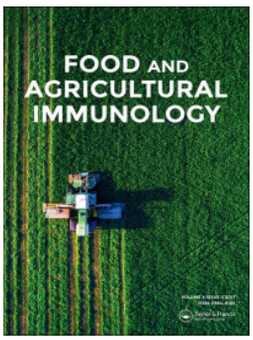

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A new high-quality elderberry plant extract exerts antiviral and immunomodulatory effects *in vitro* and *ex vivo*

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ABSTRACT

Infections of the respiratory system, including common cold and influenza, are affecting people worldwide and are more or less prone to spread depending on the season and viral load of the host. For reducing symptoms and duration of illness, treatment options to standard prescribed drugs are in demand. Natural products could provide immune-supporting treatment alternatives. Elderberry extracts have been used in traditional medicine for the treatment of respiratory infections for decades and numerous studies describe the beneficial effects of elderberries on the immune system and respiratory infectious disease. We investigated the immunomodulative and antiviral effects of a high-quality, anthocyanin-enriched elderberry fruit extract (eldosamb®). Results reveal that elderberry extract reduced the secretion of pro-inflammatory cytokines TNF-α and IFN-γ, leading to a shift towards the Th2-Helper cell response and showing antiviral efficacy against the MVA virus. Thus, with its anti-inflammatory and antiviral bioactivity the proprietary elderberry extract suggests its use as an immunomodulatory health product.

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Elderberry (*Sambucus nigra* L.) extract; TNF-α; inflammation; T-cell activation; modified vaccinia virus Ankara; IL-10

Introduction

Elderberry (*Sambucus nigra* L.), also known as black elder, is a bush or tree plant belonging to the Adoxaceae family of musk herbs and is native to Europe, Northern Africa, Western Asia and to the warmer regions of North America. Most scientific studies have been performed on the species *Sambucus nigra* L., which has blue-black berries and ivory white flowers. Elderberry is known for its beneficial effects for the prevention and treatment of common cold, influenza and upper respiratory infections for many years (Edwards et al., 2015; Mahboubi, 2020). Recently, published studies on elderberry flower and fruit extracts increased substantially, especially in the years 2020 and 2021, also due to the special interest in elderberry's antiviral effects in context with the

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global Covid-19 pandemic (Adams et al., 2020; Bartak et al., 2020; Brendler et al., 2020). Besides the interest of the scientific community, also consumer interest on botanical preparations grew noticeably, with elderberry products being increasingly sold in 2020 (Krawiek, 2021).

Elderberries contain different active ingredients and phytochemicals, including (pro)anthocyanins (Stuppner et al., 2020), flavonoids and (poly)phenolic acids (EMA, Committee on Herbal Medicinal Products, 2014; Ferreira et al., 2020), all of which are supposed to make their contributions to described antimicrobial and antiviral effects as well as immunomodulation, as shown in different preclinical studies (Barak et al., 2001, 2002; Frank et al., 2020; Kinoshita et al., 2012; Roschek et al., 2009; Torabian et al., 2019). As such, it could be shown that elderberry and in particular cyanidin 3-glucoside, its primary anthocyanin compound, reduce the infectivity of the influenza virus at different infection stages (Torabian et al., 2019) and elderberry extract reduces infectivity by binding to H1N1 virions, thereby blocking the ability to infect host cells (Roschek et al., 2009). Besides influenza, elderberry is further able to inactivate and reduce high virus loads of other viruses (e.g. rhinoviruses, HIV, coronaviruses) (Frank et al., 2020; Porter & Bode, 2017; Vlachojannis et al., 2010; Wermig-Morgan, 2020). Furthermore, the bioactivity of elderberry fruits against different pathogenic microorganisms, such as *Streptococci and Staphylococci* is suggested (Bussmann et al., 2014; Cioch et al., 2017; Krawitz et al., 2011; Wermig-Morgan, 2020).

Mechanistically, it was shown that elderberry extract exerts both pro- and anti-inflammatory properties, based on the secreted cytokines after stimulation (Barak et al., 2001, 2002), suggesting an overall modulation of the immune system.

