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In Vitro Evaluation of Deer Antler Extracts Effects on Secretion of Cytokines, Cell Proliferation and UV-Induced DNA Damage

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Abstract

Deer velvet antler (DAV) emerges as a rich source of glycosaminoglycans, uronic acid, sialic acid, and growth factors, including IGF-1, TGF-β, PGE2, and EGF. These factors contribute to documented regenerative properties, promoting bone growth, wound healing, cartilage repair, muscle growth, immune system function, and skin and hair cell growth. DAV also contains deer antler polypeptides with potential therapeutic effects. The chemical composition of antlers varies during growth, with transcriptome analysis revealing differences in gene expression and concentrations of growth factors and collagen in different parts of the antler. Despite centuries of use in traditional medicine, the mechanisms of action of deer antler extracts remain incompletely understood. The present study aims to shed light on the effects of two types of deer antler extracts on various cell types, offering valuable insights into their potential bioactivities.

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Keywords
Deer velvet antler; aging, anticancer effects, bone metabolism, DNA damage

Introduction

Deer antlers stand out as a remarkable example of mammalian regenerative capacity, representing the sole instance of an organ capable of complete regeneration following detachment from the pedicle [1-3]. This exceptional ability offers a unique opportunity to unravel the intricate mechanisms governing epimorphic regeneration in mammals, a phenomenon rarely observed in nature [4].

Antlers exhibit remarkable growth rates, reaching lengths of up to 80 cm and weights of over 15 kg [5,6]. This rapid growth, with daily increments of 1-3 cm at the tip and tine regions, is accompanied by the production of over 20 cm^2 of skin at the tip, a rate exceeding even that of cancer cells [7,8]. Despite this rapid growth, antlers exhibit remarkable resistance to tumorigenesis, with only a few reported instances of bone tumors [9-11].

Deer velvet antler (DAV) is rich in glycosaminoglycans, uronic acid, sialic acid and growth factors, contributing to its well-documented regenerative properties. Growth factors include IGF-1, TGF-β, PGE2, and EGF, which have been shown to promote bone growth and repair, wound healing, cartilage repair, muscle growth, immune system function, and skin and hair cell growth [12-15]. Antioxidative activity has been proven in biochemical and in vitro tests [16]. There have been in vitro and in vitro studies on deer antler extracts effects on osteoblast differentiation, bone inflammation and regeneration [17,18]. Extracts from different parts of the deer antlers increased proliferation and mineralization in pre-osteoblast cell cultures [19]. Additionally, DAV contains deer antler polypeptides, which regulate various cellular processes and exhibit potential therapeutic effects in various diseases [13,15].

The chemical composition of antlers varies during the growth period and differs between different parts of the antler. Transcriptome analysis has revealed differences in gene expression in different parts of the antler [20,21]. Upregulation of genes involved in mesenchymal stem cell proliferation, osteogenesis, cartilage formation, and angiogenesis has been reported. Differences have been further substantiated by analysis of chemical composition showing varying concentrations of growth factors and collagen in different antler parts [21].

While traditional medicine has utilised deer antler extracts for centuries, and various products are available on the market, there is still a need to fully elucidate the mechanisms of action of these extracts and their effects on different cell types. The present study investigates the effects of two deer antler extracts on osteoblasts, dermal fibroblasts, pancreatic adenocarcinoma cells, and monocytes, providing valuable insights into the potential bioactivities of the extracts.

