

Anti-inflammatory homoeopathic drug dilutions restrain lipopolysaccharide-induced release of pro-inflammatory cytokines: *In vitro* and *in vivo* evidence

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Abstract

Context: The lipopolysaccharide (LPS)-induced cytokine release and oxidative stress are validated experimental parameters used to test anti-inflammatory activity. We investigated the effects of homoeopathic mother tinctures, 6 CH, 30 CH and 200 CH dilutions of *Arnica montana*, *Thuja occidentalis* and *Bryonia alba* against LPS (1 µg/ml)-induced cytokine release from RAW-264.7 cells and human whole-blood culture. **Materials and Methods:** For *in vivo* evaluations, mice were orally treated with 0.1 ml drug dilutions twice a day for 5 days followed by an intraperitoneal injection of 0.5 mg/kg LPS. After 24 h, the mice were sacrificed and serum levels of pro-inflammatory cytokines and nitric oxide were determined. The extent of oxidative stress was determined in the liver homogenates as contents of reduced glutathione, malondialdehyde, superoxide dismutase and catalase. **Results:** The tested drug dilutions significantly reduced *in vitro* LPS-induced release of tumour necrosis factor- α , interleukin-1 (IL-1) and IL-6 from the RAW-264.7 cells and human whole blood culture. Similar suppression of cytokines was evident in mice serum samples. These drugs also protected mice from the LPS-induced oxidative stress in liver tissue. **Conclusions:** Our findings substantiate the protective effects of *Arnica*, *Thuja* and *Bryonia* homoeopathic dilutions against LPS-induced cytokine elevations and oxidative stress. This study authenticates the claims of anti-inflammatory efficacy of these homoeopathic drugs.

Keywords: Cytokines, Homoeopathy, Human whole blood culture, Lipopolysaccharide, RAW-264.7

INTRODUCTION

Homoeopathy has remained the most widespread and still a contentious mode of therapy. Homoeopathic medicines are prepared according to the methods endorsed in the homoeopathic pharmacopoeias. The therapeutic efficacy of these medicines is established through administration of the crude form of source material to healthy volunteers and recording their behavioural symptoms. Very minute doses of a homoeopathic dilution of a drug are considered to cure the diseases presenting similar symptoms which are induced by crude form of that drug in healthy volunteers. Apart from this, the medicines are selected dependent on the experiences as recorded in authoritative homoeopathic literature and to some extent by means of research.^[1]

The issues of standardisation and establishment of pharmacological profiles have never been systematically

settled in Homoeopathy. However, in the last few decades, claims about the efficacy of homoeopathic medicines and their high dilutions are being revisited using validated pharmacological assays. Such studies help in critically examining and scrutinising the pharmacodynamics of the homoeopathic drugs.^[2] Validated experimental models used in establishing the pharmacodynamics of modern drugs are increasingly being used in demonstrating the efficacy of the

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homoeopathic drugs and their high dilutions. Recently, we have reviewed the literature on experimental proving of the anti-inflammatory activities of homoeopathic drugs and their dilutions.^[3] These studies on high dilutions of homoeopathic drugs along with continued efforts to reproduce their findings are adding to the scientific data on efficacy of homoeopathic drugs. A recent report has established the reproducibility of claims related to the effects of high dilutions of histamine on the basophil function. This study involved a strictly standardised flow cytometry protocol and showed that low and high dilutions of histamine inhibit CD203c up-regulation in anti-IgE-stimulated basophils.^[4] Such sporadic reports give impetus to further evaluations of homoeopathic drugs through validated pharmacological assays.

Anti-inflammatory drugs predominantly used in the homoeopathic practice include *Arnica montana*, *Bryonia alba* and *Thuja occidentalis*. These plant-derived drugs have been repeatedly tested through different pharmacological assays.^[5-9]

There are discrete studies reporting the anti-inflammatory efficacy of homoeopathic dilutions of these drugs. Kawakami *et al.* have reported the anti-inflammatory activity of *Arnica* 6 CH dilution in the carrageenan induced paw inflammation in rats.^[10] Bonamin *et al.* have further substantiated the mechanisms through which *Arnica* 6 CH dilution exerts anti-inflammatory activity against acute inflammation induced by carrageenan.^[11] *Thuja occidentalis* as homoeopathic dilution is well reported to reduce hepatitis and induce apoptosis in cancer cells.^[12] Sunila *et al.* have shown that the cytotoxic potential of *Thuja* is retained even in 30CH and 200 CH dilutions.^[13] The same group of researchers have reported the anti-tumour efficacy of ultra-high dilutions (1 M) of *Thuja occidentalis*,^[14] whereas Cornu *et al.* report the clinical ineffectiveness of *Arnica* and *Bryonia* combination against the bleeding, inflammation and ischaemia after aortic valve surgery.^[15] These contrasting claims on the proofs of efficacy of homoeopathic high dilutions present further challenges of establishing the reproducibility of such results through diverse and validated assay systems.

We investigated the anti-inflammatory efficacy of the homoeopathic dilutions (Mother tincture, 6CH, 30CH and 200 CH) of *Arnica*, *Thuja* and *Bryonia* in lipopolysaccharide (LPS)-induced *in vivo* and *in vitro* cytokine release assays. The LPS-induced *in vitro* release of cytokines from RAW-264.7 mouse macrophage cell line, whole human blood culture^[16] and LPS-induced *in vivo* escalation of cytokines along with oxidative stress is used as validated pharmacological assays to test anti-inflammatory activity of drugs.^[17]

MATERIALS AND METHODS

Drugs and reagents

Murine macrophage cell line RAW-264.7 was obtained from ATCC, VA, USA. RPMI 1640 (Cat No. P04-18047) and foetal bovine serum (FBS) (Cat No. P30-8500) were procured from PAN Biotech, Germany. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) M2128 Cat no. 298-93-1,

trypsin ethylenediaminetetraacetic acid (EDTA) (10X) and antibiotic solution (100X) were procured from HiMedia, India. LPS (L2880, Lot No. #025M4040V) and thiazolyl blue tetrazolium bromide (M2128, CAS 298-93-1) were purchased from Sigma-Aldrich, USA. ELISA Kits for tumour necrosis factor (TNF)- α (Cas No. 88-7324), interleukin-1B (IL-1B) (Cas No. 88-7013) and IL-6 (Cas No. 88-7064) were obtained from eBioscience, USA. All the homoeopathic drugs and their dilutions were obtained from Sintex International Ltd., India. All the antibodies were purchased from Santa Cruz Biotechnology, Inc.

In vitro experimental protocol

Maintenance of cell line

RAW-264.7 murine macrophage cells were grown and maintained in RPMI-1640 supplemented with 10% FBS, 1.5 mM L-glutamine and 1% antibiotic (100 U of penicillin and 10 mg streptomycin/ml in 0.9% normal saline) in a humidified atmosphere of 5% CO₂ at 37°C.