Of note, several clinical studies investigated the effectiveness of elderberry in common cold, flu and influenza, proving an overall symptom reduction, shortened duration of illness or reduced use of medication. Two recent reviews and a meta-analysis summarize the available data from available clinical studies (Kong, 2009; Rauš et al., 2015; Tiralongo et al., 2016; Zakay-Rones et al., 1995, 2004) on the effects of *Sambucus nigra* L. on acute respiratory viral infections (Harnett et al., 2020; Hawkins et al., 2019; Wieland et al., 2021), attesting elderberry the ability to substantially reduce upper respiratory symptoms. Importantly, no serious adverse events after elderberry extract consumption were reported in those studies.

The purpose of the current study was to evaluate a new proprietary and high-quality elderberry extract (eldosamb®) regarding its antiviral and immunomodulatory properties. An *in vitro* viral inactivation assay and different *ex vivo* whole blood assays were applied to obtain comprehensive insights into the underlying immunological mechanisms. Results revealed that elderberry extract reduced the secretion of pro-inflammatory cytokines TNF-α and IFN-γ, leading to a shift towards the Th2 -Helper cell response and showing high antiviral efficacy against the enveloped MVA virus.

Materials and methods

Sample/study product

Freshly prepared and standardized (≥ 13% anthocyanins) powdered elderberry fruit (*Sambucus nigra* L., fructus) extract (Article Number 00-115-1016-07, eldosamb®,

Anklam Extrakt, Anklam, Germany) was used. The growing area of the used elderberry fruits is in Eastern Europe. The extract raw material was obtained from IQF (individually quick frozen) berries and was enriched in anthocyanin content in compliance with the highest GMP standards. Drying was carried out in a vertical powder dryer under vacuum (Ekato Systems, Schopfheim, Germany).

The dry extract was diluted in PBS buffer for use in the experiments. To evaluate the immunomodulatory effects in the different setups non-toxic concentrations were applied. Preliminary experiments to evaluate the maximal concentration tolerated for the *ex vivo* experiments were performed by using a lymphocyte transformation test in co-stimulation with an antigen-mix (Baehr et al., 2001).

Test setups

Ex vivo *TNF-α inhibitor assay*

Heparinized blood was used from 10 healthy voluntary donors after formal approval and signed informed consent. This study follows the principles of the Declaration of Helsinki. Whole blood was investigated for TNF-α release after stimulation with the elderberry extract in four different concentrations (72, 36, 18 and 9 µg/mL) in the supernatant with and without co-stimulation with lipopolysaccharide (LPS, Sigma Aldrich, Taufkirchen, Germany). Incubation was performed at 37°C and 5% CO_2 for 4 h. TNF-α levels were determined in the supernatant by chemiluminescence immunoassay using a fully automated analyzer IMMULITE® 1000 (Siemens Healthineers, Erlangen, Germany). The test procedure was performed in two independent runs with the blood of five donors each. The concentration of 9 µg/mL was evaluated in the second run only. TNF-α release inhibition was calculated in comparison to the respective basal value (LPS-stimulated, no extract) for each blood sample.

Ex vivo *T-cell activation assay/Determination of Th1/Th2 cytokine profile*

Heparinized blood from healthy voluntary donors was incubated *ex vivo* with a lymphocyte-specific stimulus (Concanavalin A (Con A)/staphylococcal enterotoxin B (SEB) (Sigma Aldrich, Taufkirchen, Germany)) with or without the elderberry extract in three different concentrations (72, 36 and 18 µg/mL) to investigate the modulation of the T-cellular immune reaction and determination of the Th1/Th2 cytokine profile. In addition, stimulatory effects by the elderberry extract alone were investigated. After incubation at 37°C and 5% CO_2 for 24 h, the cytokine profile (IL-2, IL-4, IL-10 and IFN-γ) was determined in the supernatant via Multiplex Assay (EMD Millipore Corporation, Billerica, MA, USA) using Luminex xMAP Technology (Luminex, Austin, TX, USA). The test procedure was performed in two independent runs with the blood of five donors each.

