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In vitro and in vivo immune stimulating effects of a new standardized *Echinacea angustifolia* root extract (Polinacea™)

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Abstract

Polinacea™ is a new standardized hydroethanolic extract obtained from *Echinacea angustifolia* roots containing echinacoside (>4%), the high molecular weight polysaccharide IDN 5405 (> 5%) and a isobutylamide fraction (<0.1%). For in vitro tests, a bacterial lipopolysaccharide-free (LPS-free) Polinacea™ has been prepared in order to avoid non-specific responses of immunocompetent cells.

LPS-free Polinacea™ enhanced the immune functions as highlighted by the proliferation rate and γ-interferon production in murine T-lymphocyte cell cultures stimulated by anti-CD3. LPS-free Polinacea™ did not have a direct role on macrophage response as measured in the nitric oxide production test using the J774 macrophage cells line. In vivo, Polinacea™ showed an immune stimulating activity by reducing the *Candida albicans* induced mortality both in normal and in cyclosporin A-treated mice.
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Keywords: *Echinacea angustifolia*; IDN 5405; Immune stimulating activity

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1. Introduction

Historical tradition of the use of *Echinacea* species is definitely related to Native Americans since several American Indian tribes, including Cheyenne, Choctaw, Comanche, Crow, Dakota, Delaware, Pawnee, Hidatsa, Sioux and others, had the common habit to treat themselves with preparations of this plant. Major disorder targets of these treatments were related to: sore mouth, toothache, colds, tonsillitis, septic diseases, snakebite, coughs and general inflammatory conditions [1–3].

During the European colonization, *Echinacea* started to be known also among settlers, it entered the American Dispensatory in 1852, and after the introduction in eclectic medical practice (1887) it became one of the most popular remedy of the following 50 years. *Echinacea* was described in the National Formulary of the United States from 1916 to 1950, and then due to the incoming prevalence of synthetic chemistry in the pharmaceutical field, it declined in the medical interest.

Nevertheless, the use of *Echinacea* still remained popular. In particular, it exploded as a dietary supplement with a peak of estimated market of about 300 millions USD [4] after the approval of the Dietary Supplement Health and Education Act (1994) in the USA.

A huge number of scientific data are available in literature and they are related to several kinds of preparations obtained from the three most relevant species of *Echinacea* spp. (*E. angustifolia*, *E. pallida*, *E. purpurea*). On the other hand, hundreds of commercial preparations are now available in pharmacies, groceries and health-food stores of most of the developed countries and consequently a rigorous parallelism between the composition of the products used in the available literature and the products on the market is an absolute need in order to avoid misleading information to the consumer.

Independently from the kind of preparation, the main indications of *Echinacea* are the prevention and treatment of common cold, flu and upper respiratory infections (URIs). The proposed mechanism of action is related to the capacity to stimulate the immune system. In a comprehensive review of 1996, Bauer [5] already stated that relevant pharmacological effects have been demonstrated in vitro and in vivo for the expressed juice of the aerial parts of *E. purpurea* and for alcoholic extracts of the roots of *E. angustifolia*, *E. purpurea* and *E. pallida*. According to the author, the effects are mainly linked to a modulation of nonspecific cellular immune system and the compounds responsible for such an effect are: polysaccharides, glycoproteins, caffeic acid derivatives and alkylamides.

Later on, stimulation of various immune cells such as macrophages, other monocytes and natural killer cells have also been demonstrated in vitro [6–10].

Same studies also demonstrated that preparations of *Echinacea* besides to stimulate these components of the immunoresponse are strongly involved in the reduction of inflammatory processes, which play a central role in the development of symptoms of primary illnesses for which the plant is used (common cold and sore throat). The phenolic components, i.e. echinacoside, of the *Echinacea* preparations are often described to be responsible for these effects [9].

