

# Water as carrier of information of heat shock and drug effect between two groups of *Adhatoda vasica* plants

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## ABSTRACT

*Adhatoda vasica* Nees plants were grown in 50 earthen pots, which were divided into 5 batches A, B, C, D, and E. Of these A, B and C, D were arranged into two separate parallel pairs. One leaf of each plant of an adjacent pair was immersed in sterile tap water in a beaker. Adjacent beakers in each pair A B or C D were connected by polythene tubes containing wet cotton threads. One leaf of each plant of A was given heat shock by immersing a leaf in hot water for 5 min. One leaf of each plant of C was treated with *Cantharis vesicatoria* 200c. Batch E served as the unstressed and untreated control. One hour after heat shock or drug treatment all the leaves were harvested and their proteins were extracted by chilled protein extraction buffer. Proteins were separated by Fast Protein Liquid Chromatography (FPLC). Protein profiles of A, B and C, D showed marked similarity with respect to expression and repression of some proteins. It is concluded that the effect of heat shock and drug treatment is transmitted through water in the capillaries of cotton threads connecting the pairs of plants. It is assumed that heat shock or drug treatment altered locally the water structure in the leaves which was propagated through global network of water structure over the protein network in the whole plants, and from there to the interfacial water in the beakers and cotton threads. A homeopathic potency is thought to be specifically structured water which influences the water structure in the treated organism.

**Key words:** *Cantharis vesicatoria*; heat-shock; water network; information transfer; homeopathy.

## Introduction

The molecules of water are thought to operate at an informational level as a function of their three-dimensional hydrogen-bonded structure, which can be preserved by ethanol as it might be the the case of the so-called potentized homeopathic drugs [1]. The effect of heat stress or *Cantharis vesicatoria* treatment on one plant leaf spreads to other parts of the plant and induces expression of heat shock proteins or protective proteins against heat shock. We demonstrated this phenomenon in *Adhatoda vasica* Nees, commonly known as basak[2]. We hypothesize that this transmission of thermal shock or *Canth* treatment from the site of application to distant parts of the plant occurs through a network of water molecules covering the surface of cell membranes and membrane proteins. If this is so, then two plants connected by water through a tube should respond in a similar way to heat shock or a drug treatment applied to either of them. To verify this hypothesis, we first performed an experiment with cowpea (*Vigna unguiculata*). Heat shock applied to a leaf of cowpea was transferred to another and unstressed plant through a water-filled tube connecting both plants. This effect was observed as the development of heat shock proteins in the leaves of both connected plants. The effect of applying *Cantharis vesicatoria* 200cH, a drug used for burn injuries, to the leaf of one cowpea plant was transmitted to another and untreated plant connected to the former by a water-filled tube. Also in this case the effect was measured as newly expressed protein, which is thought to be protective against burn

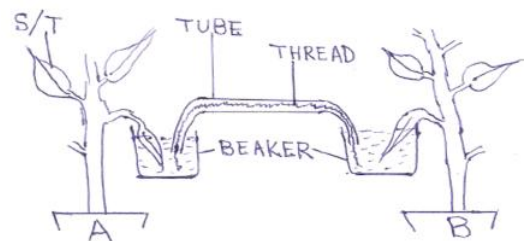
injuries [3]. High dilution 200cH was selected because it was found effective in a large number of patients and also of plants in our earlier experiments. We repeated this experiment in a controlled manner using *A. vasica*, our first plant model for heat shock and *Canth* effect. This plant has insecticidal properties that prevent attacks by insects and therefore, insect-induced expression of new proteins[2]. We anticipate expression and repression of similar proteins in two groups of water-connected plants under heat shock or *Canth* treatment applied only to one group.

## Materials and methods

### Plants

Earthen pots of 22.5 cm diameter and 22.5 cm depth were filled with a mixture of loam soil and cow dung manure in the proportion of 1:1 (v/v). The pots were treated with boiling water twice to kill soil-borne plant pathogens. Each pot was planted with a basak cutting collected from the garden of the Botany Department. The pots were divided into 5 batches, each comprising 10 seedlings, and placed over bricks in the experimental garden. Two batches (A, B) were kept side by side, 60 cm apart and named set no. I. Similarly, set no. II contained 20 pots arranged in pairs (C, D). The remaining batch (E) of 10 pots served as control. All the plants were allowed to grow for 80 days. The pots were irrigated when needed.

