Physical chemical and citotoxic evaluation of highly diluted solutions of *Euphorbia tirucalli* L. prepared through the fifty milesimal homeopathic method

Sheila Garcia¹, Rafael Cardoso Harduim², Fortune Homsani², Carlos Renato Zacharias³, Ricardo Machado Kuster⁴, Carla Holandino²

¹Laboratório de Controle de Qualidade, Departamento de Medicamentos, Faculdade de Farmácia, UFRJ. Rio de Janeiro, Brazil.

²Laboratório Multidisciplinar de Ciências Farmacêuticas, Departamento de Medicamentos, Faculdade de Farmácia, UFRJ. Rio de Janeiro, Brazil.

³Departamento de Física e Química, UNESP, Guaratinguetá, São Paulo, Brazil.

⁴Núcleo de Pesquisa em Produtos Naturais, UFRJ. Rio de Janeiro, Brazil.

ABSTRACT

Background: although Hahnemann described the fifty-milesimal (LM) method in the 6th edition of the *Organon of the Medical Art*, very little research has been carried out on the physical chemical properties of these homeopathic preparations. Furthermore, there is still no evidence allowing for the correlation between the alleged physical chemical properties and the biological effects of high dilutions. **Aims:** to evaluate physical chemical characteristics of LM preparations including electrical conductivity, pH and refraction index, and their effect on biological experimental models. **Materials and methods:** preparations tested for physical chemical analysis were dilutions 1 lm to 10 lm of *Euphorbia tirucalli* L. prepared from the latex and the juice of the plant. To rule the seasonal characteristics of this plant, 2 different populations were used, one collected in June 2007 and the other in May 2008. Furthermore, the cytotoxic effect of *Euphorbia tirucalli* 5 lm was tested on human breast cancer cells (MCF7) through MTT assay. **Conclusions:** Some differences among the two collections were observed. However, any clear correlation could be observed between physical chemical properties and biological activity.

Keywords: High dilutions, LM method, electrical conductivity, pH, refraction index, cytotoxicity, *Euphorbia tirucalli*

Introduction

Recently published literature describes some physical chemical properties of highly diluted aqueous solutions (HDs) [1-9], electrical conductivity seems a fitting indicator of structural changes in water induced by HDs [2,10-11]. However, the possible interactions between a highly diluted substance and water are still not well understood [5-6,12].

In this context, a particular method of preparation of homeopathic solutions known as fifty-milesimal (LM) has been little researched as to physical chemical properties, although it was described by Samuel Hahnemann, the founder of homeopathy, in the 6th edition of his major opus, the *Organon of the Healing Art*, finished in 1842 and published in 1925 [13-14].

Former investigations with HD of *Euphorbia tirucalli* L. showed that it can be used as a model for study physical chemical changes [10-11]. Therefore, it was used as motivation to prepare LM dilutions in order to establish potential alterations of physical chemical variables.

On the other hand, there is no data in the literature linking alleged physical chemical properties of HDs and their biological effects. For this reason, in this study we also sought to establish biological effects of HDs of *Euphorbia tirucalli* L. and attempt to correlate them with eventual changes in the physical chemical variables.

In this regard, the latex of *Euphorbia tirucalli* L. is used in folk medicine to treat chronic degenerative diseases [15-17], although the mechanisms involved in this activity are not yet known. According to the literature, the latex may have dual behavior, activating or inhibiting tumoral events [18-20].

In a previous study [21], we found interesting results on the effect of HDs of *Euphorbia tirucalli* L. on the glycolytic metabolism of cancer and normal cells (Melan A and MCF-7 cell lines), supporting the hypothesis that they interfere the metabolism of cell lines. Moreover, using mice as experimental subjects, it could be observed that the ones that received *Euphorbia tirucalli* in dilution 30cH exhibited significant increase in food consumption ($p < 0.05$) without any significant difference in weight gain, indicating an influence of this HD on animal metabolism [22].

