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Wild edible onions — *Allium flavum* and *Allium carinatum* — successfully prevent adverse effects of chemotherapeutic drug doxorubicin

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ABSTRACT

The objective of this study was to evaluate potential of two chemically characterized edible wild onion species, *Allium flavum* and *Allium carinatum*, to reduce side effects of cytostatic doxorubicin (Dox). Since Dox application is mainly limited due to its high cardiotoxicity, while there are no approved cardioprotective agents for the prevention of Dox adverse effects, new co-treatments are urgently needed. Here, we showed that methanol extracts expressed high antioxidant activity and synergistically increased Dox anticancer activity against human hepatoma (HepG2) and lung carcinoma (A549) cells, while protected normal human fibroblasts (MRC-5) from Dox cytotoxicity. Analysis of the antioxidative enzymes level (catalase and superoxide dismutases) showed that the catalase level was differently altered in cancer cells compared to normal cells upon applied treatments. *In vivo* toxicity evaluation in the zebrafish model revealed significantly lower toxicity of extracts compared to Dox, and no teratogenic effects at applied doses. We found that extracts successfully rescued the Dox-treated embryos of life-threating cardiomyopathy, while at the same time reduced developmental toxicity and neutropenia. Further analysis demonstrated that extracts had higher anti-angiogenic activity than sunitinib or auranofin, clinically used antiangiogenic drugs. In addition, angiogenesis was markedly more suppressed in Dox-extract cotreatments than upon single treatments.

1. Introduction

Despite the progress made in the development of potent chemotherapeutic drugs, their toxicity and adverse side effects are the major obstacles to successful clinical use [1]. Since cardiotoxicity is one of the most common life-threatening drawbacks of chemotherapy, the discovery of alternative treatment approaches aiming to reduce inherent cardiac muscle damages, represents important health issue.

Doxorubicin (Dox) is a highly effective, first-line chemotherapeutic agent used for the therapy of hematological malignancies and solid tumors, particularly hepatocellular, breast and lung carcinoma. However, the dose-dependent cardiotoxicity, hepatotoxicity and severe myelosuppression are commonly encountered clinical problems which limit its long-term application [2]. Due to limited selectivity, Dox targets normal tissues where induces oxidative stress and an impairment of antioxidant defense. Particularly prone to damages caused by Dox are cardiac muscle cells, in which antioxidant enzymes pool is insufficiently developed to combat oxidative stress during Dox chemotherapy, consequently leading to the cardiomyocytes apoptosis, cardiac tissue injury and even to lethal outcome [3,4]. Until now, a few synthetic and natural compounds (probucol, amlodipine, vitamins C and E) were used to prevent Dox-induced cardiotoxicity, but there is very little evidence that supports their cardioprotective effect [4]. The FDA approved only the clinical use of Dexrazoxane, but its application is limited due to interference with Dox efficacy, leading to secondary malignancies and myelosuppression in cancer survivors [5]. Therefore, there is an

Abbreviations: Dox, doxorubicin; TAC, total anthocyanin content; TFC, total flavonoid content; TPC, total phenolic content

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emerging need for discovery of alternative agents that could be used as adjuvant therapy to mitigate the life-threatening cardiomyopathy upon Dox treatment.

Plants belonging to the genus *Allium* are well known edible plants that contain diverse biologically active compounds with beneficial effects on human health. [6] Studies performed over the last years indicated the correlation between protective roles, such as cardioprotection and reduction of cancer risk, with consumption of different *Allium* species, especially *A. cepa* (onion) and *A. sativum* (garlic) [6–8]. In addition, suppression of angiogenesis, as proven strategy for cancer treatment, has been reported for the species like *A. sativum* [9], *A. aescalonicum* [10] and *A. hirtifolium* [11].

However, pharmacological properties of the majority of *Allium* species are still poorly explored. Therefore, wild-growing *Allium flavum* subsp. *flavum* L. (small yellow onion) and *Allium carinatum* subsp. *pulchelum* L. (keeled garlic), belonging to section *Codonoprasum*, were chosen to be investigated in this study. These two species are traditionally used as food, particularly in the Balkan region [12,13]. While *A. flavum* has attracted some attention in recent years as a source of potentially novel therapeutics [14–16], chemical composition and biological activities of *A. carinatum* have been poorly explored.

In this study, methanol extracts of *A. flavum* and *A. carinatum* were chemically characterized and their biological activity was tested both *in vitro* and *in vivo*. The cytotoxic activity and the potential to improve the anticancer activity of Dox were analyzed in human hepatoma (HepG2) and lung carcinoma (A549) cells. Also, the cytotoxicity and interaction with Dox were studied in normal human fibroblasts (MRC-5). Since antioxidant enzymes, superoxide dismutase (SOD1 and SOD2) and catalase (CAT) have a crucial role in cell protection from oxidative stress, such as the one induced by Dox, the effect of extracts on the level of these enzymes was also determined. Finally, the extracts were evaluated for toxicity *in vivo* as well as for anti-angiogenic, cardioprotective and myeloprotective effects upon Dox therapy in the zebrafish (*Danio rerio*) model. To the best of our knowledge, this is the first study demonstrating simultaneous myeloprotective, cardioprotective and anti-angiogenic effect of *Allium* extracts in the zebrafish model.

2. Materials and methods

2.1. Plant material and extract preparation

The whole plants of wild-growing *A. flavum* L. subsp. *flavum* and *A. carinatum* L. subsp. *pulchelum* were collected in July 2009 in Serbia at Vršački Breg and near Vlasina Lake, respectively. The voucher specimens No. 2-1769 (*A. flavum*) and No. 2-1770 (*A. carinatum*) were prepared and identified by Goran Anačkov (botanist PhD) and deposited at the Herbarium of the Department of Biology and Ecology (BUNS Herbarium), University of Novi Sad - Faculty of Sciences. The air-dried whole plants were macerated with 70% aqueous methanol (8 ml/g dry weight) during 72 h at 30 °C, filtrated, evaporated to dryness and dissolved in DMSO to the final concentration of 300 mg/mL.

2.2. Chemical characterization of Allium extracts

The *Allium* extracts were chemically characterized by spectrophotometric analysis of total phenolic content (TPC), total flavonoid content (TFC), total anthocyanin content (TAC) and quantitative LC–MS/MS analyses of 44 selected phenolic compounds. TPC and TFC were determined by methods previously described in Lesjak et al [17] and TAC by the method of Lee et al. [18] The content of 44 selected phenolic compounds (14 phenolic acids, 25 flavonoids, 3 coumarins and 2 lignans) was investigated by LC–MS/MS according to the previously reported method of Orcic et al. [19] Standards of the compounds were purchased from Sigma-Aldrich Chemie GmbH and ChromaDex (Santa Ana, USA).

