Medicinal Plant-based Biologically Active Substances and Extracts Inhibit Intestinal Autofluorescence Accumulation in Caenorhabditis elegans

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Abstract

Aging is a complex process related with the gradual diminution in cellular and physiological functions. The geroprotective effect of 10 biologically active substances (BAC – rutin, squalene, kaempferol, biohanin A, urosolic acid, chlorogenic acid, baicalin, mangiferin, quercetin and trans-cinnamic acid) and 5 crude extracts (*Ginkgo biloba, Pulmonaria officinalis, Scutellaria baicalensis, Hedysarum neglectum* and *Panax ginseng*) isolated from medicinal plants of Altai Region of Russia were evaluated for their influence on the accumulation of intestinal autofluorescence material (IAM) using *Caenorhabditis elegans* model. Gravid nematodes were synchronized, and then seeded in 96-well plates to develop to L4-stage. Each BAC in 200 µmol, 100 µmol, 50 µmol and 10 µmol concentrations and extracts with a tenth, hundredth and thousandth times-dilution were administered to each well in 6 replicates for each treatment group. On incubation days 1, 5, and 15, adult L4 nematodes underwent spectrofluorometric analysis to determine the effect of the BACs and extracts on IAM accumulation. It was found that quercetin, kaempferol, baicalin, mangiferin, *Ginkgo biloba* and *Panax ginseng* extracts exhibited the most profound inhibition of IAM accumulation compared to the control. Thus, they can be considered as important precursors or active ingredients for the pharmacosynthesis of geroprotective drugs in future research.

Introduction

By 2050, every sixth person in the globe will be over 65 years old, making up 16% of the overall population, as opposed to 2019, when only 9%, or every 11th lived past this mark[1]. Furthermore, it has been predicted that by 2050, the number of seniors (those over 65) worldwide will triple. As people age in life, there is a change in physical abilities, which may lead to unpleasant experiences, such as a decline in mental capabilities, difficulty in social interactions, or limitation in the types and quality of task execution. These transformations are manifested as one of the several age-related diseases like cardiovascular diseases, cancer, neurodegeneration (Parkinson's, Alzheimer's, and other dementia). The process of aging is intricately linked to the steady decline in physiological and cellular activities[2]. However, current research has shown that food or naturally occurring substances referred to as geroprotectors can slow down, prevent, or even reverse this age-related deterioration[3].

According to recent aging studies, bioactive compounds have exhibited enormous promise for managing even the most severe ailments and may one day replace conventional medications[4]. Nevertheless, the effects of many medicinal plant products on human are largely a mystery, despite the multitudes of research in this area. *Ginkgo biloba, Pulmonaria officinalis, Scutellaria baicalensis, Hedysarum neglectum* and *Panax ginseng* (or their active compounds) are some of the medicinal plants that are often explored for their geroprotective properties[5].

The intestinal autofluorescence material (IAM – identified by various scholars as lipofuscin, age-pigment or death fluorescence) is a non-invasive biomarker of senescence that can be monitored to evaluate the geroprotective effect of bioactive compounds in animal models over time[6,7]. It is an intralysosomal waste

material with a complex chemical composition including proteins, polyunsaturated fatty acid oxidation products, carbohydrates, and some trace elements (such as Al, Ca, Cu, Fe, Mn, and Zn) in varying concentrations[2,8,9]. In mammals and invertebrates, the build-up of IAM has been established as the most persistent and perceptible alteration throughout the aging process[2,10,11].

Although, IAM has been reported not to be biodegradable, which is further highlighted by the fact that starving cells with activated autophagy and lysosomal hydrolases are unable to break down or get rid of the pigment[12]. This could be due to the polymerization and cross linking of peptides with aldehydes that results in plastic-like structures resistant to biological degradation thereby leading to bioaccumulation over time[12]. However, there is no consensus regarding the likelihood of lysosomal IAM degeneration or its exocytosis-mediated removal. The most recognizable characteristic of IAM is its fluorescence property, which can be detected through spectrofluorimetric analysis, or laser-scanning microscopy at excitation/emission maxima of 290–420/430–650 nm[2,13,14]. The intensity of colour emission changes over time (from immature pigment to mature pigment), which makes it easy to monitor[13,14].