In vitro *infection assay of the modified vaccinia virus Ankara (MVA)*

For this quantitative suspension test, modified vaccinia virus Ankara (Institute of Animal Hygiene and Veterinary Public Health in the Centre of Veterinary Public Health of the University Leipzig) was passaged on BHK-21 cells (charge 300920, Friedrich-Loeffler Institute, Germany) and incubated for 1 and for 10 min contact time at room temperature with elderberry extract. The extract was examined as an 80% solution in the presence

of 10% interfering substance (5% (w/v) BSA Fraction V (Sigma Aldrich), 0.4% (w/v) Mucin bovine Glandula submandibularis Type I-S (Sigma Aldrich), 5% (w/v) yeast extract (Sigma Aldrich)) resulting in a final concentration of (0.24% and 1.2%). The mixture was then 10-fold serially diluted in sextuplicates and titrated onto confluent BHK-21 (*Mesocricetus auratus* kidney) target cells grown in microtiter plates in culture medium (DMEM (CCPro) with 10% FCS).

Tissue culture infectious dose 50 ($TCID_{50}$) was determined by determination of the cytopathologic effect and by counting infected wells/cell according to Spearman-Kaerber. Reduction factor as a measurement of viral clearance was determined by log10 reduction between control and plant extract. The virucidal activity was determined as the percent difference of the log titre of the virus incubated with elderberry extract compared to the log titre of the virus control.

The assay was performed according to DIN EN 14476 (DIN EN 14476, 12, 2011) and in consideration of Organisation for Economic Co-operation and Development (OECD) guidelines.

Statistics

Data are analyzed by one-way ANOVA and Dunnett's Multiple Comparison post-test (Post-test *versus* respective control for each cytokine separately). In the case of non-normal distribution, Friedman test with Dunn's Multiple Comparison Test was applied. Two-sided statistical tests were performed. A p-value < 0.05 was regarded as statistically significant.

Results

The immunomodulatory properties of elderberry extract were evaluated using different *ex vivo* blood assays.

Elderberry extract inhibits LPS-induced TNF-α release

Monocytes have phagocytic properties and mature into macrophages within the target tissue. The monocytic reaction to external stimuli can be detected by the cell's TNF-α release. In a TNF-α inhibition assay using heparinized blood, elderberry extract showed the ability to inhibit LPS-induced TNF-α release on the monocytic level. Compared to LPS, elderberry resulted in an inverse dose-dependent reduction of LPS-induced TNF-α release, which was significant for 72 and 18 µg/mL elderberry extract ($p < 0.05$; 28.1% inhibition and $p < 0.01$; 44.2% inhibition) (Figure 1).

Elderberry extract alone showed a low TNF-α induction only in its highest concentration tested (basal level: 4.9 pg/mL, stimulation with elderberry extract (72 µg/mL): 11.0 pg/mL), excluding any contamination of the plant extract with endotoxins, and excluding a general notable pro-inflammatory action of elderberry extract in the absence of a pro-inflammatory milieu. Much higher TNF-α concentrations were measured after LPS stimulation, see Figure 1.

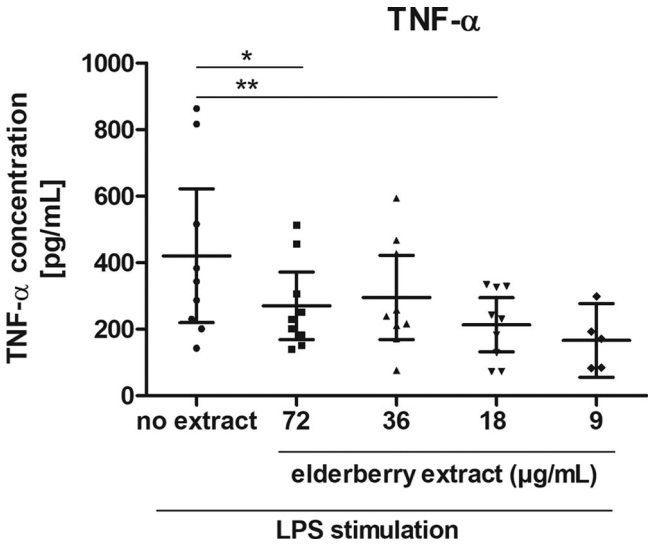

Figure 1. Inverse dose-dependent inhibition of TNF-α release after LPS-stimulation by elderberry extract. TNF-α levels (pg/mL) are shown as mean ± 95% CI ($n = 9$; $n = 5$ (9 µg/mL)).