A good level of evidence is available on the role played by the polysaccharidic fraction in the immunostimulatory effect of *Echinacea* preparations. In particular, the polysaccharides (heteroglycans) isolated from *E. purpurea* have been deeply investigated for

their capacity to activate macrophages in mice, rats and humans along with other immunological functions [7,11,12].

Data available on the lipophilic components present in *Echinacea* preparations are less consistent even if some of them such as alkylamides and in particular isobutylamides have been described to produce a strong stimulating effect on phagocyte functions [6] and on lipoxygenase-inhibiting activity [13].

The clinical evidences now available on *Echinacea* derivatives (mainly based on *E. purpurea*) have been recently reviewed by Barrett [14]. The globality of the data tentatively supports the use of these preparations in the treatment of acute URIs, which corresponds with the most widespread utilization.

On the globality of the preclinical and clinical results, two comments can be made. First, also considering the multiplicity of the effects produced by *Echinacea* preparations, a single active ingredient may not be the unique responsible for the biologic effects and, consequently, it is still reasonable to consider a properly standardized extract as "the active principle". Second, the problem of the standardization of the extract in the case of *Echinacea* has to consider at least three groups of molecules as characterizing elements for the expected pharmacological effect.

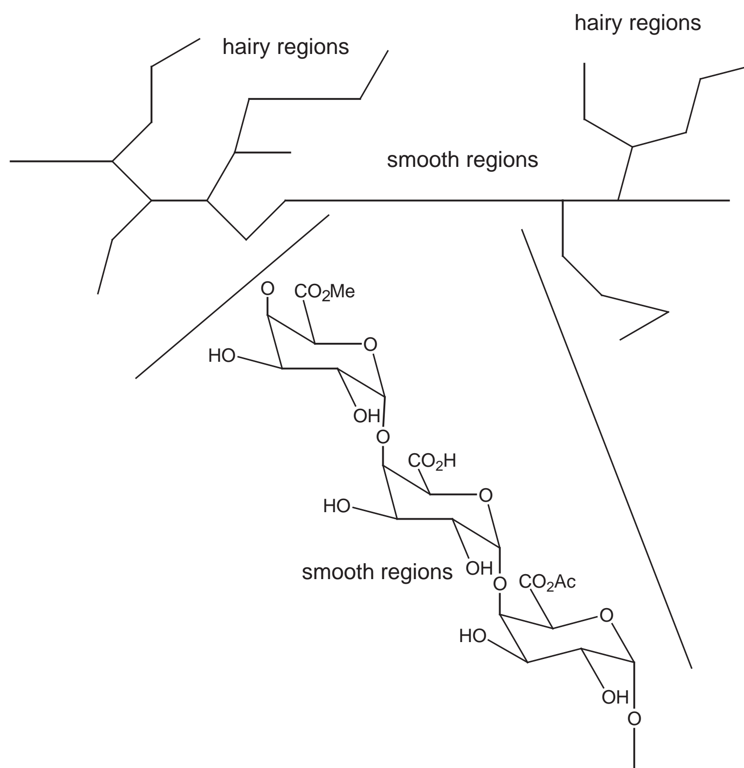

Fig. 1. IDN 5405 molecular structure.

In the present paper, a new standardized extract from the roots of *E. angustifolia* (Polinacea™) [15] has been submitted to in vitro and in vivo experimental tests to verify the immune stimulating activity. In order to avoid an unspecific response of the immune competent cells, for the in vitro studies, samples of Polinacea™ have been purified from lipopolysaccharides (LPS) of bacterial origin, which are a possible contaminant of the raw drug utilized for the extraction.

In most of the experimental tests, Polinacea™ has been compared with a purified high-molecular weight polysaccharide (IDN 5405), which is contained in it. IDN 5405 is characterized by a backbone of a polygalacturonic acid partially carboxymethylated with hairy regions of rhamnogalacturonan (Fig. 1) and it is specific to *E. angustifolia* [16].

2. Experimental

2.1. Plant material

E. angustifolia DC (Asteraceae): roots were harvested in May 2003, from a cultivated field located near Verona-Italy. A voucher specimen is kept in the Botanical Department, Indena S.p.A. Settala (Milan).

