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ORIGINAL PAPER

Heparin-binding epidermal growth factor expression in KATO-III cells after *Helicobacter pylori* stimulation under the influence of strychnos *Nux vomica* and *Calendula officinalis*

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Introduction: **Previous studies have shown the stimulating effect of *Helicobacter pylori* on the gene expression of heparin-binding epidermal growth factor (HB-EGF) using the gastric epithelial cell line KATO-III. Strychnos Nux vomica (*Nux vomica*) and *Calendula officinalis* are used in highly diluted form in homeopathic medicine to treat patients suffering from gastritis and gastric ulcers.**
Aim and method: **To investigate the influence of *Nux vomica* and *Calendula officinalis* on HB-EGF-like growth factor gene expression in KATO-III cells under the stimulation of *H. pylori* strain N6 using real-time PCR with and without addition of *Nux vomica* and *Calendula officinalis* as a 10c or 12c potency.**
Results: **Baseline expression and stimulation were similar to previous experiments, addition of *Nux vomica* 10c and *Calendula officinalis* 10c in a 43% ethanolic solution led to a significant reduction of *H. pylori* induced increase in gene expression of HB-EGF (reduced to 53.12 ± 0.95% and 75.32 ± 1.16% vs. control; $p < 0.05$), respectively. *Nux vomica* 12c reduced HB-EGF gene expression even in dilutions beyond Avogadro's number (55.77 ± 1.09%; $p < 0.05$). *Nux vomica* 12c in a 21.5% ethanol showed a smaller effect (71.80 ± 3.91%, $p < 0.05$). This effect was only be observed when the drugs were primarily prepared in ethanol, not in aqueous solutions. The data suggest that both drugs prepared in ethanolic solution are potent inhibitors of *H. pylori* induced gene expression.**
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Keywords: HB-EGF; Heparin-binding epidermal growth factor (HB-EGF)-like growth factor; KATO-3; KATO-III; *H. pylori*; *Nux vomica*; *Calendula officinalis*

Introduction

Heparin-binding epidermal growth factor (HB-EGF)-like growth factor is a growth factor protein, acting as a potent stimulus for cell proliferation and cell migration by binding to specific cell surface membrane receptors.[1] HB-EGF is synthesized as a trans-membrane protein (proHB-EGF) and cleaved on plasma membrane to yield soluble HB-EGF of 19–23 kDa.[2] HB-EGF is present in a wide range of body tissues e.g. skin, kidney, heart, brain, reproductive tissues, spleen, and lymphocytes. The ability of HB-EGF to evoke a mitogenic response from a variety of cell types and its expression in a large number of tissues suggest that it may have many roles *in vivo*.

HB-EGF plays an important role in the process of cell renewal of the gastrointestinal tract, which is important for the integrity of the mucosa. The process includes cell

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proliferation, migration and loss of gastrointestinal cells.[3] Murayma *et al.* demonstrated that HB-EGF is synthesized in parietal and gastrin cells, acting in an autocrine or paracrine fashion to regulate proliferation and differentiation of gastric mucosal cells.[4] Healing of deep gastric mucosal injury erosions and ulcerations requires epithelial cell migration to accomplish re-epithelialization and epithelial cell proliferation to provide a sufficient number of cells for both re-epithelialization and reconstruction of epithelial glandular structures within the scar.

Helicobacter pylori infection is an important cause of ulceration of the gastric mucosa.[5] Previous studies have demonstrated that *H. pylori* infections may result in delayed ulcer healing and are the main cause of ulcer recurrence.[6] This is supported by the observation that eradication of *H. pylori* infection dramatically reduces ulcer recurrence.[7] Romano *et al.* demonstrated that *H. pylori* is able to upregulate HB-EGF expression in gastric mucosal cells. Little is known about the influence of drugs on HB-EGF.[8] Komori-Masato *et al.* recently described the effect of the COX-2 inhibitor NS-398 on the interaction of HB-EGF, PGE_2 and gastrin.[9] Kayanoki-Yoshiro *et al.* described the effect of cilostazol, a cyclic nucleotide phosphodiesterase III inhibitor, on HB-EGF expression in macrophages and vascular smooth muscle cells.[10]

So far, little is known about the influence of homeopathically potentized remedies on HB-EGF expression. Strychnos Nux vomica (*Nux vomica*) and *Calendula officinalis* are both used potentized in homeopathically potentized form to treat patients suffering from gastritis and gastric ulcers. The aim of this study was to investigate the influence of *Nux vomica* and *Calendula officinalis* on HB-EGF-like growth factor expression in KATO-III cells under the stimulation of *H. pylori* strain N6. We tested the hypothesis whether homeopathically potentized homeopathic drugs act through regulation of gene expression. Since both substances are frequently used in homeopathy in ultra-high dilution, we evaluated dilutions just under and just over Avogadro's number (6.022×10^{23}). To elucidate the influence of ethanol, we compared aqueous and ethanol–water solutions.