Elderberry extract stimulates the Th2-Helper cell response

Immune responses polarized by either Th1- or Th2-Helper cell subsets result in different inflammatory effector pathways and immune reactions. The Th1/Th2 cytokine profile was determined in an *ex vivo* T-cell activation assay, measuring the secretion of key Th1- and Th2-type cytokines. We observed an elderberry extract-induced modulation of the Th1/Th2 response with a shift towards the TH2 response, mainly by reduction of IFN-γ release and increase of IL-4 secretion when co-stimulated with the lymphocyte-specific stimulant and T-cell mitogen Con A/SEB (Figure 2(A,C)). Elderberry extract (72 µg/mL) reduced IFN-γ release by 25.6% ($p < 0.01$) and increased IL-4 release by 17.1% ($p < 0.05$). In addition, IL-2 was reduced by 13% ($p < 0.01$) (Figure 2(B)), while the regulatory anti-inflammatory cytokine IL-10 was increased by 13.1% ($p < 0.05$) with 72 µg/mL elderberry extract and by 15% ($p < 0.01$) with 36 µg/mL elderberry extract in the Con A/SEB-stimulated immune response (Figure 2(D)).

The Th1 and Th2 cytokines cross-regulate each other, providing a mechanism for regulation of immune responses and fast adaptions to external stimuli. Decreased IFN-γ/IL-4 ($p < 0.001$) and IFN-γ/IL-10 ($p < 0.001$) ratios after challenge with 72 µg/mL elderberry extract indicate a significant shift towards the Th2 immune response (Figure 2(E,F)).

Of note, basal cytokine concentrations in donors as well as after stimulation with the elderberry extract alone without Con A/SEB co-stimulation, did not result in measurable cytokine secretions in most subjects, except of minor levels of IL-4 detected in some blood donors which were also present in basal measurements without the elderberry extract (data not shown).