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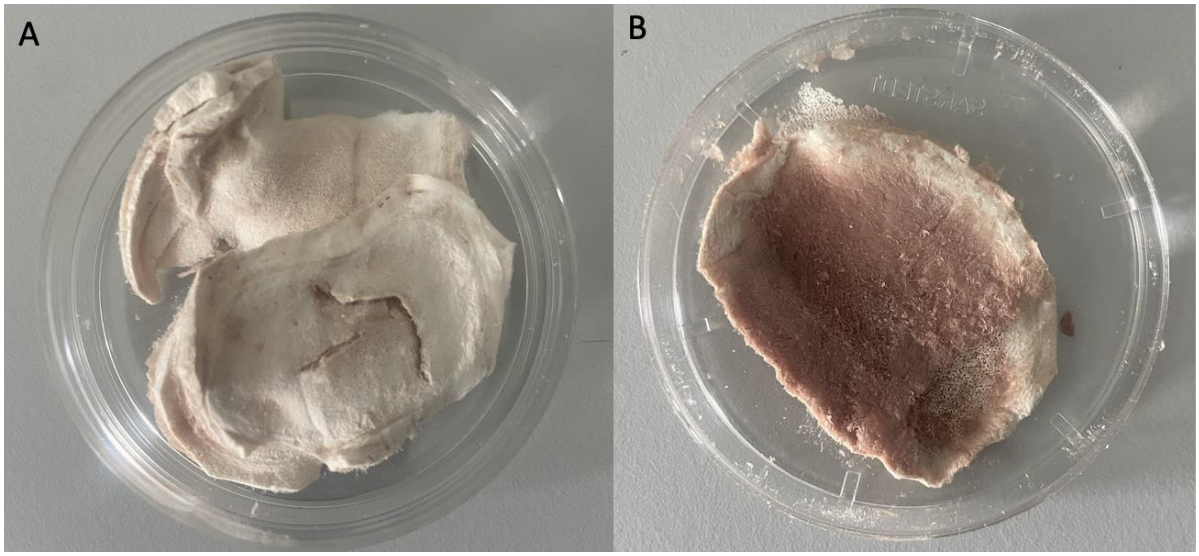

Figure 1. *Freeze dried deer antler samples. A – samples form antler tips; B – samples from middle part, red coloring indicates on the presence of red blood cells.*

Materials and methods

Sample collection

Red Deer (*Cervus elaphus*) velvet anthlers collected in May 2023. After collection alters were cut into small slices, frozen and freeze dried. Freeze dried antlers were stored at room temperature until extraction. Material form two different parts of the antler were used – tips and middle part (Figure 1).

Preparation of extracts

Freeze dried deer antlers by grinded in a powder and extracted with phosphate-buffered saline (PBS, pH 7.4) supplemented with a mixture of 1% penicillin-streptomycin (Sigma) and 1 mM phenylmethylsulfonyl phosphate (PMSF, Sigma) over a 24h period with continuous stirring. A sample to solvent ratio of 1:20 (w/v) was used. After the extraction, the samples were centrifuged for 5 min at 2000 rpm and supernatants collected. Extract samples were filtered through a 0.2 micron filter, aliquoted and stored at -80°C until further testing.

Polyacrylamide gel electrophoresis

Extract samples were subjected to polyacrylamide gel electrophoresis (PAAG) to generally assess differences in qualitative protein content. 6% polyacrylamide gel was used to separate proteins in deer antler extracts. Tris glycine 1 × and SDS 0.1% was used as the running buffer. 20 µg/sample was loaded in the gel in 25 µL of buffer 1 × (Laemmil Loading buffer, 1 ×). 10 µL of Spectra Multicolor Broad Range Protein Ladder was added in the first well. Gel electrophoresis was conducted for 45 min at 120 V. Coomassie blue staining was performed.

Comet test

The Comet test, also known as single-cell gel electrophoresis (SCGE), was used to detect deoxyribonucleic acid (DNA) damage (breaks in one or both double strands) at the individual cell level.

Primary dermal fibroblasts and human osteoblasts (MG63) were cultured in a 6-well cell culture plate in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (abbreviated as 10%FBS/DMEM). After 24 hours of incubation, deer antler extract in different variations was added to the cells. UV-A radiation was used as a DNA-damaging factor. UVA exposure was tested in two ways – cells were irradiated before (PRE) or after (POST) 24 h incubation with deer antler extract. Deer antler extract was tested at concentration 5% (v/v).