Determination of non-cytotoxic concentrations of test drugs

In the present study, we determined the non-cytotoxic concentrations of 91% ethanol (used as a vehicle for preparing homoeopathic drug dilutions) and mother tinctures of test drugs. RAW-264.7 cells were co-incubated for 48 h with serial dilutions of homoeopathic mother tinctures and ethanol ranging from 10⁰ to 10⁻¹⁶. As other dilutions were supposed to contain very minute traces of source drug (if any), their non-cytotoxic concentrations were taken to be similar to that of ethanol. Cells were plated in 96-well plates at a density of 10,000 cells per well. Cultures were treated with different concentrations of the homoeopathic drugs and its dilutions for 48 h. The cells were washed with 1 \times phosphate-buffered saline and then MTT (2.5 mg/mL) was added to each well. It was then incubated at 37°C for 6 h. Dimethyl sulphoxide solution (100 μ L) was added to each well of culture plate, and the colour intensity was measured at 570 nm using a microplate reader (PowerWave XS, Biotek, USA).^[18]

Lipopolysaccharide-induced cytokines release in RAW-264.7 cells

1 \times 10⁴ RAW-264.7 cells were seeded in 96-well plate for 70% confluence. Then, the cells were stimulated with LPS (1 μ g/ml) for 30 min followed by respective drug treatments. 1 μ l/ml of drug dilutions was added to medium for 24 h after which the supernatant was collected and processed for cytokine using ELISA following manufacturer's protocol.

After 24 h, the supernatant from each well was harvested for determination of cytokines using ELISA. The assays were carried out in duplicates.

Lipopolysaccharide-induced cytokines release in human whole blood culture

Whole blood *ex vivo* stimulation is a useful tool to investigate cytokine responses to a variety of stimuli, including bacterial endotoxin (LPS). This assay was carried out as reported earlier

by Thurm and Halsey.^[19] Human whole blood was collected from medial vein of a healthy volunteer into a sterile tube containing heparin (100 IU/ml). The volunteer had signed an informed consent to donate blood sample for this study and had no history of inflammatory conditions and had not taken any non-steroidal anti-inflammatory drugs for a fortnight.

The blood sample was diluted (1:5) with RPMI-1640 medium containing 10% foetal calf serum. Aliquots of 100 µl of the diluted whole blood were taken into sterile, 96-well microplate and stimulated with LPS at 1 µg/ml concentration for 30 min before addition of the homoeopathic test dilution (1 µl/ml). The microplates were incubated at 37°C for 24 h in a humidified CO₂ incubator. After incubation period, the supernatant was collected by centrifugation at 3000 rpm for 10 min at room temperature and stored at -20°C and analysed for cytokine content on a subsequent day. The estimation of cytokines, IL-1β, IL-6 and TNF-α was carried out following manufacturer's protocol.^[20] The assays were carried out in duplicates.

In vivo experimental protocol

Animals

Balb/C mice (7 weeks old, weighing 28–30 g) were obtained from central animal house facility of R. C. Patel Institute of Pharmaceutical Education and Research, Shirpur, (Maharashtra). The mice were kept under standard conditions (12 h light and 12 h dark), at 22°C ± 2°C with a relative humidity of 40%–80%. They were fed a standard laboratory diet and water *ad libitum*. The animals were maintained in conformity with the guidelines laid down by the Committee for the Purpose of Control and Supervision of the Experiments on Animals (CPCSEA) established under the Prevention of the Cruelty to Animals Act, 1960, Ministry of Environment and Forests, Government of India. The experimental protocols were permitted by the Institutional Animal Ethics Committee of R. C. Patel Institute of Pharmaceutical Education and Research, Shirpur, Dist-Dhule, Maharashtra, India (Protocol Approval #IAEC/CPCSEA/RCPIPER/2015/16-09). All the tests complied with the recommendations of the International Association for the Study of Pain.

Lipopolysaccharide-induced inflammation in mice

Animals were randomised by serially numbering them and then allocating them to study groups according to the sequence generated using 'Random Number Generator' available online as open source software Stat Trek®. The randomised mice were divided into 13 groups (*n* = 4). For oral administration, the test drugs and their dilutions were prepared as 10% solutions in water for injection (mixture of 0.1 ml of test drug dilution and 900 µl of water for injection). The drug treatments were given two times a day for 6 days. On the 6th day, 1 h after drug administration, the negative control group received normal saline intraperitoneally and all other groups received 0.5 mg/kg LPS solution intraperitoneally. Dexamethasone (10 mg/kg) was used as a standard drug. In this study, we included ethanol

treated group (0.1 ml ethanol mixed with 900 µl of water for injection, twice a day) as a vehicle control group. After 2 h of LPS injection, blood samples were collected by retro-orbital plexus puncture method. The sera were separated and stored at -70°C for TNF-α, IL-6 and IL-1β determinations by ELISA.^[21]

Evaluation parameters

Determination of cytokines production

The concentrations of TNF-α, IL-6, and IL-1β in cell culture supernatants, and mice serum samples were determined according to the manufacturer's protocol using ELISA kits.

Determination of nitric oxide production

For nitric oxide (NO) estimation, 500 µl of Griess reagent was mixed with 50 µl of serum sample and the absorbance was determined at 540 nm using Powerwave XS microplate spectrophotometer (Biotek, USA). Sodium nitrite was used as a standard for preparation of calibration curve.^[22] The NO concentration was expressed as µM of NO per mg of protein.

Preparation of liver homogenate

On the 6th day of study after blood collection, the mice were euthanised using CO₂ chamber and livers were quickly isolated. For estimation of total protein content and oxidative stress parameters, 10% liver homogenates were prepared in 0.1 M Tris-HCl buffer (pH 7.4).

Determination of oxidative stress

A 10% homogenate of the liver homogenate from each mouse was prepared. The homogenate was centrifuged at 2000 g for 20 min at 4°C, and the aliquots of the supernatant were used to estimate the extent of oxidative stress. The parameters indicating oxidative stress - lipid peroxidation (LPO), reduced glutathione (GSH), activities of catalase and superoxide dismutase (SOD) - were expressed per as contents per mg of the protein present in homogenate samples.

Estimation of the extent lipid peroxidation

The liver homogenate samples were treated with 3.0 ml of 1% phosphoric acid solution and 1.0 ml of aqueous solution of 0.6% thiobarbituric acid. The reaction mixtures was heated at 80°C for 45 min, cooled in an ice bath and extracted with 4.0 ml of N-butanol. The n-butanol layer was separated and the absorbance of the pink complex formed was estimated at 532 nm as an indicator of the extent of LPO.^[23]

Estimation of reduced glutathione

The GSH content in liver homogenates was determined by treating the homogenates by DTNB method.^[24] Briefly, 20 µl of tissue homogenate was treated with 180 µl of 1 mM DTNB solution at room temperature. The optical density of the resulting yellow colour was measured at 412 nm using a microplate spectrophotometer (Powerwave XS, Biotek, USA).

Determination of the catalase activity

As reported by Gore *et al.*, 2016,^[25] the liver homogenate (20 µl) was added to 1 ml of 10 mM H₂O₂ solution in the Quartz cuvette. The reduction in optical density of this mixture was

monitored using microplate spectrophotometer in UV mode at 240 nm. Rate of decrease in the optical density across 3 min from the addition of the liver homogenate was taken as an indicator of the catalase activity present in the homogenate.