The Agilent 1200 series liquid chromatograph coupled with Agilent series 6410B electrospray ionization triple-quadrupole mass spectrometer and controlled by MassHunter ver. B.03.01. the software was used for analysis. Analytes were separated using a Zorbax Eclipse XDB-C18 4.6 mm × 50 mm × 1.8 µm (Agilent Technologies) reversed-phase column. Compound-specific, optimized LC–MS/MS parameters are given in Supplementary Table S1. Additionally, fresh bulb volatiles were analyzed by headspace GC/MS (gas chromatograph Agilent Technologies 6890 N with mass spectrometer Agilent Technologies 5975B and headspace autosampler Agilent Technologies 7694E). Detailed parameters of headspace analysis are given in Supplementary material.

2.3. Antioxidant activity of Allium extracts

Antioxidant activity of *Allium* extracts was evaluated by following spectrophotometric *in vitro* assays: total antioxidant status, free radical scavenging capacity and lipid peroxidation inhibitory potential. Total antioxidant status was determined by using a commercial kit (Biorex Diagnostics Ltd, Antrim, UK). Free radical scavenging activity and lipid peroxidation inhibitory potential were determined by DPPH and TBA assays, previously described in Simin et al [16].

2.4. Evaluation of cytotoxicity by MTT assay

The cytotoxic activity of *Allium* extracts and Dox (Actavis, S.C. Sindan-Pharma S.R.L., Romania) was addressed in lung epithelial carcinoma A549 (ATCC CCL-185), hepatocarcinoma HepG2 (HB-8065), and human lung fibroblast MRC-5 (ECACC No. 05090501) cell lines by colorimetric MTT assay, as previously reported by Nikolić et al [20]. The assay was carried out after 24 h incubation of cells (2×10 [4] cells/well) with various concentrations of test substances. To address the cytotoxic effect of *Allium* extracts in combination with Dox, the cells were co-treated with a single Dox concentration (0.125 µg/ml) and different extracts concentrations (15, 30, 60 and 125 µg/ml) for 24 h. All experiments were performed three times with six replicates per treatment point.

To determine the nature of the interaction between each *Allium* extract and Dox the isobologram analysis was applied [21]. The isobole (the line of additivity) is constructed in a two-coordinate plot by setting the IC_{50} values of each single agent at the axes. The concentrations of Dox and the extract used in combination that produced 50% of cell viability are used to define the coordinates of the combinative IC_{50} point.

2.5. Western blot analysis

In order to investigate the influence of *Allium* extracts on alterations of the cell antioxidant defense system caused by Dox, the levels of SOD1, SOD2 and CAT were determined by Western blot in cancer and normal cells following procedures described in the Supplementary material.

2.6. In vivo toxicity evaluation in the zebrafish model

The *in vivo* toxicity assessment of investigated *Allium* extracts and Dox was carried out in the zebrafish *(Danio rerio)* model, as reported by Pavic et al. [22]. All experiments involving zebrafish were performed in compliance with the European directive 2010/63/EU and the Guide for Care and Use of Laboratory Animals of the Institute of Molecular Genetics and Genetic Engineering, University of Belgrade. Briefly, embryos staged at 6 h post fertilization (hpf) were treated with nine concentrations of each extract (1, 2.5, 5, 10, 20, 30, 40, 50 and 60 µg/ml) and Dox (0.1, 0.5, 1, 2.5, 5, 10, 25, 40 and 50 µg/ml). DMSO (0.1% v/v final concentration) was used as negative control. Experiments were performed three times using 20 embryos per concentration.

Apical endpoints (Supplementary Table S2) used for toxicity

Table 1
Total phenolic content (TPC), total flavonoid content (TFC), total monomeric anthocyanin content (TAC) and results of antioxidant activity assays: total antioxidant status (TAS), DPPH$^{\bullet}$ scavenging activity and lipid peroxidation (LP) inhibition ability of *A. flavum* and *A. carinatum* extracts.

Extract	TPC[a] mg/g[b]	TFC[a] mg/g[c]	TAC[a] mg/g[d]	TAS[a] mmol/g[e]	IC_{50} (DPPH$^{\bullet}$)[a] μg/ml	IC_{50} (LP)[a] mg/ml
A. flavum	13.85 ± 2.36	4.45 ± 0.38	75.0 ± 13.5	0.520 ± 0.055	72.3 ± 2.46	1.06 ± 0.198
A. carinatum	5.21 ± 0.40	1.83 ± 0.01	969 ± 89.3	0.366 ± 0.051	222 ± 2.55	> 2.91

[a] the results are given as mean ± SD of three measurements.
[b] mg galic acid equivalents / g of dry extract.
[c] mg quercetin equivalents / g of dry extract.
[d] mg cyanidin 3-*O*-glucoside equivalents / g of dry extract.
[e] mmol Trolox equivalents / g of dry extract.

evaluation were recorded at 24, 48, 72 and 96 hpf using an inverted microscope (CKX41; Olympus, Tokyo, Japan). At the 96 hpf stage, the embryos were inspected for cardiotoxic side effects - an appearance of pericardial edema and heartbeat rate (an average heartbeat rate per min), after which embryos were anesthetized by addition of 0.02% (w/v) tricaine solution (Sigma-Aldrich, St. Louis, MO), photographed and killed by freezing at −20 °C for ≥ 24 h.

In addition to the developmental toxicity, extracts were evaluated for myelotoxicity (neutropenia) in transgenic *Tg*(*mpx:EGFP*) zebrafish embryos, which express enhanced green fluorescent protein (EGFP) in neutrophils, enabling thus direct visualization of neutrophils development upon applied treatments. Embryos were exposed to different concentration at 6 hpf onwards, and evaluated for the neutrophil occurrence at 72 hpf.

2.7. Cardioprotective and myeloprotective effect of Allium extracts

Explored *Allium* extracts were further evaluated for the protective effects against Dox-induced cardiotoxicity and neutropenia in wild type and *Tg*(*mpx:EGFP*) embryos, respectively.

To address cardioprotective effect, zebrafish embryos were pretreated with 10 μg/ml of Dox (corresponding to a dose upon which all treated embryos had impaired cardiovascular functions and were malformed) at 6 hpf stage, while the extracts (1 and of 5 μg/ml) were added at 12, 24, 36 and 48 hpf. An appearance of pericardial edema and the heart beating rate were followed at the 96 hpf stage.

To address myeloprotective effect, embryos at 6 hpf were exposed to Dox, and non-toxic concentrations of each extract were added at 12 hpf, before a period of 24 hpf when neutrophils were formed and became functional. At the 72 hpf stage, embryos were anesthetized by 0.02% (w/v) tricaine solution (Sigma-Aldrich, St. Louis, MO) and the neutrophils occurrence was imaged under a fluorescence microscope (Olympus BX51, Applied Imaging Corp., San Jose, CA, USA). Experiments were performed three times using 20 embryos per concentration.