Material and methods

Bacterial strain

H. pylori N6 strain was cultivated on *H. pylori* selective plates (Becton Dickenson, Oxford, MA) under microaerobic conditions at 37°C. For stimulation experiments, N6 strain was grown in brucella broth medium supplemented with 7% fetal calf serum (FCS) (Oxoid, Basingstoke, Hampshire, United Kingdom) and *H. pylori* selective supplement (Oxoid) for 48 h at 37°C in 25 cm^2 flasks under microaerobic conditions.

Cell culture

The human gastric epithelial cell line KATO-III was obtained from the American Type Culture Collection and maintained in a humid atmosphere at 37°C in RPMI 1640 (Gibco Life Technologies BRL, Paisley, United Kingdom) supplemented with 10% FCS and 50 µg/ml gentamicin (Gibco Life Technologies BRL) in 180 ml tissue culture flasks. Cells were fed with fresh medium every 3 days and were split at approximately 1:4 when subconfluent. Before use, viability was determined by trypan blue staining. Experiments were performed only when cell viability was above 90%.

RNA isolation

2×10^6 KATO-III cells were seeded in a 6-well plate with a volume of 800 µl. Bacterial N6 strain was washed two times in PBS buffer (Gibco Life Technologies BRL) and resuspended in RPMI 1640 supplemented with 10% FCS. The estimation of bacterial number was determined by measuring the absorbance at OD_{600} using a spectrophotometer (Pharmacia, Uppsala, Sweden), and comparing the value of a standard curve generated by quantifying viable organisms from aliquots of bacteria at varying concentrations that were also assessed for absorbance. The viability of the strains was confirmed by plating them on *H. pylori* selective plates and incubating them for 2 days at 37°C under microaerobic conditions. A range finding trial was performed before selection of the quantum of KATO-III cells and the bacterial cells to be used in the mixed group. 2×10^8 bacteria (approximately 100 bacteria/epithelial cell) in a volume of 200 µl were added to the cells. Then, 10 µl of active and control treatments were added and incubated for 1 h. The final concentration of ethanol in the preparations made in 43% ethanol was thus 2.048% and in those made in 21.5% ethanol, 1.024%. These concentrations of ethanol had no significant impact on gene expression compared to plain water (see Figure 1).

RNA was extracted using the NucleoSpin RNA II Kit (Macherey & Nagel, Düren, Germany) according to the manufacturer's description. The quality of the RNA samples was determined by electrophoresis through a 1.2% agarose gel. 1 µl of the total RNA was mixed with equal volume of the gel loading buffer and denatured for 5 min at 65°C, loaded on the gel and separated for 45 min at 60 V. The 18S and 28S RNA bands were visualized under UV light after staining the gel with ethidium bromide. The amount of total RNA was assessed at OD_{260} using an UV spectrophotometer (Pharmacia).

cDNA synthesis

Reverse transcription of RNA was done using TaqMan Reverse Transcription Kit (PE Applied Biosystems, Foster City, CA) according to the manufacturer's description. The reverse transcription reaction mix was prepared with a final volume of 100 µl containing 1× RT buffer, 5.5 mM $MgCl_2$, 500 µM each deoxynucleotide triphosphate, 2.5 µM random hexamers, 0.4 U/µl RNase inhibitor, 1.25 U/µl Multiscribe reverse transcriptase and 1 µg total RNA. The samples were incubated at 25°C for 10 min, followed at 48°C for 30 min. Finally, inactivation of the reverse transcriptase was performed at 95°C for 5 min.