Estimation of superoxide dismutase activity

The superoxide dismutase activity was determined by the method reported by Dehimi *et al.*, 2016.^[26] The liver homogenate (20 µl) was added to a mixture of 20 µl of 500 mM/l of Na₂CO₃, 2 ml of 0.3% Triton X-100, 20 µl of 1.0 mM/l of EDTA, 5 ml of 10 mM/l of hydroxylamine and 178 ml of distilled water. To this mixture, 20 µl of 240 µM/l of nitroblue tetrazolium was added. The optical density of this mixture was measured at 560 nm in kinetic mode for 3 min at 1 min intervals. The rate increase in the optical density was determined as an indicator of the SOD activity.

Statistical analysis

Data were expressed as mean ± standard error mean for each group. Statistical analysis was performed using the one-way analysis of variance followed by Bonferroni's *post hoc* test with comparison of all column pairs. The data were analysed using a GraphPad, Prism software, version 6.0, GraphPad Software, Inc. USA. *P* < 0.05 was considered statistically significant.

RESULTS

Cytotoxicity of ethanol and homeopathic mother tinctures in RAW-264.7 cell line

The cell viability was measured by an MTT assay. We found that *Arnica montana*, *Bryonia alba* and *Thuja occidentalis* mother tinctures of test drugs and ethanol which is used

as vehicle in homeopathic dilutions were found to be non-cytotoxic to RAW-264.7 cells at dilutions beyond 10⁻⁴ [Figure 1]. Considering this, the effects of higher dilutions were estimated at 10⁻⁶ dilutions or 1 µl/ml of the assay medium so that cytotoxicity of ethanol and constituents of mother tinctures will not exert non-specific cellular toxicities.

Effect of homeopathic drugs on lipopolysaccharide-induced cytokine release from RAW-264.7 cells

LPS stimulation induced more than two-fold rise in the production of TNF-α, IL-1β and IL-6 from RAW-264.7 cells. As shown in Figure 2, homeopathic mother tinctures and higher dilutions of *Arnica*, *Bryonia* and *Thuja* significantly inhibited LPS-induced pro-inflammatory cytokine release from RAW-264.7 cells (*P* < 0.05). The ethanol treatment had no effect on the LPS-induced cytokine release indicating that the effects observed in case of high dilutions are not due to presence of ethanol. The inhibitory effects of higher dilutions did not show dilution-dependent effects. The suppression of cytokine release by homeopathic dilutions was similar in all the tested dilutions. All the tested mother tinctures and high dilutions of *Arnica*, *Bryonia* and *Thuja* invariably suppressed the pro-inflammatory cytokine release from RAW-264.7 cells.

Effect of homeopathic drug dilutions on lipopolysaccharide-induced cytokine release from human whole blood cultures

Similar to RAW-264.7 cells, LPS significantly induced cytokine release in whole blood culture (*P* < 0.001). We tested the effects of ethanol even in this assay to confirm its effect LPS-induced cytokine levels. However, unlike in case

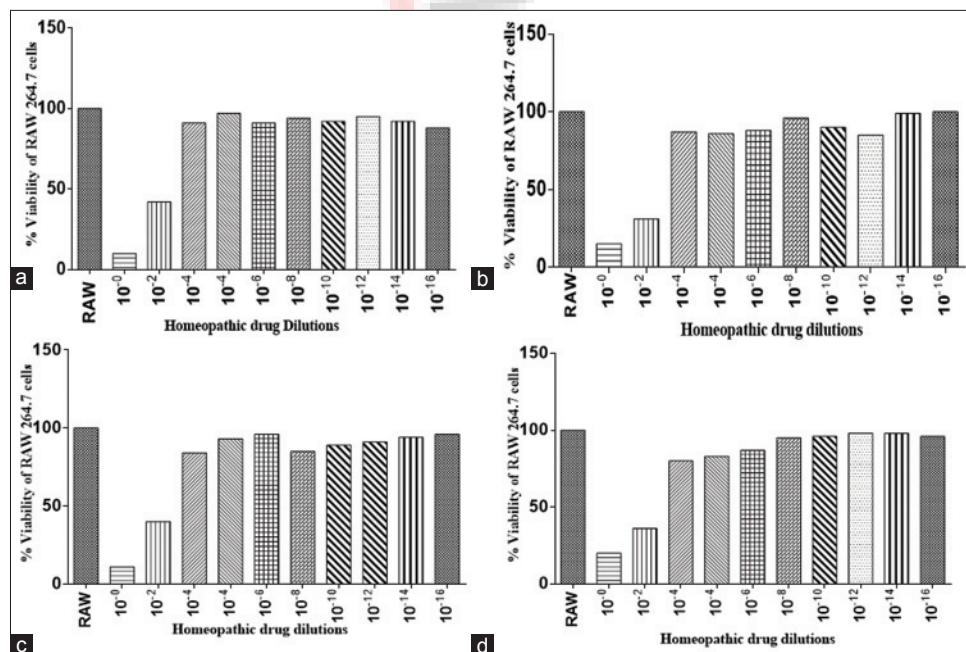


Figure 1: Estimation of non-cytotoxic concentrations of mother tinctures and ethanol in RAW-264.7 cells. (a) Ethanol and its dilutions; (b) *Arnica montana* mother tincture; (c) *Bryonia alba* mother tincture; (d) *Thuja occidentalis* mother tincture. The data were analysed by one-way analysis of variance. Alcohol and all the mother tinctures were diluted up to 10⁻¹⁶. The concentrations of all the mother tinctures up to 10⁻⁴ showed toxicity on RAW-264.7 cells; the serial dilution beyond 10⁻⁴ was safe and nontoxic when studied in RAW-264.7 cells