After 80 days of growth, each plant of batch A in set no. I was applied heat shock by immersing a mature leaf for 5 min in hot water (65-62 °C) in a beaker. Before heat-shock, each plant of batch A was connected to the corresponding plant of batch B (Figure 1) by means of a moistened cotton thread in a polythene tube for 45 min. This served to set the initial equilibrium of the plant pairs in the connected state. One leaf of a plant in batch A and its pair from batch B were immersed in two beakers filled with sterile tap water. Each end of the 80-cm long cotton thread was immersed in the one of the two beakers, thereby maintaining the water connection between both plants. While all the plants in batch A of set I were applied direct heat-shock, the corresponding connected plants of batch B of the same set I were not subjected to stress.



**Figure 1.** Diagram and photograph showing the connection between pairs of plants by means of a water-filled tube with its ends dipped in two beakers containing water. There were two cotton threads, one inside a polythene tube and another naked. One beaker contains a leaf of heat-stressed or *Canth 200cH* treated plant,

whereas the other has a leaf of unstressed and untreated plant. Stress or treatment (S/T) is given to a leaf different from that immersed in water. The photograph shows two cotton threads, one inside a polythene tube and the other is naked.

In case of set no.II, plants of batches C and D were similarly connected in pairs by means of moistened cotton threads kept in polythene tubes. Forty-five min after effecting the connection each plant of batch C was treated with *Canth* 200cH, whereas heat shock was not applied to these plants (C or D). The purpose of the treatment with *Canth* was to investigate whether this drug in high dilution alone could induce expression of new proteins able to counter the effect of heat shock. The drug was obtained from Seth Dey and Co, Kolkata, India, and diluted with sterile distilled water 1:100 (but not succussed) to minimize the effect of ethanol (original solvent). A piece of filter paper was soaked in the dilution and gently placed on a mature leaf covering an area of 1 cm of diameter. The treatment was applied on both sides of the leaf at the same spot for 1 min. Batch C was not applied heat shock, and batch D was not given pretreatment with *Canth*.

**Table 1.** Selected leaf proteins of *Adhatoda vasica* plants under heat stress and treatment with *Cantharis vesicatoria* 200cH obtained by FPLC, mean migration time (in min)  $\pm$  standard error of proteins, the molecular weight in KDa is indicated in italics below the migration time. Altogether 9 different protein types are shown in the table.

Protein type	Control	Heat Shock		Canth 200cH		F-value
		Direct A	Connected B	Direct C	Connected D	
1	17.40 $\pm$ 0.03 <b>a</b> <i>163.7</i>	16.05 $\pm$ 0.37 <b>a</b> <i>164.0</i>	17.19 $\pm$ 0.56 <b>a</b> <i>163.2</i>			3.46
2		31.85 $\pm$ 0.02 <b>b</b> <i>131.8</i>	31.75 $\pm$ 0.07 <b>b</b> <i>131.8</i>			
3	33.3 $\pm$ 0.17 <b>a</b> <i>128.8</i>			33.95 $\pm$ 0.03 <b>a</b> <i>128.8</i>	33.34 $\pm$ 0.35 <b>a</b> <i>128.8</i>	1.23
4		40.50 $\pm$ 0.52 <b>a</b> <i>75.8</i>		40.26 $\pm$ 0.33 <b>a</b> <i>75.1</i>	39.77 $\pm$ 0.33 <b>a</b> <i>75.1</i>	0.10
5		41.00 $\pm$ 0.32 <b>b</b> <i>72.44</i>	41.24 $\pm$ 0.05 <b>b</b> <i>72.44</i>			
6	42.5 $\pm$ 0.8 <b>a</b> <i>70.79</i>	43.20 $\pm$ 0.01 <b>a</b> <i>70.46</i>	43.21 $\pm$ 0.02 <b>a</b> <i>70.46</i>	42.37 $\pm$ 0.17 <b>a</b> <i>70.79</i>	42.09 $\pm$ 0.01 <b>a</b> <i>70.79</i>	0.02
7	44.7 $\pm$ 0.39 <i>69.18</i>			49.99 $\pm$ 0.72 <b>b</b> <i>64.6</i>	51.5 $\pm$ 0.8 <b>b</b> <i>65.6</i>	
8	76.4 $\pm$ 1.2 <i>28.54</i>					
9				78.21 $\pm$ 0.43 <b>b</b> <i>26.8</i>	79.5 $\pm$ 0.69 <b>b</b> <i>26.6</i>	