This material is the result of a selection/improvement work, started in 1998 and still in progress, which has been developed following the steps briefly listed here below:

- Seed collection from 23 different populations of wild *E. angustifolia* scattered within the distribution area of this species in North America.
- Cultivation of the above 23 populations of *E. angustifolia* in a trial field near Como-Italy.
- Selection based on phenotype and chemotype, among the populations and within each population, of the best performing individuals.
- Micropropagation of the best performing individuals.
- Cultivation of a small field of micropropagated plants, aimed at seed production.
- Cultivation on a larger scale for biomass production.

2.2. Extraction and products

A batch of Polinacea™ has been obtained according to a patented process [15] from *E. angustifolia* roots cultivated and selected for their high content in polysaccharides and echinacoside. The roots are exhaustively treated with 90% EtOH for echinacoside extraction and then counter-extracted with *n*-hexane for isobutylamides elimination. Wet roots were extracted with 15% aq. EtOH and then the polysaccharidic fraction obtained by precipitation from EtOH. The two dry extracts were then dissolved together and exsiccated to give Polinacea™, with the following characteristics: echinacoside 5.7%, IDN 5405 6.6% and isobutylamides 0.02% (by HPLC).

IDN 5405 has been obtained as a pure polysaccharide (ca. 200000 Da) by purification from the 15% aq. EtOH extract, according to a patented process [16].

LPS-free samples of Polinacea™ and IDN 5405 were prepared by Biosynth s.r.l. (Siena, Italy) by means of affinity-removal capacity of SAEP (synthetic anti-endotoxin peptides) according to Rustici et al. [17].

2.3. Animals

Female syngenetic Balb/c mice purchased from Charles River (Calco, ltaly) were used. They were housed in standard environmental conditions and fed on rodent diet with water ad libitum.

2.4. Pyrogen testing

The QCL-1000 (Quantitative Chromogenic CAL by Bio-Whittaker) was used to detect and quantify the presence of LPS [18] in raw or purified extracts of *E. angustifolia*. The products were suspended in pyrogen-free saline solution for intravenous injection. Different dilutions of the samples were mixed with Limulus Amebocyte Lysate and incubated at 37 °C for 10 min. A substrate solution was then added to the samples and the mixture was incubated at 37 °C for 6 min. Then the reaction was stopped with 25% acetic acid (v/v: glacial acetic acid/water). Samples containing LPS developed a yellow color. The absorbance of the sample was determined spectrophotometrically at 405–410 nm with a Behring ELISA Processor II. Since the absorbance is in direct proportion to the amount of LPS present, the concentration of LPS was calculated from a standard curve.

2.5. In vitro nitric oxide assay on isolated macrophages

J774, a murine macrophage cell line, was maintained in Dulbecco's modified Eagle medium (DMEM) (Life Technologies Italia) supplemented with 10% heat-inactivated FCS (HyClone Laboratories, Logan, UT) and 2 mM L-glutamine (Life Technologies), at 37 °C in a humidified 5% CO_2 atmosphere. Cells were plated in 96-well flat bottom microtiter plates (10^5 cells in 100 μl/well) [19].

NO production was estimated in triplicate as the amount of NO_2^- released in the culture medium. Interferon-γ (IFN-γ, 500 U/ml) and/or 1 μg/ml LPS (phenol-extracted and chromatographically purified from *Escherichia coli* serotype 0111:B4 purchased from Sigma Chemical Co., St. Louis, MO) or *E. angustifolia* products (0.1–100 μg/ml) were added to the J774 cultures. 50 μl aliquots of culture supernatants were collected after 48 h of incubation and mixed with an equal volume of Griess reagent in a 96-well flat bottom Falcon microplate. The A_{570} was monitored with a microplate reader (Behring ELISA Processor II) with background subtraction at A_{650} [20]. Quantitative analysis was performed by comparison with standard solutions of $NaNO_2$.