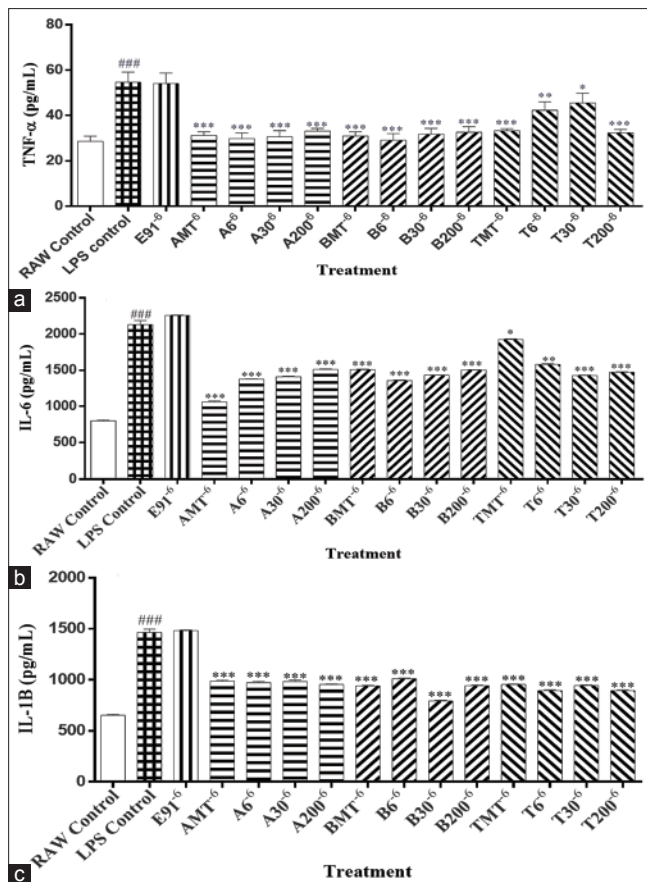


Figure 2: Effect of homoeopathic mother tinctures and high dilutions of *Arnica*, *Bryonia*, *Thuja* on lipopolysaccharide-induced pro-inflammatory cytokine release from RAW-264.7 cells. (a) Tumour necrosis factor- α ; (b) interleukin-6; (c) interleukin-1 β . Data were expressed as mean \pm standard error of mean ($n = 4$). Statistical significances were determined using one-way analysis of variance followed by Dunnett's *post hoc* test. ### $P < 0.001$ as compared to RAW control; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared to lipopolysaccharide control. The normal distribution of the data was tested by Bartlett's test ($P < 0.05$ indicated normal distribution)

of RAW cells, ethanol suppressed the LPS-induced IL-1 β and TNF- α released from the whole blood culture. Such suppressive effect was not evident in case of IL-6 and its levels were found to be similar to the LPS-stimulated control assay units. These inconsistencies in the effects of ethanol appear to cover-up the inhibitory effects of homoeopathic drugs on IL-1 and TNF- α . In the present context, it can be stated that the homoeopathic mother tinctures and high dilutions significantly inhibited the LPS-induced IL-6 release from whole blood culture ($P < 0.0001$) and it could not be concluded from present experiment whether the effects on IL-1 and TNF- α could be attributed to the effects of drug dilutions or ethanol [Figure 3].

Effect of homoeopathic drug dilutions on lipopolysaccharide-induced cytokine release in mice

LPS injection increased the serum levels of TNF- α , IL-6 and IL-1 β almost three times as compared to the control group mice ($P < 0.001$). The ethanol treatment did not reduce the LPS

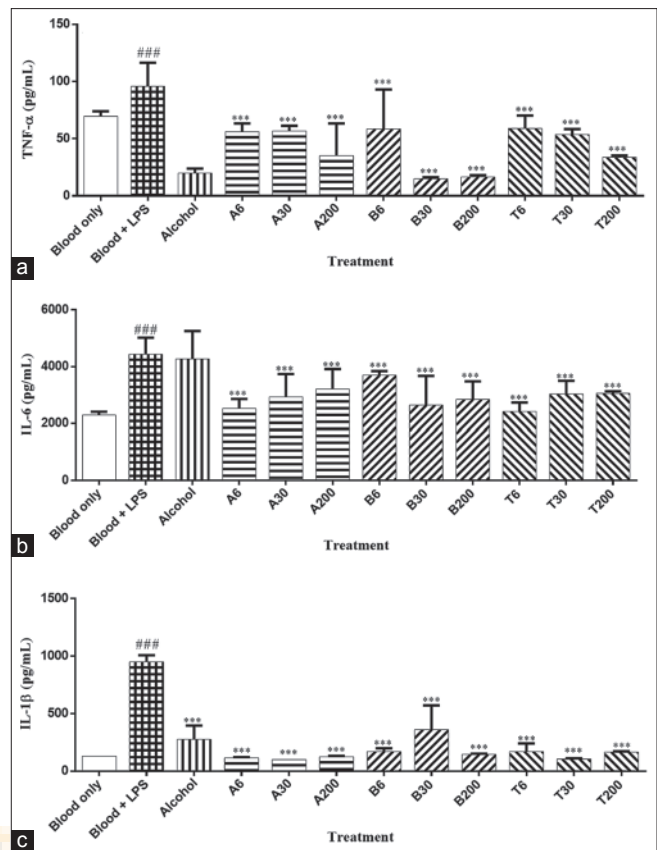


Figure 3: Effect of homoeopathic mother tinctures and high dilutions of *Arnica*, *Bryonia*, *Thuja* on lipopolysaccharide-induced release of cytokines from human whole blood culture. (a) Tumour necrosis factor- α ; (b) interleukin-6; (c) interleukin-1 β . Data were expressed as mean \pm standard error of mean ($n = 3$). Statistical significances were determined using one-way analysis of variance followed by Dunnett's *post hoc* test. ### $P < 0.001$ as compared to blood only group, *** $P < 0.001$ as compared to lipopolysaccharide-treated blood culture. The normal distribution of the data was tested by Bartlett's test ($P < 0.05$ indicated normal distribution)

challenge-induced cytokines. This indicates the lack of effects of ethanol on the LPS-induced cytokines in mice [Figure 4]. In congruence with the *in vitro* experiments, the tested homoeopathic drugs reduced LPS-induced pro-inflammatory cytokines invariably in all the tested dilutions including mother tincture ($P < 0.001$). The effects of all the tested dilutions of *arnica* were consistent and significant ($P < 0.001$) as compared to *Thuja* and *Bryonia*. The cytokine release inhibitory effects of *Thuja* and *Bryonia* were minimal for certain dilutions.

Effect of homoeopathic drugs on lipopolysaccharide-induced nitric oxide levels in serum

To investigate the effect of homoeopathic drugs *Arnica*, *Bryonia* and *Thuja* NO production, we measured the accumulation of nitrite in serum. Administration of LPS significantly increased serum levels of NO as compared to the control group mice. Pretreatment with the homoeopathic drug dilutions significantly reduced the LPS-induced rise in serum NO levels ($P < 0.01$). Of all tested drug dilutions,

