Journal of Clinical Pharmacy and Therapeutics (2005) **30**, 363–369

ORIGINAL ARTICLE

Echinacea intake induces an immune response through altered expression of leucocyte hsp70, increased white cell counts and improved erythrocyte antioxidant defences

L. L. Agnew* BSc, S. P. Guffogg* BSc, A. Matthias† PhD, R. P. Lehmann† PhD, K. M. Bone†,‡ BSc and K. Watson* PhD

**School of Biological, Biomedical and Molecular Sciences, University of New England, Australia, †MediHerb Research Laboratories, University of Queensland, Brisbane, Australia and ‡School of Health, University of New England, Australia*

SUMMARY

Objective: **To study the effect of Echinacea tablets on the expression of leucocyte heat shock protein 70 (hsp70), erythrocyte haemolysis, plasma antioxidant status, serum chemistry, haematological values and plasma alkylamide concentrations.**

Method: **Eleven healthy individuals (26–61 years of age) were evaluated at baseline (day 1) and on day 15 after consuming two commercially blended Echinacea tablets daily for 14 days.**

Results: **Echinacea supplementation enhanced the fold increase in leucocyte hsp70 expression after a mild heat shock ($P = 0{\cdot}029$). White cell counts (WCC) were also increased ($P = 0{\cdot}043$). We also observed a preventative effect against free radical induced erythrocyte haemolysis ($P = 0{\cdot}006$) indicative of an antioxidant effect.**

Conclusion: **The pilot study suggests that Echinacea may invoke an immune response through altered expression of hsp70 and increased WCC.**

***Keywords*: antioxidant, Echinacea, heat shock protein 70, immunomodulation**

INTRODUCTION

The heat shock or stress proteins, which are among the most highly conserved proteins in nature, are found in all organisms from bacteria to humans and are expressed constitutively as well as induced in response to a mild, generally non-lethal stress. These proteins were originally observed in cells exposed to a mild heat shock and hence the name heat shock proteins (hsps). Later experiments reported that these proteins were also induced on exposure of cells to environmental stressors other than heat. These stress factors include inflammation, microbial infection (viral and bacterial), oxidative stress and cytotoxins. The more general term 'stress protein' has thus been applied to this set of proteins (1).

An important physiological function for the hsps is their role in the assembly and transport of newly synthesized proteins within cells as well as in the removal of denatured proteins. The hsps are therefore important in both preventing damage and in cellular repair processes after injury. There is well documented evidence that increased production of hsps protects cells against subsequent lethal stress induced by a number of conditions including oxidative stress, cytotoxins, heat stress and cellular damage after ischaemia or sepsis-induced injury (2). Additionally, there is increasing evidence that the hsps play key roles as prominent antigens in the humoural and cellular immune responses mediated by antibodies and T cells respectively (3, 4). The involvement of altered hsp expression in a number of disease states has emphasized the important role of these highly conserved proteins in the modulation of the immune response (5).

Many compounds have been postulated to modulate the immune response including the constituents found in Echinacea preparations. Echinacea, also known as purple coneflower and *Rudbeckia*, is a flowering plant member of the Compositae family that is mainly used for the treatment of upper

Received 15 February 2005, Accepted 17 March 2005
Correspondence: K. Watson, School of Biological, Biomedical and Molecular Sciences, University of New England, Armidale 2351, New South Wales, Australia. Tel.: +61 2 6773 3125; fax: +61 2 6773 3267; e-mail: kwatson2@une.edu.au

respiratory tract infections. It is, however, also used prophylactically as an immunomodulator and antioxidant and for the treatment of urinary tract infections, eczema and psoriasis as well as to aid wound healing (6). Although Echinacea and its effects on the immune system have been studied since the late 1930s, its mode of action is still unclear. It has been concluded that there is a need for improved scientific evidence and quality control of Echinacea preparations with respect to acceptance by the medical community as to its efficacy as an immune stimulant (7, 8).

The present study was a pilot scale clinical trial involving 11 subjects (five male, six female), orally dosed with the tablet form of an ethanolic extract of two species of *Echinacea purpurea* and *Echinacea angustifolia*. The aim of the study was to investigate the potential immunomodulatory effects of Echinacea in healthy subjects by measuring leucocyte hsp70 expression as a biomarker of the immune response. The kinetics of erythrocyte haemolysis induced by the hydrophilic free radical generator AAPH [2,2-azobis(2-amidinopropane) dihydrochloride] and plasma total antioxidant status (TAS) were measured as indicators of antioxidant effects of Echinacea.

