

## The effect of Sambucol, a black elderberry-based, natural product, on the production of human cytokines: I. Inflammatory cytokines

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### Summary

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**Summary** : *Sambucus nigra* L. products – Sambucol – are based on a standardized black elderberry extract. They are natural remedies with antiviral properties, especially against different strains of influenza virus. Sambucol was shown to be effective *in vitro* against 10 strains of influenza virus. In a double-blind, placebo-controlled, randomized study, Sambucol reduced the duration of flu symptoms to 3-4 days. Convalescent phase serum showed a higher antibody level to influenza virus in the Sambucol group, than in the control group. The present study aimed to assess the effect of Sambucol products on the healthy immune system – namely, its effect on cytokine production. The production of inflammatory cytokines was tested using blood – derived monocytes from 12 healthy human donors. Adherent monocytes were separated from PBL and incubated with different Sambucol preparations i.e., Sambucol Elderberry Extract, Sambucol Black Elderberry Syrup, Sambucol Immune System and Sambucol for Kids. Production of inflammatory cytokines (IL-1b, TNF-a, IL-6, IL-8) was significantly increased, mostly by the Sambucol Black Elderberry Extract (2-45 fold), as compared to LPS, a known monocyte activator (3.6-10.7 fold). The most striking increase was noted in TNF-a production (44.9 fold). We conclude from this study that, in addition to its antiviral properties, Sambucol Elderberry Extract and its formulations activate the healthy immune system by increasing inflammatory cytokine production. Sambucol might therefore be beneficial to the immune system activation and in the inflammatory process in healthy individuals or in patients with various diseases. Sambucol could also have an immunoprotective or immunostimulatory effect when administered to cancer or AIDS patients, in conjunction with chemotherapeutic or other treatments. In view of the increasing popularity of botanical supplements, such studies and investigations *in vitro*, *in vivo* and in clinical trials need to be developed.

**Keywords** : Sambucol, inflammatory cytokines, IL-1b, TNF-a, IL-6, IL-8.

### Pictures

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## INTRODUCTION

There is a growing interest in medicinal botanicals as complementary medicine all over the world.

*Sambucus* species were reported to have been used for years by native Americans, mostly against rheumatism and fever [1, 2]. Sambucol is a natural remedy with antiviral properties, especially against the human influenza virus. The main ingredient of the formulation is an extract of the black elderberry (*Sambucus nigra* L.) [1]. The preparation also contains smaller amounts of Echinacea and Propolis. The black elderberry, which is widely used in Europe for juices and preserves, and designated as a "natural antibiotic product" contains high levels of three bioflavonoids and anthocyanins [3, 4]. Bioflavonoids possess a wide spectrum of biological activities, including antioxidant properties. The antioxidant and prooxidant activities of an elderberry extract on low density lipoproteins have been demonstrated [5]. Antioxidants disarm free radicals, thus decreasing tissue damage. They also prevent damage caused by irradiation [6, 7]. Extracts of plants containing bioflavonoids and purified flavonoids were active against *Herpes* virus Type 1, respiratory syncytial virus, parainfluenza and influenza viruses [7]. Sambucol was shown to inhibit the hemagglutination and the replication of different human influenza viruses, both type A and B. It was also shown *in vitro* to be effective against 10 strains of the influenza virus [8]. In a double-blind, placebo-controlled, randomized study, Sambucol reduced the duration of flu symptoms to 3-4 days. Convalescent phase serum antibodies showed higher antibody levels to influenza virus in the group treated with Sambucol, as compared to the control group [8]. The prevention of flu like symptoms by Sambucol was demonstrated as well in a colony of chimpanzees in the Jerusalem Zoo [9].

Echinacea, the purple coneflower, is used as a herbal medicine against the common cold and bronchitis [1, 10]. Studies have shown that preparations of this plant affect the phagocytic immune system but not the acquired immune system or the production of cytokines [11-13].

Propolis is produced by the honey bee from plant resins. It has been used as an anti-bacterial material for wound cleaning and as an anti-inflammatory remedy for over 2,000 years. Propolis is considered to be an anti-oxidant, having a protective effect on the liver [14]. It has also been shown to reduce damage caused by irradiation [15].

The aim of the present study was to determine the immune enhancing potency of Sambucol preparations by studying their effect on the production of inflammatory cytokines by human peripheral blood monocytes of healthy individuals.