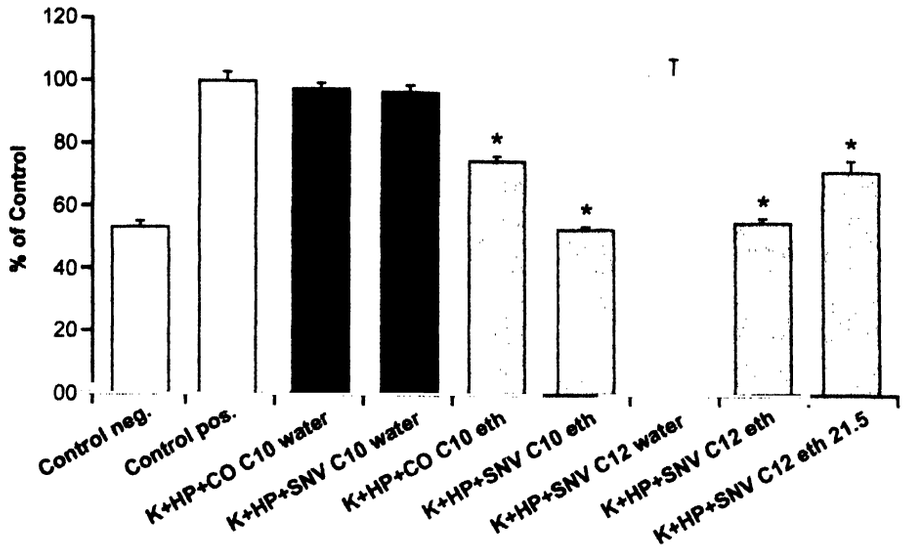

Figure 1 Gene expression of HB-EGF in KATO-III cells. Control negative: Control in unstimulated control in 43% ethanol; Control positive: Control after *H. pylori* wild type (N6) stimulation; K + HP + CO 10c water: after stimulation with *H. pylori* wild type (N6) + *Calendula officinalis* 10c in aqueous solution K + HP + SNV 10c water: after stimulation with *H. pylori* wild type (N6) + *Nux vomica* 10c in aqueous solution K + HP + CO 10c ethanol: after stimulation with *H. pylori* wild type (N6) + *Calendula officinalis* 10c in 43% ethanol K + HP + SNV 10c ethanol: after stimulation with *H. pylori* wild type (N6) + *Nux vomica* 10c in 43% ethanol. K + HP + SNV 12c water: after stimulation with *H. pylori* wild type (N6) + *Nux vomica* 12c in aqueous solution K + HP + SNV 12c ethanol: after stimulation with *H. pylori* wild type (N6) + *Nux vomica* 12c in 43% ethanol K + HP + SNV 12c ethanol 21.5%: after stimulation with *H. pylori* wild type (N6) + *Nux vomica* 12c in 21.5% ethanol. Data are expressed as percentage of positive control. $^*p < 0.05$ vs. positive control (control positive). There was no difference between positive Controls in either aqueous or ethanolic (43 and 21.5%) solution.

Realtime RT-PCR

The exact amount of total RNA and its quality (level of degradation) must be determined for each sample. Therefore, transcripts of the β-actin housekeeping gene were quantified as the endogenous control (reference), with each unknown sample normalized to β-actin content. The relative target gene expression level was also normalized to a calibrator, an unstimulated sample that contained the smallest quantifiable amount of the target polyA$^+$-RNA. The calibrator thus indicates the limit of assay quantification of the target which corresponds to a target C_t value of 37.

Generation of PCR-primers and TaqMan probes for real-time RT-PCR

The PCR-primers and TaqMan probes, to amplify and detect the HB-EGF-like growth factor, were designed using the Primer Express software version 1.0 (PE Applied Biosystems, Foster City, MA). The forward and reverse primers were designed to lie in adjacent exons (separated by a long intron) to prevent amplification of genomic DNA that may be contained in samples. TaqMan probes were labeled with 6-carboxy-fluorescein (FAM) as the reporter dye at the 5′ end and 6-carboxy tetramethyl-rhodamine (TAMRA) as the quencher fluorescent at the 3′ end. The fluorochrome VIC was chosen as reporter dye at the 5′ end of the β-actin probe. BLASTN searches against dbEST and nr (nonredundant set of GenBank, EMBL, DDBJ database sequences) were conducted to confirm the total gene specificity of the nucleotide sequences chosen for the primers and probes and the absence of DNA polymorphisms (Table 1).

