, USA), 3 independently prepared samples
484   were analyzed in triplicate.

485   *4.2. Animals*

486   The experiments were carried out using adult male New Zealand rabbits with an average
487   body weight of 2.5-3.0 kg. The animals received human care in compliance with "Principles
488   of Laboratory Animal Care" by EU Directive 2010/63/EU for animal experiments. The
489   duration of the adaptive feeding was 2 weeks. The rabbits were provided with laboratory
490   rodent chow, or chow enriched with 2.0% cholesterol (Jurasko, Debrecen, Hungary), or
491   cholesterol-supplemented chow containing 2% wild garlic leaf lyophilisate (WGLL) daily
492   for 8 weeks *ad libitum*. *Allium ursinum* lyophilisate-containing chow was produced in the
493   laboratory of Dept. of Pharmaceutical Technology, University of Debrecen. Comparison of
494   behavior and general health status of animals provided with unsupplemented rabbit chow
495   versus feed containing other components showed no observable differences, with no
496   indication that administration of feed acted as a confounder to the experiments conducted.

497   *4.3. Echocardiograpy.*

498   Echocardiographic examination of animals was conducted under light anesthesia
499   (ketamine 15 mg/kg, xylazine 3 mg/kg (i.m.) at the 8-week timepoint of the study [47]. The
500   chest of each animal was shaved, and the rabbit was positioned in a dorsal decubitus position.
501   Echocardiographic measurements were performed using a Siemens Acuson 512 sonograph,
502   with 7V3c probe at 7 MHz, with fundamental imaging (Figure 4). Conventional
503   measurements were carried out in 2D and M-mode. Parasternal long axis views were
504   obtained and recorded to ensure that the mitral and aortic valves, as well as the apex, were
505   visualized. The parasternal short axis views were recorded at the mid-papillary muscle level.
506   M-mode tracings were performed at the mid-papillary muscle level, either in parasternal long
507   or short axis views. M-mode for visualization and quantification of wall motion in
508   cardiovascular research was used; single line acquisition allows for the very high-temporal
509   (1000 fps) resolution necessary for analysis of LV function. Echocardiographic
510   measurements included interventricular and left ventricular free-wall thickness in diastole
511   and systole (IVSs, IVSd) and left ventricular internal diameter at end-diastole (LVIDd) and
512   end-systole (LVIDs). End-systolic volume (ESV), end-diastolic volume (EDV), stroke
513   volume (SV), and left ventricular mass were calculated. Fractional shortening was computed
514   by using the equation [(LVIDd – LVIDs) / LVIDd] × 100%, and ejection fraction was
515   calculated by using the following formula (Teiholz): $EF = (LVEDD^2 - LVESD^2)/LVIDD^2$.
516   Mitral and tricuspid annular peak systolic excursions (MAPSE and TAPSE) were assessed
517   with M-mode, measuring the distance of mitral or tricuspid annular movement between