2.8. Anti-angiogenic potential detection in the zebrafish model

The angiogenesis inhibitory activity of *Allium* extracts was evaluated using transgenic zebrafish *Tg*(*fli1:EGFP*) embryos with EGFP-labelled endothelial cells, as described by Pavic et al. [22]. Briefly, zebrafish embryos at 6 hpf were exposed to the range of non-toxic concentrations of each extract and incubated at 28 °C. After treatments, embryos were anesthetized with 0.02% tricaine and subsequently photographed. The development of intersegmental blood vessels (ISVs) and dorsal longitudinal anastomotic vessels (DLAVs), as well as of subintestinal vessels (SIVs) were inspected and imaged in embryos at 48 hpf and 72 hpf, respectively, under a fluorescence microscope (Olympus BX51, Applied Imaging Corp., San Jose, CA, USA). Auranofin (Sigma-Aldrich) and sunitinib-malate (Suten, Pfizer, New York), clinically used drugs with anti-angiogenic activity, were used as the positive controls. Anti-angiogenic efficacy of the combination treatment with Dox and extracts has also been explored in developing embryos, where Dox and extracts were simultaneously applied to embryos at 6 hpf.

2.9. Statistical analysis

The experimental results were expressed as mean values ± SD of three different trials. The differences between samples were evaluated using the one-way ANOVA followed by a comparison of the means by Bonferroni test ($P < 0.05$). All analyses were performed using SPSS 20 (SPSS Inc., Chicago, IL) software.

3. Results and discussion

3.1. Chemical composition of Allium extracts

Phytochemical profile of examined *Allium* extracts was determined by spectrophotometric analysis of total phenolic content (TPC), total flavonoid content (TFC), total anthocyanin content (TAC) and quantitative LC–MS/MS analyses of 44 selected phenolic compounds. The results showed that TPC and TFC were more than double in *A. flavum* compared with *A. carinatum* extract, while TAC was much higher in *A. carinatum* extract (Table 1). In LC–MS/MS analysis, the MRM mode was applied as the preferred acquisition method. According to the results shown in Table 2, methanol extracts of examined species differ in both qualitative and quantitative phenolic composition. The most abundant flavonoids in both extracts were rutin, quercetin 3-*O*-glucoside and kaempferol-3-*O*-glucoside, while the aglycones quercetin and isorhamnetin were detected only in *A. flavum* extract. Among phenolic acids, ferulic, caffeic and vanillin acids were dominantly present in both extracts, although their content was much higher in *A. flavum* extract.

GC–MS analysis revealed that the only volatile compound present in investigated species is dimethyl-disulfide (relative abundance is 100%; chromatograms are given in Supplementary Figure S1). These data suggest that *A. flavum* and *A. carinatum*, unlike many other *Allium* species, are poor in organosulfur compounds, which is in accordance with their weak odor and flavor.

3.2. Antioxidant activity of the extracts

Antioxidant potential was determined by measuring total antioxidant status, DPPH$^{\bullet}$ radical scavenging capacity and lipid peroxidation inhibition potential. The results have shown that both extracts exhibited antioxidant activity, but in all three assays the activity of *A. flavum* extract was much higher (Table 1). It was expectable, since *A. flavum* had higher TPC and polyphenols have been recognized as the main carriers of antioxidant activity in plants [1,23]. Individual phenolics, differing in structure and quantity, can act as reducing agents, hydrogen donors and singlet oxygen quenchers, contributing to the total antioxidant capacity of the extract.

Table 2
Concentrations of selected phenolic compounds in *A. flavum* and *A. carinatum* extracts determined by LC–MS/MS analysis.

	Content of selected phenolics (μg/g of dw)[a]	
Compound	*A. flavum*[b]	*A. carinatum*
Phenolic acids		
p-Hydroxybenzoic acid	318 ± 10.6	101 ± 3.35
Protocatechuic acid	404 ± 0.46	127 ± 5.87
Vanillic acid	1168 ± 43.1	90.9 ± 3.35
Gallic acid	9.41 ± 0.30	< 5[c]
Syringic acid	521 ± 25.1	42.7 ± 2.06
p-Coumaric acid	279 ± 6.18	168 ± 5.54
Caffeic acid	639 ± 13.0	275 ± 8.2
Ferulic acid	1434 ± 60.6	647 ± 27.3
Sinapic acid	48.8 ± 2.62	< 15
5-O-caffeoylquinic acid	13.6 ± 0.55	< 3.5
Flavonoids		
Isorhamnetin	762 ± 18.5	< 0.75
Kaempferol	160 ± 3.41	67 ± 1.43
Kaempferol 3-*O*-glucoside	1991 ± 34.4	4960 ± 85.9
Chrysoeriol	17.5 ± 0.4	< 0.75
Luteolin	10.2 ± 0.41	4.15 ± 0.17
Quercetin	465 ± 16.8	< 6
Quercetin 3-*O*-glucoside	17658 ± 612	4190 ± 145
Rutin	23685 ± 535	1920 ± 43.4
Other phenolics		
Aesculetin	184 ± 3.7	157 ± 3.15
Secoisolariciresinol	543 ± 24.5	< 12.5

[a] Results are given as concentration (μg/g of extract dry weight) ± standard error of repeatability (as determined by method validation).
[b] Chemical composition of *A. flavum* extract was previously published in [16].
[c] Lower of quantification limit.

3.3. Cytotoxic potential of Allium extracts

Evaluation of the cytotoxic potential of *Allium* extracts and Dox in HepG2, A549 and MRC-5 cells revealed remarkably higher cytotoxicity of Dox in comparison to the extracts towards all three cell lines (Table 3, Supplementary Figure S2). According to determined IC_{50} values, HepG2 cells were the most sensitive to both Dox and the extracts, followed by MRC-5 and then A549 cells (Table 3). Moderate cytotoxicity of the extracts could be attributed to phenolic constituents, such as ferulic and caffeic acids, kaempferol, quercetin-3-*O*-glucoside and rutin, for which cytotoxic activity is documented previously [1,23,24]. Accordingly, stronger cytotoxicity of *A. flavum* than of *A. carinatum* could be due to higher content of total phenolics, higher amounts of ferulic and caffeic acids, as well as to isorhamnetin and quercetin presence only in *A. flavum* extract [14,24,25].

In order to explore the potential of *Allium* extracts to modulate the cytotoxic activity of Dox, we further examined cancer and normal cell survival following combination treatment, where Dox was applied at a sub-lethal concentration for MRC-5 cells (0.125 μg/ml). As shown in Fig. 1 and Table 3, the usage of extracts in combination treatment with Dox markedly reduced the needed dose of Dox to achieve IC_{50}, especially in HepG2 cells (up to 10 times), while protected normal cells (MRC-5) from Dox-induced cytotoxicity. *A. flavum* extract more highly sensitized cancer cells - when applied with Dox decreased the HepG2 cells survival even up to 8%, contrary to their survival of 80% upon Dox alone. This is particularly important since Dox hepatotoxicity limits its permissible therapeutic doses for conventional treatment of hepatocellular carcinoma [2]. On the other hand, *A. carinatum* exhibited better protection in normal MRC-5 cells - it raised the MRC-5 cells survival to

Table 3
In vitro cytotoxicity of *Allium* extracts and doxorubicin (Dox) on cancer and normal cell lines in single and combinative treatments.