2.6. In vitro IFN-γ determination in isolated T lymphocytes

Lymphocytes were obtained from spleens of mice. Spleens aseptically removed were teased apart in cold buffered salt solution supplemented with 5% FCS and the cell

suspension was enriched for T lymphocytes by elution from nylon wool columns as described by Julius et al. [21]. The resulting cell suspension contained more than 90% T cells, as determined by cytofluorimetric analysis after labeling with anti-CD3 FITC-conjugated monoclonal antibodies using a FACScan (Becton-Dickinson, Mountain View, CA).

Aliquots of 0.1 ml containing 4×10^5 murine T lymphocytes were plated in triplicate in 96-well microtiter round bottom plates (Falcon) and 0.1 ml of medium containing Polinacea™ or IDN 5405 (0.1–10 μg/ml) and/or anti-CD3 moAb (Pharmingen, Milano, ltaly) were added. Cultures were incubated for 72 h at 37 °C in a humidified 5% CO_2 incubator.

The titer of IFN-γ in the supernatants of T cells treated as above indicated for 72 h was assessed with a commercial ELISA kit (Amersham) [19].

2.7. Experimental candidosis

The method described by Martinez et al. [22], slightly modified, has been used.

Candida albicans (strain CA2) was kindly supplied by Dr. A. Cassone (Istituto Superiore di Sanità, Rome, Italy) and was grown by weekly transfer onto fresh Sabouraud's dextrose agar (Biolife, Milano). CA2 is an agerminative strain and grows as a pure yeast form in vitro at 28 °C or at 37 °C in conventional mycologic media.

For experiments, *C. albicans* cells were harvested from Sabouraud plates, suspended in PBS, counted in a Thoma chamber and diluted to obtain a concentration of 1.5×10^6 cells/ml. Volumes of 0.2 ml of the suspension were injected in the lateral caudal vein of mice.

Polinacea™ was administered in mice at dose of 1 g kg^{-1} day^{-1} p.o. and at 0.1 g kg^{-1} day^{-1} i.p. for 1 day before and 6 consecutive days after infection. Cyclosporin A (Sigma) was diluted in olive oil and administered p.o. at the dose of 10 mg kg^{-1} day^{-1} for 1 day before and 4 consecutive days after infection.

Mortality in each group following infection and treatment was monitored daily up to 14 days after challenge with *C. albicans.*

Table 1
In vitro effect of Polinacea™ (not deprived of LPS) on NO_2^- production by J774 macrophages stimulated by IFN-γ (500 U/ml)

Co-treatment	NO_2^- (μM)	
	–	IFN-γ
Control	3.1	14.9
LPS	3.4	79.1*
Polinacea™ 100 μg/ml	4.8	56.4*
Polinacea™ 10 μg/ml	2.7	44.7*
Polinacea™ 1 μg/ml	3.0	21.0
Polinacea™ 0.1 μg/ml	3.2	11.1

* $P < 0.01$.

Table 2
In vitro effect of Polinacea™ (LPS-deprived) on NO_2^- production by J774 macrophages stimulated by IFN-γ (500 U/ml)

Co-treatment	NO_2^- (μM)	
	–	IFN-γ
Control	0.0	21.8
LPS	5.4	49.3*
Polinacea™ 100 μg/ml	0.0	24.6
Polinacea™ 10 μg/ml	0.0	20.9
Polinacea™ 1 μg/ml	0.0	21.4
Polinacea™ 0.1 μg/ml	0.0	16.1

* $P<0.01$.

2.8. Statistical analysis

In experimental candidosis tests, chi-square test was used on the final number of survivors. In the other tests, ANOVA and Dunnett's multiple comparison test were utilized.