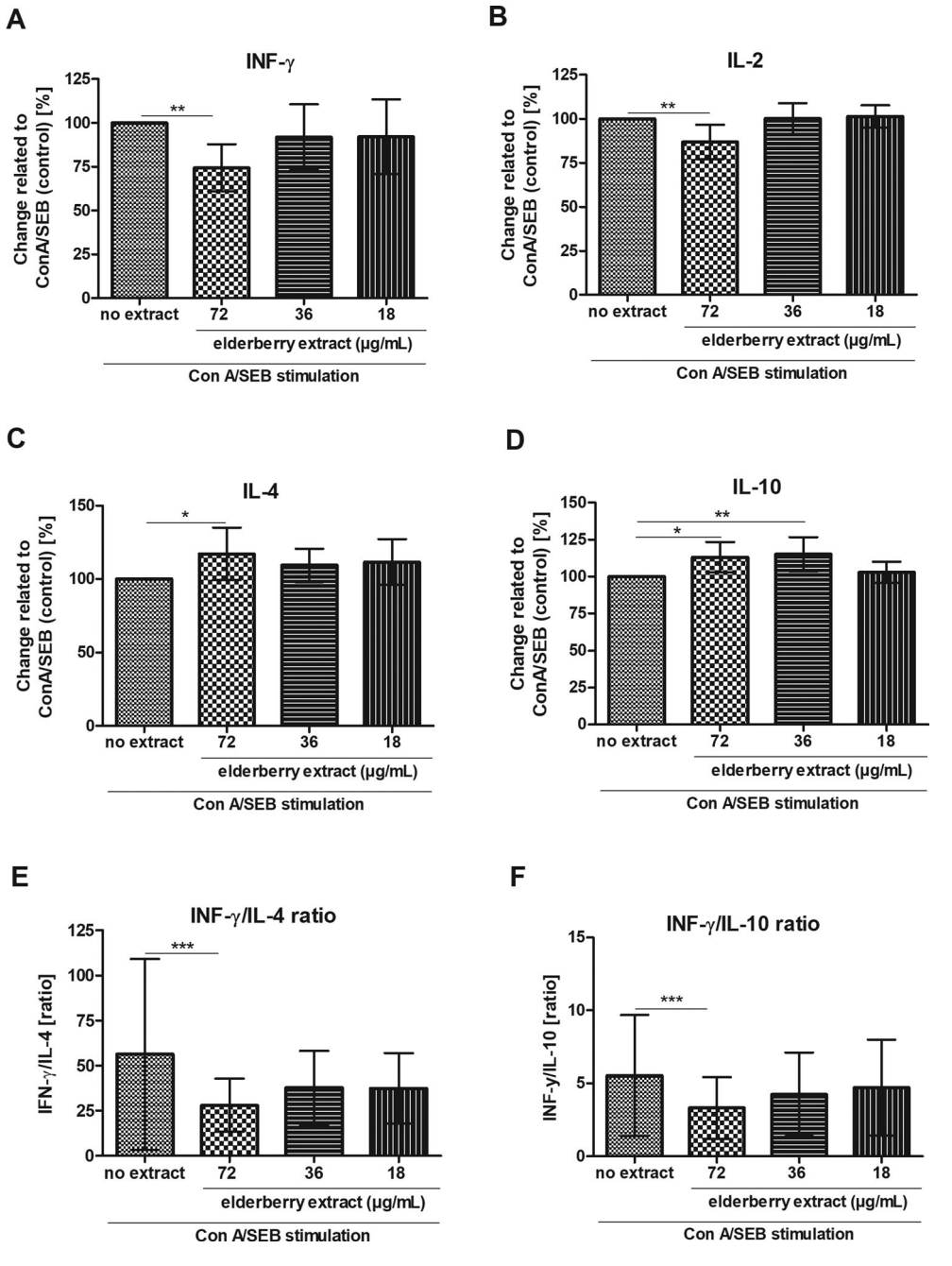

Figure 2. Modulation of Th1/Th2-related cytokine release by elderberry extract, co-stimulated with Con A/SEB. Inhibition of IFN-γ (A) and IL-2 (B) release, induction of IL-4 (C) and IL-10 (D) release and decrease of the IFN-γ/IL-4 ratio (E) and IFN-γ/IL-4 ratio (F) by elderberry extract. A-D are depicted as % change compared to Con A/SEB (control treatment) as mean ± 95% CI ($n = 9$ (IFN-γ), $n = 10$ (IL-2, IL-4, IL-10)). Ratio levels in E-F are depicted as mean ± 95% CI ($n = 9$).

Elderberry extract possesses antiviral activity against the modified vaccinia virus Ankara (MVA)

To assess the antiviral activity of elderberry extract, MVA viruses produced in BHK-21 cells were chosen as a surrogate for enveloped viruses and were tested in an *in vitro* infection assay. In this assay, the capability of elderberry extract in reducing MVA virus load was investigated with respect to viral clearance after incubation of BHK-21 cells with the virus-extract mixture. Results indicate a substantial infection reduction by 95.3%, when using a 1.2% elderberry extract with 10 min virus contact time, compared to cells infected with the virus alone (Figure 3). Of note, also a reduced contact time between the virus and the elderberry extract (1 min) resulted in a 32.4% reduction of the infectious virus titre. Less concentrated elderberry extract (0.24%) showed a reduction of the virucidal activity by 32.4% (1 min contact time) and 68.3% (10 min contact time) (Figure 3, Table 1).

Discussion

The results of this study show that the investigated anthocyanin-enriched *Sambucus nigra* L. extract (eldosamb®) does not act as a direct immunostimulant, but rather as an immunomodulator through immune cell activation in a pro-inflammatory environment. By investigating the underlying immunological aspects in *ex vivo* cytokine secretion assays, we showed that the pro-inflammatory cytokines TNF-α secreted by monocytes and IFN-γ stimulating and secreted by Th1 cells were significantly reduced by the investigated elderberry extract. TNF-α is an indicator for macrophage activation,

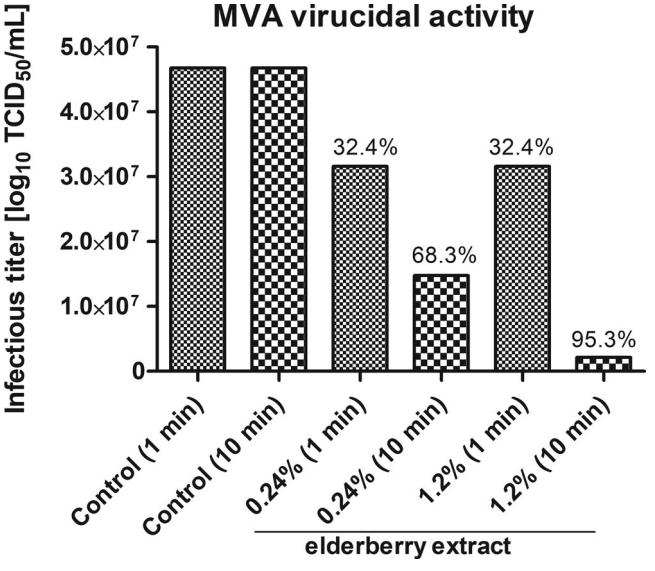

Figure 3. Virucidal activity of elderberry extract against MVA. MVA was incubated with elderberry extract (0.24% and 1.2%) for 1 min and for 10 min before serial titration and incubation of target cells. The decrease of infectious titre ($TCID_{50}$) is indicated as percent decrease compared to the control (virus alone; not incubated with elderberry extract). Data are presented as mean ($n = 6$).