METHODS

Echinacea Premium tablets

Echinacea Premium tablets containing 675 mg of *E. purpurea* root extract and 600 mg of *E. angustifolia* root extract, prepared by ethanol extraction, were obtained from MediHerb, Warwick, Australia.

Study participants

Eleven individuals (five male and six female) aged between 26 and 61 years with body mass indexes ranging from 18·8 to 30·0, participated in the study. All participants were in good health and not taking any other medications. The University of New England Human Research Ethics Committee (HE03/075) approved all procedures and all participants gave written consent to the study. On day one, a fasting, baseline, heparinized blood sample was collected. Participants then consumed two MediHerb Echinacea Premium tablets per day for 14 days, a total of 28 tablets per subject. A further fasting, heparinized blood sample was collected on the morning of day 15.

Expression of hsps

Leucocytes from 20 mL of venous blood were isolated using Ficoll-Paque gradient centrifugation and incubated at either 37 °C (control) or 42·5 °C (heat shock) for 1 h. Leucocytes were then allowed to recover at 37 °C for 3 h and proteins were extracted and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Hsp70 expression was measured as previously described (9) using western immunoblots (with β-actin as an internal control). Fold-increase in leucocyte hsp70 expression was calculated by densitometric analysis and expressed as the ratio of hsp70 expression at 42·5 °C to that at 37 °C.

Erythrocyte haemolysis

Approximately 4 mL of whole blood was centrifuged at $600 \times g$ for 10 min. Plasma was removed by aspiration and frozen in aliquots at −80 °C for later analysis. The buffy coat and platelets were removed by aspiration and discarded. Erythrocytes were washed twice with five volumes of phosphate-buffered saline (PBS) [136·9 mM NaCl, 2·68 mM KCl, 10 mM Na_2HPO_4, 1·76 mM K_2HPO_4; pH 7·4] by centrifugation at $600 \times g$ for 5 min. Erythrocyte aliquots (200 μL) were combined with 800 μL PBS and 1 mL 100 mM AAPH [2,2-azobis(2-amidinopropane) dihydrochloride] in PBS, pH 7·4. The suspension was carefully mixed and incubated at 37 °C in a shaking water bath for 6–7 h. The optical densities of 50 μL aliquots of erythrocyte suspension in 1·95 mL 0·15 M NaCl were measured spectrophotometrically at 540 nm every 30 min. Per cent haemolysis was calculated against erythrocytes suspended in double distilled water which results in 100% haemolysis.

Antioxidant effects

Analysis of TAS in plasma was determined using the Calbiochem Total Antioxidant Status Assay Kit (Cat. No. 615700), in accordance with the manufacturer's instructions.

Blood chemistry and haematology

Blood samples were collected for standard haematological analyses (haemoglobin, white cell counts, platelets, red cell counts, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, red blood cell distribution width, differential white cell count) and for liver function tests (protein, albumin, calcium globulin, bilirubin, γ-glutamyltransferase, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase). These analyses were conducted in the pathology laboratory at the Armidale and New England Hospital, Armidale, NSW, Australia.

Alkylamide concentrations

Blood samples were taken 1 h after ingestion of one Echinacea tablet and were collected into tubes containing lithium heparin. Plasma was separated immediately by centrifugation and stored frozen (−80 °C) for subsequent analysis of Echinacea active components. Alkylamides were separated from the plasma and concentrated by passage through a solid phase extraction cartridge using methanol as the elution solvent, essentially as previously described (10). Alkylamide concentrations in samples were determined by LC-MS using a gradient HPLC system (Shimadzu LC10AT, Shimadzu Corporation, Tokyo, Japan) coupled to a quadrupole mass spectrometer (Shimadzu Corporation, Tokyo, Japan) operating in SIM mode [using the molecular ions (m/z) according to the masses of the individual compounds] with a positive ion electrospray interface. The mobile phase was a mixture of water and acetonitrile and was applied using a linear gradient.

All values are expressed as mean ± SE. Paired student's t-tests were used for comparisons between baseline and after supplementation values.

RESULTS

Expression of hsp70

Expression of hsp70 in leucocytes was measured by western immunoblot. Consistent with the literature, hsp70 expression increased in all samples after a mild heat shock (Fig. 1). The expression of hsp70 (relative to β-actin, the internal control) measured at 37 °C at baseline was 0·39 ± 0·10 and this did not differ significantly after Echinacea supplementation (0·29 ± 0·08). The expression of hsp70 (relative to β-actin) after heat shock at 42·5 °C was also not significantly different with 0·66 ± 0·12 at baseline and 0·68 ± 0·13 after Echinacea intake. The ratio of control (37 °C) vs. heat shock (42·5 °C) hsp70 expression was subsequently examined as a fold-increase. Mean hsp70 fold-increase at baseline was 2·21 ± 0·35 and this was significantly different after Echinacea intake at 3·25 ± 0·67 (P = 0·029).