After incubation, cells were detached from the culture surface by treatment with 0.25% trypsin/EDTA and attached to an agarose gel on a microscope slide. Slides were placed in cold lysis buffer (2.5 M NaCl/0.1 M EDTA/10 mM Tris-HCl/1% Triton-100/dH_2O) overnight at 4°C. The next day, samples were placed in electrophoresis buffer (300 mM NaOH/1 mM EDTA/dH_2O, pH 13). After 20 min of electrophoresis, samples were neutralized in 0.4 M Tris-HCl (pH 7.5) and fixed overnight in ice-cold 96% ethanol. To assess DNA damage, samples were stained with ethidium bromide and analyzed using a fluorescence microscope (Zeiss Axio). The degree of DNA damage is determined by evaluating comet-like structures on electrophoresis. The intensity of the comet's "tail" relative to the head reflects the amount of DNA damage.

Analysis of IL-10 and TNF-α secretion in U937 cell line

To analyze the effect of deer extracts on the secretion of inflammatory factors, U937 cells were seeded in a 24-well plate at $3x10^5$ cells per well in RPMI (Gibco) medium supplemented with 10% fetal bovine serum (Sigma) and 1% penicillin/streptomycin (Sigme). Deer antler extracts were added at concentrations 0.5%, 1% and 5% (v/v) to unstimulated cells and cells timulated with 5 µg/ml lipopolysaccharide (Sigma). After 24h incubation cell cultivation media was collected for quantification of IL-10 and TNF-α.

Quantification of secreted IL-10 and TNF-α in U937 cell culture media was done using R&D Systems ELISA reagent sets (Human IL-10 DuoSet and Human TNF-α DuoSet) according to the manufacturer's recommended protocol. The results were expressed as the concentration of the secreted analyte in pg/ml and also as the relative changes in the secretion compared to the corresponding controls were analyzed.

Evaluation of effects on viability and proliferation of MG-63, MC3T3-E1 and HpafII cell lines

The effects of deer antler extracts on murine preosteoblast (MC3T3-E1), human osteoblast (MG-63) and pancreatic adenocarcinoma (HpafII) cell proliferation and viability were evaluated.

Cell lines were seeded in 24-well plates at concentration 2.5 x 10^4 cells per well and cultured for 24h before the addition of extracts. DMEM (Gibco) cultivation media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin was used for cultivation of MG-63 and HpafII cells. MC3T3-E1 cells were cultivated in α-MEM (Gibco) media without ascorbic acid supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The extracts were added to cell cultures at concentrations 0.5%, 1% and 5% (v/v) and the cells were cultured for 48 hours at 37 oC in a 5% CO_2 atmosphere. Cells without added extracts were used as control. After the end of the incubation time, 0.5% MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) solution in 10%FBS/DMEM medium was added to the cell cultures and incubated for 1 hour 37 oC in a 5% CO_2 atmosphere. After incubation, the MTT-containing medium was removed, and formazan was formed by viable cells dissolved in 200 mL of dimethyl sulfoxide. Plates were incubated on a shaker for 10 min at room temperature to allow complete dissolution of the dye. Absorbance was measured using a TECAN Infinite 200 PRO spectrophotometer at 570 nm and relative changes in cell viability compared to control were calculated using the formula:

$$Viability\,(\%) = \frac{Abs_{570}(extract) - Abs_{570}(background)}{Abs_{570}(control) - Abs_{570}(background)}\, x100\%.$$

Statistical analysis

Experimental data were analyzed using GraphPad Prism 9 software. Average ± standard deviation (SD) was used to express the experimental values. One-way analysis of variance (ANOVA) with Tukey's multiple comparison test was used for statistical analysis. A p-value < 0.05 was considered to be statistically significant (*$p < 0.05$; ** $p < 0.01$; *** - for $p < 0.001$).