Short-lived creatures like fruit flies, zebra fish, mice, rats, and nematodes accumulate the IAM at a greater rate than long-lived species thus validating their use as model animals for aging studies. This is particularly because of their highly active mitochondria, which produce more superoxide and hydrogen peroxide radicals than long-lived species[15,16]. These models make it easier to thoroughly research how newly created medications affect the aging process. However, the model organism *C. elegans* is gaining increasing popularity in these studies because of its numerous advantages. They include short lifespan, easy lab maintenance, transparent body for real-time imaging, advanced genetic, genomic and molecular tools and manipulations, high genetic homology with human etc[17]. Autofluorescence has long been recognized as a correlate of aging in *Caenorhabditis elegans*[14]. Similar to mammalian cells, *C. elegans* intestinal cells frequently exhibit intracellular granules of lysosomal origin, which are the primary source of autofluorescence[18].

In this study, 10 biologically active substances (BACs) namely rutin, squalene, kaempferol, biohanin A, ursolic acid, chlorogenic acid, baicalin, mangiferin, quercetin and trans-cinnamic acid isolated from medicinal plants of Altai Region of Russia and 5 crude extracts (*Ginkgo biloba, Pulmonaria officinalis, Scutellaria baicalensis, Hedysarum neglectum* and *Panax ginseng*) were evaluated for their capacity to delay the onset of aging by monitoring the dynamics of IAM accumulation using *C. elegans* model.

Results

Dose - dependent mode of BAC influence on IAM accumulation. The administration of 50 µmol and 100 µmol of rutin significantly ($p < 0.05$) decreased the rate of IAM accumulation by 0.8 and 1.2-folds when compared to the control (Fig. 1a). In addition, the difference in RFU of IAM accumulation in *C. elegans* treated with 200 and 100 µmol of quercetin were 1.8 and 2.1 folds respectively (Fig. 1a). Similar to rutin, the administration of 200 and 50 µmol of kaempferol also significantly ($p < 0.05$) decreased the rate of

IAM accumulation when compared to the control (Fig. 1a). Furthermore, the 200 µmol of baicalin and chlorogenic acid facilitated a reduction in IAM accumulation whereas the lower doses did not (Fig. 1a). The treatment with squalene and biohanin A at all experimental doses did not have any significant effect in the rate of IAM accumulation when compared to the control (Fig. 1b). Although, the accumulation of IAM was decreased at all doses investigated for ursolic acid, trans-cinnamic acid and mangiferin (Fig. 1b), the decreases by mangiferin at all treatment doses (Fig. 1b) were particularly noteworthy.

Furthermore, the efficacy of kaempferol, baicalin and chlorogenic acid were only significant ($p < 0.05$) at 200 µmol whereas quercetin was more effective at 100 µmol (Fig. 1a). Mangiferin was the only compound to exhibit a significant ($p < 0.05$) decrease in IAM accumulation at the extremely low dose of 10 µmol (Fig. 1b). Also, the IAM accumulation lowering effect of mangiferin at 50 µmol and 10 µmol was evidently visible from Day 5 whereas a higher dose (200 µmol) of quercetin and baicalin were required to produce a similar effect.

Lowering of IAM signals in the presence of medicinal plants extracts. Next, the 10-x of *Gingko biloba* and 1000-x of *Pulmonaria officinalis* significantly ($p < 0.05$) decreased the accretion of IAM by 2.8 and 1.8-folds respectively (Fig. 2). *Scutelleria baicalensis* at all treatment doses did not have a significant ($p < 0.05$) effect on the rate of IAM accumulation, when compared to the control (Fig. 2). In addition, *C. elegans* pretreated with the 100-x concentration of *Hedysarum neglectum* and 10-x concentration of *Panax ginseng* exhibited 1.25 and 1- fold decrease in IAM accumulation respectively (Fig. 2).

Relative to the dynamics of IAM accumulation, the highest (10-x) concentration of *G. biloba* exhibited the most significant ($p < 0.05$) decrease in the rate of IAM accumulation while the lower doses were ineffective (Fig. 2). In contrast, the lowest (1000-x) concentration of *P. officinalis* was required to produce a similar IAM lowering effect (Fig. 2). However, the 10-x concentration of *P. ginseng* exhibited the most significant decrease in IAM accumulation relative to the control. *Gingko biloba* produced a swift effect, noticeable on Day 5 whereas the effect of *P. officinalis* was not evident until Day 15 (Fig. 2).