Experimental approach

Real-time RT-PCR was performed on an ABI PRISM 7700 Sequence Detector, using the ABI PRISM 7700 Sequence Detector Software 1.6 (PE Applied Biosystems). The amplification reaction was performed in a total volume of 25 µl containing 1× universal master mix (PE Applied Biosystems), 600 nM forward primer, 600 nM reverse primer, 200 nM TaqMan probe and 1 ng cDNA. For the endogenous control 1× universal master mix, 1× predeveloped TaqMan assay reagent β-actin (hu β-actin PDAR; PE Applied Biosystems) and 1 ng cDNA was used. The reaction was performed in a 96-well microtiter plate with thermal cycling conditions including 2 min at 50°C, proceded with 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was analyzed in triplicate, and the

Table 1 Primers and probes used in real-time RT-PCR to quantify gene expression

Primer and probes	*Sequence (5′ → 3′) location*	*PCR product*
HB-EGF forward	AGAATGCAAATATGTGAAGGAGCTC	609–633
HB-EGF reverse 80 bp	GCCCATGACACCTCTCTCCAT	688–669
HB-EGF probe	CTCCTGCATCTGCCACCCGGG	642–662

ΔR_n and C_t were averaged from the values obtained in each reaction.

Normalization

The comparative C_t-method was used for quantification of HB-EGF polyA$^+$-RNA levels. This method eliminates the need for standard curves; it uses arithmetic formulas to achieve the same result for relative quantification.

Relative quantification using the comparative C_t-method

The ΔC_t value is determined by subtracting the average β-actin C_t value from the average HB-EGF C_t value. The calculation of $\Delta\Delta C_t$ involves subtraction by the C_t calibrator value. This is a subtraction of an arbitrary constant. The relative expression of HB-EGF is determined by evaluating the expression with the formula $2^{-\Delta\Delta C_t}$. This formula is derived from the equation which describes the exponential amplification of PCR $X_n = X_0 (1 + E_x)^n$, where X is the initial number of target molecules, E_x is the efficiency of target amplification, and n is the number of cycles.

Nux vomica, *Calendula officinalis*, and Controls

Nux vomica (synonyms: poison nut, semen strychnos) belongs to the family Loganiaceae. It grows in India, in the Malay Archipelago, the dried ripe seeds are used for medical purposes. It is a medium-sized tree with a short, crooked, thick trunk, the wood is white hard, close grained, durable and the root very bitter. The fruit is about the size of a large apple with a smooth hard rind or shell, which when ripe is a lovely orange color, filled with a soft white jelly-like pulp containing five seeds covered with a soft woolly-like substance, white and horny internally. The seeds are removed when ripe, cleansed, dried and sorted. The seeds are used for drug preparation.[11] *Nux vomica* seeds contain alkaloids in a concentration of 2–3%. The content of the alkaloid Strychnine is between 1.1 and 1.5%, and of Brucine between 1.1 and 2.1%. There are several less important alkaloids in a total concentration of about 1%. The content of oil lies between 4 and 5%, mainly linolic acid (62%).[12]

The properties of *Nux vomica* are substantially those of the alkaloid Strychnine. The powdered seeds are employed in atonic dyspepsia. The tincture of *Nux vomica* is often used in mixtures – for its stimulant action on the gastrointestinal tract. It is especially useful in nausea, several forms of gastric complaints, including gastritis and gastric ulcer. In the mouth it acts as a bitter, increasing appetite: it stimulates peristalsis, and may be helpful in chronic constipation.[13]

Calendula officinalis (Synonym: Marigold) belongs to the family Compositae. It consists of the dried ligulate florets of the common marigold. It is an annual herb indigenous to Southern Europe and widely cultivated. Flowers are collected when fully open and dried. *Calendula* contains triterpensaponines, a relative simple glycoside of oleanic acid. Sterols are found in all parts of the plant. *Calendula* contains 20% fatty oil, which consists of calendula acid, an octadecatrien acid, in a concentration of about 60%. It is found in the USA, Middle and Southern Europe, and Western Asia, and is used for treatment of gastric and duodenal ulcer.[14]

