Mechanism of action

In this guide

20. The exact mechanism by which Echinacea preparations exert their beneficial effect on the treatment and prevention of common cold is not known. Antiviral, immunomodulatory and anti-inflammatory effects of Echinacea were demonstrated in in vitro, in vivo and human studies discussed in more detail below. However, their relevance to clinical efficacy is not known and exact pharmacodynamic mechanism cannot be established (EMA, 2014).

21. The information presented below collates the data available in the EMA reports on E. purpurea (L.) Moench. (EMA, 2014), E. angustifolia DC, radix (EMA, 2012) and E. pallida (Nutt.) Nutt., radix (EMA, 2018), as well as data from a literature search performed by the Secretariat. For further details on the literature search methodology, please see Annex A.

22. Where information was available about the bioactive components present in the particular Echinacea preparation used in the studies, this has been included in this paper.

In vitro and Animal Studies

Antiviral effects

23. The stems, leaves, and flowers of E. purpurea (L.) Moench were fractionated and the fractions evaluated for antiviral activity in vitro against herpes simplex virus (HSV) Type I, influenza virus and rhinovirus (Vimalanathan et al., 2005). Ground dried E. purpurea aerial parts were extracted with 70% ethanol and fractionated 3 times with equal parts n-hexane, resulting in n-hexane and hydroalcoholic fractions. The latter were fractionated twice with ethyl acetate. E. purpurea dried parts were also extracted with water either 40°C or 80°C and the extracts were filtered. H-1 cells, a subclone of HeLa cells that is particularly sensitive to rhinovirus replication, were used for the propagation of the influenza virus and rhinovirus, whilst HSV was propagated in Vero cells. Cytopathic effects (CPE) end point assay was used, with the end point defined as the highest dilution of extract giving complete elimination of viral CPE produced by 100 infectious units of virus (MIC100). Some of the experiments were designed to test for photosensitisers and the MIC100 was calculated in the presence and absence of light exposure. The chemical composition of the fractions was also determined and described below.

24. Echinacoside was absent from all the fractions, whilst cynarin was found in the 80°C water extract from the flower parts. The aqueous fractions from all aerial parts (total herb, flower, stem and leaves) contained caftaric, caffeic and chicoric acid; the concentration of these bioactive components was 3-40 times higher in the 80°C water fractions compared to the 40°C ones. All the aqueous extracts exhibited potent activity against HSV and influenza virus with MIC100 of 1.2-23.5 µg/mL and contained no photosensitiser compounds as determined by measurement of the MIC in the presence and absence of light exposure. The ethanol extracts of the total herb and stems/leaves and their ethyl acetate fractions were also active against HSV and influenza virus (MIC100 of 1.5-31.2 µg/mL) and contained photosensitising compounds. On the other hand, the ethanol extracts of the flower parts had only marginal antiviral activity (MIC100 of 100-125 µg/mL) with no photosensitising compounds. The crude ethanol extracts of the total herb, flower part and stems/leaves contained high levels of chicoric (2,879 – 7,340 µg/mL), caftaric (767-919 µg/mL), caffeic (220-421 µg/mL) and

chlorogenic acid (45-208 µg/mL). Vimalanathan et al. (2005) stated that whilst other studies (Binns et al., 2002) attribute the antiviral activity of Echinacea to chicoric acid and other caffeic acid derivatives, there was no clear correlation between phenolic concentrations and relative activity observed, suggesting synergy between bioactive compounds and/or the presence of other antiviral compounds. The authors concluded that the aerial parts of E. purpurea contain multiple antiviral compounds, including more than one water-soluble compound, and at least one caffeic acid derivative, plus at least one ethyl acetate–soluble component that was a photosensitiser and was absent from the flower extract. In addition, none of the extracts tested were active against rhinovirus, which suggested a possible membrane target for the antiviral agents as the rhinovirus does not have a membrane, whilst HSV and influenza do.

25. A study with a similar design by the same authors evaluated the antiviral activity of E. purpurea, E. pallida and E. angustifolia root extracts in vitro (Hudson et al., 2005). The antiviral activity of E. purpurea roots was predominantly in the aqueous fractions with MIC100 of 1.2-2.5 µg/mL against HSV Type I and influenza virus compared to MIC100 of 88-500 µg/mL with the ethanol and ethyl acetate extracts. Again, no activity against rhinovirus was observed. Interestingly, the aqueous fraction had the lowest concentration of chicoric acid, caffeic acid and echinacoside, suggesting that the compounds responsible for the observed potent antiviral effects of the aqueous fraction are not the major phenolic constituents associated with previously reported antiviral activity of Echinacea. It was speculated that the antiviral activity of the E. purpurea root aqueous fraction was attributable to cell wall extractable polysaccharides or glycoproteins. In contrast, the aqueous fractions of E. angustifolia root had no antiviral activity, but the ethanol and ethyl acetate fractions had moderate activity against all three viruses, including rhinovirus. The authors stated that this correlated very well with the presence of alkylamides, but not with echinacoside, the principal phenolic constituent. None of the E. pallida fractions demonstrated any antiviral activity, consistent with the lack of alkylamides in these fractions. The study concludes that based on these results, commercial preparations of Echinacea roots can be expected to differ widely in their antiviral activities.