The aims of this study were to verify whether the electrical conductivity, pH and refraction index of HDs of *Euphorbia tirucalli* L. prepared according to the LM method present significant changes in different degrees of dilution and to establish whether electrical conductivity correlates with biological parameters, in this case the cytotoxicity of *Euphorbia tirucalli* on human breast cancer cells (MCF7 line).

Materials and Methods

Preparation of HDs

The experiments were carried out with the juice of the whole plant and the latex of *Euphorbia tirucalli* L. gathered from the garden of *Núcleo de Pesquisa de Produtos Naturais* of *Universidade Federal do Rio de Janeiro*, Brazil. The plants were gathered at the beginning of June 2007 and in mid-May 2008, in order to avoid seasonal variations.

The juice and the latex were grinded separately in lactose up to 3 cH as described by Hahnemann and the Brazilian Homeopathic Pharmacopeia [13,23]. Then, it was taken 1 grain (63mg) from the 3cH preparation and it was dissolved, with gentle shaking, in 400 drops of distilled water and 100 drops of alcohol 96% (v/v), totalizing 500 drops. This is the standard starting potency in the LM method whose ratio is 1 grain (63mg) to 500 drops. One drop (0.025ml) from the starting potency was mixed with 100 drops (2.5ml) of alcohol 96% (v/v). This solution was agitated by 100 handmade succussions. This is the first degree in the lm method (1 lm). 500 microglobules (weighing 315 mg) were impregnated with the solution 1 lm, homogenized and left to dry naturally, each one of these being 1 lm. One microglobule was placed in an amber-hued vial (capacity 4.5ml) and 1 drop of distilled water was added to dissolve it. Then 100 drops of alcohol 96% (v/v) were added in the vial and hand succussed 100 times to obtain the second degree. One drop of the latter was used to impregnate 500 microglobules, which were homogenized and left to dry naturally (2 lm). The process was thus repeated to obtain dilutions 3 lm to 10 lm.

As controls, we used distilled water (DW) and diluted-agitated lactose (LDLM), following the same procedure.

Physical chemical analysis

Electrical conductivity, pH and refraction index were evaluated in the HDs of *Euphorbia tirucalli* 1 lm to 10 lm prepared from both the latex and the juice of the full plant. Electrical conductivity and the pH measurements were made with Mettler-Toledo MPC 227 at 25° C.

A Zeiss Refractometer was used to measure the refraction index. For these assays 3 microglobules were placed in 3 different amber glass vials (1 microglobule in each vial) and solubilized with 30ml of distilled water.

Cytotoxicity assay

Euphorbia tirucalli 5 lm was prepared from the plants gathered in May 2008, and its cytotoxicity was evaluated by MTT assay in MCF-7 cells. For that purpose, confluent MCF7 cells (5×10^4 cells) were seeded in 96 well plates with 5% and 10% HDs and their respective controls and kept in incubation for 24 hours [22]. At the end of treatments, 20 μ l of 5 mg/ml solution of tetrazolium reagent (3,4,5-dimethiazol-2,5-diphenyl-tetrazolium bromide, Sigma) were added to each well. Culture plates were incubated for further 3 hours at 37°C in the absence of light. Supernatant was removed and formazan crystals were dissolved with 200 μ l of dimethylsulfoxide. Absorbance was measured in each well in dual wave-length, according to Mosmann [24].

Statistical analysis

Student's test was used to evaluate the significance of results. In all analyses, confidence interval was 95% and p values less than 0.05 were considered significant.

Results and Discussion

Physical chemical parameters of HDs prepared from plants gathered in June 2007

The results obtained in the measurement of pH and refraction index of all LM dilutions of *Euphorbia tirucalli* exhibited no statistical difference ($p > 0.05$) (data not shown). On the other hand, the measurement of electrical conductivity in the latex and whole plant juice of the plants collected in June 2007 (Figure 1) presented statistically significant differences, as described in Table 1.