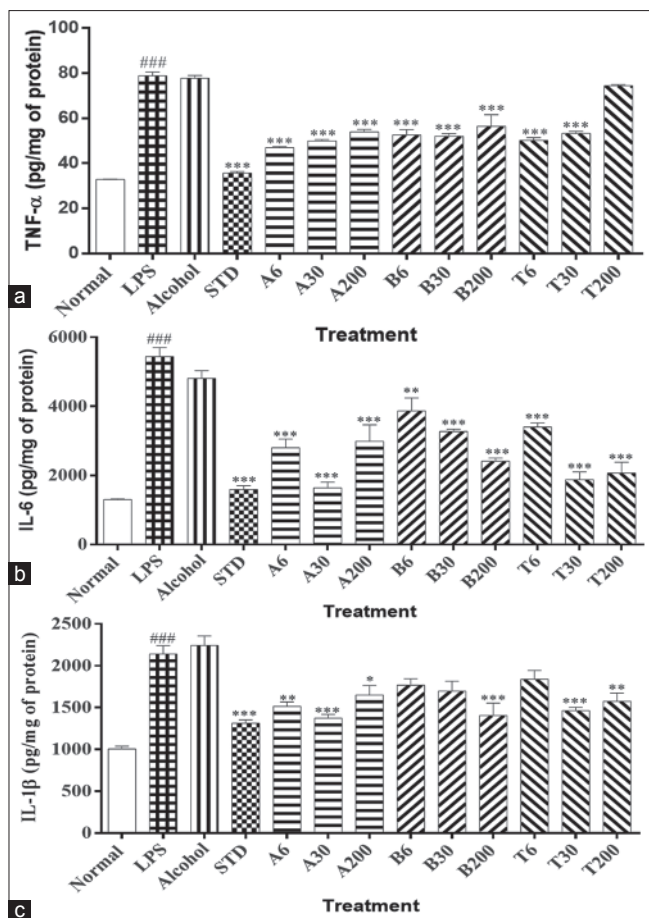


Figure 4: Effect of homoeopathic drugs *Arnica montana*, *Bryonia alba*, *Thuja occidentalis* with its ultra-high dilutions on lipopolysaccharide-induced release of cytokines in mice. (a) Tumour necrosis factor- α ; (b) interleukin-6; (c) interleukin-1 β . Data were expressed as mean \pm standard error of mean ($n = 6$). Statistical significances were determined using one-way analysis of variance followed by Dunnett's *post hoc* test. ### $P < 0.001$ as compared to normal group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared to lipopolysaccharide-treated control group. The normal distribution of the data was tested by Bartlett's test ($P < 0.05$ indicated normal distribution)

Thuja (200 CH) had minimal inhibitory effect on the NO levels [Table 1].

Effect of homoeopathic drugs on lipopolysaccharide-induced oxidative stress in mice

We used liver as a representative organ to estimate the extent of LPS-induced oxidative stress and effects of homoeopathic drugs on it. LPS injection induced a state of marked oxidative stress as evident from a significant increase in MDA level ($P < 0.001$) and a significant decrease in the GSH and the activities of SOD and catalase ($P < 0.001$) in the liver tissue homogenates. The mice treated with 6 CH dilutions of *Arnica*, *Thuja* and *Bryonia* were maximally protected from the induced by LPS. The protection against LPO was less prominent in case of the dilutions. The levels of GSH and the enzymatic activities SOD and catalase were significantly reduced by LPS treatment. All homoeopathic drug dilutions significantly

consistently improved the GSH levels and activities of catalase and SOD. However, there was no dilution-dependent protection against oxidative stress.

DISCUSSION

Homoeopathy is used worldwide as an economic alternative to allopathy. However, in the last few decades, homoeopathic medicines have been bitterly criticised for lack of efficacy in clinical trials. There are striking discrepancies in the proofs of efficacy even in the preclinical trials. Further, the most controversial aspect of Homoeopathy is use of drug dilutions to such an extent that just infinitesimal quantities drug substances are administered as a portion. In case of ultra-high dilutions, the drug is diluted even beyond Avogadro's number, and hence, it can be deduced that no drug is actually administered to the patient. The efforts to validate the claims on efficacy of homoeopathic drug dilution have yielded inconsistent results. Still, there are certain reports which reproducibly indicate that the biological effects of high dilutions can be demonstrated. The extensive research on the high dilutions of histamine and dexamethasone support the claims that high dilutions of drugs may possess biological activities and such activities can be proved using validated *in vitro* and *in vivo* assays.^[27,28] However, certain judiciously planned experimental evaluations of homoeopathic drugs and their dilutions have reproducibly proved the efficacy of homoeopathic drugs and even their high dilutions. Such scientific proofs necessitate preclinical evaluations of homoeopathic drugs through validated pharmacological assays.

The present investigation is an effort to substantiate the efficacy of three anti-inflammatory homoeopathic drugs and their high dilutions using validated experimental models of *in vitro* LPS-induced cytokine release from murine macrophage cell line (RAW-264.7) and whole human blood culture. The effects observed in these *in vitro* assays were further confirmed using *in vivo* LPS stimulation-induced cytokine release and oxidative stress in mice. We observed that the homoeopathic dilutions including mother tinctures, 6 CH, 30 CH and 200 CH dilutions of *Arnica*, *Bryonia* and *Thuja* significantly inhibited the LPS induced *in vitro* cytokine release from RAW-264.7 cell line and also from the whole blood culture. The *in vivo* evaluation proved that these dilutions significantly reduced LPS-induced cytokines and oxidative stress in mice.

In the pilot study, we studied the effects of ethanol on the RAW-264.7 to estimate the concentration of ethanol that affects the survival and activity of these cells. We used dilutions of alcohol (vehicle) ranging from 10^0 to 10^{-16} dilutions in the cell culture medium. After co-incubation of these ethanol dilutions for 48 h, we observed that the dilutions beyond 10^{-6} are non-toxic and do not affect the cellular activity (cytokine release in the present case). Up to 10^{-4} dilutions, the ethanol exerted cytotoxic effects on RAW cells. Hence, for further studies, we diluted all the homoeopathic drug dilutions (which contained 91% ethanol as vehicle) up to 10^{-6} in cell culture

Table 1: Effect of homoeopathic drug dilutions on nitric oxide levels and oxidative stress in lipopolysaccharide-induced oxidative stress in mice liver homogenates

Treatment	NO (μM)	LPO ($\mu\text{g}/\text{mg}$ of protein)	GSH ($\mu\text{g}/\text{mg}$ of protein)	SOD (U/mg of protein)	Catalase (U/mg of protein)
Normal	30.3 \pm 5.07	27.32 \pm 2.05	4.05 \pm 0.47	0.47 \pm 0.02	0.24 \pm 0.01
LPS	225.4 \pm 10.23 ^{###}	104.3 \pm 4.97 ^{###}	0.63 \pm 0.05 ^{###}	0.01 \pm 0.005 ^{###}	0.06 \pm 0.01 ^{###}
Ethanol	226.2 \pm 9.12	106.5 \pm 6.46	0.63 \pm 0.05	0.017 \pm 0.004	0.06 \pm 0.009
Standard	45.9 \pm 2.11 ^{***}	44.40 \pm 2.56 ^{***}	3.89 \pm 0.14 ^{***}	0.33 \pm 0.03 ^{***}	0.23 \pm 0.02 ^{***}
A6	86.07 \pm 5.41 ^{***}	66.87 \pm 2.16 ^{***}	2.14 \pm 0.45 ^{***}	0.16 \pm 0.04 ^{***}	0.23 \pm 0.01 ^{***}
A30	125.4 \pm 7.84 ^{***}	83.87 \pm 5.56	1.98 \pm 0.17 ^{***}	0.10 \pm 0.03*	0.24 \pm 0.007 ^{***}
A200	145.1 \pm 8.29 ^{***}	90.34 \pm 8.82	3.59 \pm 0.40 ^{***}	0.18 \pm 0.09	0.21 \pm 0.008 ^{***}
B6	103.3 \pm 14.48 ^{***}	58.80 \pm 5.15 ^{***}	0.44 \pm 0.15	0.10 \pm 0.007*	0.15 \pm 0.01**
B30	162.3 \pm 4.82 ^{**}	80.77 \pm 3.63 ^{**}	0.81 \pm 0.15	0.10 \pm 0.03*	0.21 \pm 0.01 ^{***}
B200	132.0 \pm 11.23 ^{***}	94.32 \pm 7.43	1.29 \pm 0.05 ^{**}	0.17 \pm 0.06 ^{**}	0.21 \pm 0.02 ^{***}
T6	101.6 \pm 17.22 ^{***}	65.53 \pm 5.43 ^{***}	2.75 \pm 0.32 ^{***}	0.19 \pm 0.02 ^{**}	0.20 \pm 0.02 ^{***}
T30	63.93 \pm 14.10 ^{***}	69.57 \pm 1.20 ^{***}	0.58 \pm 0.09	0.08 \pm 0.02	0.17 \pm 0.013 ^{***}
T200	186.1 \pm 7.00	71.10 \pm 3.42 ^{***}	1.45 \pm 0.08 ^{**}	0.07 \pm 0.02	0.15 \pm 0.01*