	IC_{50} (μg/ml)		
	HepG2	A549	MRC-5
Single treatment			
A. flavum	126.2	153.3	146.6
A. carinatum	195.3	572.1	286.9
Dox	0.6	6.2	1.0
Combinative treatment[a]			
A. flavum	12.5	56.6	nd
A. carinatum	19.9	125	nd

nd – not determined due to antagonism (the protective activity of extracts).
[a] in combinative treatment Dox was administered in a concentration of 0.125 μg/ml.

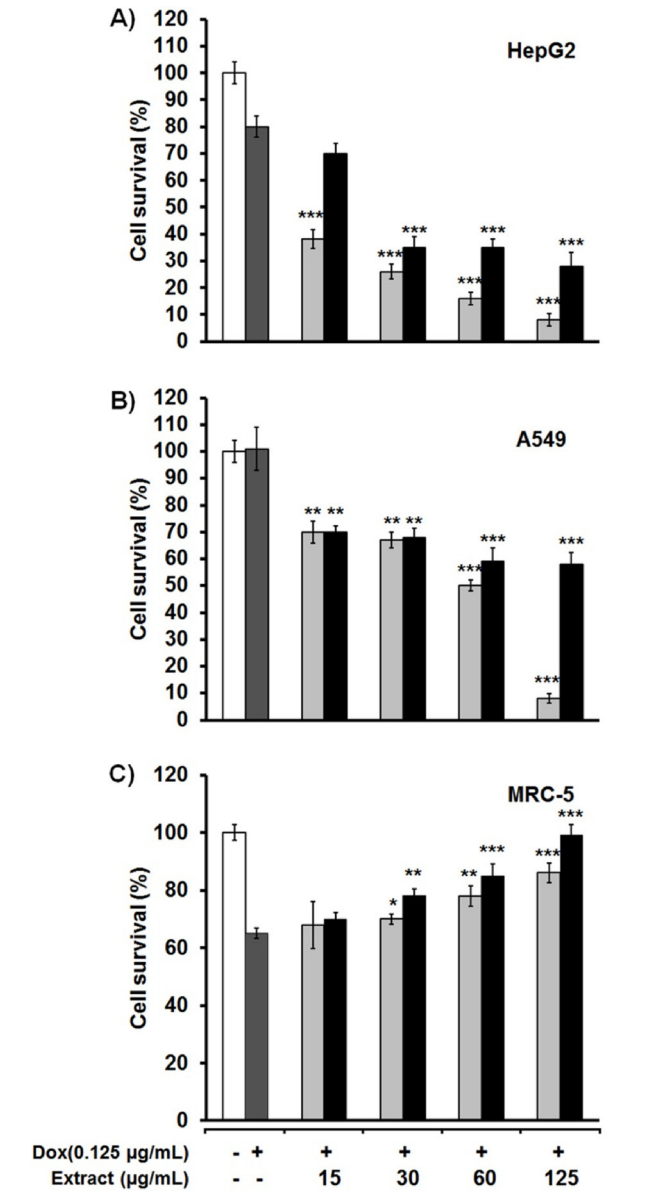

Fig. 1. *In vitro* cytotoxicity of combination treatment by Dox and *Allium* extracts in human hepatocellular carcinoma HepG2 (A), human lung carcinoma A549 (B) and human lung fibroblast MRC-5 (C) cells. Solvent control (DMSO) - white bars; Dox alone - dark grey bars; *A. flavum* - light grey bars; *A. carinatum* - black bars. While extracts improved the cytotoxic activity of Dox against HepG2 and A549 cancer cell lines in a dose-dependent manner, they exerted a protective effect against Dox toxicity in the normal cell line (MRC-5). Data are presented as the mean ± SE of three independent experiments with six replicates per a treatment point. Statistically significant differences between the cell survival upon combination treatment and upon Dox alone (0.125 μg/ml) are denoted with the asterisks (*P < 0.5, **P < 0.01;*** P < 0.001).

99% (Fig. 1C). Isobologram analysis revealed strong synergistic effect of extracts and Dox in cancer cells, but antagonistic in MRC-5 cells (Supplementary Figure S3). Literature data indicate that in case of *A. flavum* extract, both synergism with Dox against cancer cells and protective effect from Dox toxicity in normal cells could be attributed, at least partially, to the presence of quercetin and isorhamnetin. They were shown to potentiate Dox-induced apoptosis in liver cancer and increase Dox concentration in the multidrug-resistant cancer cells, while attenuated Dox cytotoxicity in normal liver cells and cardiomyocytes by reducing oxidative stress, mitigating mitochondrial dysfunction and decreasing DNA double-strand breaks [1,25,26].

3.4. Effect of Allium extracts on antioxidant enzymes

The excessive ROS level and/or attenuation of the cells antioxidant defense can disrupt their redox homeostasis and induce the cell death. Cancer cells are often characterized by an intrinsic oxidative stress and have developed sophisticated adaptation systems, involving the rearrangement of the antioxidant functions, increased antioxidative enzymes level and upregulation of pro-survival molecules [27]. CAT and SODs have a central role in the cancer cells protection of intrinsic oxidative stress, thus usage of their inhibitors can contribute to the resensitization of cancer cells to ROS-induced apoptosis. Since phytochemicals are potential inhibitors of these enzymes [27]. In this study the effect of *Allium* extracts - alone (10 μg/ml) or in combination with Dox (0.125 μg/ml) – on levels of SOD1, SOD2 and CAT in both cancer and normal cells was evaluated by Western blot analysis. The concentration of extract was selected based on our previous finding that 10 μg/ml of *A. flavum* extract modulate SOD and CAT level in MRC-5 cells [14]. Results obtained after 24 h treatment indicated diverse responses depending on the cell line. In A549 cells, we found a significant decrease of all monitored antioxidant enzymes, which could contribute to cytotoxicity of *Allium* extracts and their combinations with Dox (Fig. 2). In the case of HepG2 cells, the level of SOD enzymes was increased by all treatments, but the CAT level was significantly decreased, indicating that reduced capacity of hydrogen peroxide elimination could contribute to the obtained cytotoxicity (Fig. 2). In the cascade of antioxidant defense the function of all components must be coordinated, and therefore a significant decrease of one enzyme in the pathway, in this case CAT, could collapse the whole system. In MRC-5 cells the level of SOD2 was reduced by all treatments, as well as of CAT (except by *A. flavum* alone), while the level of SOD1 varied depending on the treatment: it was increased by *A. flavum* alone and *A. carinatum*/Dox, but decreased by *A. flavum*/Dox. Considering that Dox induces apoptosis in normal cells mainly through the induction of oxidative stress [28], the protective effect of *Allium* extracts on MRC-5 cells could be, at least partially, explained by its ability to neutralize Dox-induced ROS. Moreover, the CAT levels in MRC-5 cells treated with extract/Dox combinations were higher compared to Dox alone, thus contributing to protection of cells from Dox toxicity.