Figure 1: Results of conductivity measurement in latex and juice of *E. tirucalli* collected in June 2007 and respective controls. Three measurements were made independently (n=3).

Statistical treatment was made using the values obtained from the latex and the juice, considering “p value” with 95% confidence interval. The dilutions exhibiting more significant differences were analysed (Table 1).

Table 1: Statistical treatment of the dilutions chosen from the latex and the juice of *E. tirucalli* collected in June 2007, considering “p value” with 95% confidence interval.

Dilutions	Latex (p-value)	Juice (p-value)
1 lm vs 3 lm	0.054	0.078
1 lm vs 5 lm	0.101	0.016*
1 lm vs 8 lm	0.046*	0.117
1 lm vs 9 lm	0.092	0.032*
3 lm vs 5 lm	0.106	0.977
5 lm vs 9 lm	0.129	0.837

*Statistically significant difference

Results of the statistical analysis of the changes in the measurements of electrical conductivity obtained from the latex were not significant enough as to exclude the possibility of chance (they presented high standard deviations - Figure 2), excluding *1 lm vs. 8 lm, which exhibited statistically significant change (Table 1). For juice samples, t-test showed statistically significant change between *1 lm vs 5 lm and *1 lm vs 9 lm (Table 1).

The results for the control HD lactose (LDLM) prepared exactly in the same way as the latex and the juice are shown in Table 2. There is statistically significant difference between lactose and latex, and lactose and juice with 3 lm, 5 lm, 7 lm, 8 lm and 9 lm.

Table 2: Statistical treatment of conductivity measurements between lactose and latex, and lactose and juice of plants gathered in June 2007, considering “p value” with 95% confidence interval.

Dilutions	Lactose vs Latex (p-value)	Lactose vs Juice (p-value)
3 lm	0.014*	0.014*
4 lm	0.084	0.064
5 lm	0.021*	0.030*
6 lm	0.360	0.947
7 lm	0.018*	0.013*
8 lm	0.015*	0.023*
9 lm	0.039*	0.011*
10 lm	0.290	0.182

*Statistically significant difference

As pH values did not change significantly over all LM potencies, we could not correlate them with the values for electric conductivity.

Physical chemical parameters of HDs prepared from plants gathered in May 2008

The refraction index and pH values all HDs tested did not differ significantly (data not shown), as it was also the case of the plants collected in June 2007. Electrical conductivity showed a similar profile of values as the one obtained with the plants gathered in June 2007 (Figure 2 and Table 3).

FIGURE 2: Results of conductivity measurement from latex and juice of the plants gathered in May 2008 and respective controls. Three measurements were made independently (n=3).

The differences observed in the profiles obtained from both sets of plants should be attributed to the phytochemical composition of this *Euphorbiaceae*. Varricchio quantified the latex of *E. tirucalli* with high performance liquid chromatography coupled with UV absorption spectrometry (CLAE/UV) for 1 year [25], and the results indicate that the phytochemical composition of this latex has seasonal variations. Moreover, this study confirmed that the maximum synthesis of phorbol esters occurred during the morning period in the month of May [25]. Additionally, the comparative study made between plants gathered in May and in June showed different cytotoxic properties induced by dilution 30cH on tumor cells [26]. Seasonal aspects might be involved in the phytochemical properties of *E. tirucalli*, and consequently affect the properties its HDs.

TABLE 3: Statistical treatment of the dilutions chosen from the latex and juice of *E. tirucalli* gathered in May 2008, considering “p value” with 95% confidence interval.

Dilutions	Latex (p-value)	Juice (p-value)
1 lm vs 3 lm	0.056	0.028
1 lm vs 5 lm	0.006*	0.006*
1 lm vs 8 lm	0.569	0.117
1 lm vs 9 lm	0.004*	0.013*
3 lm vs 5 lm	0.013*	0.018*
5 lm vs 9 lm	0.119	0.526

*Statistically significant difference

T-test showed statistically significant change between *1 lm vs 5 lm, *1 lm vs 9 lm and *3 lm vs 5 lm. The comparisons of latex and juice with the control HD lactose (LDLM) is in table 4. There is statistically significant difference between lactose and latex, and lactose and juice with 5 lm, 7 lm, 8 lm and 9 lm.