Table 1. Infectious titre (log_{10} $TCID_{50}$), reduction factor (representing viral clearance) and reduction of the infectious titre compared to the control are depicted for elderberry extracts (0.24% and 1.2%) for the two contact times (1 and 10 min). $TCID_{50}$: Tissue culture infectious dose 50.

Elderberry extract (contact time)	Control (1 min)	Control (10 min)	0.24% (1 min)	0.24% (10 min)	1.2% (1 min)	1.2% (10 min)
log_{10} $TCID_{50}$/mL ± 95% CI	7.67 ± 0.54	7.67 ± 0.54	7.50 ± 0.47	7.17 ± 0.42	7.50 ± 0.47	6.33 ± 0.33
Reduction factor ± 95% CI			0.17 ± 0.71	0.50 ± 0.68	0.17 ± 0.71	1.33 ± 0.63
Reduction compared to control [%]			32.4	68.3	32.4	95.3

often involved in chronic inflammation, triggering the activation and recruitment of further immune cells, e.g. cytotoxic activity of granulocytes and lymphocytes. IFN-γ is a marker for lymphocyte-based inflammation, which in turn activates macrophages and can trigger TNF-α and IL-1 secretion.

Results further showed that levels of the secreted interleukins IL-4 and IL-10 increased due to the elderberry extract. IL-4 is a marker cytokine for Th2-cells, which stimulates B-cells for antibody synthesis, thus connecting the innate with the adaptive immune system. IL-10 is an important immunomodulative cytokine secreted by Th2 cells, mainly acting immune suppressive and exerting important regulatory functions by controlling excessive inflammatory processes and thus mitigating possible cytokine storms after infection. During a cytokine storm, the stimulation of the immune system overshoots to the point of no return, thereby worsening the condition rather than improving it. For any immunomodulatory substance, such as the herein tested elderberry extract, the induction of a cytokine storm is thus to be excluded. In this study, we showed that a pro-inflammatory cytokine storm caused by elderberry is unlikely, which is in line with other authors (Wermig-Morgan, 2020; Wieland et al., 2021). This finding adds to safety profile of elderberry preparations, which is still controversially discussed, mainly since consumption of other plant parts or processing of unripe elderberries contain possibly harmful substances, which can be overcome by using ripe fruits free of stems and panicles (Ulbricht et al., 2014).

Taken together, the presented findings show an immune response shift towards the Th2-based T-Helper cell response, demonstrated by a reduced IFN-γ/IL-10 ratio. Immune regulation upon infection is crucially affected by the Th1/Th2 balance, and shifts towards one of the two response pathways direct the host's pathogen combat. While Th1 cells drive cellular immunity to fight intracellular pathogens and cancerous cells, Th2 cells involve humoral immunity by up-regulating antibody production to fight extracellular organisms (Kidd, 2003). In situations of acute infection, elderberry may thus exert beneficial immunomodulating properties, counteracting overshooting pro-inflammatory reactions while activating antibody production via humoral immunity.

Confirming the herein presented findings, elderberry extract-induced downregulation of TNF-α gene expression in macrophages was demonstrated earlier, as was the expression of the pro-inflammatory cytokine IL-6 (Zielińska-Wasielica et al., 2019). These anti-inflammatory effects were also seen by other authors demonstrating a dose-dependent reduction of UVB-induced IL-6 (David et al., 2014; Lin et al., 2019) and

LPS-induced TNF-α and IL-6 (Olejnik et al., 2015) by elderberry extract treatment of macrophages. In contrast, other *in vitro* studies showed that elderberry rather induced TNF-α and not being able to inhibit pro-inflammatory actions (Grunz-Borgmann et al., 2015) and that elderberry fruit extract increases IL-6 and TNF-α expression (Barak et al., 2001; Torabian et al., 2019). Moreover, a study in diabetic rats also showed increased production of TNF-α und IFN-γ after elderberry consumption (Badescu et al., 2015).