3. Results

3.1. NO production by J774 after 48 h of treatment with Polinacea™ and IDN 5405

NO production, signal of macrophage activation, was tested in a model of co-stimulation with IFN-γ (500 U/ml). Polinacea™ demonstrated to be a good dose-dependent activator of macrophage functions (Table 1); anyway, the product was heavily contaminated by LPS (1000 ppm). After LPS cleaning, as shown in Table 2, Polinacea™ demonstrated to be totally ineffective on macrophage NO releasing capability. Moreover, the pure polysaccharidic fraction (IDN 5405) resulted very active before LPS cleaning (data not shown) and totally ineffective when tested as LPS-free product (Table 3). These results strongly suggest that the presence of LPS is a factor to be considered when experimenting on in vitro models with botanical extracts, at least in this field of activity.

Table 3
In vitro effect of IDN 5405 (LPS-deprived) on NO_2^- production by J774 macrophages stimulated by IFN-γ (500 U/ml)

Co-treatment	NO_2^- (μM)	
	–	IFN-γ
Control	0.0	16.8
LPS	6.4	50.0*
IDN 5405 100 μg/ml	0.0	18.0
IDN 5405 10 μg/ml	0.0	16.0
IDN 5405 1 μg/ml	0.0	17.8
IDN 5405 0.1 μg/ml	0.0	17.7

* $P<0.01$.

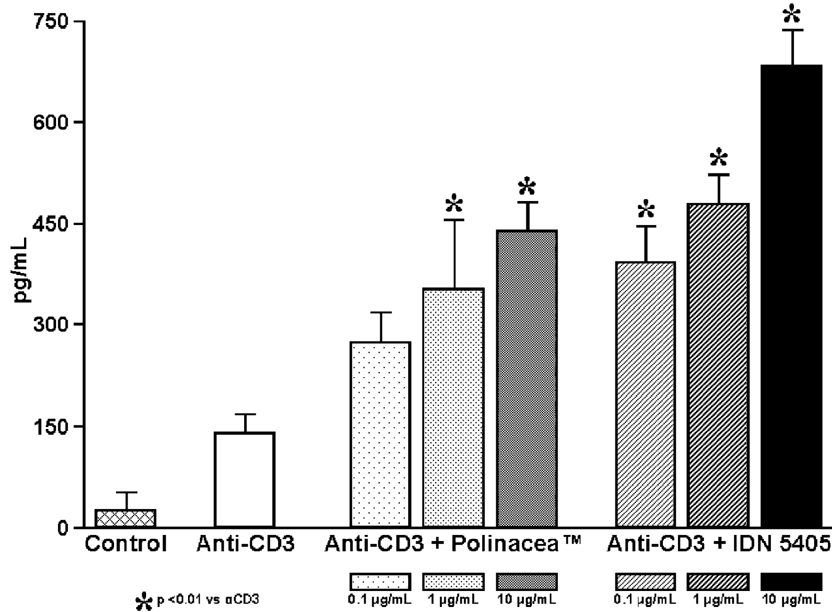

Fig. 2. Effect of Polinacea™ and IDN 5405 on IFN-γ production by CD3-treated human T lymphocytes.

3.2. IFN-γ production in purified murine spleen T lymphocytes stimulated with anti-CD3

Polinacea™ and IDN 5405 (both LPS-deprived) dose-dependently stimulated anti-CD3-treated isolated T lymphocytes to produce and release IFN-γ (Fig. 2). These results were paralleled by a good response in term of cell proliferation (data not shown).

3.3. Survival in C. albicans-infected mice

Polinacea™, orally administered at the dose of 1 g kg^{-1} day^{-1} for 7 days, was effective (30%) in counteracting the mortality induced by *C. albicans* infection; even in the case of cyclosporin (CsA)-induced immunosuppression, Polinacea™ was able to prevent animal death (40%) (Table 4).

Table 4
Effect of oral administration of Polinacea™ on survival in normal and immunosuppressed mice infected with *Candida albicans*

Treatment	No.	Survivors
C. albicans	20	0
C. albicans + Polinacea™ (1 g kg^{-1} day^{-1} × 7 days)	20	6*
C. albicans + CsA (10 mg kg^{-1} day^{-1} × 5 days)	10	0
C. albicans + CsA (10 mg kg^{-1} day^{-1} × 5 days) + Polinacea™ (1 g kg^{-1} day^{-1} × 7 days)	10	4*

* $P < 0.01$.