26. The antiviral effects and mode of action of a Echinaforce were investigated against influenza viruses human Victoria (H3N2) and PR8 (H1N1), avian strains KAN-1 (H5N1) and FPV (H7N7), and the pandemic S-OIV (H1N1) in Madin-Darby canine kidney cells (MDCK) or embryonated chicken eggs (PR8) (Pleschka et al., 2009). Echinaforce is a standardised E. purpurea root and herb preparation derived by ethanol extraction of freshly harvested E. purpurea herb and roots

(95:5). The preparation contains caftaric acid (264.4 µg/mL), chlorogenic acid (40.2 µg/mL), chicoric acid (313.8µg/mL), echinacoside (6.9 µg/mL), chlorogenic acid (40.2 µg/mL) and alkylamides (PID 8/9 at 36.3 µg/mL) in 65% ethanol concentration (Sharma et al., 2009). The MIC100 of the Echinacea extract was determined using cytopathic effects (CPE) end point assay, which involves incubating the cells with serial dilution of the herb extract and the virus until CPE are complete in the control wells containing untreated virus. The MIC100 was defined as the maximum dilution of the Echinacea at which CPE were inhibited. Concentrations of Echinacea ranging from the recommended oral daily dose of 1.6 mg/mL to 1:1,000 dilution (1.6 µg/mL) achieved over 99% inactivation of the H3N2 virus with a viral load of 105 PFU/mL. The authors state that similar results were obtained with human H1N1 and avian FPV (H7N7), but the data is not shown. It was noted that direct contact between the virus and the Echinacea extract was required for the inhibitory effect since Echinacea treatment of cells before or after the virus infection resulted in substantially less inhibition than pre-incubation of the virus particles with the extract. The authors conclude that the anti-viral effect of Echinacea manifests at a very early stage in the infection process.

27. This was confirmed by a hemagglutination assays, which demonstrated inhibition of viral hemagglutinin by Echinacea leading to prevention of the entry of virus into the cells (Pleschka et al., 2009). The interaction between viral hemagglutinin and cellular sialic acid containing receptor is the first step of influenza virus entry in the cell. The receptor binding can be measured by the ability of the virus to agglutinate chicken erythrocytes. The assay was performed by pre-incubating 5 concentrations of Echinacea extract with the virus prior to the addition of chicken erythrocyte suspension. Plates were the incubated for a further 1 or 4 hours and the wells were visually inspected for presence or absence of hemagglutination. E. purpurea inhibited the hemagglutination activity of the avian strains KAN-1 (H5N1), FPV (H7N7) and the pandemic S-OIV (H1N1) in a concentration and time-dependent manner.

28. The same study also determined the production and intracellular localisation of viral ribonucleoprotein (RNP) in MDCK cells infected with KAN-1 (H5N1), in the presence and absence of Echinacea treatment (Pleschka et al., 2009). The intracellular production, pattern of migration and localisation of the viral RPN were similar between untreated virally infected cells and cells treated with Echinacea after infection. However, the overall number of cells positive for viral RNP was significantly reduced when cells were infected with Echinacea treated virus. This suggests that Echinacea treatment is effective at a very early

stage before the virus enters the cells. Once the viral particles are in the cells, the replication and spread are not affected by the treatment.

29. The antiviral activity of a Echinaforce, standardised ethanol extract of freshly harvested E. purpurea herb and roots, was evaluated in vitro against influenza (strain H3N2, human isolate), HSV Type I, rhinovirus types 1A and 14, adenovirus types 3 and 11, respiratory syncytial virus (RSV) and feline calicivirus (FCV) (Sharma et al., 2009). This preparation has high levels of caftaric and chicoric acid, moderate levels of chlorogenic acid and echinacoside, but no caffeic acid or cynarin (see paragraph 25 for composition of Echinaforce preparation). Antiviral activity was tested using the CPE end-point assay with either pre-incubating the extract with the virus prior to adding it to the cells for calculating virucidal MIC100 or incubating the cells with the extract before adding the virus for calculating the intracellular MIC100. The influenza virus and HSV were very sensitive to Echinacea with virucidal MIC100 < 1.0 µg/mL.and so was the RSV with MIC100 2.5 µg/mL. The non-membrane viruses rhinovirus, adenovirus and feline calicivirus resistant to the highest concentration tested (800 µg/mL), although the rhinoviruses showed partial inhibition of CPE at this concentration. The intracellular MIC100 values were two orders of magnitude higher than the virucidal MIC for HSV, influenza and the RSV indicating that the Echinacea had a direct virucidal effect on membrane containing viruses, rather than inhibition of virus replication.

30. The study also tested the ability of the Echinacea extract to inhibit the induction of cytokines in response to a viral infection in human bronchial epithelial cells (BEAS-2B cells), A549 cells, and primary human skin fibroblasts (Sharma et al., 2009). Viruses were added to the cells at 1 pfu/cell for 1 h, followed by the addition of Echinaforce at 160 µg/mL. Culture supernatants were then harvested at 24 and 48h post infection and the cytokine concentration of IL-6, IL-8 and TNF-α determined by ELISA. A fluorescent antibody array assay was also performed for 20 cytokines and inflammation-related mediators. The Echinacea preparation was effective at inhibiting the induction of IL-6, IL-8 and TNF-α by the virus infection with all viruses tested in the three human cell lines.

31. The antiviral and immunomodulatory properties of E. purpurea aerial parts extracts were evaluated in female C57BL6 mice intranasally infected with 500 PFU influenza A/WSN/33 (H1N1) strain (Fusco et al., 2010). The E. purpurea aerial used in this study contained significant amounts of polysaccharides and little to none of other known immunomodulatory components (alkylamides, chicoric acid and cynarin). In total 59 mice were used in the study and split into 4 groups:

infected, untreated (n=24), infected, treated (n=16), uninfected, treated (n=9) and uninfected, untreated (n=10). E. purpurea aerial parts including leaves, stems and flower heads were homogenised, extracted with 20% aqueous ethanol, the extract lyophilised and dissolved in deionised water. The treated groups received 10 mg of this preparation administered daily by oral gavage for 5 consecutive days starting on the day of the infection. The mice were weighed daily, viral titres and cytokine analysis were performed throughout the study.