BACs and extracts inhibit autofluorescence age pigment during C. elegans adulthood. The rate of IAM inhibition exhibited by the 200 µmol of quercetin, kaempferol, baicalin and mangiferin on Day 15 of adulthood were 19.94%, 18.88%, 20.60% and 43.41% respectively when compared to the control (Fig. 3). Similarly, the crude extracts of *G. biloba* and *P. ginseng* exhibited 16.21% and 24.12% inhibition of IAM accumulation respectively (Fig. 3).

Discussion

Several research have shown that plant-derived nutraceuticals can increase the average lifespan of *C. elegans*[19–21]. Also, experimental data suggests that the accretion of IAM which is inversely proportional to longevity can be modulated[22–24]. A cell culture experiment revealed that IAM formation was hampered by exposure to iron chelators, antioxidants and 8% oxygen medium. In contrast, the production rate of

IAM was accelerated by oxidative stressors (such as UV lights, free radicals and heat), as well as the exposure to extremely low-molecular-weight iron compounds and 40% oxygen[22].

A steady increase in the intracellular build-up of IAM has been reported to proliferate oxidative injury in cells via the disruption of lysosomal activity, causing a decline in the antioxidant defence system, lysosomal hemochromatosis, dysfunctional mitochondria and cell death[2,25]. Consequently, any substance that can slow down the rate of accretion of the IAM would make a good therapeutic option for healthy aging.

The BACs and plant extracts adopted in this study are potential geroprotectors with their influence on longevity, antioxidant and other age-related ailments already established in open scientific literature. For instance, quercetin was reported to exhibit a significant extension of lifespan[26], stimulate motility in heat-stressed and aged nematodes *via* the modulation of *hsf-1* activity, p38-MAPK and IIS pathways[27]. Baicalein has been shown to influence longevity and stress tolerance in *C. elegans via skn-1*, whereas it does not affect *daf-16*[28]. Chlorogenic acid and its isomers (4-caffeoylquinic acid and 5-caffeoylquinic acid) acted on the IIS pathway's upstream of *akt*, and then its life-extending and anti-aging properties were primarily mediated through *daf-16* and its downstream stress factors, *hif-1*, *skn-1*, and *hsf-1*[29]. The life extension and oxidative stress tolerance effect of trans-cinnamic acid was also reported by Fedorova et al.[30]. *Gingko biloba* has been reported to prevent mitochondrial dysfunction through the suppression of caspase-9 activation, mitochondrial ROS generation as well as the expression of toll-like receptors thereby mitigating cell necrosis[31–33]. Similarly, various extracts of ginseng have been reported to sustain healthy aging and lifespan in *C. elegans*[34–36].

The aggregation of the aging marker-IAM increases with time which can be aggressive in a number of age-related debilitating diseases, thus leading to cellular and organofunctional deterioration[2,22,37]. Hence, our research was designed to investigate the influence of our BACs and crude extracts on the dynamics of IAM accumulation.

The results of our study suggests that the BACs – quercetin, kaempferol, baicalin and mangiferin as well as the crude extracts of *G. biloba* and *P. officinalis* exhibited the most profound effect in the lowering of IAM accumulation albeit at varying concentrations. In contrast, our study could not find a substantial amount of evidence to support the effectiveness of rutin and squalene as they rather stimulated the process of IAM production while biohanin A was rather inconclusive.

Furthermore, during aging, there is an unwanted decline in the rate of auto-phagocytosis, proteolysis, lysosomal degradation, mitochondrial function, exocytosis, and lipid metabolism all of which partly or in combination promotes the intracellular aggregation of the IAM[38–40].