## **MATERIALS AND METHODS**

### ***Healthy donors blood monocyte cultures***

Twelve donors (young, healthy students with no inflammatory conditions and currently receiving no medication) participated in this study. Heparinized peripheral blood (50 ml) was obtained from each subject and diluted 1:1 with phosphate-buffered saline (PBS). The mononuclear cells were separated by Ficoll-Hypaque sedimentation (400 g, 30 min) and washed three times in PBS (175 g, 10 min) to remove platelets. An aliquot of cells was counted in a hemocytometer, and viability was checked using the trypan blue exclusion method. The adherent cells were > 90% monocytes by beta-naphthyl acetate non-specific esterase staining [16]. The cells were suspended in RPMI 1640 media, supplemented to a final concentration of 1 mM sodium pyruvate, 50 U/ml penicillin, 50 mg/ml streptomycin, 2 mM L-glutamine, 1/100 MEM-vitamins and 2% inactivated human AB serum. The cells were plated in 24-well culture dishes at a concentration of  $4 \times 10^6$  cells per well and incubated for 90 min at 37° C in a humidified atmosphere containing 5% CO<sub>2</sub>. Non-adherent cells were removed by aspiration and the wells washed three times with PBS. The adherent cells were cultured for 24 hours in RPMI media without the addition of serum. Different Sambucol formulations (1/10 dilution, 10 µl/well) or LPS (100 ng/ml) as a control monocyte stimulator, were added to the monocytes. At the end of the culture period (24 hours), the supernatants were harvested, centrifuged (300 g, 10 min) and stored at -70° C until assayed for cytokines, as previously reported [17, 18].

### ***Sambucol preparations***

A standardized extract of the black elderberry, (E.Ex.) is the main ingredient of different Sambucol preparations. *Sambucol Black Elderberry Syrup* (B.E.) contains 38% E.Ex., glucose, raspberry extract, citric acid and honey. *Sambucol Active Defense* (A.D.), in the USA known under the name *Immune System Formula*, contains also 38% E.Ex., glucose, raspberry extract, citric acid, honey, *Echinacea angustifolia*, *Echinacea purpurea*, propolis, ascorbic acid and zinc gluconate. *Sambucol for Kids* (Kids) contains half the amount of E.Ex. (19%) used in the other formulations and in addition - glucose, raspberry extract, citric acid, *E. angustifolia*, *E. purpurea* and propolis.

The anthocyanin content of the Sambucol formulations was assessed by measuring the absorbance at 526 nm (not less than 0.6 for B.E, A.D. and 0.3 for Kids [1, 7].

### ***Cytokine assays***

Levels (pg/ml) of inflammatory cytokines (IL-1beta, IL-6, IL-8 and TNF-alpha) in supernatants of monocyte cultures were measured by a solid phase ELISA (R&D, Minneapolis, USA). This assay employs the quantitative "sandwich" enzyme immunoassay technique. A monoclonal antibody specific for the cytokine molecule was pre-coated onto the polystyrene microtiter plate. Standards and samples were introduced into the wells where the immobilized specific antibodies bind the cytokines. After washing away any unbound proteins, the second enzyme-linked polyclonal or monoclonal antibody specific for the cytokine was added to the wells to "sandwich" the cytokine immobilized during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells, causing the development of a color that was proportional to the amount of cytokine bound in the initial step. The color development was stopped and the intensity of the color was measured. A standard curve plotting the optical density *versus* the concentration of a given cytokine was prepared, and used to determine the concentration of the cytokine in unknown samples, as previously reported [17-20].

### ***Evaluation of Sambucol's effect***

The effect of the different formulations of Sambucol on the induction of inflammatory cytokines production (in pg/ml) was also expressed as:

$$\text{Stimulation index} = \frac{+ \text{Sambucol cytokine production}}{- \text{Sambucol}}$$

### ***Statistical analysis***

Comparison between groups was performed by Mann-Whitney's test. A value of  $p < 0.05$  was considered significant.

## **RESULTS**

The Sambucol E.Ex. and its three formulations (B.E., A.D., Kids) significantly stimulated the production of the inflammatory cytokines IL-1beta, IL-6, IL-8 and TNF-alpha by normal human monocytes.

The effects of the Sambucol formulations and of LPS (the well known positive control for monocyte activation) expressed as range (minimum and maximum induction levels) and as mean  $\pm$  SE of stimulation indexes, for each of the cytokines tested by 12 healthy donors' monocytes, are presented in [Table 1](#): IL-1beta ([Table 1a](#)), TNF-alpha ([Table 1b](#)), IL-6 ([Table 1c](#)) and IL-8 ([Table 1d](#)).

In addition, the effect of Sambucol preparations on the actual amounts of cytokines produced (expressed as pg/ml) in comparison to the untreated control monocytes, which represent physiological levels, are presented in [Table 2](#): IL-1beta ([Table 2a](#)), TNF-alpha ([Table 2b](#)), IL-6 ([Table 2c](#)) and IL-8 ([Table 2d](#)).