a, a, a = No significant difference in rows by ANOVA (one way).

b, b = No significant difference in rows by Student's t-test.

## Separation, quantification and precipitation of proteins

The leaves were harvested 1 hour after heat shock, and 1 hour after treatment with *Canth*. The purpose in this case was to investigate the individual effect of heat shock and *Canth* on the protein profile. Leaves immersed in water and those given heat shock or *Cantharis vesicatoria* treatment were left untouched. The leaves that were applied direct heat shock dried up and fell on the ground in 2-3 days. The leaves of the control plant were also harvested at the same time.

One mature leaf of each plant of a batch was randomly collected. Leaves of each batch, A, B, C, D and E were homogenized separately with chilled extraction buffer (1g leaf tissue /3cm<sup>3</sup> of buffer) containing 50 mM Tris-HCl, 2% β-mercaptoethanol, 1 mM ethylenediaminetetraacetic acid disodium salt (EDTA-Na<sub>2</sub>), 5% sucrose, 1.5% PVPP, 1 mM phenylmethylsulfonyl fluoride (PMSF) at pH adjusted to 8.0 with 1M HCl. The mixture was centrifuged at 15,000 g for 20 min at 4° C. The supernatant of each sample was kept at -80° C until analysis. The concentration of each protein sample (representing the average protein profile of a batch) was measured using the Lowry method [4], and protein was precipitated by Laemmli acetone precipitation method [5]. The apparent molecular weight of the separated proteins was determined by comparison to the relative migration rates of marker proteins (Hyper PAGE Prestained protein marker).

## FPLC analysis

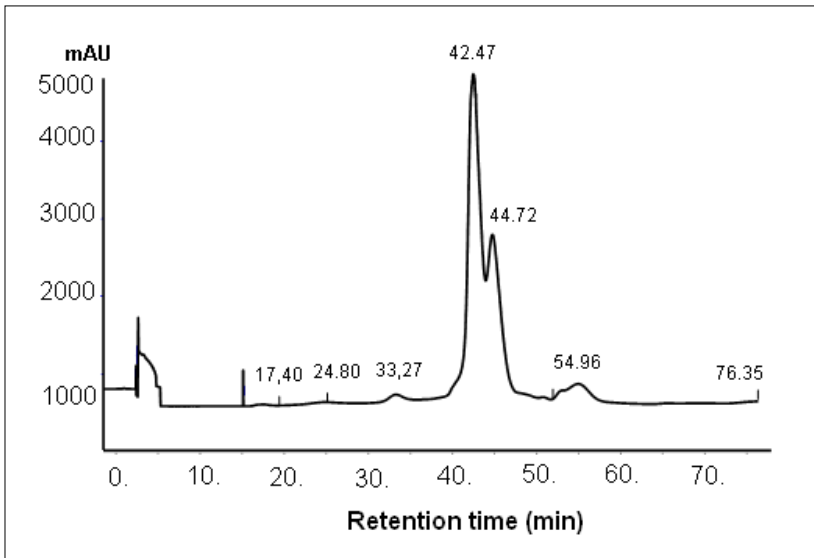
Each sample of the extract of leaves, 1ml in vol, was injected into column Superose<sup>TM6</sup> 10/300GL. Protein separation was performed by means of fast protein liquid chromatography (FPLC) using the mobile phase of 0.5 M Tris -HCl buffer (pH 7.5) with protease inhibitors at a flow rate 0.5 ml/min at 25 ° C with UV detector fixed at 280 nm wavelength. The device used was GE healthcare, AKta purifier, model 10. Leaf extract and buffer were filtered through Millipore filter (0.45 μm) to remove any suspended particles before the FPLC run. The chromatograms were monitored and printed. Five replications were performed for each sample. The mean migration times of proteins in a column were analyzed by Student's t-test and one-way ANOVA to investigate eventual significant differences between proteins in a column (Table 1).