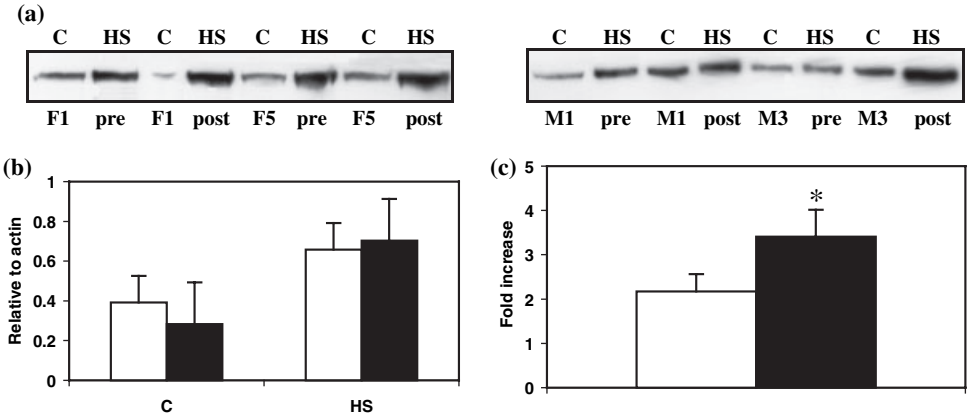

Fig. 1. Heat shock protein 70 (hsp 70) expression. (a) Representative Western immunoblots for hsp70 expression in leucocytes. C, control (37 °C), HS, heat shock (42·5 °C), pre, before Echinacea intake, post, after 2 weeks Echinacea supplementation. (b) Leucocyte hsp70 expression. Measured by Western immunoblot and expressed relative to β-actin. Pre-Echinacea (□), after Echinacea (■). Values are mean ± SE for n = 11. (c) Increase in Hsp70 expression. Data is expressed as fold increases of the ratio of hsp70 expression at 42·5 °C relative to that measured at 37 °C. Pre-Echinacea (□), after Echinacea (■). Values are mean ± SE for n = 11. *P = 0·029.

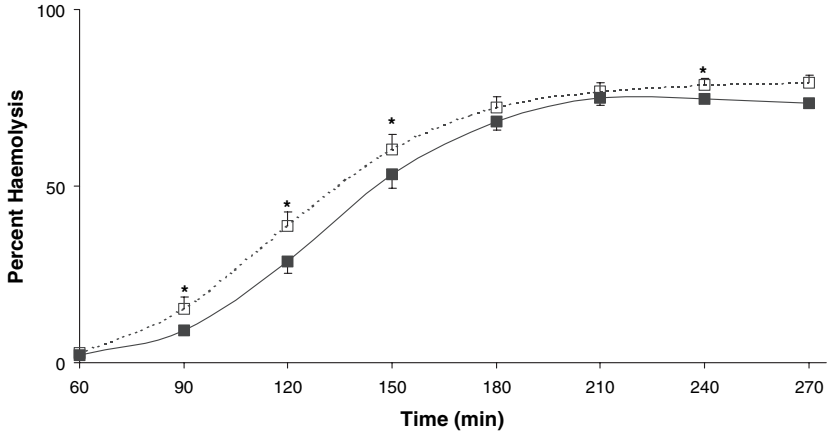

Fig. 2. Erythrocyte haemolysis. The kinetics of erythrocyte haemolysis as induced by the free radical generator AAPH [2,2-azobis(2-amidinopropane) dihydrochloride]. Each point represents the mean ± SE (n = 11). Pre-Echinacea (□), after Echinacea (■). *P < 0·05 for pre compared with post for the given time points.

Erythrocyte haemolysis

Figure 2 illustrates the kinetics of erythrocyte haemolysis as determined by progressive haemoglobin release after challenge with the free radical generator AAPH. As a result of the differences observed in per cent haemolysis at a number of the examined time points, the 50% haemolysis time increased from 135 ± 6 to 146 ± 5 min after Echinacea supplementation (P = 0·006).