The highest stimulation index was observed with the E.Ex., followed by B.E. and A.D. which contain the same amount of elderberry extract (E.Ex.) and had similar stimulatory effect on these cytokines. Kids, which contains the lowest amount of extract, showed indeed the lowest stimulation index. For the E.Ex., the highest mean stimulation index (44.9) was observed for the induction of TNF-alpha, followed by IL-6 (19.5), IL-1beta (6.4) and IL-8 (3.3).

The effect of Sambucol preparations and of LPS (tested in 7 out of 12 donors) expressed as mean stimulation indexes, on the production of inflammatory cytokines for all individual donors, are shown in [Figures 1-4](#):

IL-1beta ([Figure 1](#)), TNF-alpha ([Figure 2](#)), IL-6 ([Figure 3](#)) and IL-8 ([Figure 4](#)).

E.Ex. was the strongest activator for all 4 cytokines and was statistically significantly different from the other Sambucol formulations, except for IL-8 ( $p < 0.05$ ). However, there were no statistically significant differences between effects of B.E., A.D. and Kids. LPS induction of all cytokines was lower than that induced by E.Ex ( $p < 0.05$ ). For IL-8, the effect of LPS was similar to that of E.Ex. and a little higher than the other 3 formulations. For IL-6, the effect of LPS was higher than B.E., A.D. and Kids. For IL-1beta, the effect of LPS was higher than B.E., A.D. and Kids. For TNF-alpha the effect of LPS was a little higher than that of B.E., A.D. and Kids. There was no significant difference between levels of cytokines activated by B.E. and A.D.

A representative assay performed on monocytes from one single donor (K.L.) showing cytokine production is presented in [Figure 5](#).

When the Sambucol preparations were mixed together with LPS to activate the monocytes, a synergistic effect was found, especially for E.Ex. (data not shown).

## DISCUSSION

The results of this study show that all Sambucol elderberry formulations have a strong stimulatory effect on inflammatory cytokine production by human normal monocytes. With the exception of IL-8, the stimulatory activity was dose-dependent, *i.e.*, the E.Ex. with the highest extract concentration of Sambucol showed the highest cytokine stimulation, while the less concentrated B.E. and A.D. caused a less pronounced stimulation. The less concentrated formulation, Kids, had the lowest effect on the production of inflammatory cytokines. The most impressive effect of E.Ex. was on TNF-alpha production. The extremely high amounts of TNF-alpha produced by Sambucol, were even higher than those produced by LPS. TNF-alpha is a multipotential mediator of cellular immune responses with a wide variety of biological activities. Under different conditions, TNF-alpha can exhibit favorable or unfavorable effects on the host immune response. The unfavorable effects may occur with high concentrations of TNF-alpha or with low concentrations - for longer periods [21]. The Sambucol preparations, especially the E.Ex., when added together with LPS, had a synergistic inducing effect.

*Echinacea*, propolis, ascorbic acid and zinc gluconate, which are included in the A.D. formulation, but not in the B.E., did not enhance the stimulatory effect of the E.Ex. and even appeared to reduce it (data not shown).

Our results are in accordance with the studies which showed that the extracts of *Echinacea* are not able to induce increased cytokine production [10, 11]. Levels of the cytokines (IL-1alpha, IL-1beta, IL-2, IL-6, TNF-alpha and IFN-gamma) were measured in culture supernatants of stimulated whole blood cells derived from 23 tumor patients undergoing a 4-week oral treatment with an *Echinacea* complex. No significant alteration in the production of cytokines was detected [11]. In healthy volunteers, two out of five studies performed with different preparations of *Echinacea* showed an enhancement in the phagocytic activity of polymorphonuclear neutrophil granulocytes [21].

Propolis was shown to enhance murine macrophage spreading and mobility [22]. Its influence on the production of cytokines has not yet been reported.

In addition to Sambucol's antiviral properties [7, 8], the E.Ex. and the other Sambucol formulations were shown in this study to activate the immune system by strongly increasing inflammatory cytokines production. In preliminary studies, we have shown that Sambucol formulations significantly induce the production of both Th1 (IFN-gamma and sIL-2R) and Th2 (IL-10) cytokines, as well as the production of the hematopoietic growth factor GM-CSF by monocytes and lymphocytes from healthy individuals. (unpublished results).

Sambucol may therefore be beneficial to the stimulation of the immune system in healthy individuals, as well as in patients with influenza, cancer and HIV, which have decreased immune functions. As we have shown previously, breast cancer patients undergoing chemo/hormonotherapy and provided with an addition of interferons, which activated the immune system, had a better therapy response concomitant to changes in cytokine production [24]. We believe therefore, that Sambucol may be beneficial to cancer patients receiving chemotherapy. The immunoprotective or immunostimulatory effects of Sambucol, in

various patients will be assessed in future clinical trials.

## CONCLUSION

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As the Israeli Cytokine Standardization Laboratory, we have performed many studies dealing with various activation pathways of human cytokines.

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