Some of the controversial results of the different elderberry extracts could be attributed to unknown LPS contamination of the tested elderberry preparations. Particularly in *in vitro* and *ex vivo* models, in which products are directly used for the immunomodulatory experiments without gastrointestinal digestion, any contamination could influence the experiments. In contrast, due to different protection systems, LPS contamination of plant extracts e.g. during the production process is not of relevance in human or animal studies. Additionally, the controversial findings might be attributed to different compositions of the elderberry extracts used, including preparations containing multiple plant substances or different enrichment systems. Thus, comparability between studies is hampered.

Despite these inconsistent findings that require further clarification, a general potent modulating activity of the immune system by elderberry extract is clearly shown in numerous studies and is affecting various immune cell types (Ho et al., 2017; Młynarczyk et al., 2018; Sidor & Gramza-Michałowska, 2015; Vlachojannis et al., 2010).

The herein applied *ex vivo* test setups to investigate immunomodulatory effects are accepted methods, which are also used in routine diagnostic to screen different compounds for their stimulatory or inhibitory effects in individuals. It is known that the response to plant extracts is quite individual and heterogeneous between individuals, with varying immune patterns, which is all the more illustrated by the different observed disease outcomes in the case of a COVID-19 infection. Based on this background, one outlier subject had to be excluded from data evaluation regarding some study parameters. Nevertheless, the presented experiments clearly demonstrate the immunomodulatory effect and potential of the elderberry extract in the majority of subjects.

Besides the above-described effects on pro- and anti-inflammatory cytokines, this study aimed to investigate the potential antiviral efficacy of the eldosamb® elderberry extract. MVA virus was used as a surrogate for enveloped viruses, including the family of Coronaviridae and Orthomyxoviridae (influenza viruses). This study shows a reduction of virus load and moderate diminishing of viral infectivity upon reaction with elderberry constituents in target cells. These results are in line with a recent study having investigated MVA, SARS-CoV-2 and influenza virus A, showing a susceptibility of MVA (79–86% reduction of infectious titre) and influenza virus A, but not of SARS-CoV-2 to elderberry juice (Frank et al., 2020).

Several modes of action are discussed for the antiviral effect of *Sambucus nigra* L., for instance that main anthocyanin constituent cyanidin-3-sambubioside binds to and inactivates the viral neuraminidase enzyme, thus reducing pathogenicity (Swaminathan et al., 2013), or that elderberry extracts blocks influenza virus glycoproteins (Torabian et al., 2019).

The herein described effects *in vitro* and *ex vivo* may be even stronger in humans due to an *in vivo* interplay of immunomodulatory effects in different cells and tissues. Further

clinical studies, e.g. also involving immune-compromised persons are highly recommended to further clarify the potential of elderberry products in treatment of respiratory infects, especially in light of a recent RCT study that could not detect a clinical benefit of elderberry on duration or severity of influenza (Macknin et al., 2020), contradicting the above-mentioned studies with positive treatment outcomes.

Conclusion

All in all, this study describes the herein studied high-quality elderberry preparation (eldosamb®) as mainly anti-inflammatory through reduction of pro-inflammatory cytokines TNF-α, IFN-γ and IL-2. eldosamb® might therefore qualify as an immune modulator especially in situations of present or emerging inflammation. Moreover, the elderberry extract exerts significant antiviral bioactivity, shown by a high virucidal activity against the MVA virus, reducing its infectious titre by up to 95%. These results suggest the use of the eldosamb® elderberry extract as an immune health product.

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Disclosure statement

The authors have no conflict of interest regarding the content of this manuscript. All authors have read and approved the final article. BioTeSys GmbH and IMD Institut für Medizinische Diagnostik are independent third party research institutes that were responsible for performance of the experiments, and data analyses. The study was initiated and sponsored by Anklam Extract GmbH. Realization, data analysis, and reporting were undertaken independently from the sponsor. B.B. is employee of Anklam Extract GmbH. I.P. is a consultant of Anklam Extract GmbH..

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Data availability statement

The data that support the findings of this study are available on request from the corresponding author.

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