Quercetin a prominent food antioxidant was able to mitigate the accretion of IAM by 2.1-folds which was consistent with the findings by Kampkötter et al.[41]. The ameliorative effect of quercetin on mitochondrial function and lipid metabolism has been well established as it can restore mitochondrial membrane

potential, scavenge reactive oxygen species, and stimulate AMP-activated protein kinase (AMPK) activity[42–44]. Also, quercetin has been attributed with the restoration of lysosomal function and autophagy flux in pancreatic β-cells through the modulation of lysosomal membrane permeability[45]. Thus, the reversal of lysosomal dysfunction, improvement of lipid metabolism, stimulation of mitochondrial function and autophagocytosis could be some of the plausible mechanisms responsible for the effective reduction in IAM accumulation exhibited by quercetin as early as day-5 according to our findings. The steady decline up to day-15 suggests that the rate of elimination exceeds the rate of accumulation, resulting in a net offset of the IAM.

Both *in vivo* and *in vitro* studies suggests that the activation of AMPK facilitates lipolysis and suppresses lipid biosynthesis[46]. *Scutelleria baicalensis* and its active constituent baicalin play integral role in the prevention and management of metabolic syndrome through the upregulation of the AMPK-facilitated modulation of glucose and lipid metabolism[47]. Similarly, kaempferol in combination with cinnamaldehyde ameliorated lipid metabolism disorders *via* the activation of AMPK[48]. Thus, kaempferol and baicalin might have acted to subjugate the accretion of IAM *via* the regulation of lipid metabolism. Also, baicalin exhibits iron-chelating property thus, precluding iron overloading which promotes IAM production[49]. The IAM accumulation mitigatory effect exhibited by kaempferol in our study corroborates the findings by Kampkötter et al.[50] where treatment with fisetin and kaempferol led to a reduction in IAM accumulation.

The tremendous health benefit of mangiferin has been reported, ranging from antioxidative, antiaging, antidiabetic, immunomodulatory, antiviral amongst others[51]. Mangiferin shields the cell from oxidative stress through the expression of antioxidant enzymes catalase, superoxide dismutase, glutathione peroxidase and mitigates lipid peroxidation[51]. Also, mangiferin stimulates the production of proteins such as cytochrome c oxidase subunit 1, important for mitochondrial biogenesis and suppresses the production of proteins such as acetyl-coA carboxylase 1, integral for lipogenesis[52]. Hence, the protective effect of mangiferin towards the aggregation of IAM particularly at the lowest concentration (10 µmol) implies that it must have acted through multiple mechanisms. Also, it can be adduced from our findings that a trace amount is enough to stimulate the myriads of molecular intervention critical for the inhibition of IAM biogenesis including the restoration of AMPK-mediated autophaghy, oxidative balance, lysosomal and mitochondrial function, modulation of lipid and carbohydrate metabolism[51–53].

The incapability of rutin (a glycosylated variant of quercetin – quercetin-3-O-rutinoside) to delay the accumulation of IAM has also been reported by Kampkötter et al.[41]. This could be attributed to the absence of more reactive centres in rutin, thus reducing its antioxidant potential, when compared to its aglycone variant – quercetin[54].

Also, the ineffectiveness of squalene (a long chain fatty acid-$C_{30}H_{50}$ and precursor for cholesterol synthesis) could be due to the absence of the cholesterol-synthesis branch of the mevalonate pathway in *C. elegans*[55]. This could lead to bioaccumulation and probably serve as lipid-source for IAM production,

hence the increased production reported in this study. Unlike rutin and squalene, the geroprotective effect of biohanin A is still unclear. However, the solubility and bioavailability of biohanin A has been reported and its ineffectiveness in the mitigation of IAM accumulation could simply be attributed to its poor absorption by the nematodes to elicit its biological activity[56].

Ursolic acid and trans-cinnamic acid were able to slowdown the rate of IAM accumulation at all doses investigated. However, their effects were not in folds which might imply a poor pharmacokinetics between the drug(s) and organism or the dose range adopted in this study is outside the optimal dosage required to stimulate a pharmacological response comparable with quercetin and mangiferin.