3.5. Toxicity assessment in zebrafish embryos

In order to evaluate the safety of *Allium* extracts *in vivo*, we further explored them for the *in vivo* toxicity and teratogenicity in the zebrafish model and compared them with Dox. Results of this assay, performed over a 96-h period, revealed that both extracts were markedly less toxic than Dox (Supplementary Figure S4), with LC_{50} values determined at 55.8 μg/ml, 50.3 μg/ml and 8.51 μg/ml for *A. carinatum*, *A. flavum* and Dox, respectively. Moreover, embryos treated with extracts showed neither visible skeletal malformations, nor cardiovascular disorders (changed heart morphology, declined contractility and pericardial edema) irrespective to applied extracts' dose (Fig. 3, Supplementary Figure S4). Interestingly, the lack of blood circulation was observed in some part of the tail region suggesting the possible anti-angiogenic activity of explored extracts.

On the other side, it is known that severe oxidative stress and impairments of antioxidant defense induced by Dox chemotherapy lead to many adverse side effects, such as cardiac tissue injury, hepatotoxicity and myelosuppression [3,4]. In our study all Dox-treated embryos suffered from multiple teratogenic defects already at 2.5 μg/ml dose (Supplementary Fig. 4). At a dose of 10 μg/ml, Dox markedly reduced the embryos' survival (by 60%), while all survived embryos developed the life-threating cardiovascular and morphological abnormalities (Supplementary Figure S4). The Dox-treated embryos showed severe cardiovascular damages, such as large pericardial edema, accompanied with markedly decreased heartbeat rate (Fig. 3C) and reduced/absent caudal circulation. They had significantly shorter body, severe skeletal deformities (scoliosis, small head with the reduced size of otocysts/otoliths and eyes, reduced jaw length and malformed tail) and signs of tissue necrosis over the entire body (Fig. 3A). Also, the yolk consumption was significantly reduced in these embryos compared to the control group, probably due to hepatotoxicity and an impairment of yolk sac circulation, contributing to the embryos' growth retardation. The most of these embryos did not survive up to 114 hpf (data not shown).

3.6. Allium extracts successfully rescued the embryos against lethal cardiotoxicity of Dox

Due to all observed harmful side effects of Dox, the adjuvant therapeutic agents that can reduce these effects are very welcome. Given that there are no approved therapies for the treatment of doxorubicin cardioxicity, a zebrafish model of doxorubicin induced cardiomyopathy has recently been used to discover new cardioprotective compounds [29]. The zebrafish has emerged as a versatile platform for the drug discovery and toxicity assessment of bioactive compounds due to their high molecular, genetic, physiological and immunological similarities with humans, and to high correlation in response to pharmaceuticals [30,31].

To examine the possible protective effect of *Allium* extracts on Dox-caused cardiotoxicity, the zebrafish embryos were pretreated with 10 μg/ml of Dox (corresponding to a dose upon which all embryos were affected) and exposed to extracts (1 and 5 μg/ml) at 12, 24, 36 and 48 hpf. Embryos survival, cardiovascular functions (pericardial edema, caudal circulation and heartbeat rate) and the appearance of teratogenic malformations were inspected. To the best of our knowledge, this is first time that the extracts of *Allium* sp. plants have been examined for the cardioprotection in the zebrafish model. The obtained results revealed strong protection at 5 μg/ml of both extracts. The extracts raised the survival of Dox-treated embryos to 100% irrespective of application time. Furthermore, they had potential to totally alleviate cardiotoxic and teratogenic defects caused by Dox (Fig. 3), but this depended on the time of extracts application. While administration of extracts up to 36 hpf rescued the embryos from all skeletal deformities (Fig. 3A) and cardiovascular defects, such as pericardial edema (Fig. 3B) and heartbeat rate ($P < 0.001$; Fig. 3C), the addition of extracts at 48 hpf resulted in weak embryos' protection, manifested as partially restored heartbeat rate and an appearance of pericardial edema and some skeletal deformities (Fig. 3B, C). It suggests that the time of extracts administration was crucial for the cardioprotection during Dox treatment: the total rescue of cardiac dysfunctions is obtained if extracts were applied up to 36 hpf. This indicates that *Allium* extracts could be beneficial if they were administered early during Dox chemotherapy, before irreversible damages of the heart muscle had occurred.

3.7. Allium extracts successfully protected the Dox-treated embryos against neutropenia

Since severe myelosuppression, especially neutropenia, is a commonly encountered problem in patients receiving Dox therapy, the potential of *Allium* extracts to alleviate Dox-caused neutropenia was