32. The authors reported that Echinacea treated mice had better clinical outcomes than untreated mice after influenza infection based on the percentage body weight change, which was used as a surrogate marker for disease severity. The weight loss caused by influenza infection was attenuated by Echinacea administration with the mean weight loss being significantly reduced in treated vs untreated mice in repeated measures analysis ($p < 0.001$). The viral titers in lung homogenates of treated and untreated mice were measured on days 2-7 post-infection. No significant differences in the viral titers were observed between the Echinacea treated and untreated mice. In the absence of influenza infection, mice treated with Echinacea had similar serum and lung cytokine profiles compared to untreated mice. In the influenza infected mice, there was a significant decrease in the serum IL-10 (73%, $p = 0.015$), serum IFN-γ (74%, $p = 0.01$) and lung IL-10 (67%, $p = 0.003$) in Echinacea treated compared to untreated mice on day 7 post-infection. Levels of IL-1β, IL-2, IL-4, and TNFα were similar between treated and untreated mice. The authors concluded that that Echinacea improves the clinical outcomes of influenza infection in mice by modulating the immune response rather than exerting a direct antiviral effect.

33. There have been several recent studies evaluating the antiviral effect of Echinacea against Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2). The antiviral activity of Echinaforce preparation (see paragraph 25 for composition) was tested in vitro against common cold coronavirus 229E (HCoV-229E) and highly pathogenic coronaviruses (SARS-CoV-1, SARS-CoV-2 and MERS-CoV) (Signer et al., 2020). The virucidal and antiviral effects of Echinaforce against HCoV-229E were determined by pre-treatment of the virus particles or cells with the extract or post-infection treatment of the cells. The residual viral infectivity was determined using a limiting dilution assay to determine tissue culture infectious dose (TCID50), defined as the dilution of a virus required to infect 50% of a given cell culture. The results of the study showed that direct exposure of HCoV-229E to the extract led to a statistically significant permanent inactivation that could not be reverted by extensive washing at all the doses tested (1,10 and 50 µg/mL); complete inactivation of the viral infectivity was

observed at 50 µg/mL. In contrast, pre-treatment of cells had no influence on HCoV-229E infectivity or replication. Post-treatment of the cells showed a small reduction in the virus titer at the highest concentration tested (50 µg/mL). The effects observed against MERS-CoV, SARS-CoV-1 and SARS-CoV-2 were comparable to HCoV-229E, with complete inactivation of virus infectivity after pre-treatment of the viral particles with 50 µg/mL Echinacea. This is consistent with previous studies reporting that a direct contact between Echinacea and the virus is required for the antiviral activity of the extract.

Immunomodulatory effects

34. The immunomodulatory properties of Echinacea and its constituents have been extensively studied and reviewed in the literature. Recent review articles report that Echinacea activated macrophages leading to increased cytokine production and phagocytosis, promotes dendritic cell maturation, activates natural killer (NK) cells and leads changes in the percentage of immune cell populations, including T lymphocytes and NK cells (Burlou-Nagy et al., 2022; Rondanelli et al., 2018). Some of the primary studies on the immune system effects of Echinacea are discussed in more detail below.

35. Commercial preparations of E. purpurea fresh and dried juice (EchinaFresh) were tested for their ability to induce cytokine production by human macrophages in vitro (Burger et al., 1997). EchinaFresh is Echinacea extract made from the aerial portion of E purpurea and standardized for a content of 2.4% soluble β-1,2-D-fructofuranosides. Human peripheral blood macrophages were collected from a 50-year-old female. The fresh (0.05-10 µg/mL) and dried (0.01-10 µg/mL) Echinacea juice was mixed with 1 × 106 macrophages and supernatants were collected at 18 h for IL-1 determination and at 36 and 72 h for TNF-α, IL-6 and IL-10 determinations enzyme-linked immunosorbent assay (ELISA). Experiments were performed in triplicate. Cells incubated in media only were used as negative control and cells incubated with 5 µg/mL bacterial lipopolysaccharide (LPS). Production of IL-1, TNF-α, IL-6 and IL-10 by the human macrophages at all concentrations of Echinacea tested was significantly higher than in the media only control ($p < 0.05$). A dose-response trend in the production of cytokines by Echinacea stimulated macrophages was also observed. The levels of cytokines induced by LPS fell within the range of those induced by Echinacea leading the authors to conclude that the immunostimulatory ability of E. purpurea pressed juice is comparable to the LPS endotoxin.

36. Various Echinacea preparations were tested in vitro for their effects on murine macrophages and human peripheral blood mononuclear cells (PBMCs) (Rininger et al., 2002). Echinacea samples were either dissolved in dimethyl sulfoxide (DMSO) or subjected to a simulated digestion protocol. Simulated digestion was performed with the Echinacea by adding 750 mg raw material to 15 mL simulated gastric fluid, incubating at 37°C in a shaker incubator for 2 h, neutralizing the gastric fluid and incubating with simulated intestinal fluid for a further 2h. Macrophage activation assays were performed by plating the cells (RAW267.7 murine macrophages) at a density of 1 × 106 cells/mL. The test agent was added after 24 hours and the supernatant was assayed for TNF-α, 24 h posttreatment, indicative of macrophage activation. Sample supernatants were taken at 24, 30, and 48 h post-treatment and assayed for cytokine production using an ELISA. Cell viability was routinely monitored by visual inspection and confirmed with a cell proliferation assay kit. Nitric oxide (NO) production was determined by assaying for the presence of nitrites (NO_2) in culture medium. Lipopolysaccharide (LPS) from E. coli was also tested as a positive control inducer of cytokine production.