518   end-diastole to end- systole. All measurements were averaged over three to five consecutive
519   cardiac cycles.
520   Doppler (PW) imaging of the mitral valve and aortic valve were obtained from the apical
521   4-chamber view and the apical 5-chamber view. From the mitral inflow velocity image, the
522   following measurements were obtained: peak E and peak A waves (mitral early and late
523   filling velocities), E to A ratio (E/A), deceleration time of early filling velocities (DecT).
524   Aortic and left ventricular outflow tract (LVOT) parameters were also calculated:
525   LVOTVmax, LVOT maxPG, and LVOTEnvTi.
526   Tissue velocity imaging (TVI) measurements were analyzed from the apical 4-chamber
527   view and from the parasternal long axis and short axis views as well. A 5-mm tissue sampling
528   volume was obtained at the mitral annulus from both septal and lateral walls. From the
529   acquired images, the following functional parameters were measured: S', E'/A' wave
530   velocities, E/E' (early diastolic mitral inflow velocity divided by average value of lateral
531   and septal tissue Doppler early diastolic velocities), and E'/A' (tissue Doppler early and
532   late diastolic velocity ratio) [48].
533
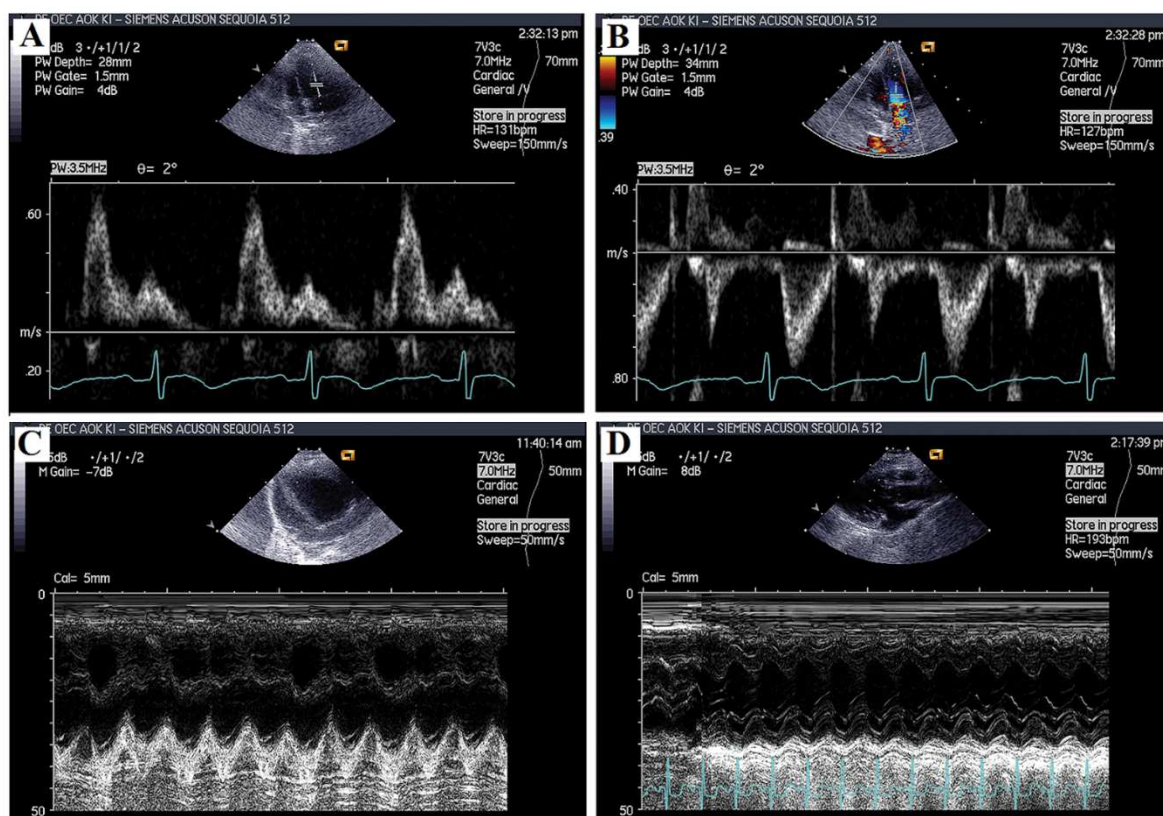
535   **Figure 5. Representative images of echocardiographical measurements.** A:
536   Doppler image, mitral inflow velocities; B: color Doppler image, velocity of left
537   ventricle outflow tract; C: M-mode image, tricuspidal annular plane systolic
538   excursion (TAPSE); D: M-mode image, parasternal long axis view (PLAX) of left
539   ventricle.

4.4. Measurement of Serum Parameters.

541   Blood samples were collected with EDTA-K2 evacuated tubes (BD Vacutainer, USA)
542   from the marginal ear vein of the animals, at the endpoint of the treatment. The samples were
543   *collected* and processed aseptically to minimize hemolytic activity. Serum level of total
544   cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C), low
545   density lipoprotein cholesterol (LDL-C), and value of Apolipoprotein A-I (ApoA),

546 Apolipoprotein B (ApoB), C reactive protein (CRP), Aspartate transaminase (also called
547 Glutamic oxaloacetic transaminase, GOT), Lactate dehydrogenase (LDH), and Creatine
548 kinase (CK) were detected by automated analyzers in the Department of Laboratory
549 Medicine at the University of Debrecen.