Data was expressed as mean \pm SEM ($n=6$). Statistical significance was determined by one-way ANOVA followed by Dunnett's *post hoc* test. ^{###} $P<0.001$ as compared to control group; * $P<0.05$, ** $P<0.01$, *** $P<0.001$ as compared to LPS-treated group. ANOVA: Analysis of variance; SEM: Standard error of mean; LPS: Lipopolysaccharide; NO: Nitric oxide; LPO: Lipid peroxidation; GSH: Glutathione; SOD: Superoxide dismutase

medium so as to avoid the interference of effects of vehicle. We had used the succused alcohol as vehicle in this study. It is suggested that for investigations involving homoeopathic drugs, the vehicle (ethanol) may be succused in the same manner and to the extent as the test drug dilution being studied.

Systemic inflammation is involved in the pathogenesis of many diseases. LPS is a prototypical endotoxin, which can directly activate macrophages,^[29] and induces production of inflammatory cytokines such as IL-1, IL-6, TNF- α , and inflammatory mediators including NO and prostaglandin E2.^[30] The LPS-induced Toll-like receptor 4-dependent systemic inflammation is a classic model used in the evaluation of anti-inflammatory efficacy of drugs.^[31] The modulation of macrophage response to bacterial LPS, which is a well characterised, widely used and efficient model of evaluation of inflammatory response *in vitro*.^[32,33] LPS-induced macrophage inflammatory response involves the activation of the MAPKs ERK, p38 and JNK.^[34] MAPK activation then stimulates transcription factors including nuclear factor- κ B (NF- κ B) and AP-1. In particular, the p38 MAPK is an important mediator of stress-induced gene expression and plays a key role in LPS-induced signal transduction pathways, leading to cytokine synthesis. The p38 MAPK activation was reported to be involved in inducible NO synthase expression in TNF- α and IL-1-stimulated mouse astrocytes, as well as in LPS-stimulated mouse macrophages. The LPS-induced cytokine release and oxidative stress in mice are used as a method to test efficacy of drugs intended to be used in the sepsis and rheumatoid arthritis.^[35]

In the present study, we tested mother tinctures, 6 CH, 30 CH and 200 CH dilutions of *Arnica*, *Thuja* and *Bryonia*. The dilutions up to 30 CH are contemplated to contain at least few molecules of drug substances. However, higher dilutions can be deduced to contain no active components of the starting material. Our study results indicate that *Arnica*, *Bryonia*

and *Thuja* dilutions even up to 200 CH exert significant inhibition of LPS-evoked cytokine release and oxidative stress. These results are in congruence with earlier sporadic reports where these drugs, as homoeopathic medicines, either alone or in combination with other drugs, are reported to exert anti-inflammatory activity. The *Arnica* 6 CH dilution is reported to significantly inhibit the carrageenan-induced paw oedema in rats. In this report, the authors highlight the need to consider inter-individual differences in the rats' responses to carrageenan-induced inflammation while determining the efficacy of *Arnica* homoeopathic dilutions.^[9] In our study, 6 CH, 30 CH and 200 CH dilutions of *Arnica* significantly reduced the release of IL-1 β , IL-6 and TNF- α from the LPS-stimulated RAW-264.7 cells *in vitro*. These effects also extended to the *in vivo* experiments where 5 days pre-treatment with *arnica* homoeopathic dilutions significantly reduced serum levels of pro-inflammatory cytokines in the LPS-challenged mice. The LPS-induced oxidative stress in the liver tissues was also significantly reduced by all the tested dilutions of *Arnica*. The *Arnica montana* extracts and its active constituents are reported to inhibit binding between NF- κ B and DNA and inhibit of NF- κ B-dependent gene expression.^[36,37] Another study has revealed that the anti-inflammatory activity of active constituents of *Arnica* involves inhibition of transcription factor NF- κ B.^[38] The *Arnica* flower extracts and active constituents such as helenalin are reported to inhibit carrageenan-induced inflammation and the chronic adjuvant-induced arthritis^[39] in experimental models. Hence, it will be of great significance to check whether similar alterations in the NF- κ B transcription pathway are induced by high dilutions of *arnica*. The findings of the present study indicate that 6CH, 30CH and 200 CH dilutions of *Arnica* retain the anti-inflammatory efficacies of active constituents of *Arnica*. As reviewed by Bonamin *et al.*, there are certain studies which indicate that the *arnica* mother tincture induces local irritation and exerts phylogistic effects similar to carrageenan. The observed pro-inflammatory effects

of the *Arnica* mother tincture and anti-inflammatory activity of the higher dilutions have been proposed to reinforce the homeopathic principle of similitude.^[11] Recent research on effects of arnica on gene expression in macrophages proves that 1–15 CH dilutions of *Arnica* modulates the expression of gene related to the process of inflammation.^[40] These findings are in congruence with the observed anti-inflammatory activity of the *Arnica* dilutions in the present study.

Bryonia alba is an important anti-inflammatory drug widely used by homeopaths. The major active components of the *Bryonia* extract are cucurbitacin glycosides and trihydroxy octadecadienoic acids. A homeopathic formulation containing *Arnica* and *Bryonia* called *Canova* has been investigated through multiple *in vitro* and *in vivo* models of immune response and inflammation. This formulation containing *Bryonia* is reported to reduce oxidative metabolism in mouse macrophages.^[41] A randomised controlled clinical study has concluded that the treatment of chronic low-back pain due to osteoarthritis using homeopathic *Bryonia* has revealed that the combination of physiotherapy and homeopathic drug treatment has beneficial effects as compared to the placebo treatment. This study has stated the need for further investigations using larger sample sizes.^[42] Another study on the homeopathic formulation containing *Bryonia* (Healwell VT-6) has proved its effectiveness in the treatment of mastitis in cattle and has projected it as an economical alternative to allopathic anti-inflammatory drugs.^[43] The finding of the present study further consolidate these claims and indicate that further investigations elucidating the molecular mechanisms of such effects of high dilutions of *Bryonia* might provide insights into its novel mechanisms.