37. DMSO dissolved Echinacea did not stimulate the production of TNF-α and NO. Both the E. purpurea herb processed through simulated digestion and LPS (0.1 µg/mL) produced comparable time- and dose-dependent induction of TNF-α, NO, IL-1α, IL-1β, and IL-6, with TNF-α and NO being the most sensitive biomarkers. At 24 hours, levels of TNF-α and NO induced by Echinacea were significantly higher than the control at doses as low as 5 µg/mL ($p < 0.001$). The levels of IL-1α and IL-1β were significantly higher at Echinacea doses of 80 µg/mL and above ($p < 0.01$), whilst with IL-6 the significance was only observed at the highest dose tested (320 µg/mL). The TNF-α levels produced by E. purpurea herb stimulation peaked at approximately 30 h post-stimulation and then declined sharply by 48 h, whereas TNF-α levels from LPS stimulation did not drop dramatically. In contrast, IL-1β, IL-6, and NO continued to rise over the 48-h time-course with both LPS and Echinacea. These immunostimulant effects are in agreement with those reported by Burger et al. (1997), with the exception that Burger et al. (1997) found Echinacea pressed juice (EchinaFresh) to be more potent at stimulating macrophage cytokine synthesis than bacterial endotoxin LPS. Rininger et al. (2002) highlight that the immunostimulatory effects of Echinacea on murine macrophages observed in their study were more transient than with LPS and occurred at 3,200-fold higher concentrations. The authors (Rininger et al., 2002) also comment that it is unlikely for Echinacea preparations to have greater potency at stimulating macrophage cytokine than LPS without resulting in significant toxicities in vivo such as endotoxin shock. It must be noted

that Burger et al. (1997) used LPS at 5 µg/mL, whilst Rininger et al. (2002) at a 50 times lower concentration of 0.1 µg/mL.

38. Different E. purpurea root and herb samples were also tested by Rininger et al (2002), but it was found that only 2/7 herb samples and 1/5 root samples activity were similar to the initial herb material, suggesting a high degree of variability between different preparations. Human PBMCs were also treated with the different Echinacea preparations and it was reported that Echinacea materials that stimulated TNF-α production in RAW264.7 macrophage cells significantly enhanced the viability of PBMCs in vitro, whilst preparations that did not stimulate macrophage TNF-α production did not enhance PBMC viability.

39. Echinacea extracts were studied for their ability to stimulate macrophage function in the lung and spleen of male Sprague-Dawley rats (Goel et al., 2002). E. purpurea aerial parts or roots were extracted with aqueous ethanol and four different fractions with different concentrations of chicoric acid, polysaccharide and alkylamides were produced. The authors determined a ‘basal dose level’ of each component through a survey of various products, but further details for the basal dose level determination are not available in the article. One of the fractions contained basal dose level of chicoric acid, polysaccharides and alkylamides, whilst the other fractions contained the three components at levels 3, 20 and 50 times the basal dose level. Male Sprague-Dawley rats were divided into 5 groups (n=6) and administered 100 µL of each fraction, plus a control, twice a day for 4 days via oral gavage. The animals were sacrificed, and alveolar macrophages were obtained from the animals by bronchoalveolar lavage, whilst the immune cells were isolated from the spleen by pressing them through nylon mesh. Phagocytosis assay was performed by adding latex beads to the alveolar macrophages in a ratio of 10 beads/cell and incubating for 1h at 37°C. The cells were washed and the percentage of infected cells and the phagocytic index (number of beads per infected cell) were determined by microscopic examination. Nitric oxide (NO) production by alveolar and spleen cells was determined by measuring nitrite production in colorimetric reaction using Griess reagent. Concentrations of TNF-α, IFN-γ and IL-2 in cell culture supernatant were measured using an ELISA.

40. The weight gain and food intake of the rats were unaffected by the oral administration of Echinacea for 4 days. The percentage of alveolar macrophages in actively phagocytosis and the phagocytic index increased in a dose dependent manner with increase in the concentration of the three components in the Echinacea fraction. The phagocytic index obtained by treating the macrophages

with the fractions containing 20 and 50 times the basal dose levels of chicoric acid, polysaccharides and alkylamides was significantly higher ($p < 0.05$) than the phagocytic index produced by the fractions containing the basal levels and 3 times the basal levels of these components. Dose related increases in the NO production and by alveolar macrophages were also observed with increasing the concentrations of the 3 components in the Echinacea fractions. The rats administered the fraction with 50 times the basal dose level resulted in an 80% increase ($p < 0.05$) in the NO production by the lung macrophages compared to the control. A dose-dependent increase in the TNF-α secretion from alveolar macrophages was observed with only the fractions containing 3 and 20 times the basal dose level of the three components as TNF-α produced from the 50 times fraction was not significantly different the basal level and the negative control. Increasing the concentrations of chicoric acid, polysaccharides and alkylamides in the Echinacea fractions led to increases in the release of TNF-α and IFN-γ by the spleen macrophages in a dose-dependent manner, but no effect on the IL-2 production was observed.

41. E. purpurea extracts have been shown to promote phenotypic and functional maturation of murine dendritic cells via modulating the activation of JNK (c-Jun N-terminal kinase), p38-MAPK (mitogen activated protein kinase), and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathways (Li et al., 2017). E. purpurea extract with a defined chemical composition of chicoric acid (3.045%), caftaric acid (1.575%), chlorogenic acid (0.065%), dodeca-2E, 4E, 8Z, 10E/Z-tetraenoic acid isobutylamide (1.635%) was used. Bone marrow-derived dendritic cells (BMDCs) were derived from femur and tibia of 6–8-week-old female C57BL/6 mice. The cells were treated with phosphate buffer saline (PBS), Echinacea extract or bacterial LPS for 48 hours and the expression of cell surface molecules was determined by flow cytometry. A phagocytosis assay was conducted to quantify the uptake of fluorescently labelled dextran by the BMDCs following pre-treatment with 400 µg/mL Echinacea for 48 h. A cytokine assay was also performed by pre-treating the cells with NF-κB, p38-MAPK or JNK inhibitor for 1 hour, followed by an incubation of the cells with 400 µg/mL Echinacea extract for 24 hours and analysing the supernatant for cytokines such as IFN-γ, IL-12p70, IL-10 and TGF-β1 using an ELISA.