Table 4: Statistical treatment between the conductivity values of lactose and latex, and lactose and juice in plants gathered in May 2008, considering “p value” with 95% confidence interval.

Dilutions	Lactose vs Latex (p-value)	Lactose vs Juice (p-value)
3 lm	0.874	0.541
4 lm	0.458	0.325
5 lm	0.003*	0.018*
6 lm	0.198	0.756
7 lm	0.028*	0.041*
8 lm	0.483	0.016*
9 lm	0.017*	0.057
10 lm	0.526	0.732

*Statistically significant difference

The results obtained with the latex from the plants gathered in May 2008 showed a different pattern. In this analysis, HDs 5 lm and 9 lm showed statistically significant difference (Figure 3, Table 3), indicating that the moment of gathering influenced the phytochemical composition of *E. tirucalli*, and also possibly the properties of its HDs.

Cytotoxicity assay

Results obtained by MTT assay showed significant difference ($p < 0.05$) in cell viability when MCF7 cells were treated with 5% (Figure 3-A) and 10% (Figure 3-B) *E. tirucalli* 5 lm prepared from the latex and juice of plants gathered in May 2008. There was statistical difference among the groups that received HDs of *E. tirucalli* (juice and latex 5 lm), lactose 5 lm and distilled water by comparison to the control group (cells without treatment).

The results obtained by MTT assay after incubation with 5% HDs of *E. tirucalli* (Figure 3A) showed increase in MCF-7 viability by comparison to the respective controls (DW and LDLM). However, when the cells were incubated with 10% HDs of *E. tirucalli* and controls there was significant increase ($p < 0.018$) in cell viability induced only by the latex (Figure 4B), probably due to variations in the osmolarity of the culture medium.

Figure 3A

Figure 3B

Figures 3 (A and B): Viability of MCF7 cells incubated for 24 hours with 5% and 10% *E. tirucalli* 5 lm (juice and latex) and respective controls (distilled water – DW, lactose 5 lm –LDLM, control), prepared from plants gathered in May 2008. A: MCF7 citotoxicity induced by 5% *E. tirucalli* 5 lm; B: MCF7 citotoxicity induced by 10% *E. tirucalli* 5 lm. Values are means $\pm$ SD of 3 independent experiments performed in quintuplicate (n = 15).

Table 5 describes the statistical results considering “p value” with 95% confidence interval for 5% and 10% *E. tirucalli* 5 lm gathered in May 2008.

T-test showed statistically significant differences between *lactose 5 lm vs control cells, *latex 5 lm vs control cells (5% and 10%), *juice 5 lm vs control cells, *water vs control cells, *lactose 5 lm vs water, *latex 5 lm vs water, juice 5 lm vs water.

Table 5: Statistical analysis of HDs from latex and juice of *E. tirucalli* gathered in May 2008, considering “p value” with 95% confidence interval, extracted from MTT graphs.

Samples	Concentration 5% (p-value)	Concentration 10% (p-value)
Lactose 5 lm vs Control	<0.001*	0.279
Latex 5 lm vs x Control	<0.001*	0.018*
Juice 5 lm vs Control	0.002*	0.122
Water vs Control	<0.001*	0.231
Latex 5 lm vs Lactose 5 lm	0.910	0.225
Juice 5 lm vs Lactose 5 lm	0.463	0.769
Lactose 5 lm vs Water	0.006*	0.593
Latex 5 lm x Water	0.006*	0.768
Juice 5 lm vs Water	0.002*	0.802

*Statistically significant difference

Our results differ from those obtained by Aquino *et al.* in a previous work [21]. These authors showed a significant fall ($p < 0.05$) in MCF7 viability between cells incubated with *E. tirucalli* 30cH and the corresponding HD of ethanol. Considering the presence of molecules of *E. tirucalli*, Aveloz 30 cH and Aveloz 5 lm are both highly diluted, but for the presence of lactose remnants. Thus, we could not explain this different pattern evaluating only the tumoral metabolic aspect. Nevertheless, our results suggest that the effects observed might not be due to the solvent, but to the process of preparation, a hypothesis that must be verified in future works. Also, our findings might be attributed to a specific behavior of high-diluted systems whose dynamics are not completely clear yet.