550 *4.5. Isolated Working Heart Preparation (Langendorff Method).*

551 Each of the animals were anaesthetized with a mixture of ketamine/xylazine (50/5
552 mg/kg, intramuscularly). A bolus of heparin was administered (1,000 U/kg of body weight,
553 intravenously) 20 minutes before thoracotomy, to avoid thrombosis. Next, the chest cavity
554 was opened and the pericardium incised. The heart was excised and immediately transferred
555 to ice-cold modified Krebs-Henseleit (mKH) buffer (pH 7.4 on 37°C, gassed with 95% O2
556 and 5% CO2 mixture) [49]. The aorta was then cannulated and the heart perfused for 10
557 minutes, retrogradely in Langendorff mode with mKH buffer to clear residual blood from
558 each harvested organ. The perfusate had the following composition: NaCl, 118 mmol/l;
559 NaHCO$_3$, 25 mmol/l; KCl, 4.8 mmol/l; CaCl$_2$, 1.8 mmol/l; Mg$_2$SO$_4$, 1.2 mmol/l; KH$_2$PO$_4$,
560 1.2 mmol/l; and 10 mM glucose. A dual-headed peristaltic pump controlled the rate of
561 perfusion of mKH buffer. The left atrial appendage was incised, and the pulmonary veins
562 were ligated. A small incision was made at the bifurcation of pulmonary arteries; thus all
563 coronary effluent was collected by the pulmonary artery. Next, the circulation was switched
564 to anterograde perfusion, in order to set the baseline parameters in working heart mode for 20
565 minutes.
566 The following parameters were recorded and resulting data analyzed using a pressure
567 transducer attached to the aortic outflow line: aortic pressure (AoP), heart rate (beats/min),
568 left ventricular developed pressure (LVDP). Aortic flow (AF, ml/min) and coronary flow
569 (CF, ml/min) were measured by using flowmeter. Hearts were then subjected to 30 minutes
570 of global ischemia, then perfused for 15 minutes in Langendorff mode and converted to
571 working heart mode for 105 minutes. The above-listed outcomes were measured and
572 recorded during the reperfusion at the 30-, 60-, 90-, and 120-minute timepoints. Immediately
573 following 120 minutes of reperfusion, small myocardial biopsies from LV heart tissue were
574 removed and frozen for subsequent molecular biological analysis.

575 *4.6. Histological Analysis of the Aortic Root.*

576 Lipid staining was carried out with Oil red O (Sigma Diagnostics, St. Louis, MO, USA)
577 by use of the following protocol: aortic tissues were frozen in OCT medium (Thermo Fisher
578 Scientific Inc., Waltham, MA, USA). Cryostate tissue sections were cut to a thickness of 6.0
579 µm and applied to Superfrost Plus slides (Daiggers, Vernon Hills, IL, USA). Atherosclerotic
580 lesions in the aortic root were examined at 3 locations and each separated by 120µm. 4 to 5
581 serial sections were prepared from each location, starting beyond the aortic arch. The
582 sections were stained, as described previously, with Oil red O, followed by analysis of the
583 lipid composition of the lesions, by calculating the percentage of Oil red O positive area,
584 relative to the total cross-sectional vessel wall area. Nuclei were counterstained with
585 hematoxylin (Sigma Diagnostics), using routine methods. All images were captured with a
586 binocular light microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with a
587 video camera and analyzed using Scion Image software (Scion Corp., Torrance, CA, USA).

588 *4.7. Extraction of Myocardial Protein.*

589 300 mg frozen tissues from rabbit left ventricular myocardium were homogenized in 800
590 µl Buffer A (25 mM Tris-HCl, pH 8, 25 mM NaCl, 4 mM Na-orthovanadate, 10 mM NaF, 10
591 mM Na-Pyrophosphate, 10 nM Okadaic acid, 0.5 mM EDTA, 1 mM PMSF, and 1x Protease
592 inhibitor cocktail (Sigma-Aldrich, St. Louis, Missouri, U.S.A.) in a Polytron-homogenizer.

593　Homogenates were centrifuged at 2,000 rpm at 4°C for 10 minutes. Supernatant from the
594　above centrifugation was further centrifuged at 10,000 rpm at 4°C for 20 minutes, and the
595　resulting supernatant was used as cytosolic extract. The nuclear pellet was resuspended in
596　400 µl of Buffer A with 0.1% Triton-X-100 and 500 mM NaCl, then lysed by incubation for
597　one hour on ice. Homogenates were then centrifuged at 14,000 rpm at 4°C for 10 minutes,
598　and the supernatant was used as a mitochondrial lysate. Cytosolic mitochondrial extracts
599　were aliquoted, snap frozen, and stored at -80°C for further investigations. The total protein
600　concentration was assayed using bicinchoninic acid (BCA) method with bovine serum
601　albumin as the standard (Pierce, Rockford, IL, USA).