The 10-x concentration of *G. biloba* and 1000-x concentration of *P. ginseng* exhibited the most profound reduction in IAM accumulation whereas *P. officinalis, S. baicalensis* and *H. neglectum* exhibited a moderate reduction which can be attributed to their constituent BACs. Quercetin and kaempferol were isolated from *G. biloba* while mangiferin was isolated form *H. neglectum.* Furthermore, age-related deterioration involves multiple pathologies that act in synchronization to facilitate aging. As such, the multitudes of BACs present in the crude extract could exert antiaging effect via pharmacodynamic synergy with various compounds acting on different receptor targets of the aging pathways[57]. Thus, the swift and profound effect of the crude extracts of *G. biloba* reported in this study can be attributed to the abundance and potential synergistic effect of these BACs towards the repression of IAM production. A similar effect was not observed in *H. neglectum* treatment group probably due to the presence of other impurities that might have acted as antagonist for the bioactive function of mangiferin. Our findings on the effect of *P. ginseng* on IAM accumulation are consistent with current available literature[34–36]. Ginsenoside, a bioactive component of *P. ginseng* has been reported to extend lifespan and reduce IAM accumulation via the modulation of lipid metabolism and activation of the stress response biomolecules[34]. The rate of IAM clearance was also facilitated in *C. elegans* treated with the 1000-x concentration of *P. officinalis*. This can be attributed to the mitigation of oxidative stress via the removal of mitochondrial ROS which culminates in enhanced mitochondrial function[58].

The intestinal autofluorescence material is not only a hallmark of aging, but also a facilitator of age-related deterioration, thus any substance capable of IAM clearance and/or slow down its rate of accretion will constitute an important supplement towards healthy aging. Our finding has identified baicalin, quercetin, kaempferol, and mangiferin as potential geroprotectors that could be subjected to further studies towards the realisation of the UN decade goal of healthy aging[59]. Their probable mechanism of clearance includes the enhancement of intra-lysosomal degradation, autophagocytosis, mitochondrial function and lipid metabolism. In addition, *Ginkgo biloba* and *Panax ginseng* can be considered as important sources for geroprotectors development in future research, for a potential breakthrough in antiaging-drug discovery.

Methods

Biologically Active Compounds and Crude Extracts

Stock solutions of all the BACs (10 mmol) and crude extracts were provided by the Laboratory of Bioassay of Natural Nutraceuticals, Kemorovo State University, Kemorovo, Russia. They were prepared as described by Faskhutdinova et al.[5] and stored at 4 ℃. The BACs and crude extracts were dissolved in sterilized deionized water to arrive at the concentrations of 200 µmol, 100 µmol, 50 µmol and 10 µmol of each BAC and; a ten-, hundred- and thousand-times dilution of each crude extract used in this study.

Nematode Growth Conditions

The nematodes (*Caenorhabditis elegans*) – wild type N2 Bristol strain was provided by the Swammerdam Institute of Life Sciences, Department of Molecular Biology and Microbial Food Safety, University of Amsterdam, Netherlands. They were seeded with 100 µl of *Escherichia coli* OP50 (Engelhardt Institute of Molecular Biology, Moscow, Russia) pre-coated on nematode growth medium (NGM) plates and housed in a Binder Growth Chamber (KBWF 240, GmbH Tuttlingen, Germany) at 20 °C[60].

Nematodes Synchronisation

Adult gravid nematodes were synchronised using sodium hypochlorite solution to collect eggs, which were left overnight in S-media to produce L1 nematodes. The young nematodes were inoculated with 100 mg/ml of *Escherichia coli* OP50 and 120 µl of the *C. elegans/E.coli OP50* solution were dispensed in 96-well plates, where they were allowed to mature to adult L4 stage. Forty-eight hours later, an aliquot (15 µl) of 5'-fluoro-2-deoxyuridine (1.2 mmol FUDR) was added into each well. Finally, after 24 hours, 15 µl of each BACs and crude extracts at varying concentrations were added to each well to make up a total volume of 150 µl/well. Following the procedures described by Pincus et al.,[14] the dynamics of IAM accumulation was evaluated per-treatment group on days 1, 5 and 15. All experiments were conducted with 6 repeats for each treatment condition. The average nematode count per well for the BACs treatment is 28.75 ± 3.11 (Suppl. Table S1) while the average nematode count per well for the crude extract is 37.82 ± 5.87 (Suppl. Table S2).