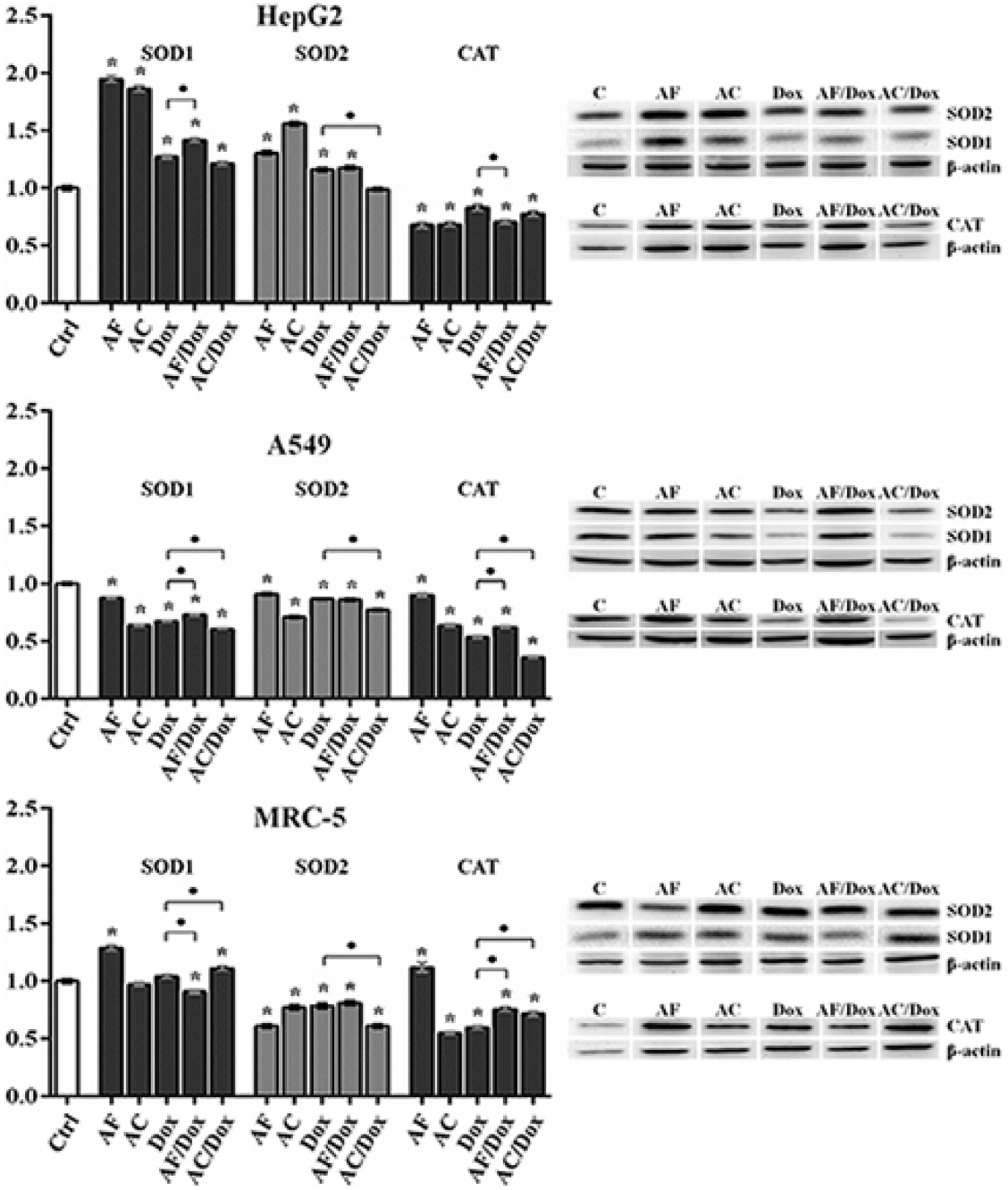

Fig. 2. The effect of *Allium* extracts, Dox and their combinations on the amounts of SOD1, SOD2 and CAT in HepG2, A549 and MRC-5 cells. Ctrl - control (untreated cells); AF - *A. flavum*; AC - *A. carinatum*; Dox - doxorubicin; AF/Dox - a combination of *A. flavum* and Dox; AC/Dox - a combination of *A. carinatum* and Dox. Data are presented as the mean ± SE of three independent experiments. Statistically significant differences compared to the control are denoted with the asterisks above bars (*P < 0.5). Statistically significant differences between different treatments are denoted with the symbol above brackets (•P < 0.5).

also explored. To address this issue, we used the embryos of transgenic *Tg*(*mpx*:*EGFP*) zebrafish line with GFP-labelled neutrophils, enabling thus direct visualization and fluorescence quantification of neutrophils. To the best of our knowledge, this is the first study demonstrating the myeloprotective effect of plant extracts, including those of *Allium* genus, in the zebrafish model.

Obtained results showed that none of the extracts alone caused neutropenia ($P > 0.5$), while Dox decreased the neutrophils occurrence by 51% compared to the control group ($P < 0.001$; Fig. 4). In combination treatments, both extracts applied at 5 μg/ml, demonstrated strong protective effect against Dox-caused neutropenia (Fig. 4A), especially *A. flavum* extract ($P < 0.0001$; Fig. 4B). In addition, these embryos developed without pericardial edema contrary to the group upon Dox alone, suggesting simultaneous myeloprotective and cardioprotective effect of tested *Allium* extracts. This is particularly important if we consider that Dexrazoxane, the only FDA-approved cardioprotective agent for Dox chemotherapy, even worsens myelosuppression in cancer patients [32].

3.8. *Allium extracts have the anti-angiogenic activity*

While controllable and balanced angiogenesis is essential for normal physiological processes, an excessive angiogenesis leads to numerous pathological conditions, including cancer, where the growth, invasion