Results

Analysis of genoprotective activity

Comet test was done in dermal fibroblast and MG-63 osteoblast cell lines. UV-A radiation was used as a DNA-damaging factor. UVA exposure was tested in two ways – cells were irradiated before (PRE) or after (POST) 24 h incubation with deer antler extract. Different treatments were used to assess if extracts have protective activity if used before exposure to DNA damaging factors, or they have regenerative activity, reducing negative effects of the DNA damaging agent after exposure.

The results are presented in Figure 2. In a dermal fibroblast cell line (Figure 3) incubated with deer antler extract, it can be observed that cells incubated with the extract before UV irradiation have almost half as much DNA damage as the sample where the extract was added after irradiation. Samples irradiated with UVA without incubation with deer antler extract showed the same trend. No DNA damage was detected in control samples not exposed to UVA.

Similar results regarding damage in pre incubation versus incubation in the presence of extracts after exposure to UVA radiation were observed in the osteoblast cell line MG-63. In the case of osteoblasts, it should be emphasized that a reduction in

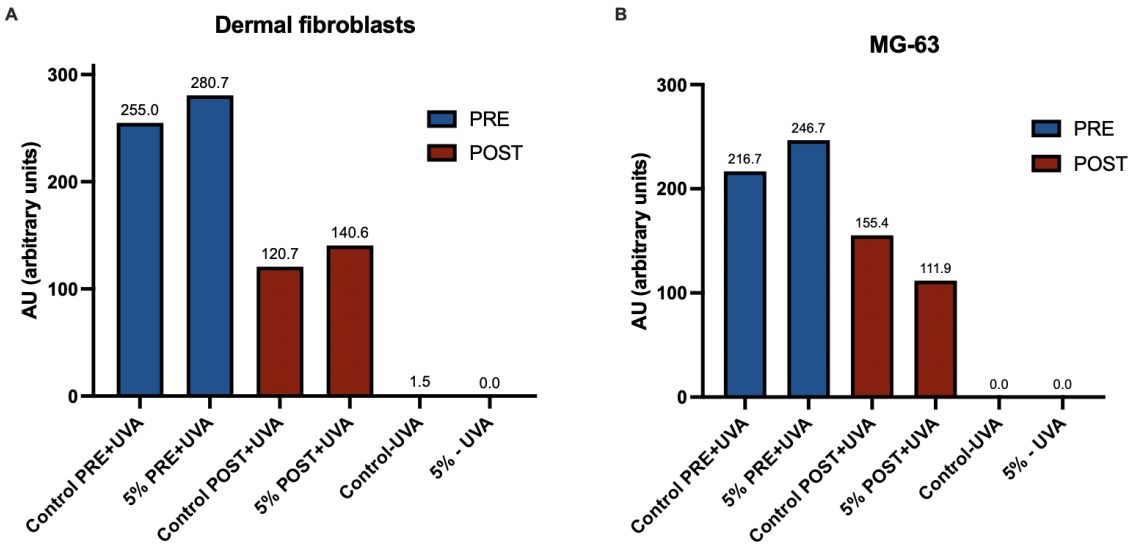

Figure 2. *Evaluation of DNA damage in a dermal fibroblast (A) and MG-63 osteoblast cell lines (B) after incubation with 5% deer antler extract before (PRE) and after (POST) UVA irradiation.*

DNA damage was observed in cell cultures where a 5% extract sample was added after irradiation compared to the control. This result potentially indicates the ability of the extract to stimulate repair mechanisms in cells in response to DNA damage.

Secretion of IL-10 and TNF- α

The obtained results are reflected in Figures 3 and 4.

Regarding the secretion of IL-10, in the case of the light horn extract, it was observed that as the concentration of the tested extract increases, the concentration of IL-10 decreases compared to the control. Dark antler extract also had reduced levels of IL-10 compared to control cell cultures, a reduction observed at all concentrations tested. Addition of deer antler extracts together with bacterial endotoxin (LPS) to cell cultures resulted in a decrease in IL-10 concentrations for both extract samples at all concentrations tested.