Both substances were prepared by the homeopathic method of potentization: step-wise dilution and hand-succussion according to the European Pharmacopeia (Maria Treu Pharmacy, Vienna, Austria; batch numbers 021 to 131/03). We used ultra-high dilutions (10c potency) and a concentration well beyond Avogadro's number (12c potency). Both were prepared in aqueous and organic solutions. *Calendula officinalis* 10c and *Nux vomica* 10c were prepared in a solvent containing 43% ethanol as used therapeutically. Both were also prepared in an aqueous solution (distilled water). *Nux vomica* 12c was prepared in 43% ethanolic solution, in distilled water without ethanol, and in a 1:1 mixture of ethanolic solution and distilled water (final concentration: 21.5% ethanol). Comparison was with matching Controls without *Nux vomica* or *Calendula officinalis*, which were potentized (diluted and succussed) following the same procedure made by the same pharmacy and contained matching ethanol concentrations. For Controls, expression of HB-EGF in the gastric epithelial cell line KATO-III after *H. pylori* wild type (N6) stimulation (positive control) was compared to a stimulation of a 43% ethanolic solution without *H. pylori*. There was no difference between positive Controls in either aqueous or ethanolic (43 and 21.5%) solution. Therefore, Figure 1 displays Control in 43% ethanolic solution only.

Statistical analysis

Three replicates and repeats were done with each concentration. Controls were set at 100% after normalization to the house keeping gene expression. A two-way univariate analysis of variance with Bonferroni correction, using SPSS software (SPSS 10.0) was performed. Statistical significance was set at $p < 0.05$.

Results

Using real-time PCR, no amplification occurred when total RNA or human genomic DNA was used as template. After 1 h of incubation, the expression of HB-EGF in the gastric epithelial cell line KATO-III was significantly higher after *H. pylori* wild type (N6) stimulation (positive control) compared to a stimulation of a 43% ethanolic solution without *H. pylori* (negative control; 100.0 ± 2.9 vs. 53.3 ± 1.9%; $p < 0.05$; $n = 9$ for each result).

The incubation of KATO-III cells stimulated by *H. pylori* strain N6 with *Calendula officinalis* 10c diluted in ethanol reduced the HB-EGF gene expression to 75.3 ± 1.2% ($p < 0.05$) of positive control, whereas *Nux vomica* 10c diluted in ethanol led to a reduced HB-EGF expression of 53.1 ± 1.0% ($p < 0.05$) after *H. pylori* strain N6 stimulation (Figure 1). No significant effect was observed when the two treatments were prepared in aqueous solutions (97.6 ± 1.9 and 97.1 ± 2.0%, respectively).

With a concentration beyond Avogadro's number, *Nux vomica* 12c in a 43% ethanolic solution reduced the *H. pylori* wild type (N6) stimulated HB-EGF gene expression significantly to 55.8 ± 1.1% ($p < 0.05$), whereas *Nux vomica* in the same concentration (12c) diluted in water without ethanol had no effect (102.5 ± 6.0%; p = n.s.). A 1:1 mixture of *Nux vomica* (50% *Nux vomica* 12c diluted in 43% ethanol and 50% of *Nux vomica* 12c diluted in water; final concentration 21.5% ethanol) showed an intermediate effect (71.8 ± 4.0%, $p < 0.05$).

Discussion

Few studies have investigated possible drug or osmotic influence on HB-EGF expression. Koh *et al.* investigated osmotic stress on HB-EGF expression in Ca^{2+} signal cascades in rat aortic smooth muscle cells.[15] These findings indicate that osmotic stress activates Janus kinases 1 over calcium signaling cascades inducing HB-EGF gene expression.

Previous studies using real time PCR have investigated the effect of *H. pylori* infected tissue on the gene expression of HB-EGF-like growth factor in gastrointestinal tissue samples.[16] Our results confirm the stimulating effect of *H. pylori* wild type N6 on HB-EGF expression, showing that the homeopathically potentized drugs *Calendula officinalis* as well as *Nux vomica* are able to reduce this stimulating effect. The reduction may be due to blockade of the stimulating effect of *H. pylori* and/or on HB-EGF expression by epithelial cell line KATO-III (Figure 2). However, the mechanism of action of the homeopathic drugs in alteration of the gene expression remains unknown. *Nux vomica* induced a stronger decrease as compared to *Calendula officinalis*. This down-regulating effect could be observed even with *Nux vomica* in dilutions exceeding Avogadro's number. The gene expression appeared to be modulated by the homeopathic preparations. The results of the present work support the hypothesis that potentized homeopathic drugs act through regulation of gene expression.