## Results

The protein profile of the leaves of the control plants is depicted in Figure 2, of the heat-stressed plants in Figure 3a, of the unstressed but connected to heat-stressed plants by moistened cotton threads in Figure 3b, of the *Canth*-treated plants in Figure 4a, and of the untreated but connected plants in Figure 4b. The molecular weights of the proteins separated by FPLC are presented in Table 1.

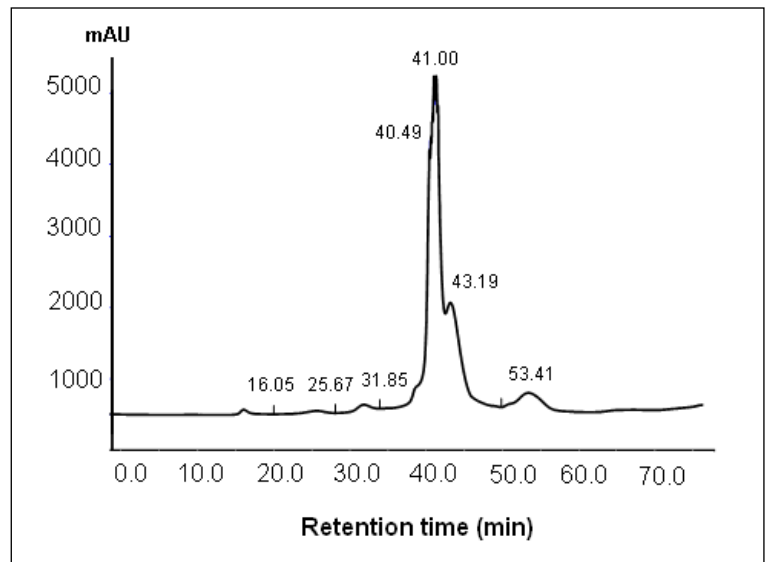
The plants that were applied direct heat shock, and the ones connected to them through capillary water in cotton threads show marked similarity in regard to expressed 132 KDa and 72.4 KDa proteins. These proteins are not present in any other group of tested plants.(Table 1). Proteins of 128.8 KDa are present in all other groups of plants, but conspicuously absent in heat-stressed (A) and connected (B) plants (Table 1, Fig. 2, 3ab, 4ab).

Proteins of 75KDa, 70.8KDa, 64KDa and 26.6KDa are common in both groups of plants, which were treated directly with *Cantharis vesicatoria* 200c (C) and their connected counterparts (D). (Table 1, Fig 4ab). Of these proteins, two of 64KDa and 26.6KDa are exclusive to the *Cantharis vesicatoria* -treated (C) and its corresponding connected (D) group (Table 1, Fig. 4ab). Proteins of 163 KDa and 27.5KDa, which are present in all other groups, are conspicuous by their absence in these groups C, D (Table1). Proteins of 69.2 KDa and 28.54KDa are exclusive to the control group (E).