Intestinal Autofluorescence Accumulation Assay

The level of IAM accumulation was measured spectrofluorimetrically at excitation - emission maxima of 340–430 nm using CLARIOstar® *Plus* multimodal plate reader (BMG Labtech, Ortenberg, Germany). The measurements were recorded on days 1, 5 and 15 post-administrations of the BACs and crude extracts. Each experiment was repeated three times.

Data Analysis

The data obtained were expressed as the difference between the mean relative fluorescence units-RFU (Suppl. Tables S3-S8) of IAM accumulation at each day of measurement compared to the first day (Eq. 1) for the mean value of six replicates per treatment group. The statistical analysis was conducted using Microsoft Office Professional Plus 2021 (Microsoft Cooperation, Redmond, WA, USA) and GraphPad

Prism 9.0 (GraphPad Software, Boston, MA, USA). The results were considered to be statistically significant at $p < 0.05$ and the error bars on each graph depicts the standard error of means.

$$RFU difference = \{MeanRFU for Day5(\, or Day15) - MeanRFU for Day1\}$$

Declarations

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Competing interests

The author(s) declare no competing interests.

Data availability

The datasets generated and/or analysed during the current study are included in this published article and its supplementary information files.

Author contributions

S.S.S performed the experiments, data collected and analysed, search literature, and wrote the manuscript; **M.E.I** designed the study, interrelated results, edited manuscript; **L.S.V** design the research, supported study, financing and supplies, edited manuscript. All the authors read and approve the final manuscript.

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Figures

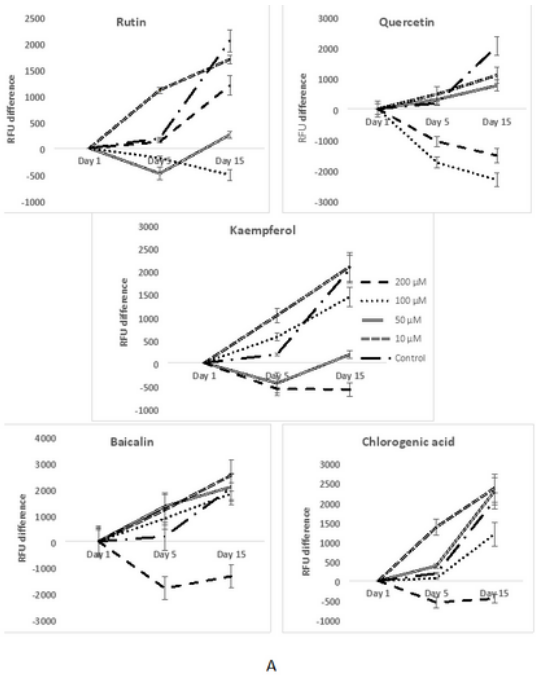

Figure 1

a. Effect of rutin, quercetin, kaempferol, baicalin, and chlorogenic acid compounds on intestinal autofluorescence accumulation in *C. elegans*. Rutin, quercetin, kaempferol, baicalin, and chlorogenic acid were added to *Caenorhabditis elegans* in each well of 96-well plate in concentrations 10 µmol, 50 µmol, 100 µmol, and 200 µmol on day 5st of L4 stage. The intestinal autofluorescence material (IAM) was measured on days 1, 5 and 15 after treatment with BACs. The IAM accumulation results presented as a

difference value between the mean relative fluorescence unit (RFU) of 6 replicates on the day of measurement (5^{st} and 15^{st}), compared to day 1^{st} value. Data obtained are considered to be statistically significant at ($p < 0.05$).

b. Effect of squalene, biohanin A, ursolic acid, trans-cinnamic acid, and mangiferin on intestinal autofluorescence accumulation in *C. elegans.* Squalene, biohanin A, ursolic acid, trans-cinnamic acid, and mangiferin were added to *Caenorhabditis elegans* in each well of 96-well plate in concentrations 10 µmol, 50 µmol, 100 µmol, and 200 µmol on day 5^{st} of L4 stage. The intestinal autofluorescence material (IAM) measured on days 1, 5 and 15 after treatment with BACs. The IAM accumulation results presented as a difference value between the mean relative fluorescence unit (RFU) of 6 replicates on the day of measurement (5^{st} and 15^{st}), compared to day 1^{st} value. Data obtained are considered to be statistically significant at ($p < 0.05$).