42. Li et al. (2017) reported that the percentage of CD40, CD80, CD83 and CD86 markers, associated with antigen presentation and T-cell immunity induction, expression was significantly upregulated ($p < 0.05$) in the Echinacea treated (400 µg/mL) groups compared to controls. The endocytosis of the fluorescently labelled dextran was markedly reduced in the Echinacea treated

cells. Similar results were obtained with LPS treatment of the cells. No significant cytotoxicity was observed in the BMDC following 100-400 µg/mL E. purpurea treatment for up to 48 hours. Echinacea treatment was also reported to increase the phosphorylation levels of the MAPKs JNK and p38 and increase the nuclear translocation of NF-κB subunit p65, suggesting that E. purpurea can activate these pathways in BMDCs. Cytokine assay results showed that Echinacea also increased the secretion of IFN-γ, IL-12, IL-10, and TGF-β1 by BMDCs. Moreover, pre-treating the cells with JNK pathway inhibitor decreased the production of IFN-γ by the Echinacea treated cells, whilst inhibition of p38-MAPK down-regulated IL-10 and TGF-β1 levels. NF-κB inhibition decreased the Echinacea-induced production of IFN-γ, IL-12 and TGF-β1, but not IL-10. The authors conclude that E. purpurea treatment promoted phenotypic maturation of the murine dendritic cells by up regulating the expression of key accessory molecules. Functional maturation was also observed via activation of the JNK, p38-MAPK and NF-κB pathways and subsequent cytokine production by the BMDCs.

43. E. purpurea extracts were evaluated ex vivo for their effects on NK cells from healthy patients and patients with either the chronic fatigue syndrome or the acquired immunodeficiency syndrome (See et al., 1997). PBMC were isolated from 20 healthy patients, 20 patients with chronic fatigue syndrome (CFS) and 20 patients with acquired immunodeficiency syndrome (AIDS). The dried E. purpurea was homogenised in Roswell Park Memorial Institute (RPMI) cell culture medium, filtered and used fresh on the day. The cytotoxicity of the extract was tested by incubating the PBMC with various concentrations of the extract and assessing the cells for viability using trypan blue staining. The NK-cell activity was assessed by adding the PBMC (effector cells) to NK sensitive cell line K562 (target cells) labelled with 51-sodium chromate at an effector: target ratio 40:1, 20:1, 10:1 and 5:1, followed by a 10-fold increase in concentrations of the Echinacea extracts from 0.001 to 1000 pg/mL or medium alone (negative control). After 4 h incubation at 37°C, radioactivity was determined by scintillation counting for 51Cr release and the cytotoxic activity calculated in terms of lytic units (LU), where one LU is defined as the number of effector cells required to achieve 20% specific lysis of 5 x 103 target cells per 107 effector cells. The antibody-dependent cellular cytotoxicity (ADCC) activity was also assessed by incubating 51-sodium chromate labelled target cells (K562) infected with human herpesvirus 6 (HHV-6) virus with high titers of polyclonal antibodies against HHV-6. Effector cells (PBMC) were then added in triplicate atex effector:target ratios of 40:1, 20:1, 10:1 and 5:1 with or without serial 10-fold concentrations of the E. purpurea extracts from 0.001 to 100 µg/mL.

44. Concentrations of E. purpurea of up to 1000 µg/mL were not associated with diminished viability of PBMC after 4 h from either normal controls or patients with CFS or AIDS. The NK cell function was significantly decreased for the 20 patients with CFS ($p < 0.01$) and AIDS ($p < 0.001$) compared to healthy controls. The addition of Echinacea at concentrations ≥ 0.1 µg/mL led to a significant increase in the NK cell activity from healthy patients and those with CFS and AIDS in a concentration dependent manner. In addition, the ADCC was significantly increased in all three patient groups when E. purpurea was present at concentrations ≥ 1 µg/mL in a concentration dependent fashion and statistical significance was greatest with the cells of AIDS patients in the presence of the highest concentration of Echinacea tested (100 µg/mL). The authors (See et al., 1997) concluded that increasing concentrations of E. purpurea enhance the immune function of PBMC in normal subjects and in patients with either CFS or AIDS.

45. The effects of E. purpurea extracts on the natural killer (NK) cells present in human PBMCs were examined using flow cytometry (Gan et al., 2003). E. purpurea was dissolved in water and filtered to prepare the Echinacea water soluble extract used in the study. Human PBMCs were treated with the Echinacea extract overnight and analysed for CD16, CD45, CD56, and CD69 markers by flow cytometry. The NK cell-mediated cytotoxicity was assessed by incubating various Echinacea concentrations with peripheral blood lymphocytes (effector cells) for 10 minutes and adding the target cells GL40 cells (2×105/mL), a fluorescently labelled K562 cell line which is sensitive to NK cell cytotoxicity. Effector: target ratios of 20:1, 10:1, 5:1 and 2.5:1 were used. Fluorescence is emitted when the target cells are alive, and the NK induced cytotoxicity was measured by flow cytometry and validated by the standard 51Cr release assay. Four separate experiments, with each experiment performed in triplicate, were conducted. The cytotoxic activity was represented in lytic units LU20/106 cells, where one LU20 represents the concentrations of effector cells that kill 20% of the target cells.