In the case of TNF- α secretion (Figure 3) in unstimulated cell cultures, the secretion did not differ from the control. Addition of LPS to the cell cultures along with deer antler extracts showed differences from control cells supplemented with LPS alone. The secretion of TNF- α increased in the case of light horn extract, however, the changes were not statistically significant (Figure 3). In the case of dark deer antler extract, a decrease in TNF-a secretion was observed in LPS-stimulated cells compared to control. Secretions decrease more markedly by increasing the concentration of the extract. The result shows the ability of dark horn extract to reduce the secretion of inflammatory mediators, thus indicating the potential to regulate inflammatory reactions.

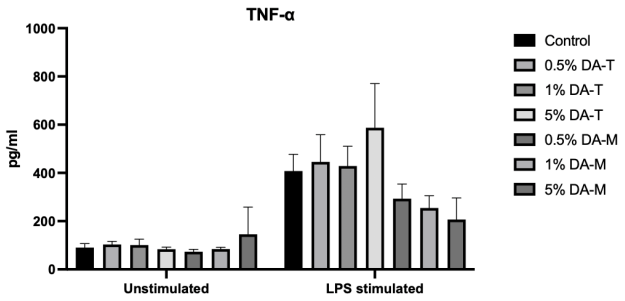

Figure 3. *TNF- α secretion in unstimulated and LPS stimulated U937 cell culture after 24h incubation with deer antler extracts (DA-T – extract from the tip; DA-M – extract from the middle part), n=3*

Analysis of IL-10 secretions on U937 cells supplemented with deer antler extracts showed no increase in secretion.

On the other hand, the analysis of TNF-α secretions in U937 cells to which light horn extracts were added showed an increase in values, the highest observed in cell samples to which light 0.5% horn extract was added, but in samples where LPS was added to the cell line, the highest increase in values was observed in samples where the cells added 5% DA-T + LPS.

Analysis of TNF-α secretions in U937 cells, where dark deer antler extracts were added, an increase in values compared to the control was observed only in samples where 5% dark deer extracts were added.

The results indicate that the addition of deer antler extracts stimulates the immune response, including cell survival, differentiation and proliferation, TNF-alpha concentrations increase.

Effects of deer antler extracts on the viability and proliferation of different cell lines

In osteoblast and pre-osteoblast cell lines, the light horn extract was evaluated. Comparing the effect of the extract on pre-osteoblasts (MC3T3-E1) and osteoblasts (MG63), it was observed that the extract has an activity promoting pre osteoblast division, while in the osteoblast cell line, this extract showed a small but statistically significant division-inhibiting effect at the highest tested concentration. The results indicate that the extract could potentially stimulate the division of progenitor cells, which can be evaluated as a positive effect, for example, in the regeneration of bone tissue. MG-63, on the other hand, is an immortalized cell line derived from osteosarcoma and the anti-proliferative effect of the extract could indicate a potentially desirable effect against tumor cells. Additional in vitro tests are recommended to more specifically characterize the mechanisms of this effect (Figure 5).

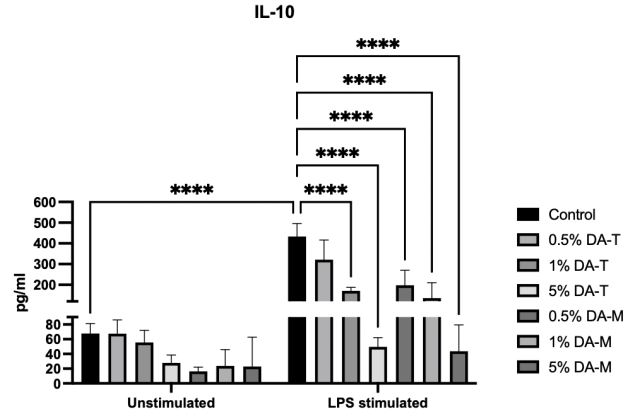

Figure 4. *IL-10 secretion in unstimulated and LPS stimulated U937 cell culture after 24h incubation with deer antler extracts (DA-T – extract from the tip; DA-M – extract from the middle part), n=3, 2-way ANOVA, **** p<0.0001.*