Thuja occidentalis homeopathic preparations are reported to exert antineoplastic and immunomodulatory activity in experimental models. Oral administration of 30 CH, 200 CH and 1 M dilutions of *Thuja* for 10 days to Balb/C mice significantly enhanced the haematological parameters including total white blood cell count, hematopoietic parameters such as bone marrow cellularity and the number of α -esterase-positive cells. The higher dilutions of *Thuja* increased circulating antibody titre and the number of plaque forming cells. *Thuja* also increased proliferation of B- and T-lymphoid cells.^[44] Another study by Mukherjee *et al.*, 2013 reports that *Thuja* 30 CH dilution increases the lung cell viability in benzo(a) pyrene intoxicated mice. It reduces the oxidative stress and increases GSH content in the mouse lung cells and possesses striking ability to repair DNA damage induced by benzo(a) pyrene.^[45] A study by Lee *et al.*^[46] reports that aqueous extract of *Thuja* exerts anti-inflammatory activity in human umbilical vein endothelial cells. The possible mechanisms of this activity include reduction in the TNF- α induced expression of intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and E-selectin. In addition to this, *Thuja* extract also attenuated TNF- α -induced p65 NF- κ B translocation into the nucleus and phosphorylation of I κ B- α . These effects indicate that *Thuja* crude extract exerts significant

anti-inflammatory activity through multiple mechanisms including inhibition of NF- κ B transcription pathway.^[46,47]

Though cytokine inhibitory and antioxidant effects of tested drug dilutions were significant, there were certain inconsistencies. Particularly, in case of the whole blood culture assay, we came across inhibitory effects of ethanol on LPS-induced TNF- α and IL-1 β release. In the same assay medium, the concentrations of IL-6 remained unsuppressed. The differential regulation of cytokine release and variations in the patterns of IL-1 β , IL-6 and TNF- α release are reported earlier.^[48,49]

However, the present study results are insufficient to state how whole blood culture responds to ethanol. Such ethanol-induced differential suppression of TNF- α and IL-1 was not evident in the RAW-264.7 cell line. Hence, it can be postulated that LPS-induced cytokine release from RAW-264.7 cell line can be considered a better assay method for testing the activities of alcoholic solutions of anti-inflammatory drugs rather than whole blood culture assay.

CONCLUSIONS

These findings indicate the modulatory effects of anti-inflammatory homeopathic drugs on LPS-induced cytokine release. These effects were not dilution dependent but consistent and reproducible in both *in vitro* and *in vivo* experiments. Our results pave the way for further investigations into the reproducibility of these effects through other experimental models and also highlight the possibility of delineating the mechanisms of anti-inflammatory effects of *Arnica*, *Thuja* and *Bryonia*. As LPS-induced cytokine release is mediated through different pathways including NF- κ B pathway,^[47] it will be interesting to know how the homeopathic dilutions of these drugs affect NF- κ B transcriptions pathway.

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Conflicts of interest

None declared.

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दाहक समर्थक साइटोकीन्स के लिपोपॉलीसाकाराइड उत्प्रेरित रिलीज पर दाहक विरोधी होम्योपैथिक औषध डाइल्यूशन्स का नियंत्रण: इन विट्रो और इन विवो प्रमाण

सार

संदर्भ— दाहक विरोधी गतिविधि को जाँचने के लिए लिपोपॉलीसाकाराइड (एलपीएस)—उत्प्रेरित साइटोकीन रिलीज और आक्सीडेटिव स्ट्रेस मान्य प्रयोगात्मक मापदंड है। आरएडब्ल्यू (आरएडब्लू – 264.7) सेल्स और मानव होल ब्लड कल्चर से एलपीएस (1एमजी/एमएल)—उत्प्रेरित साइटोकीन रिलीज के प्रतिकूल हमने होम्योपैथिक मदर टिंक्चर, आर्निका मोंटाना, थूजा आक्सीडेन्टालिस और ब्रायोनिया अल्बा के 6सीएच, 30सीएच और 200सीएच डाइल्यूशन के प्रभाव की जाँच की।

सामग्रियाँ और विधियाँ: इन-विवो मूल्यांकन के लिए, चूहों को मुख से 0.1 मि.लि. ड्रग डाइल्यूशनस पाँच दिन तक दिन में दो बार दी गई, इसके बाद 0.5 मि.ग्रा/कि.ग्रा. एलपीएस का एक इंद्रापैरिटोनियल इंजेक्शन दिया गया। 24 घंटे बाद, चूहों की मृत्यु हो गई और प्रो-इंफ्लेमेटरी साइटोकीन्स के सीरम स्तर और नाइट्रिक ऑक्साइड का निर्धारण किया गया। ऑक्सीडेटिव स्ट्रेस का निर्धारण लीवर होमोजेनेट्स में ग्लूटाथियोन, मालोनडाइलडिहाइड, सुपरआक्साइड, डिस्म्यूटेज और कैटैलेज के घटने से किया गया।

परिणाम: परीक्षित ड्रग डाइल्यूशन ने मानव होल ब्लड कल्चर और आरएडब्ल्यू-264.7 सेल्स से ट्यूमर नैकरोसिस फैक्टर- α , इंटरल्यूकिन-1 (आईएल-1) और आईएल-6 के इन-विट्रो एलपीएस उत्प्रेरित रिलीज में काफी हद तक कमी की। चूहों के सीरम नमूनों में भी साइटोकीन्स का ऐसा दबाव स्पष्ट था। इन ड्रग्स ने लीवर टिशू में एलपीएस उत्प्रेरित ऑक्सीडेटिव स्ट्रेस से चूहों की रक्षा की।

निष्कर्ष: हमारे जाँच परिणामों से एलपीएस—उत्प्रेरित साइटोकीन एलीवेशन और आक्सीडेटिव स्ट्रेस के प्रतिकूल होम्योपैथिक डाइल्यूशनस, ब्रायोनिया, थूजा और आर्निका के सुरक्षात्मक प्रभावों की पुष्टि होती है। यह अध्ययन इन होम्योपैथिक औषधियों की दाहक विरोधी प्रभाविकता के दावों को प्रमाणित करता है।

Entzündungshemmende, homöopathische Arzneimittelverdünnungen die die LPS-induzierte Freisetzung von proinflammatorischen Zytokinen hemmen: in vitro- und in vivo-Evidenz

Abstrakt

Kontext: Die Lipopolysaccharid (LPS)-induzierte Zytokin-Freisetzung und der oxidative Stress sind validierte, experimentelle Parameter, um entzündungshemmende Aktivitäten zu testen. Wir untersuchten die Wirkung von homöopathischen Urtinkturen sowie der Potenzen C 6, C 30 und C 200 von Arnica montana, Thuja occidentalis und Bryonia alba gegenüber LPS (1 μ g/ml)-induzierter Zytokin-Freisetzung aus RAW-264.7-Zellen und menschlichen Vollblutkulturen.