602　*4.8. Western Blot Assays for Protein Expression in Cardiac Tissue.*

603　　　Western blot analysis was used to evaluate left ventricular myocardial tissue for
604　expression level of the following proteins: heme-oxygenase 1 (HO-1), superoxide-dismutase
605　1 (SOD1), vascular endothelial growth factor A (VEGF), cytochrome c oxidase III (COXIII),
606　cytochrome c oxidase IV (COXIV), and glyceraldehyde 3-phosphate dehydrogenase
607　(GAPDH). The total protein concentration in cytosolic and mitochondrial extract was
608　determined using the BCA Protein Assay Kit. Next, the protein was diluted with Laemli
609　buffer and heated to 100°C for 10 minutes. The denatured samples were separated by
610　SDS/polyacrylamide gel electrophoresis (SDS-PAGE) at 120 V for 90 minutes and
611　transferred onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, California,
612　U.S.A.) at 100 V for 1 hour. Precision plus Protein Kaleidoscope standards (Bio-Rad
613　Laboratories, California, U.S.A.) were used as molecular-weight standards. The membranes
614　were blocked in 5% low fat milk blocking buffer for 90 minutes and then incubated overnight
615　at 4°C with primary antibodies (Sigma-Aldrich, St. Louis, Missouri, U.S.A.). After being
616　washed with tris-buffered saline containing Tween 20 (TBS-T) three times for 10 minutes,
617　the membranes were incubated with horseradish peroxidase-labeled secondary antibody
618　diluted 1:2,000 in TBS-T and 1% (wt/vol) nonfat dry milk for 90 minutes at room
619　temperature. Enhanced chemiluminescent substrate (ECL, Litmus Scientific) was used to
620　identify bands. Detection was made by autoradiography for variable lengths of time with
621　Medical X-Ray Film (Agfa-Gevaert N.V., Belgium). Quantitative analysis of scanned blots
622　were carried out using Scion for Windows Densitometry Image program version Alpha
623　4.0.3.2 (Scion Corporation, Maryland, USA). Signal intensity for bands corresponding to
624　each protein of interest was estimated and reported in arbitrary units ± SEM.

625　*4.9. Statistical Analysis.*

626　　　All data are presented as average magnitudes of each outcome in a group ± standard
627　error of the mean (SEM). Statistical analysis was performed using one-way analysis of
628　variance (ANOVA) followed by Kruskal-Wallis multiple comparison tests with GraphPad
629　Prism software for Windows (GraphPad Software Inc., CA, USA). Probability values (*P*)
630　less than 0.05 were considered statistically significant.

631　**5. Conclusions**

632　　　Outcomes of the present report demonstrate that wild garlic leaf lyophilisate improves
633　cardiac functions in isolated hearts harvested from WGLL-treated rabbits. The
634　improvements observed include significantly better post-ischaemic values of aortic flow in
635　treated animals compared to the HC group (p<0,05). Coronary flow measurements showed
636　similar trends. Echocardiographic measurements showed improved diastolic heart functions
637　in animals that ate an *Allium ursinum* lyophilisate-containing high-cholesterol diet.
638　relaxation expressed as DecT and E/A ratios was found in HC animals, while parameters
639　measured in WGLL treated animals reached normal values. Systolic function expressed as

640 FS and EF was also found significantly decreased in HC animals, and the process was
641 counteracted by WGLL treatment. Interestingly, better right ventricle functions were
642 measured in treated animals (higher peak E-wave velocity, and higher TAPSE values).
643 Tissue staining showed significantly decreased artherosclerotic plaque formation in animals
644 treated with HCT compared to the HC group. Total blood cholesterol levels in animals fed
645 with 2% cholesterol-containing diet showed a dramatic increase after the 8-week period,
646 while the values of the control group remain in the physiologic range. Cholesterol levels in
647 animals treated with *Allium ursinum* lyophilisate were significantly lower compared to the
648 HC group (p<0,05). WGLL also had notable beneficial effects on the other monitored
649 parameters (GOT, LDH, CK). Important novelties of this present report include the
650 that increased dietary cholesterol intake may raise the level of SOD1 in cardiac tissue,
651 is associated with increased ROS-dependent tissue damage and this may be counteracted by
652 WGLL treatment, furthermore, our work revealed that WGLL supplementation could
653 the activity of HO-1-mediated cardioprotective pathway in hypercholesterolemic
654 circumstances.

Author Contributions

668 Mariann Bombicz: Isolated Working Heart Preparation, Protein Isolation and Western
669 Blot, Histology; Daniel Priksz: Echocardiography, Isolated Working Heart Preparation;
670 Balazs Varga: Animal Treatment, Histology, Manuscript Preparation; Rudolf Gesztelyi:
671 Isolated Working Heart Preparation; Attila Kertesz: Echocardiography; Peter Lengyel:
672 Statistical Analysis, Research Plan; Peter Balogh: Statistical Analysis, Research Plan; Dezso
673 Csupor: Bioanalytics; Judit Hohmann: Sample lyophilization; Harjit Pal Bhattoa:
674 Measurement of Serum Parameters; David D. Haines: Native English Speaker, Data analysis
675 and Manuscript preparation; Bela Juhasz: Echocardiography, Corresponding Author.

676 **Conflict of Interest:** The authors declare no conflict of interest.

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