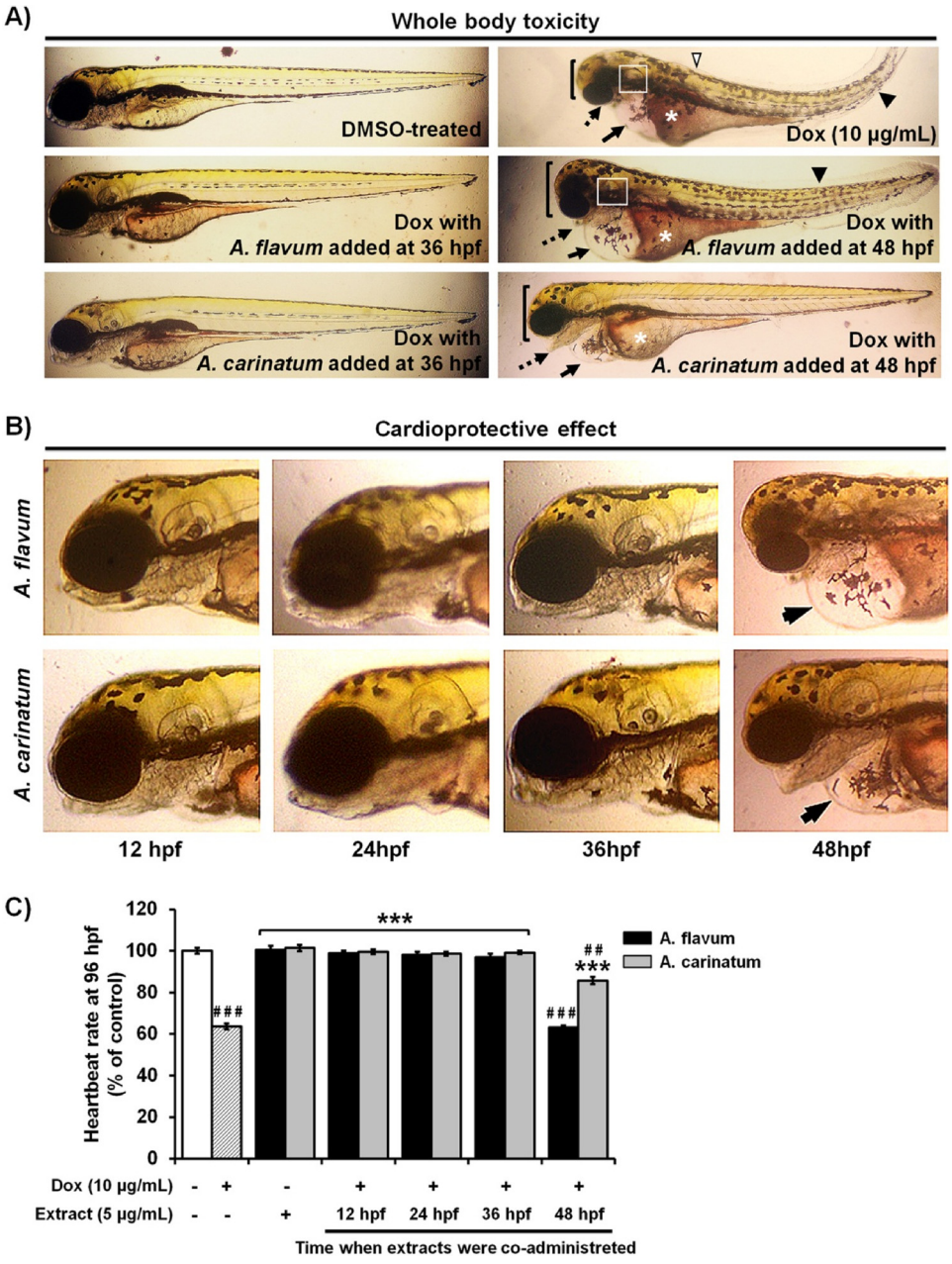

Fig. 3. The time-dependent protective effect of *Allium* extracts on Dox-induced whole body toxicity (A) and cardiotoxicity (B, C) in zebrafish embryos. Embryos were treated with Dox (10 µg/ml) at 6 hpf and extracts (5 µg/ml) at a different time up to 48 hpf. When applied up to 36 hpf, both extracts rescued Dox-treated embryos from skeletal deformities (A) and pericardial edema (B); but if applied at 48 hpf, embryos developed pericardial edema (arrow), malformed head (bracket), jaw (dashed arrow) and otoliths (boxed), and scoliosis (arrowhead). Cardioprotective effect of *Allium* extracts has also been manifested by the improved heartbeat rate of Dox-treated embryos, as evaluated at 96 hpf (C). Data are presented as the mean ± SD of three independent experiments. Statistically significant difference in the heartbeat rate between the Dox-treated group and the control group (### $P < 0.001$), and between embryos upon the combination treatments and Dox alone (*** $P < 0.001$) are denoted.

and metastasis are fully dependent on the neovascularization [33]. Accordingly, the blockade of new blood vessels formation is a proven clinical strategy for the treatment of cancers [34]. Combined with chemotherapy it increases its efficacy and provides significantly better survival rate of the cancer patients [35].

Prompted by the observed pattern of reduced caudal circulation in zebrafish embryos upon treatments with *Allium* extracts, we explored their anti-angiogenic potential. The suppression of angiogenesis was studied using Tg(*fli1*:*EGFP*) transgenic zebrafish embryos, in which the endothelial cells expressing EGFP can be directly observed by a fluorescence microscopy. In normally developing embryos, 6–9 arcades in the SIV basket-like structure and 28–30 ISVs were present. The anti-angiogenic phenotype was defined as the reduced number/length of subintestinal vessels (SIVs) or intersegmental vessels (ISVs) along the whole body, or as disrupted dorsal lateral vessels (DLAVs). The obtained results revealed that extracts inhibit angiogenesis in dose-dependent manner (Fig. 5, Supplementary Figure S5), efficiently suppressing both ISVs and SIVs development even at dose of 2.5 µg/ml ($P < 0.001$; Fig. 5B, C). *A. flavum* was more effective than *A. carinatum* for both ISVs and SIVs suppression, achieving 78% and 98% regression in ISVs length and SIVs basket length (Fig. 5B, C). Interestingly, the *A. flavum*-treated embryos had reduced size of whole SIVs basket, while the embryos upon *A. carinatum* primarily had less SIVs arcades, suggesting probably different molecular mechanisms of their anti-angiogenic activity (Supplementary Figure S5). This is the first study demonstrating anti-angiogenic activity of plants extracts of *Allium* genus in the zebrafish model. So far, the blood vessels suppression has been reported only for a few edible *Allium* species: *A. sativum* [9], *A. aescalonicum* [10] and *A. hirtifolium* [11].

The anti-angiogenic potential of *Allium* extracts was compared with the approved anti-angiogenic drugs - auranofin and sunitinib-malate. At the highest applied dose (15 µg/ml) overall anti-angiogenic effect of *A. flavum* extract was significantly higher than that of both drugs ($P < 0.001$, for both ISV and SIV length), while effect of *A. carinatum* was higher ($P < 0.001$, for both ISV and SIV length) than that of auranofin and comparable ($P > 0.05$, for both ISV and SIV length) to

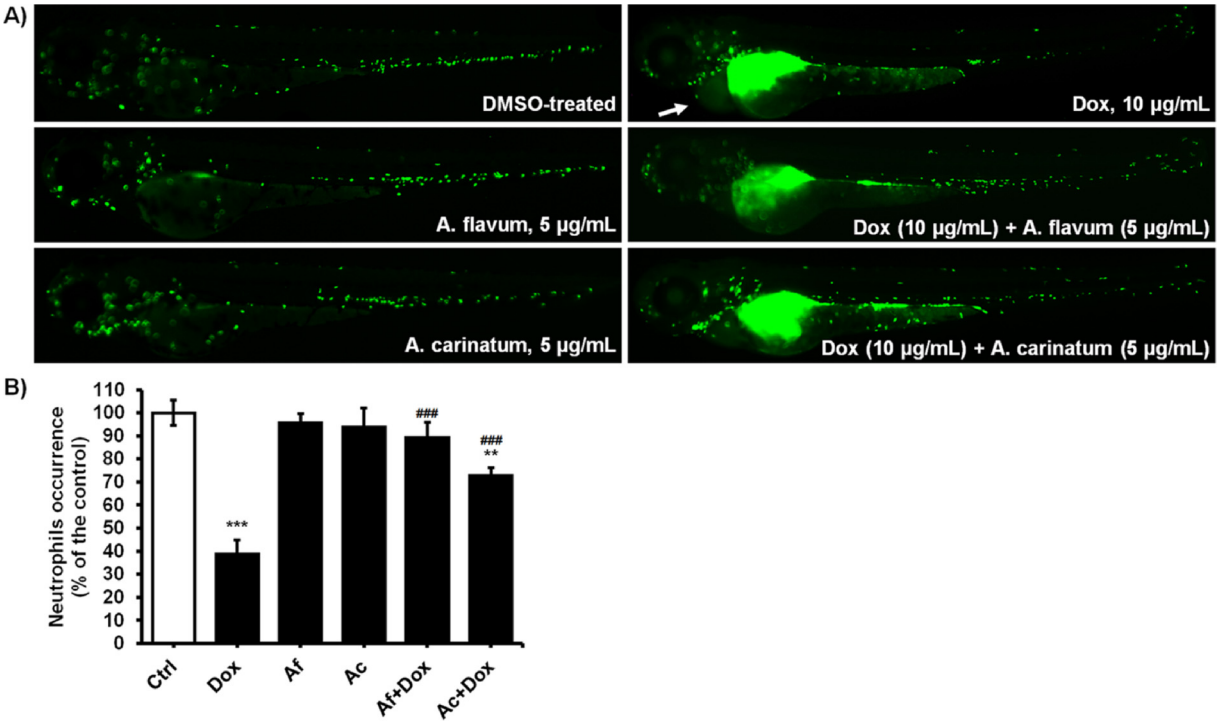

Fig. 4. Myeloprotective effect of *Allium* extracts against Dox toxicity in *Tg*(*mpx*:EGFP) zebrafish embryos. Neutrophils occurrence (A) and the fluorescence intensity (B) upon different treatments are shown. While Dox caused severe neutrophils depletion (neutropenia) and large pericardial edema (arrow) at 10 μg/ml, extracts applied at a dose of 5 μg/ml had no toxic effect on neutrophils, while exerted both myeloprotective and cardioprotective effect in the combination treatments. Strong fluorescence in the yolk sac region in Dox-treated embryos originates from Dox accumulation. Statistically significant differences in neutrophil occurrence between the control (0.1% DMSO-treated) group and the groups upon single treatments (*P < 0.5, **P < 0.01; ***P < 0.001), and between the Dox-treated group and the groups upon the combination treatments (#P < 0.5, ##P < 0.01; ###P < 0.001) are denoted.