Materialien und Methoden: Für in vivo Auswertungen wurden Mäuse mit 0,1 ml Arzneimittelverdünnungen zweimal täglich oral fünf Tage lang behandelt, gefolgt von einer intraperitonealen Injektion von 0,5 mg/kg LPS. Nach 24 Stunden wurden die Mäuse getötet und die Serumspiegel von proinflammatorischen Zytokinen und Stickstoffmonoxid bestimmt. Das Ausmaß des oxidativen Stresses wurde in den Leberhomogenaten als Gehalt an reduziertem Glutathion, Malondialdehyd, Superoxiddismutase und Katalase bestimmt.

Ergebnisse: Die getesteten Arzneimittelverdünnungen reduzierten die in vitro LPS-induzierte Freisetzung von TNF- α , IL-1 und IL-6 aus den RAW-264,7-Zellen und der menschlichen Vollblutkultur signifikant. Eine ähnliche Hemmung von Zytokinen zeigte sich bei Mäuseserumproben. Diese Arzneien schützten die Mäuse auch vor dem LPS-induzierten oxidativen Stress im Lebergewebe.

Fazit: Unsere Erkenntnisse begründen die schützenden Wirkungen homöopathischen Verdünnungen von Arnica, Thuja und Bryonia gegen LPS-induzierte Zytokin-Erhöhung und oxidativen Stress. Die Studie authentifiziert die Ansprüche der entzündungshemmenden Wirksamkeit dieser homöopathischen Arzneien.

Las diluciones de medicamentos homeopáticos antiinflamatorios restringen la liberación de citocinas proinflamatorias inducidas por lipopolisacárido: evidencias *in vitro* e *in vivo*

Resumen

Contexto: La liberación de citocinas inducida por lipopolisacáridos (LPS) y el estrés oxidativo son parámetros experimentales validados que se utilizan para examinar la actividad antiinflamatoria. Hemos investigado los efectos de las tinturas madre y las potencias de 6cH, 30cH y 200cH de *Arnica montana*, *Thuja occidentalis* y *Bryonia alba* frente a la liberación de citocinas inducida por LPS (1 µg/ml) a partir de las células RAW-264.7 y el cultivo de sangre completa humana.

Materiales y métodos: Para las evaluaciones *in vivo*, se administró un tratamiento oral a los ratones con 0,1 ml de dilución del medicamento, dos veces al día durante 5 días, seguido de una inyección intraperitoneal de 5 mg/kg de LPS. Tras 24 horas, se sacrificaron los ratones y se determinaron los valores séricos de las citocinas proinflamatorias y el óxido nítrico. La medida del estrés oxidativo se determinó en homogenizados hepáticos como reducción del contenido en glutatión, malondialdehído, superóxido dismutasa y catalasa.

Resultados: Las diluciones medicamentosas examinadas redujeron significativamente la liberación *in vitro* inducida por LPS de TNF- α , IL-1 e IL-6 a partir de las células RAW-264.7 y los cultivos de sangre completa humana. Se evidenció una supresión similar de las citocinas en las muestras séricas murínicas. Estos medicamentos también protegieron a los ratones frente al estrés oxidativo inducido por LPS en el tejido hepático.

Conclusiones: Nuestros hallazgos evidencian los efectos protectores de las diluciones homeopáticas de *Arnica*, *Thuja* y *Bryonia* frente al aumento de las citocinas inducido por LPS y el estrés oxidativo. Este estudio es prueba de la eficacia antiinflamatoria de estos medicamentos homeopáticos.



Les dilutions de médicaments homéopathiques anti inflammatoires limitent la libération de cytokines pro inflammatoires induite par les lipopolysaccharides: évidence *in vitro* et *in vivo*

Résumé

Contexte: La libération de cytokines induite par les lipopolysaccharides (LPS) et le stress oxydatif sont des paramètres expérimentaux validés utilisés pour tester l'activité anti-inflammatoire. Nous avons étudié les effets des teintures-mères homéopathiques, les dilutions de 6 CH, 30 CH et 200 CH d'*Arnica montana*, de *Thuja occidentalis* et de *Bryonia alba* contre la libération de cytokines induite par les LPS (1 µg/ml) provenant des cellules RAW-264.7 et de la culture du sang total humain.

Matériels et Méthodes: Pour les évaluations *in vivo*, des souris ont été traitées par voie orale avec les dilutions des médicaments à 0,1 ml deux fois par jour pendant 5 jours, suivies d'une injection intrapéritonéale de LPS à 0,5 mg/kg. Après 24 heures, les souris ont été sacrifiées et les taux sériques de cytokines pro-inflammatoires et d'oxyde nitrique ont été déterminés. L'étendue du stress oxydatif a été déterminée dans les homogénats de foie en termes de teneurs en glutathion, malondialdéhyde, superoxyde-dismutase et catalase réduits.

Résultats: Les dilutions des médicaments testées ont considérablement réduit la libération induite par les LPS *in vitro* du facteur de nécrose tumorale- α , de l'interleukine 1 (IL-1) et de l'IL-6 à partir des cellules RAW 264.7 et de la culture du sang total humain. Une suppression similaire des cytokines était évidente dans les échantillons de sérum des souris. Ces médicaments ont également protégé les souris du stress oxydatif induit par les LPS dans le tissu hépatique.

Conclusions: Nos résultats confirment les effets protecteurs des dilutions homéopathiques d'*Arnica*, de *Thuja* et de *Bryonia* contre les élévations de cytokines induites par les LPS et le stress oxydatif. Cette étude authentifie les affirmations d'efficacité anti-inflammatoire de ces médicaments homéopathiques.

抗發炎順勢療法稀釋藥物可抑制LPS-誘發的促炎細胞因子釋放：體外和體內實驗的證據摘要

內文：脂多糖(LPS)誘發細胞因子釋放和氧化壓力是已被驗證的實驗參數，可用來測試抗炎活性。我們研究了山金車、側柏和瀉根，各自在順勢療法母酊、6cH、30cH和200cH對從RAW-264.7 細胞和人類全血培養物釋放的脂多糖 (1 µg/ml) - 誘發細胞因子的影響。

物料和方法：在體內評估中，老鼠每天兩次口服0.1毫升稀釋藥物，持續5天，然後在腹膜內注射0.5mg/kg LPS。24小時後，老鼠會被殺死，並測定血清內促炎細胞因子和一氧化氮的水平。氧化壓力水平則取決於肝均質液，包括其還原型穀胱甘肽、丙二醛、超氧化物歧化酶和過氧化氫酶。

結果：測試中的稀釋藥物明顯減少從 RAW-264.7 細胞和人類全血培養物中釋放體外LPS-誘發的TNF- α 、IL-1 及 IL-6。在老鼠血清樣本中，有類似的細胞因子抑制。這些藥物還保護了老鼠免受肝臟組織中LPS誘發的氧化壓力。

結論：我們的研究證實了順勢療法稀釋的山金車、側柏和瀉根對於LPS誘導的細胞因子升高和氧化壓力有保護作用。這項研究證實了這些順勢療法藥物有抗炎療效。