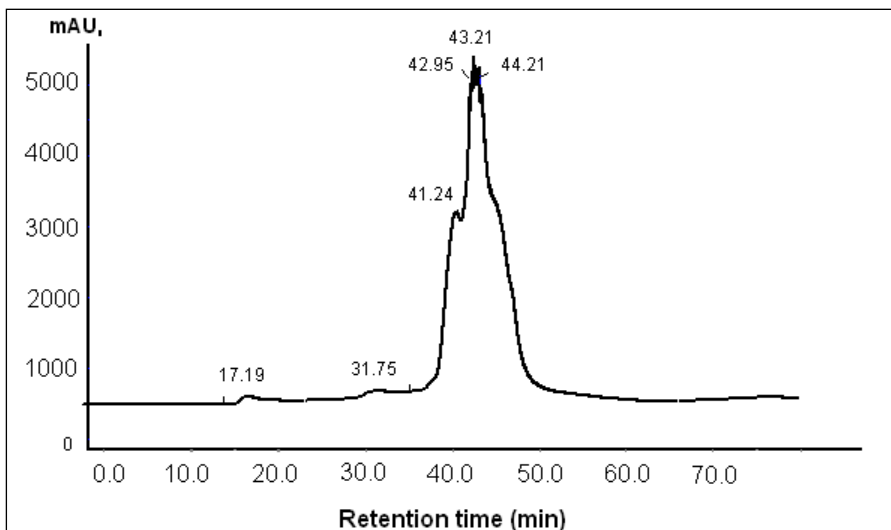


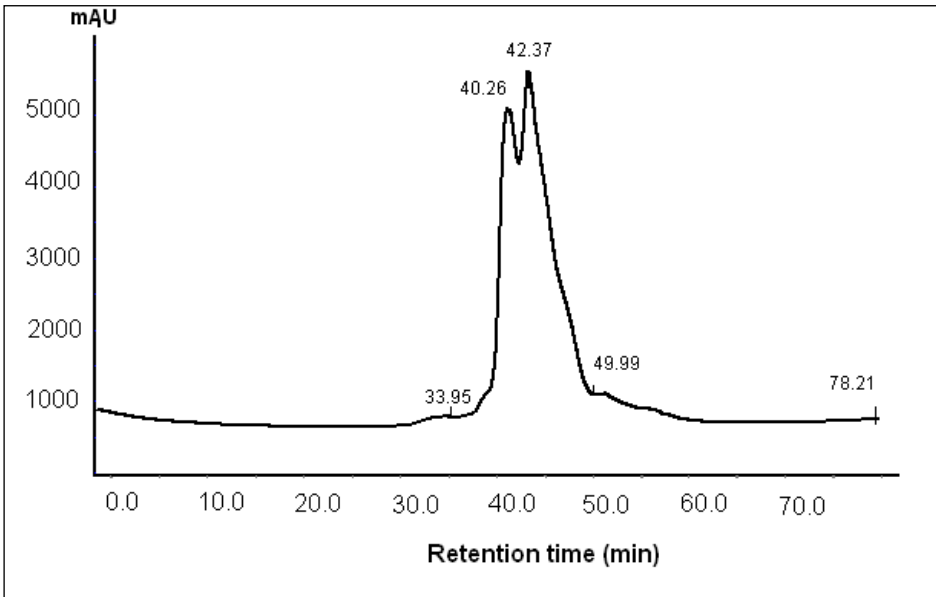
**Figure 2.** Proteins separated by FPLC in unstressed and untreated leaves of basak plants, (control) N=10 plants.

**Figure 3a.** Proteins separated by FPLC from leaves of basak plants given heat stress through hot water (65-62 °C for 5 min) to one leaf in each plant. N=10 plants.