46. The study reports that E. purpurea extract increased the NK-mediated cytotoxic activity in a concentration dependent manner. At the maximum concentration of Echinacea used in the assay (10 µg/ml), there was a 93.3% increase in LU20 compared to the 121% increase observed with IL-2, the control activator of NK cell activity used. Treating the PBMCs with Echinacea extract decreased the frequency and mean fluorescence intensity of CD16 expression by the lymphocytes as a function of the Echinacea concentration used. In the CD16 positive populations, the frequency of CD69 expression increased as a function of the Echinacea concentration used and at 10 µg/ml Echinacea concentration over

90% of the CD16+ cells expressed CD69. The authors (Gan et al., 2003) concluded that water soluble extracts of E. purpurea lead to activation of NK cells marked by decrease in CD16 expression and an increase in CD69. In addition, the study considered Echinacea to be a potent activator of NK cytotoxic function.

47. The immunomodulatory effects of E. purpurea on human T-cells and their cytokine response were evaluated in vitro using human T-cell line Jurkat E6-1 (Fonseca et al., 2014). Fresh E. purpurea aerial parts were ground with water into a slurry, passed through a hydraulic press, the filtrate mixed with 70% ethanol and freeze dried to produce fine powder. The mono and polysaccharide composition of the extract, along with the total amino acid content and the concentrations of bioactive small molecules such as cynarin, chicoric acid, caftaric acid and (2E)-N-isobutylundeca-2-ene-8, 10-diynamide were determined. The human T-cell line Jurkat E6-1 (0.5 × 106 or 5 × 106 cells/mL) was incubated with Echinacea, chicoric or caftaric acids for 40 min at 37 °C, followed by another incubation with/without phorbol 12-myristate 13-acetate (PMA) and/or ionomycin for 24 hours. The cell culture supernatant was then assessed for secretion of IL-2 and IFN-γ using Beadlyte Human Multi-Cytokine Beadmaster Kit. The cells were evaluated for expression of the IL-2 receptor (CD25) using flow cytometry. All experiments were performed in triplicate.

48. The chromatographic analysis of the E. purpurea extract revealed that it contained several phenolic compounds, including chicoric and caftaric acids, as well as cynarin, but not alkylamides. The effects of Echinacea on cytokine secretion by T cells was dependent on the T-cell density. At high cell density, the IL-2 secretion in response to PMA and ionomycin was lower compared to low cell density conditions ($p < 0.001$). Pre-treatment with Echinacea had a greater impact on IL-2 secretion in high T-cell density than low density conditions. A dose-response relationship was observed with IL-2 secretion by the high-density T-cell group after E. purpurea treatment, with effect of the 250 µg/mL dose being significantly greater than the 100 µg/mL dose ($p < 0.01$). A similar pattern was observed with IFN-γ secretion, whereby low-density T-cells produced significantly more IFN-γ than high density ones. Treatment with both 100 µg/mL and 250 µg/mL doses of Echinacea caused a five-fold increase in the secretion of IFN-γ in the high-density condition, whilst a small suppression in the IFN-γ secretion was observed in the low-density population. Echinacea treatment had no effect on the expression of the IL-2 receptor CD25 in high density conditions, but in the low density conditions it reduced the percentage of T cells expressing CD25 ($p < 0.001$). Neither chicoric nor caftaric acid showed effects on IL-2 or IFN-γ response by the T cells leading the authors to conclude that the principle source of T cell

enhancement in the study was the polysaccharide component of the preparation. (Fonseca et al., 2014)

49. Alcohol extracts of E. purpurea, E. angustifolia and E. pallida were investigated for immunomodulating properties in mice (Zhai et al., 2007). E. angustifolia, E. pallida, and E. purpurea root extracts were prepared by extracting the ground powder with ethanol and evaporating to dryness. Eight-week-old male BALB/c mice were split into five groups with groups 1-3 dosed with Echinacea preparations by gavage, group 4 dosed with vehicle control by gavage and group 5 serving as a no gavage group. The Echinacea preparations were given at a dose of 130 mg/kg of body weight once daily for 7 consecutive days and the authors state that this regimen was chosen based on the extrapolation of dose recommended for humans (4 g of powder/day for an average 65 kg human for 1 week). The mice were weighed at the beginning and end of the study, euthanised and the blood was collected for haematological analysis. The spleen was removed, weighed and the splenocytes were enumerated and analysed for CD19+ and CD49+ subsets using flow cytometry. The splenic NK cell cytotoxicity was assessed using the chromium (51Cr) release assay with three effector/target cell ratios, 25:1, 50:1, and 100:1, assessed in triplicate.

50. The study (Zhai et al., 2007) reports differences in the levels of phytochemicals in the three Echinacea preparations. Echinacoside was found to be the main caffeic acid derivative in preparations of both E. angustifolia and E. pallida, whereas no echinacoside was detected in the E. purpurea preparation. Chicoric acid was the main caffeic acid derivative in E. purpurea. Cynarin was detected in the E. angustifolia preparation, chlorogenic acid in the E. angustifolia and E. pallida preparations, and caftaric acid in the E. purpurea and E. pallida preparations. No adverse effects, weight differences or changes in behaviour were observed in the mice with the 7-day administration of the Echinacea preparations. No significant changes in any of the haematological parameters, including leukocyte number, red blood cell number and haemoglobin level, or the spleen parameters such as spleen weight, spleen-to-body weight ratio, and total spleen cell number per mouse, were observed with oral administration of any of the three Echinacea preparations as compared to the vehicle control and the no gavage control. However, the three Echinacea preparations significantly increased the percentage of lymphocytes in peripheral blood over the vehicle control group and the no gavage control (62.7% vs. 58.0%; $p = 0.001$). No significant differences were observed for other peripheral blood leukocyte populations such as neutrophils, monocytes, eosinophils, and basophils. Echinacea treatment groups also led to a significant increase in the percentage of

the splenic lymphocyte subpopulation when compared to the vehicle control plus the no gavage control (83.3% vs. 81.1%; p = 0.004).