Antioxidant effects

Mean plasma TAS was not significantly altered by Echinacea supplementation with values of 0·832 ± 0·039 mM found under baseline conditions and 0·804 ± 0·029 mM measured after supplementation.

Blood chemistry and haematology

The serum chemistry and haematological values for subjects after Echinacea intake, did not vary significantly from baseline levels (Table 1) with the exception of WCC. The mean baseline WCC was 6·6 ± 0·4 × 10^9/L whereas after supplementation it was 7·2 ± 0·3 × 10^9/L (P = 0·043). Differential WCC were measured to determine whether any particular cell types showed significant increases or whether the trend was consistent across all cell types. Most cell types displayed modest increases after Echinacea intake, although only the lymphocyte sub-population approached significance (P = 0·056).

Alkylamide concentrations

Plasma alkylamide concentrations were determined following ingestion of one Echinacea tablet. Only the major alkylamide found in the ingested Echinacea preparation was clearly identified in all individuals. Alkylamide concentrations were found to be 11·5 ± 2·0 ng equiv/mL plasma (n = 7).

DISCUSSION

Echinacea was found to affect the immune system by increasing WCC and the hsp70 response in leucocytes to heat shock as well as decreasing erythrocyte haemolysis after a 2-week dosing regimen.

The immunomodulatory effects of Echinacea are considered to be via non-specific activation of the immune system. Several possible modes of action have been implicated, including macrophage stimulation, phagocytic activity and NK cell activity (7, 11, 12). Our data showed that although neither basal nor heat shock (mild stress) hsp70 levels in leucocytes were different there was a greater fold-increase in hsp70 expression (the ratio of expression at 42·5 °C compared with expression at 37 °C) after Echinacea supplementation. This is indicative of an improved leucocyte stress response. The stress response is a highly conserved, adaptive response that is present in both unicellular and multicellular organisms and it confers tolerance and stress resistance at both cellular and whole organism levels (13). This enhanced stress response is also indicative of an improved immune response given that increases in hsp expression following cellular stress may play

Table 1. Blood chemistry and haematological values

	Before Echinacea	After Echinacea
Liver function		
Protein (g/L)	74 ± 2	74 ± 2
Albumin (g/L)	41 ± 1	42 ± 1
Calc. Glob. (g/L)	33 ± 1	32 ± 1
Bilirubin (μmol/L)	7 ± 1	7 ± 1
GGT (U/L)	17 ± 1	23 ± 5
Alk. Phos. (U/L)	71 ± 5	77 ± 10
ALT (U/L)	30 ± 2	31 ± 5
AST (U/L)	20 ± 1	23 ± 4
Haematology		
Haemoglobin (g/L)	146 ± 4	146 ± 4
WCC (10^9/L)	6·6 ± 0·4	7·2 ± 0·3*
Platelets (10^9/L)	276 ± 20	272 ± 15
RCC (10^{12}/L)	4·84 ± 0·15	4·81 ± 0·16
Haematocrit (L/L)	0·45 ± 0·01	0·45 ± 0·01
MCV (fl)	92·9 ± 0·8	92·9 ± 0·8
MCH (pg)	30·2 ± 0·3	30·3 ± 0·3
MCHC (g/L)	325 ± 1	326 ± 1
RDW (%)	11·4 ± 0·1	11·3 ± 0·2
Neutrophils (10^9/L)	3·6 ± 0·3	4·0 ± 0·3
Lymphocytes (10^9/L)	2·2 ± 0·1	2·3 ± 0·1
Monocytes (10^9/L)	0·4 ± 0·0	0·5 ± 0·0
Eosinophils (10^9/L)	0·3 ± 0·1	0·3 ± 0·0
Basophils (10^9/L)	0·0 ± 0·0	0·0 ± 0·0

All values are mean ± SE (n = 11).
calc. glob., calcium globulin; GGT, γ-glutamyltransferase; alk. phos., alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; WCC, white cell count; RCC, red cell count; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHE, mean corpuscular haemoglobin concentration; RDW, red blood cell distribution width.
*P = 0·043.

critical roles in antigen presentation and lymphocyte effector function (reviewed in 14), cytokine (15) and β-chemokine induction (16), dendritic cell maturation (17) and danger signalling (18).