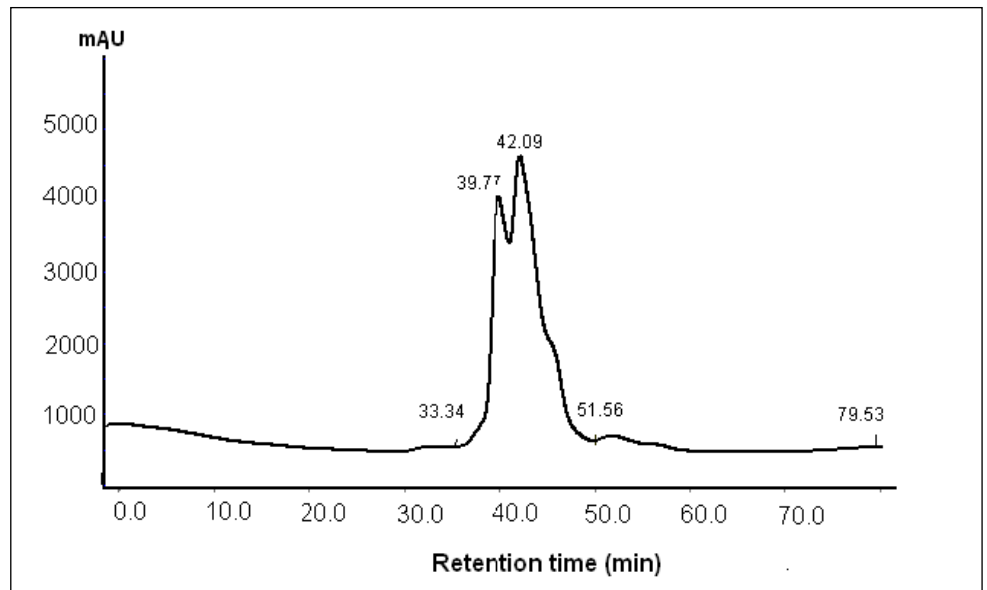
**Figure 3b.** Proteins separated by FPLC from the leaves of unstressed basak plants connected by water to heat stressed plants. N=10 plants.





**Figure 4a.** Proteins separated by FPLC from the leaves of basak plants treated with *Cantharis vesicatoria* 200cH. The drug was diluted with mili Q water 1:100 (not succussed) soaked in sterile filter paper and applied by gentle touch to one leaf in each plant. N=10 plants.

**Figure 4b.** Proteins separated by FPLC from the leaves of untreated basak plants connected by water to plants treated with *Cantharis vesicatoria* 200cH. N=10 plants.



## Discussion

The results suggest that proteins of 131.8 KDa and 72.4 KDa are somehow related with heat-shock, either directly or indirectly through capillary water in cotton threads (Table 1). Proteins of 128.8 KDa were repressed in groups A, B (Table 1). Heat-shock might induce expression of heat-shock proteins and repression of some normal cellular proteins [3]. Similarly, two new proteins were related with *Canth* treatment, either directly or indirectly through capillary water in wet threads (Table 1). Two proteins were repressed by *Canth* in groups C, D (Table 1). These results agree with our earlier work on cowpea plants, where we showed that water serves as a carrier of information of heat-shock and *Canth* treatment between two groups of plants [2]. This agreement concerns the similarity of the protein profiles of two groups of plants connected one to the other by water.

The difference in the protein profiles between the A,B and C,D groups (Table 1) may be due to different effects of heat-shock and *Canth* in regard to the expression and repression of proteins. However, the expression and repression of proteins is similar within each connected group (A,B or C,D). This shows that the water connection between each connected pair plays an important role in maintaining this similarity. The two connected plants behaved as one single plant with different branches. While some proteins are common to the control and the two pairs of investigated groups, some proteins are exclusive to the control group as rows 7 and 8 (Table 1). This is partially due to the effect of heat stress and *Canth* resulting in the repression of certain proteins.

We assume that the stimulus of heat shock or *Cant* on a small area of a leaf might first induce local change in the metabolic activity of that area. This change may be propagated through the global molecular network (GMN) of water covering the cell membranes and the GMN of protein molecules [2, 6].

The existence of global metabolic structures was verified in some organisms, whereas the self-organized enzymatic configuration seems to be common to all cellular organisms [7, 8]. The metabolic network is a dynamic superstructure that integrates different dynamic subsystems, i.e. the metabolic subsystems [9]. Water serves as a continuous medium that covers the cells and all molecules inside and outside them. Different non-covalent forces may locally change the structure of water, and this effect is likely to propagate to all other parts [1, 10].

Water molecules adsorbed on cell membranes, protein networks, inner surfaces of glass beakers and cotton threads may play an important role in the transmission of information from one plant to another. There is evidence that the physicochemical properties and structure of water close to hydrophobic surfaces differ from those of bulk water [11]. It has been hypothesized that homeopathic dilutions represent specifically structured water with characteristic molecular oscillation, which may act on the water structure over macromolecules of cells and change their pattern of oscillations [2, 7]. Molecular oscillations occur spontaneously in enzymatic networks and involve fundamental metabolic processes including gene expression [12, 13].