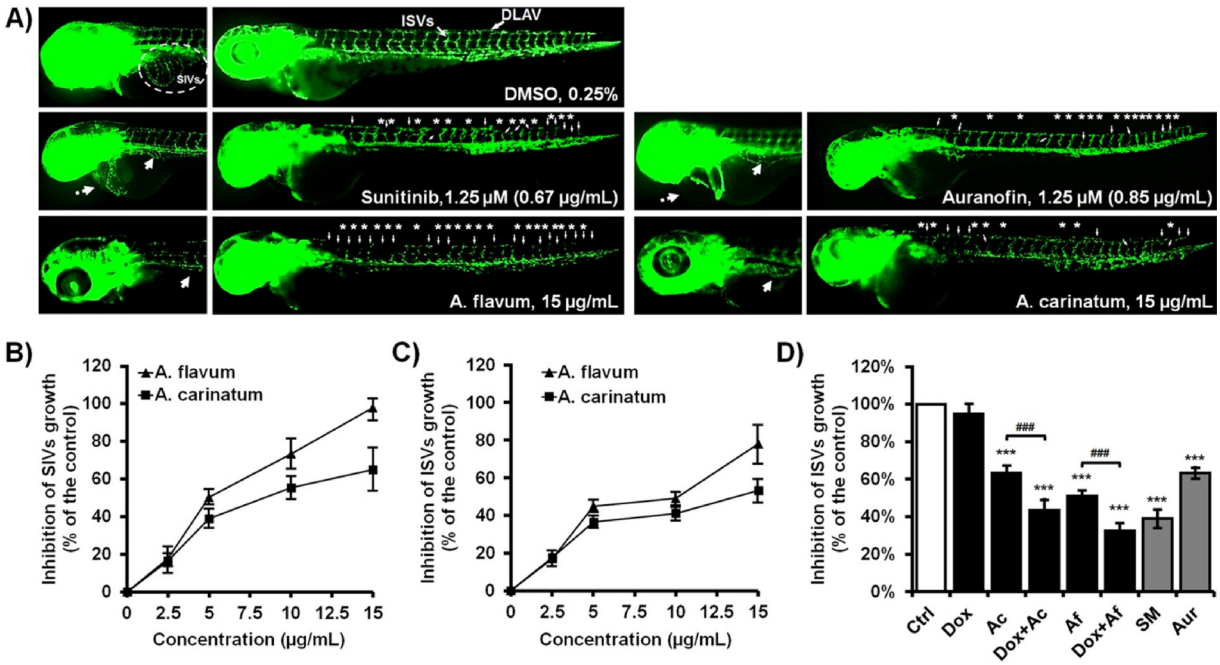

Fig. 5. *In vivo* anti-angiogenic effect of *Allium* extracts applied alone (A and B) and in combination with Dox (C). Effects of the treatments on intersegmental vessels (ISVs) and dorsal longitudinal anastomotic vessels (DLAVs), as well as on subintestinal vessels (SIVs) development in *Tg*(*fli1*:EGFP) zebrafish embryos were assessed at 48 hpf and 72 hpf, respectively. Normally developed ISVs, DLAVs and SIVs are designated in the control (0.1% DMSO-treated) embryo, while disrupted DLAVs (asterisk), thinner or reduced ISVs (arrow) and reduced SIVs (arrowhead) are designated upon applied treatments. Statistically significant differences in ISVs length between the control group and the groups upon treatments (***P < 0.001) as well as between the Dox-treated group and the groups upon the combination treatments (###P < 0.001) are denoted.

sunitinib effect. It is important to highlight that extracts caused no developmental defects in zebrafish embryos, while both auranofin and sunitinib have induced serious cardiotoxic effects (Fig. 5A), retarded the embryos' growth and reduced their survival (data not shown).

Prompted by these findings, we further assessed the anti-angiogenic efficacy of *Allium* extracts in combination with Dox. The dose of extracts causing suppression of SIVs length for 40–50% (5 μg/ml) was used. Since SIVs development was not possible to measure due to strong green fluorescence caused by Dox accumulation in the yolk sac region, we analyzed only ISVs development. As shown in Fig. 5C and Supplementary Figure S6, the suppression of neovascularization by the extracts was markedly higher in the combination treatment with Dox compared to single treatments ($P < 0.001$), with no signs of cardiotoxicity. This strongly implies that *A. flavum* and *A. carinatum* have great potential to be used in adjuvant chemotherapy with Dox.

Further studies are required to elucidate molecular mechanisms of observed anti-angiogenic, cardioprotective and myeloprotective effects of explored extracts. The antioxidant activity probably contributes to these pharmacological activities. Moderate ROS level triggers the endothelial cell proliferation and new blood vessels formation [36], while excessive ROS production leads to cardiovascular damages and myelosuppression [3]. Therefore, neutralization of ROS by the extracts could suppress angiogenesis and prevent above mentioned adverse side effects. This is consistent with higher anti-angiogenic and myeloprotective potential of *A. flavum* compared to *A. carinatum*. In fact, the majority of phenolic acids and flavonoids found in *A. flavum* and *A. carinatum* extract, besides their notable antioxidant potential, were shown to inhibit angiogenesis *in vitro* and/or *in vivo* [37,38], and their synergistic activity could be responsible for demonstrated anti-angiogenic activity. Likewise, cardioprotective effect has been evidenced *in vivo* [25,39] for caffeic acid, rutin, quercetin, isorhamnetin, kaempferol and chrysoeriol, all constituents of *A. flavum* and *A. carinatum* extracts. Also, modulation of apoptosis, the effect on mitochondria and calcium ion regulation, have been reported for the above-mentioned compounds [39], and may be involved here.

4. Conclusion

Since cardiotoxicity is a serious life-threating problem of many approved cytostatics and anti-angiogenic drugs, like Dox and sunitinib, particularly when they are applied in combination therapy, the new protective agents from cardiotoxicity are needed. Our results strongly suggest that wild edible onions *A. flavum* and *A. carinatum* could be great candidates for that purpose. Detailed analysis of their chemical composition revealed that they are rich in phenolic compounds. Further *in vitro* and *in vivo* analysis demonstrated the broad spectrum of pharmacological activities of both extracts including: synergistic improvement of Dox anticancer activity, an effective protection of normal cells from Dox toxicity, protective effect from Dox cardiac toxicity and neutropenia and high anti-angiogenic activity. All these data imply possible application of examined extracts as adjuvants in Dox chemotherapy to increase survival rate and improve quality of cancer patient's life. Forthcoming research should be focused on preclinical testing of combined treatment of Dox and *A. flavum* or *A. carinatum* extracts against different cancers in animal xenografts, particularly against human hepatocellular carcinoma that exceptionally highly upregulate angiogenic factors.

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Conflicts of interest

There are no conflicts to declare.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.biopha.2018.11.106.

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