51. Seven days of oral administration of E. purpurea resulted in a significant increase in the percentage of both CD49+ and CD19+ splenic cells. The percentage of CD49+ cells, but not of CD19+ cells, was significantly increased in the E. angustifolia group compared with the vehicle control. No significant effect of E. pallida on splenic CD49+ and CD19+ subsets was seen. The authors (Zhai et al., 2007) stated that since Echinacea treatment altered the CD49+ subset in spleen, NK cell cytotoxicity analysis was covaried for the percentage of CD49+ cells measured in each individual. When compared to the vehicle control, only the E. pallida group demonstrated a significant increase in NK cell cytotoxicity ($p < .035$).

52. Mitogen-induced proliferation assay was also performed by Zhai et al. (2007) using whole spleen cells and whole blood cells using [3H]-thymidine incorporation in the presence or in the absence of bacterial lipopolysaccharide (LPS) or concanavalin A (Con A). The levels of IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12 (p40), IFN-γ and TNF-α were measured using ELISA in the cultures of mitogen-stimulated mouse splenocytes ex vivo. The proliferation of peripheral blood and splenic lymphocytes was not affected by Echinacea in the presence of LPS treatment. Con A stimulation of splenic lymphocytes from Echinacea treated mice led to a significant increase in their proliferation ($p < 0.034$), particularly for E. angustifolia and E. pallida, but had no effect on peripheral blood lymphocytes. On the other hand, there was a significant stimulation of baseline blood lymphocyte proliferation by the Echinacea treatment in comparison to both controls in non-mitogen stimulated lymphocytes, particularly for the E. angustifolia and E. pallida groups. The levels of TH2 cytokines (IL-4, IL-6, and IL-10) were mainly influenced by E. angustifolia and E. pallida treatment, with E. angustifolia and E. pallida leading to increased IL-4 ($p = 0.046$) and IL-10 ($p = 0.057$) production over the vehicle control in Con A stimulated spleen cells. E. angustifolia also significantly ($p = 0.13$) increased IL-4 production from non-mitogen stimulated spleen cells. The Echinacea treatment significantly increased TH1 cytokines (IFN-γ and IL-2) production in baseline cultures of splenocytes ($p < .035$) and IFN-γ production by Con A-stimulated splenocytes ($p = 0.005$), whilst only E. angustifolia produced a significant increase in IL-2 production in Con A-stimulated splenocytes ($P = 0.037$). The three Echinacea preparations significantly decreased the production of macrophage cytokines IL-1β ($p=0.007$) and TNF-α ($p = 0.004$) by LPS stimulated splenocytes. The authors commented that this is suggestive of anti-inflammatory activity and that the reduced production of IL-1β and TNF-α might

be associated with the increased production of IL-4, as IL-4 supports differentiation of CD4+ cells into TH2-type cells and supresses the development of macrophage activating TH1 cells. It is worth noting that E. purpurea induced a significant increase in IL-1β production compared to the vehicle control ($p = 0.006$) in non-mitogen stimulated splenic lymphocytes.

Anti-inflammatory effects

53. Alkylamides from the roots of E. purpurea (L.) Moench were examined for anti-inflammatory activity in an in vitro cyclooxygenase-inhibition model system (Clifford et al., 2002). The E. purpurea roots were extracted using dichloromethane, followed by the addition of methanol with the methanol-soluble fraction concentrated under vacuum and dissolved in chloroform. Hexane was added and the supernatant, containing crude alkylamides, was separated through preparative high-performance liquid chromatography (HPLC). The alkylamides were tested for inhibition of both COX-I and COX-II using arachidonic acid as a substrate and monitoring the oxygen uptake. Aspirin, ibuprofen, naproxen, celecoxib and rofecoxib were used as positive controls. At 100 µg/mL, several E. purpurea alkylamides inhibited COX-I and COX-II enzymes in the range of 36-60% and 15-46%, respectively, as compared to controls. As a comparison, 80% inhibition of COX-1 was observed with aspirin, 70-90% inhibition of COX-2 by celecoxib and rofecoxib and 30-70% inhibition of both isoforms by ibuprofen and naproxen.

54. A study investigated the anti-inflammatory activity of Echinacea by testing 5-lipoxygenase (5-LOX)-inhibiting activity of root extracts of five wild and three commercially used species including E. purpurea, E. pallida and E. angustifolia (Merali et al., 2003). Their roots were extracted in ethanol, filtered and n-hexane added to the filtrate followed by a rotary evaporation of the solvent to 0.5 g/mL by dry root weight. Rat basophilic leukaemia cells (RBL-1) were incubated with the extracts for 15 minutes, stimulated with calcium ionophore and supernatants metabolite levels analysed by HPLC. The percent inhibition of 5-LOX was determined in comparison to leukotriene B4 (LTB4) standard, which is the product of 5-LOX. A vehicle control was run with each sample to determine the baseline level of enzyme activity. The root extracts of three commercial Echinacea species inhibited the 5-LOX activity with IC50 values of 0.444 µg/mL for E. angustifolia, 0.642 µg/mL for E. purpurea and 1.08 µg/mL for E. pallida. The 5-LOX inhibition was attributed to the presence of alkylamides in the Echinacea preparations.