Conclusions

The results obtained in this and in previous studies indicate that physical chemical parameters and biochemical features of MCF-7 cells are modified, even in the absence of molecules of *E. tirucalli*. Besides, the LM scale might have a different behaviour in the conductivity measurements and cytotoxicity features when compared with the centesimal scale. Further studies are under way to correlate the physical chemical properties of highly diluted solutions of other homeopathic medicines to improve the understanding about dynamized systems.

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RESUMO

Motivação: Embora Hahnemann tenha descrito o método LM na 6ª. Edição do *Organon da Arte de Curar*, poucas pesquisas tem sido feitas sobre as propriedades físico-químicas destas preparações. Além disso, ainda não existem evidências que apontem para a correlação entre propriedades físico-químicas e os efeitos biológicos das altas diluições. **Objetivos:** determinar características físico-químicas das preparações LM tais como condutividade elétrica, pH e índice de refração, bem como o efeito biológico em modelos experimentais. **Materiais e Métodos:** foi analisada uma série de diluições desde 1 lm até 10 lm de *Euphorbia tirucalli* L., preparada a partir do látex e do suco da planta. Para considerar as características sazonais desta planta, usamos 2 populações diferentes, colhidas uma em Junho de 2007 e outra em Maio de 2008. Além disso, o efeito citotóxico da *Euphorbia tirucalli* L. 5 lm foi testado em células humanas cancerosas (MCF7) através de ensaio MTT. **Conclusões:** Algumas diferenças entre as duas populações foram observadas. No entanto, nenhuma correlação clara pode ser observada entre as propriedades físico-químicas e a atividade biológica.

Palavras-chave: altas diluições, método LM, condutividade elétrica, pH, índice de refração, citotoxicidade, *Euphorbia tirucalli* L.

Evaluación físico-química y citotóxica de soluciones altamente diluídas de *Euphorbia tirucalli* L. preparadas por el método homeopático cincuenta milésimal (LM).

RESUMEN

Motivación: Aunque Hahnemann describió el método cincuenta milésimal (LM) en la 6ª edición del *Organon del Arte Médico*, muy poca investigación se ha realizado sobre las propiedades físico-químicas de estos preparados homeopáticos. Por otra parte, todavía no hay pruebas que permitan la correlación entre las presuntas propiedades físico-químicas y los efectos biológicos, de las altas diluciones. Objetivos: evaluar las características físico-químicas de los preparados LM, incluyendo conductividad eléctrica, pH y el índice de refracción, y su efecto en modelos biológicos experimentales. Materiales y métodos: las muestras para el análisis físico-químico fueron diluciones 1 a 10 lm de *Euphorbia tirucalli* L. preparadas a partir del látex y el jugo de la planta. Para descartar las características sazonales de esta planta, dos poblaciones diferentes fueron utilizadas, una recogida en junio de 2007 y otra en mayo de 2008. Además, el efecto citotóxico de *Euphorbia tirucalli* 5 lm fue probado en células humanas de cáncer de mama (MCF7) a través del ensayo MTT. Conclusiones: Algunas diferencias entre las dos colecciones se observaron. Sin embargo, ninguna correlación clara se observó entre las propiedades físicas- químicas y actividad biológica.

Palabras claves: altas diluciones, método LM, conductividad eléctrica, pH, índice de refracción, citotoxicidad, *Euphorbia tirucalli*

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Correspondence author: Carla Holandino, cholandino@yahoo.com; Sheila Garcia, sheiloka@globo.com

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