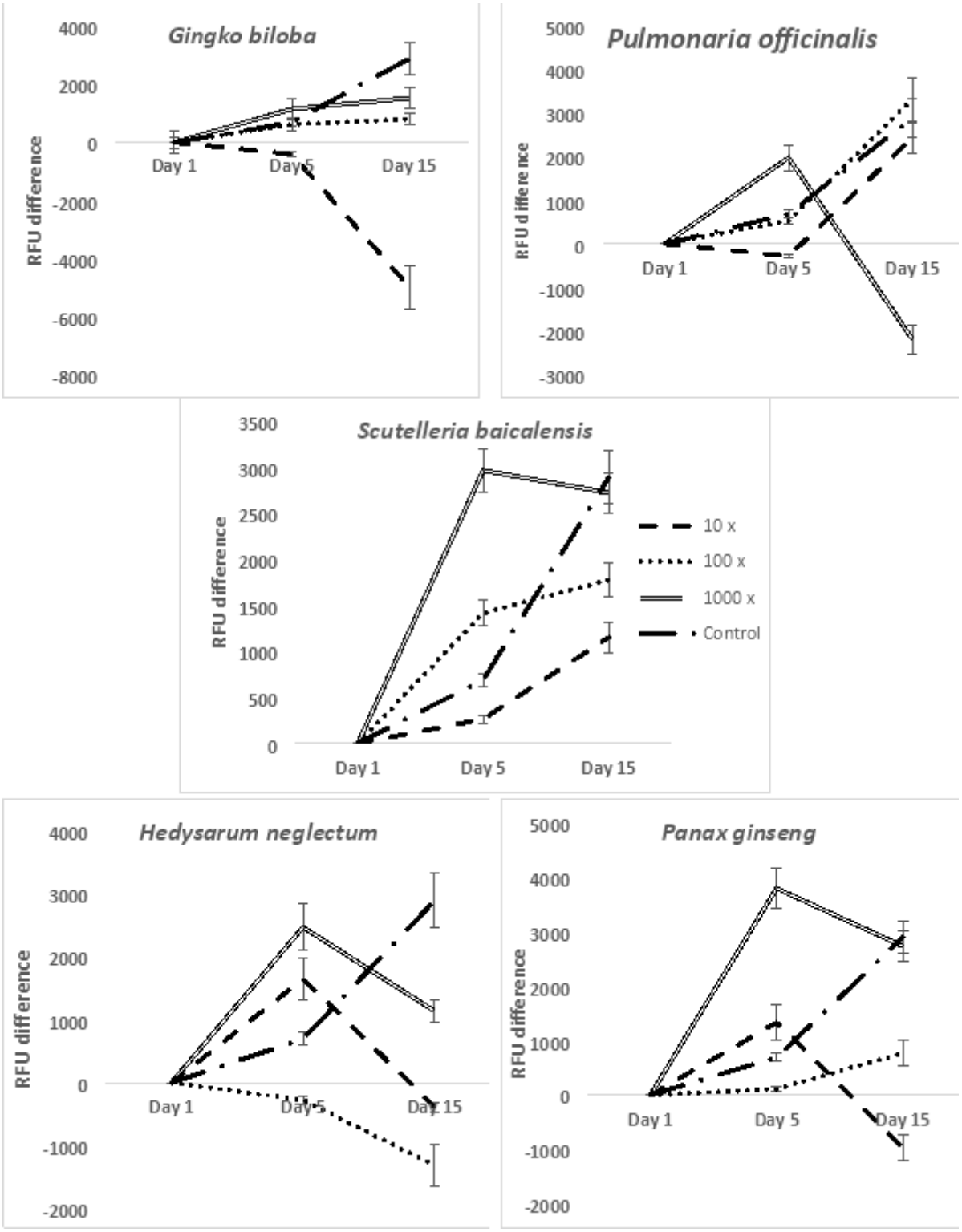

Figure 2

Effect of the crude extracts on intestinal autofluorescence accumulation in *C. elegans*. The result of intestinal autofluorescence material (IAM) accumulation presented as a difference between the first day of measurement and the 5st or 15st day after treatment of nematodes with 10-x, 100-x and 1000-x concentrations of extracts isolated from *G. biloba, P. officinalis, S. baicalensis, H. neglectum* and *P.*

ginseng plants. The data represents the mean ± standard error of means for six replicates and are considered to be statistically significant at ($p < 0.05$).