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## Água como veículo de informação sobre choque térmico e efeito medicamentoso em dois grupos de plantas *Adhatoda vasica*

### RESUMO

Plantas *Adhatoda vasica* Nees foram cultivadas em 50 vasos de argila e divididos em 5 lotes A, B, C, D e E. Os lotes A, B e C, D foram arranjados em dois pares paralelos separados. Uma folha de cada planta dos pares adjacentes foi submergida num béquero com água corrente estéril. Os béqueros de cada par, A, B e C, D foram interconectados através de tubos de polietileno contendo fio de algodão umedecido. Uma folha de cada planta no lote A recebeu choque térmico através de imersão em água quente por 5 minutos. Uma folha de cada planta no lote C foi tratada com *Cantharis vesicatoria* 200c. O lote E foi utilizado como controle não submetido nem a estresse nem a medicação. Uma hora depois da aplicação de choque térmico ou tratamento medicamentoso, todas as folhas foram coletadas e suas proteínas foram extraídas através de tampão de extração de proteínas frio. As proteínas foram separadas através de cromatografia líquida rápida de proteínas (CLRP). Os perfis proteicos dos lotes A, B, C e D mostraram similaridade considerável quanto à expressão e repressão de algumas proteínas. Conclui-se que o efeito do choque térmico e do tratamento medicamentoso foi transmitido através da água nos capilares de fio de algodão conectando pares de plantas. Assume-se que o choque térmico e o tratamento medicamentoso alteraram localmente a estrutura da água nas folhas, que se propagou através da rede global da estrutura da água até a rede proteica nas plantas inteiras e dali para a água interfacial nos béqueros e fios de algodão. Considera-se que uma potência homeopática é água especificamente estruturada que influencia a estrutura da água no organismo tratado.

**Palavras-chave:** *Cantharis vesicatoria*; choque térmico; rede da água; transferência de informação; homeopatia.



## Agua como vehículo de información sobre shock térmico y efecto medicamentoso en dos grupos de plantas *Adhatoda vasica*

### RESUMEN

Plantas *Adhatoda vasica* Nees fueron cultivadas en 50 macetas de tierra y divididas en 5 lotes A, B, C, D y E. Los lotes A, B y C, D fueron distribuidos en dos pares paralelos separados. Una hoja de cada planta de pares adyacentes fue sumergida en un vaso de precipitados con agua corriente estéril. Los vasos de cada par A, B e C, D fueron interconectados mediante tubos de polietileno conteniendo hilo de algodón humedecido. Una hoja de cada planta del lote A recibió shock térmico por inmersión en agua caliente durante 5 minutos. Una hoja de cada planta del lote C fue tratada con *Cantharis vesicatoria* 200c. El lote E fue utilizado como testigo no sometido a estrés ni medicación. Una hora después de la aplicación de shock térmico o tratamiento medicamentoso, todas las hojas fueron recogidas y sus proteínas extraídas mediante de tampón de extracción de proteínas frío. Las proteínas fueron separadas mediante cromatografía líquida rápida de proteínas (CLRP). Los perfiles proteicos de los lotes A, B, C e D mostraron considerable semejanza en la expresión y represión de algunas proteínas. Se concluye que el efecto del shock térmico y del tratamiento medicamentoso fue transmitido por el agua en los capilares de hilo de algodón conectando pares de plantas. Se asume que el shock térmico y el tratamiento medicamentoso alteraron localmente la estructura del agua en las hojas, con propagación por la red global de la estructura del agua hasta la red proteica en las plantas enteras y de allí hasta el agua de interface en los vasos de precipitados e hilos de algodón. Se considera que una potencia homeopática es agua específicamente estructurada que influencia la estructura del agua en el organismo tratado.

**Palabras llave:** *Cantharis vesicatoria*; shock térmico; red del agua; transferencia de información; homeopatía.

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