Table 5
Effect of intraperitoneal administration of Polinacea™ and *E. purpurea* extract on survival in normal mice infected with *Candida albicans*

Treatment	No.	Survivors
C. albicans	10	0
C. albicans + LPS (2 μg kg^{-1} day^{-1} × 7 days)	10	1
C. albicans + Polinacea™ (0.1 g kg^{-1} day^{-1} × 7 days)	10	4*

* $P < 0.01$.

Polinacea™, intraperitoneally administered at the dose of 0.1 g kg^{-1} day^{-1}, was effective (40%) in counteracting mortality induced by *C. albicans* infection (Table 5). This effect was also supported by the fact that LPS administered at a dose (2 μg kg^{-1} day^{-1}) corresponding to the amount administered in LPS-containing Polinacea™ was practically ineffective.

4. Discussion

According to the results obtained in the present study, some aspects of the immune stimulating activity of *Echinacea* preparations could be related with the fact that botanical derivatives can be contaminated by lipopolysaccharide (LPS), a high molecular weight product coming from bacterial cell wall and characterized by a very strong capacity, even at a very low concentration (ppm), to activate many of the in vitro and in vivo immunological responses. LPS, in fact, in mammalian phagocytes directly interacts with LPS-binding protein, CD14-receptor and the recently identified Toll-like receptor 4 (TLR4)-MD-2 complex leading to the rapid activation of an intracellular signaling pathway, which results in the release of pro-inflammatory mediators [23,24]. This clearly means that many of the botanical extracts currently available (especially those where the solvents used concentrate polysaccharides and therefore LPS) give rise to false positive results in terms of immune stimulation if tested either in vitro or in vivo throughout intravenous or intraperitoneal administration.

In line with this, LPS-containing Polinacea™ resulted very effective in activating in vitro macrophages, measured in terms of NO production; on the contrary, after LPS elimination, we did not observe any activity. On T cells, results sounded differently: the good results obtained with LPS-containing Polinacea™ (data not shown) were also observed after LPS elimination showing that T cells could be considered the real target for Polinacea™. Interestingly, the purified polysaccharide IDN 5405 showed the same profile of activation on T cells suggesting its pivotal role in the immunomodulating activity of Polinacea™. These data showing the central role of T cells in Polinacea™ activity are also confirmed by the total negative results we got in experimental model where the pure cell types analyzed were B cells, natural killer cells and granulocytes (data not shown).

On the basis of the in vitro results on the T cells, we focused our interest on those in vivo tests, such as candidosis in normal and in immunosuppressed mice, where a T-cell response plays a detrimental role. Polinacea™, administered by oral route in this

experimental model, showed to be very effective in counteracting *C. albicans* infection suggesting the potentiality for its usage as an immunostimulant.

In the same experimental model, Polinacea™ showed very interesting results when administered by intraperitoneal route. Also in this case, the effect can be considered specific since LPS tested at a dose correspondent to the amount administered with the extract was completely ineffective.

In conclusion, our study demonstrates that:

1. products claimed to be effective in vitro or in vivo after parenteral administration on immune system cells (mostly on macrophages) should be checked first for LPS contamination;
2. Polinacea™ activity on the immune system seems to be primarily mediated by interaction with T cells;
3. it is likely that most of the activity played on T cells by Polinacea™ is due to the presence of the high molecular weight polysaccharide IDN 5405.

Polinacea™ and the polysaccharide IDN 5405 are now undergoing additional biochemical and pharmacological investigations aimed to more exhaustively define its biological profile. Preliminary safety studies have been already performed (acute toxicity in rodents, and sub-acute toxicity in rats and dogs) demonstrating a good tolerability of the product and allowing future clinical testing.

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