The model used is a well described method for investigating HB-EGF expression.[17] The role of HB-EGF-like growth factor in inflammatory processes is also well documented.[18] Murayma *et al.* demonstrated that HB-EGF is synthetized in parietal and gastrin cells, acting in an autocrine or paracrine fashion to regulate proliferation and differentiation of gastric mucosal cells.[4] It is a potent mitogen for these cells, which substantiated the hypothesis that HB-EGF may play a crucial role in mucosal repair of the stomach.[19] Further studies reported that the other EGF-r related peptides like EGF, TGF-α and amphiregulin also play pivotal roles in the gastrointestinal physiology and pathophysiology like tissue repair and ulcer healing.[20] The predominant effects of these peptides are in suppression of gastric acid secretion, gastric cytoprotection, and stimulation of DNA synthesis and proliferation.

Nux vomica and *Calendula officinalis* are both used in homeopathic medicine for treating patients suffering from gastritis and gastric ulcers. We chose the model of KATO-III cells stimulated by *H. pylori* under the influence of these two substances. The drugs showed a significant decrease of the expression of HB-EGF which seems to be in accordance with the intended medicinal effect of the two substances tested, namely to counteract the stimulatory effect of *H. pylori*.

Unlike the ethanol preparation, *Nux vomica* in an aqueous diluent did not show an effect on the expression of HB-EGF. The effect of *Nux vomica* therefore appears in ethanol preparations only. Resch and Gutmann have discussed the special properties of water–ethanol mixtures with respect to the hierarchic layers.[21] Ethanol may create a protective shell which protects the potentized substance. It is well documented that various drugs display a different effect depending on solubilization.[22] Ahmed *et al.* compared the impact of different hydrophilic carriers on the properties

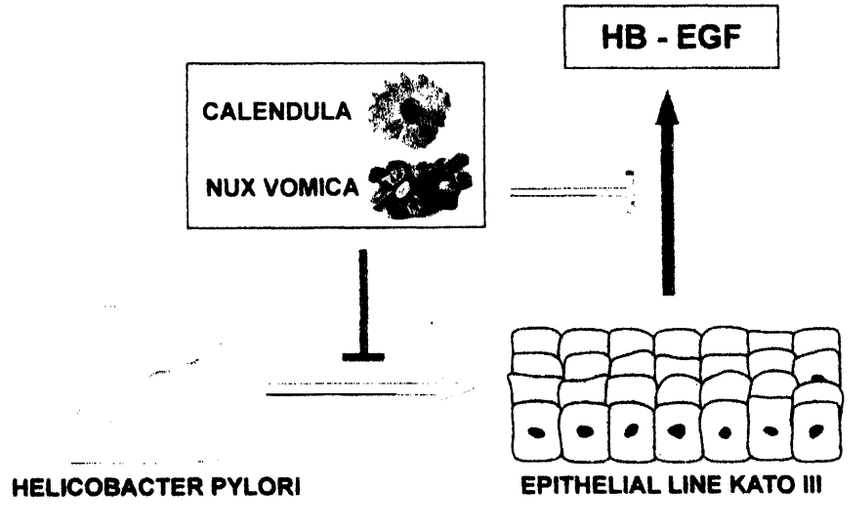

Figure 2 The reduction may be due to blockade of the stimulating effect of *H. pylori* or on HB-EGF expression by epithelial cell line KATO-III. However, the mechanism of action of the homeopathic drugs in alteration of the gene expression remains unknown.

of piperazine.[23] The highest solubilizing efficiency for the study drug was due to a larger hydrophobic substance. These studies support our findings of different results with ethanolic and aqueous solutions.

There are several studies on the effects of Nux vomica.[24–27] Sukul *et al.* administered potentized Nux vomica 30c to mice to evaluate its effect on ethanol-induced sleep time.[24] Nux vomica 30c was prepared with either distilled water, pure absolute ethanol or 90% ethanol with successive dilution and sonication. Only Nux vomica 30c prepared with 90% ethanol was effective in reducing the sleep time in mice. The authors concluded that the solution structure of ethanol–water mixture carries the specificity of the Nux vomica at ultra-high dilution.

In conclusion, our data suggest that *Nux vomica* and *Calendula officinalis* highly diluted in ethanol are potent inhibitors of *H. pylori* induced gene expression in KATO-III cells. This *in vitro* effect may explain their therapeutic effects.

Conflict of interest

The work was funded by the Institute for Homeopathic Research Vienna, Austria. The authors have no conflict of interest.

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