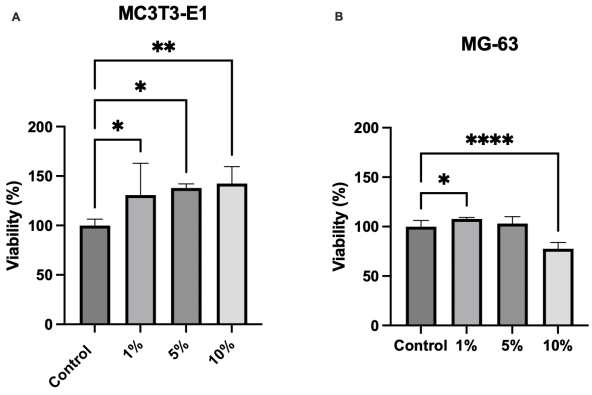

Figure 5. *Effects of deer antler extract DA-T on viability and division of preosteoblasts MC3T3-E1 (A) and osteoblasts MG-63 (B).*

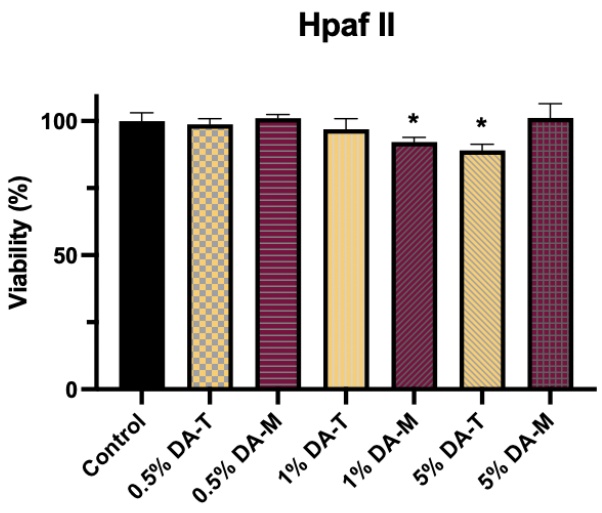

Figure 6. *Effects of deer antler extracts on the viability of the pancreatic adenocarcinoma cell line HpafII.*

The effects of both extracts were evaluated in the pancreatic adenocarcinoma cell line HpafII. The results show that both types of extracts have little effect on cell viability. Although a decrease in viability was observed in the presence of 1% dark horn extract and 5% light horn extract, the change did not exceed 11%, which cannot be considered a cytotoxic effect (Figure 6). None of the tested concentrations showed a stimulatory effect on cell division.

Discussion

Deer antler extracts do not increase secretion of the anti-inflammatory cytokine IL-10 by a monocyte cell line. A decrease in the secretion of this interleukin was observed upon LPS stimulation. In the case of the inflammatory cytokine TNF-α, differences were found between light and dark horn extracts. The ability of dark horn extracts to reduce TNF-α secretion indicates their ability to reduce inflammatory processes.