There is also increasing evidence to suggest that non-specific activation of the immune system by Echinacea may be mediated by increased monocyte secretion of cytokines (19, 20). It has been established that monocytes also express more hsp70 than any other leucocyte subtype (21, L.L. Angew and K. Watson unpublished data) and, whilst the leucocyte populations used in the current studies were not exclusively monocytes, it is likely that the observed enhanced stress response is primarily related to monocyte expression of hsp70. This increased stress response was not apparently due to an increase in monocyte numbers *per se*, as demonstrated by no differences in monocyte counts before and after Echinacea intake (Table 1). It is therefore hypothesized that non-specific activation of the immune system by Echinacea may be mediated by monocytes synthesizing increased amounts of hsp70 following cellular stress.

The absence of significant changes in serum chemistry and haematological values for subjects post-supplementation is consistent with other human Echinacea clinical trial results (22). Despite this lack of significant change however, the observed increases in circulating white cell numbers after Echinacea supplementation indicates an enhanced immune response.

There was no difference in the levels of plasma antioxidants as measured by the ABTS 2, 2′-Azino-di-[3-ethylbenzthiazoline sulfonate] method. It should be noted however, that this assay only measures the water-soluble antioxidants in the plasma and not the lipid-soluble antioxidant levels. The latter are more likely to be incorporated into plasma membranes. Nevertheless, we found that Echinacea intake provided some protection against oxidative damage to erythrocytes. As the erythrocyte membrane contains high concentrations of polyunsaturated fatty acids that are susceptible to free radical induced peroxidation (23), this experimental system was a useful tool to measure the ability of Echinacea to protect membrane lipids against free radical damage. A number of other *in vitro* studies have also demonstrated that Echinacea root extracts are a good source of antioxidants that possess free radical scavenging activities (24–26). The antioxidant effects appear to be similar for each of the three species of Echinacea. Given that the combinations of constituents vary considerably between species, it has been postulated that these effects are cumulative, rather than being attributable to individual active constituents (26). As free radical damage is a well-established, causative factor in many disease states (27, 28) and dietary antioxidant supplementation has been reported to improve cell-mediated immunity in humans (29), the benefits of therapeutic agents such as Echinacea that improve the antioxidant status of an individual should be more closely evaluated.

CONCLUSIONS

The results of this pilot study suggest that supplementation with Echinacea may induce an immune response through altered expression of leucocyte hsp70 and increased WCC. There is also some support for the protective effects of Echinacea against free radical induced damage to erythrocytes.

COMPETING INTERESTS

Linda Agnew, Sharon Guffogg and Kenneth Watson have no declared interests. Anita Matthias and Reg Lehmann are employed by MediHerb Pty Ltd. Kerry Bone is a consultant to MediHerb Pty Ltd.

AUTHORS' CONTRIBUTIONS

LLA and SPG participated in clinical studies. AM participated in obtaining the alkylamide experimental data and clinical data analysis. LLA, AM and KW drafted the manuscript. KW, RL and KMB conceived of the study and participated in its design and coordination. All authors read and approved the final manuscript.

ACKNOWLEDGEMENTS

This research was supported in part by a University of New England research grant and MediHerb Pty. Ltd. Echinacea Premium Tablets were supplied by MediHerb Pty. Ltd. We thank Neil Horton and staff at the Pathology Department, Armidale and New England Hospital and the volunteers who participated in the study.