Human Studies

55. A meta-analysis of randomised control trials concluded that Echinacea can potently lower the risk of recurrent respiratory infections and its immunomodulatory, antiviral and anti-inflammatory effects contribute to the observed clinical benefits (Schapowal et al., 2015). Systematic literature search identified 949 potentially relevant publications and after the exclusion of 848 pre-clinical/non-human studies, the remaining 101 abstracts were reviewed. Eighty-nine studies were excluded because they did not include respiratory tract infections as indications, studied pharmacodynamic effects, lacked appropriate placebo control, or had inappropriate endpoints. Twelve clinical studies on the use of Echinacea in the prevention of respiratory tract infections were reviewed and 6 were excluded due to low methodological quality (1), duplication (2) or investigating experimentally induced infections (3). The included studies varied in the Echinacea preparations and doses administered. Four studies employed ethanol/glycerol extractions from E. purpurea/E. angustifolia (500–4,000 mg extract/day), and two used pressed juices from E. purpurea (6,200–10,000 mg/day). Data on recurrent infections were available from all the six clinical trials included and a total of 2,458 participants, who received a variety of Echinacea extracts for up to 4 months. The cold symptoms were initially self-reported by patients during the observation period and then confirmed by physicians or study staff.

56. Pooling all the included clinical studies together yielded an overall relative risk (RR) of recurrent respiratory tract infection in the Echinacea treated groups of 0.649 (95% CI 0.545–0.774, $p < 0.0001$). The meta-analysis also looked at a sub-group of patients with increased susceptibility to respiratory tract infections with risk factors including exposure to stress (perceived stress score, PSS-10), being an active smoker, poor sleep, with presumed immune weakness due to low helper: suppressor T cells T4/T8 ratio <1.5, and a history of >2 colds/year. The risk for contracting a recurrent respiratory tract infection in these patients was reduced by treatment with Echinacea (RR = 0.501, 95% CI 0.380–0.661; $p < 0.0001$). Complications including conjunctivitis, sinusitis, otitis media/externa, tonsillitis/pharyngitis, bronchitis, and pneumonia were reported in three of the studies analysed. The overall complication incidence was effectively reduced by 50% with Echinacea (RR 0.503, 95% CI 0.384–0.658; $p < 0.0001$), with the reduction in pneumonia being the most prominent with 64.9% decrease. The authors of the meta-analysis (Schapowal et al., 2015) conclude that Echinacea is an effective option for the management of recurrent respiratory tract infections and their related complications. They state that people with presumed lower immune function and high susceptibility to infection may benefit most. They attribute the increased resistance to viral infections observed in the human

studies to the reported immunomodulatory effects of Echinacea in in vitro and in vivo studies.

57. The results from five placebo-controlled randomised studies investigating the immunomodulatory activity of Echinacea extracts in volunteers are described and discussed (Melchart, 1995). A total of 134 (18 female and 116 male) healthy volunteers between 18 and 40 years of age were studied. Two of the studies used intravenous homeopathic preparation containing E. angustifolia administered once daily for 4-5 days. One study asked volunteers to take liquid ethanolic extracts of E. purpurea roots at doses of 333 mg three times daily (999 mg daily dose) for 5 days, whilst another study administered the ethanolic extracts of E. purpurea or E. pallida roots at 380 mg three times daily (1,140 mg daily dose) for 5 days. The last study used 70% liquid ethanolic extract of E. purpurea (95% herb and 5% root) at 800-900 mg three times daily (2,400 – 2,700 mg daily dose) for 5 days. The primary outcome measure for immunomodulatory activity was phagocytic activity of polymorphonuclear neutrophil granulocytes (PNG) measured using a microscopic method (2 studies) or cytometric methods (3 studies). The secondary outcome measure was the number of leukocytes in peripheral venous blood.

58. Melchart et al. (1995) report that two of the studies found a significantly enhanced phagocytic activity of PNG compared to placebo, whilst the other three studies did not observe a significant effect. The studies with significant findings involved the intravenously administered homeopathic preparation of E. angustifolia and the study with oral ethanolic extracts of E. purpurea root taken at 333 mg three times daily for 5 days, with maximal stimulation of phagocytic activity of 22.7% (95% CI 17.5-27.9%) and 54.0% (95% CI 8.4- 99.6%), respectively. Peripheral blood leukocyte number was not significantly changed in any of the studies. The review authors state that it is difficult to draw a conclusion on the effects of Echinacea on the PNG activity from these five studies due to the use of different methods for measuring phagocytosis, the small sample sizes and the lack of chemically defined standardised Echinacea preparations.

59. A human study with 10 healthy subjects (5 male and 5 female) evaluated the immunomodulatory effect of a standardised E. angustifolia root extract (Polinacea) by measuring the mRNA and protein levels of the cytokines IL-2, IL-8, IL-6 and TNF-α in plasma samples (Dapas et al., 2014). The subjects took 10 mL, equal to 100 mg E. angustifolia root extract, daily for 4 weeks. The syrup contained 4.7 mg/10 mL of echinacoside and 8.0 mg/10 mL of high molecular weight polysaccharides. Blood samples were taken once a week, with the first sample taken a week before the Echinacea supplementation started. Lympho-

monocytes were isolated, their total RNA extracted, and quantitative PCR performed for cytokines IL-2, IL-8, and TNF-α gene expression. The protein levels of the cytokines in the blood samples were measured by flow cytometry. The expression levels of IL-2 and IL-8 were upregulated after Echinacea treatment, whilst a downregulation of the pro-inflammatory cytokines TNF-α and IL-6 occurred. The maximal differential gene expression for the cytokines was observed after 14 days of Echinacea treatment. The up-regulation of the IL-2/IL-8 gene expression and the downregulation of IL-6 detected at mRNA level positively correlated with the protein levels detected in the plasma. The authors acknowledge the study limitations such as small sample size and the lack of comparison to other Echinacea preparations.