The pre-osteoblast stimulatory effect of the light antler extract indicates its potential to promote bone tissue regeneration, while the inhibitory effect in the culture of immortalized osteoblasts could indicate the ability to inhibit the division of malignant cells. In the third cell line used in the study, pancreatic osteosarcoma cells, no effect on cell viability and division was detected

Deer antler extract does not show genoprotective activity in a primary cell line, dermal fibroblast culture, but has a damage-reducing effect on the genetic material in the MG-63 immortalized osteoblast line when the extract is added after exposure to a genotoxic agent (in this case, UV radiation). No positive effect was observed when the extract was added before UV irradiation. These observations indicate a potential ability of the extract to stimulate reparative processes, most likely by improving DNA repair pathways. Exposure of genomic DNA to UV light leads to the formation of multitude of types of damage (depending on wavelength and exposure time) that are removed by effectively working repair pathways. DNA double-strand breaks (DSB) and single-strand breaks are NOT formed as a consequence of the direct absorption of UV radiation by DNA. Rather, they are formed as the consequence of the attempted repair of UV radiation-induced base damage in DNA.

The genome of a cell is continuously damaged, which is inevitable because DNA damage often arises as a result of normal cellular processes. A DSB can be caused by environmental exposure of radiation, various chemical agents and ultraviolet light (UV). By-products of the cell's own metabolism such as reactive oxygen species can damage DNA bases and cause lesions that can block progression of replication. The result is double-strand breaks (DSBs) in the chromosome. The good news is that organisms have evolved checkpoint mechanisms (responses that facilitate repair or damage tolerance by arresting cell cycle progression) that inspect the genome for damage. The cell then goes through a series of repair pathways such as base excision repair (BER), nucleotide excision repair (NER), and double-strand break repair (DSBR) [22,23]. DSBs are particularly troublesome because they can lead to cell death if not repaired. And, if not repaired correctly, DSBs can cause deletions, translocations, and fusions in the DNA. These consequences are collectively referred to as genomic rearrangements, and they are commonly found in cancerous cells. Several genes involved in double-strand break repair are significantly upregulated after UV-C irradiation [24].

Genomic instability is an important driver of ageing. The accumulation of DNA damage is believed to contribute to ageing by inducing cell death, senescence and tissue dysfunction [25]. The accumulation of somatic mutations is a driver of cancer and has long been associated with ageing.

Studies across species have also found that longer lived species have lower somatic mutation rates, though these could be explained by selective pressures to reduce or postpone cancer as longevity increases. Overall, with a few exceptions like cancer, results from recent DNA sequencing studies do not add weight to the idea that somatic mutations with age drive ageing phenotypes and the phenotypic role, if any, of somatic mutations in ageing remains unclear. Recent studies in patients with somatic mutation burden and no signs of accelerated ageing further question the role of somatic mutations in ageing.

Thus the ability to reduce mutational burden is a vital and unique property of DAV extract.

The results of the study indicate potential positive properties of the extracts. In order to more concretely and specifically describe the biological activity in future studies, it is necessary to evaluate the effects on bone tissue cells in depth, as well as the different effects in different cancer cell lines.

Conclusions

DNA damage affects most if not all aspects of the ageing phenotype making it a most likely unifying cause of ageing. Hence, targeting DNA damage and its mechanistic links with the ageing phenotype will provide a logical rationale for developing interventions to counteract age-related dysfunction and disease in concert.

Scientific data suggest that DAV extract contains bioactive compounds with tumor suppressor properties. The expected mechanism of action is ensuring the stability of the genome, activating repair processes, reducing the number of mutations. Reducing the mutation rate, reduces gene loss.

Maintenance of a stable genome is a prerequisite for preserving biological function of a cell and hence the organism. Data unveil common mutational processes across mammals, and suggest that somatic mutation rates are evolutionarily constrained and may be a contributing factor in ageing. So we can conclude that DAV extract could be a promising longevity drug candidate targeting genome stability as a therapeutic target.

Author Contributions

Conceptualization, U.K., LP. and E.E.G.; methodology, L.P., U.K.; formal analysis, E.E.G. and U.K.; investigation, D.P., M.P.; writing—original draft preparation, U.K.; project administration, M.P. All authors have read and agreed to the published version of the manuscript.

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Declaration of conflict of interest

The authors declare no conflicts of interest regarding the publication of this article.

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