REFERENCES

1. Watson K (1990) Microbial stress proteins. *Advances in Microbial Physiology*, **31,** 183–223.
2. Jolly C, Morimoto RI (2000) Role of the heat shock response and molecular chaperones in oncogenesis and cell death. *Journal of National Cancer Institute*, **92,** 1564–1572.
3. Zugel U, Kaufmann SHE (1999) Role of heat shock proteins in protection from and pathogenesis of infectious diseases. *Clinical Microbiology Reviews*, **12,** 19–39.
4. Basu S, Srivastava PK (2000) Heat shock proteins: the fountainhead of innate and adaptive immune responses. *Cell Stress and Chaperones*, **5,** 443–451.
5. Srivastava PK (2002) Interactions of heat shock proteins with peptides and antigen presenting cells: chaperoning of the innate and adaptive immune responses. *Annual Reviews of Immunology*, **20,** 395–425.
6. Anonymous (2001) Monograph: Echinacea. *Alternative Medicine Review*, **6,** 411–414.
7. Barrett BP (2003) Medicinal properties of Echinacea: a critical review. *Phytomedicine*, **10,** 66–86.
8. Melchart D, Linde K, Fisher P, Kaesmayr J (2000) Echinacea for preventing and treating the common cold. *Cochrane Database Systematic Review 2000*, (2):CD000530 Review.
9. Rao DV, Watson K, Jones GL (1999) Age-related attenuation in the expression of the major heat shock proteins in human peripheral lymphocytes. *Mechanisms of Ageing and Development*, **107,** 105–118.
10. Matthias A, Addison RS, Penman KG, Dickinson RG, Bone KM, Lehmann RP (2005) Echinacea alkamide disposition and pharmacokinetics in humans after tablet ingestion. *Life Sciences* (in press).
11. Burger RA, Torres AR, Warren RP, Caldwell VD, Hughes BG (1997) Echinacea-induced cytokine induction by human macrophages. *International Journal of Immunopharmacology*, **19,** 371–379.
12. Gan XH, Zhang L, Heber D, Bonavida B (2003) Mechanism of activation of human peripheral blood NK cells at the single cell level by Echinacea water soluble extracts: Recruitment of lymphocyte-target conjugates and killer cells and activation of programming for lysis. *International Immunopharmacology*, **3,** 811–824.
13. Lindquist S, Craig EA (1988) The heat shock proteins. *Annual Reviews of Genetics*, **22,** 631–677.
14. Prohaszka Z, Fust G (2004) Immunological aspects of heat-shock proteins – the optimum stress of life. *Molecular Immunology*, **41,** 29–44.
15. Asea A (2003) Chaperokine-induced signal transduction pathways. *Exercise Immunology Reviews*, **9,** 25–33.
16. Lehner T, Bergmeier LA, Wang Y, Tao L, Sing M, Spallek R, van der Zee R (2000) Heats hock proteins generate β-chemokines which function as innate adjuvants enhancing adaptive immunity. *European Journal of Immunology*, **30,** 594–603.
17. Kuppner M, Gastpar R, Gelwer S, Noessner E, Ochmann O, Scharner A, Issels RD (2001) The role of heat shock protein (HSP70) in dendritic cell maturation: Hsp70 induces the maturation of immature dendritic cells but reduces DC differentiation from monocyte precursors. *European Journal of Immunology*, **31,** 1602–1609.
18. Matzinger P (1998) An innate sense of danger. *Seminars in Immunology*, **10,** 399–415.

19. Rininger J, Kickner S, Chigurupati P, McLean A, Franck Z (2000) Immunopharmacological activity of Echinacea preparations following simulated digestion on murine macrophages and human peripheral blood mononuclear cells. *Journal of Leukocyte Biology*, **68,** 503–510.
20. Goel V, Chang C, Slama J, Barton R, Bauer R, Gahler R, Basu T (2002) Echinacea stimulates macrophage function in the lung and spleen of normal rats. *Journal of Nutritional Biochemistry*, **13,** 487–492.
21. Bachelet M, Mariethoz E, Banzet N *et al.* (1998) Flow cytometry is a rapid and reliable method for evaluating heat shock protein 70 expression in human monocytes. *Cell Stress and Chaperones*, **3,** 168–176.
22. Randolph RK, Gellenbeck K, Stonebrook K, Brovelli E, Qian Y, Bankaitis-Davis D, Cheronis J (2003) Regulation of human immune gene expression as influenced by a commercial blended Echinacea product: preliminary studies. *Experimental Biology and Medicine*, **228,** 1051–1056.
23. Miki M, Tamai H, Mino M, Yamamoto Y, Niki, E (1987) Free-radical chain oxidation of rat red blood cells by molecular oxygen and its inhibition by α-tocopherol. *Archives of Biochemistry and Biophysics*, **258,** 373–380.
24. Pellati F, Benvenuti S, Magro L, Melegari M, Soragni F (2004) Analysis of phenolic compounds and radical scavenging activity of *Echinacea* spp. *Journal of Pharmaceutical and Biomedical Analysis*, **35,** 285–301.
25. Hu C, Kitts DD (2000) Studies on the antioxidant activity of Echinacea root extract. *Journal of Agricultural and Food Chemistry*, **48,** 1466–1472.
26. Sloley BD, Urichuk LJ, Tywin C, Coutts RT, Pang PK, Shan JJ (2001) Comparison of chemical components and antioxidants capacity of different *Echinacea* species. *Journal of Pharmacy and Pharmacology*, **53,** 849–857.
27. Rice-Evans CA, Diplock AT (1993) Current status of antioxidant therapy. *Free Radicals in Biology and Medicine*, **15,** 77–96.
28. Halliwell BH, Gutteridge JM (1999) *Free radicals in biology and medicine*, 3rd edn. Oxford: Oxford University Press.
29. Meydani M (1999) Dietary antioxidants and modulation of aging and immune-endothelial cell interaction. *Mechanisms of Ageing and Development*, **111,** 123–132.