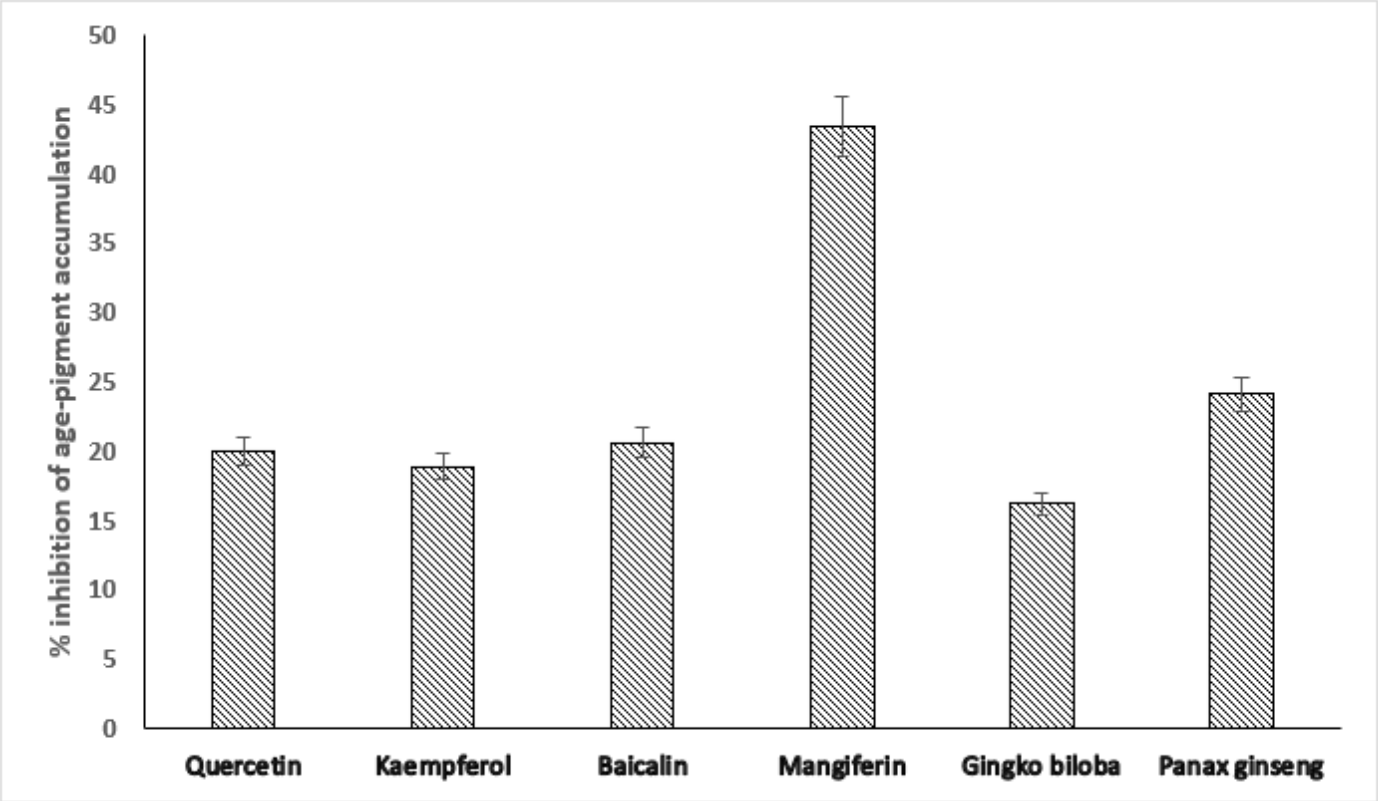

Figure 3

Inhibitory effect of BACs and extracts on age-pigment accumulation in *C. elegans* body on 15st day of treatment. The percentage of intestinal autofluorescence material (IAM) accumulation inhibition calculated relatively to the control group of nematodes at the same conditions of 15st day of treatment by BACs or extracts from medicinal plants.

Supplementary Files

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- Supplementaryfile.docx