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Effects of Commonly Used Solubilizing Agents on a Model Nerve-Muscle Synapse

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SUMMARY. Solubility represents a limiting factor when testing new compounds in animal experiments, since solubilizing agents generally have pharmacological effects that can interfere with the studied substance. Vehicles are commonly used for solubilizing certain substances including apolar and polar extracts obtained from medicinal plants. In this study, fifteen vehicles were investigated on mice neuromuscular preparations. A known *in vitro* neuroblocker myotoxin from *Bothrops jararacussu* venom, bothropstoxin-I, was used as a pharmacological tool for testing the medicinal potential of apolar and polar extracts (hexane, dichloromethane, ethyl acetate and methanol) obtained from *Casearia sylvestris* Sw. leaves, which in turn were used for testing their solubility and concomitantly to produce no change on basal response of indirectly stimulated mouse phrenic nerve-diaphragm preparations. Taken together *in vitro* biological system and extracts solubility, our results showed that dimethyl sulphoxide and polyethylene glycol 400 were the better vehicles, and methanol extract solubilized on PEG 400 was the only one able to act against the paralysis induced by the myotoxin. Thus, this study points out to the relevant role that vehicles exhibit for extracting the potential pharmacological activity of plants in a given test system.

INTRODUCTION

The plant kingdom represents an enormous reservoir of biologically active molecules and so far only a small fraction of plants with medicinal activity have been assayed, although nearly 50% of drugs used in medicine are of plant origin. The classical chemical procedure for obtaining organic constituents from dried plant tissue is to continuously extract powdered material in a Soxhlet apparatus with a range of solvents. Generally, the chemical constituents of plants are classified based on their biosynthetic origin, the presence of certain key functional groups and solubility properties [1].

In this context, solubility properties are not always enough for studying the pharmacological activity of plants, mainly when testing in animal experiments [2]. Depending on the animal model adopted for pharmacological screening, the obtained extract can not be administered according to its solubility or simply the vehicle utilized can interfere in a given test system, difficulting the data interpretation. Conceptually, a solubilizing agent makes hydrosoluble one substance that is endowed with little or no solubility in water; moreover, it must be stable and cause no interference in the stability or effectiveness of the active substance [3,4].

A current experimental model used pharmacologically for testing new compounds is the isolated mouse phrenic nerve-diaphragm preparation [5], modified for mice. Anatomically it represents the nerve-muscle synapse, whereas physiologically, the muscular contraction. Pharmacological events can be shown by blockade, facilitation or contracture, among other possibilities. For example, bothropstoxin-I (BthTX-I), a myotoxin isolated from *Bothrops jararacussu* crude venom [6] produces an irreversible neuromuscular blockade [7-9].

KEY WORDS: Medicinal plant extract, Neuromuscular junction, Solubility.

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The aim of this study was to test different solvents able to solubilize apolar and polar extracts of *Casearia sylvestris,* and which, simultaneously, cause no interference on the normal physiology of the mouse phrenic nerve-diaphragm preparations, using BthTX-I as pharmacological tool for evaluating the medicinal potential of different extracts of *C. sylvestris*.

MATERIALS AND METHODS

Casearia sylvestris Sw. extracts

The leaves of *C. sylvestris* were harvested from adult plants growing in an orchard at the University of Sorocaba (UNISO). A voucher specimen was deposited in the UNISO herbarium after identification by the Instituto de Botânica at São Paulo city (Brazil). Soxhlet containing 75 g leaves powder (after drying using a forced air circulation dryer model Marconi®, Brazil; and crushing, 10 Mesh, 1.70 mm, using macro mill Wiley type, MA 340 model, Marconi®) were filled successively with 350 mL each hexane, (HE, Synth®, Brazil), dichloromethane (DME, Synth®), ethyl acetate (EAE, Synth®), and methanol (ME, Ecibra®, Brazil). The extracts were them evaporated until dryness and stored at room temperature, protected from light and humidity until assayed.

Solubility tests

Since the obtained *C. sylvestris* HE-, DME-, EAE- and ME- extracts were poorly soluble in the nutritive Tyrode solution used in the incubation bath for nerve-muscle *in vitro* preparations, it was necessary to choose a solvent which besides presenting good solubility in the Tyrode, did not produce undesirable effects which could interfere with the basal response of the neuromuscular preparation. In this sense, fifteen solvents were tested taking into account the polarity of each solvent as well as its ability to solubilize 1 mg of each type of extract (Table 1).

Animals

Male Swiss white mice (26-32 g) were supplied by the Animal Services Unit of the State University of Campinas (UNICAMP). The animals were housed at 25 ± 3 °C on a 12 h light/dark cycle and had access to food and water *ad libitum*. This project (protocol number 644-1) was approved by the Institutional Committee for Ethics in Animal Experimentation (CEEA-IB, UNICAMP) and the experiments were done within the guidelines established by the Brazilian College for Animal Experimentation (COBEA).

Mouse phrenic nerve-diaphragm muscle (PND) preparation

The diaphragm and its phrenic nerve branch were obtained from mouse anesthetized with chloral hydrate (300 mg/kg, i.p.) and sacrificed by exsanguination. The diaphragm was removed according to Bülbring [5], modified for

Extracts from *C. sylvestris* Sw.	Solvents
Hexane (HE)	Soy lecithin (Calbiochem®, USA and Canada) Dimethyl sulphoxide (DMSO, Sigma®) DMSO
Dichlormethane (DME) and Ethyl Acetate (EAE)	Polyethylene glycol (PEG 400, Synth®), Silicone oil (Audaz®, Brazil) Mineral oil (Audaz®) Coco fatty acid diethanolamine (Audaz®) Triethanolamine (Merck®) Triethanolamine lauryl sulphate (Audaz®) Acetic acid (Ecibra®) Sulphuric acid (Synth®) Coco amido propyl betaine (Audaz®) Polyoxyethylene-20-sorbitan monolaurato - Tween 20 (Audaz®) PEG 400
Methanol (ME)	Polyoxyethylene-80-sorbitan mono oleate – Tween 80 (Audaz®) Sodium lauryl sulphate (Synth®) Propylene glycol (Nuclear®, Brazil)

Table 1. Solvents tested for solubilizing *Casearia sylvestris* leaves extracts.

mice, and mounted under a tension of 5 g in a 5 mL organ bath containing Tyrode solution with the following composition (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.49, NaH_2PO_4 0.42, $NaHCO_3$ 11.9 and glucose 11.1. After equilibration with the carbogen aeration mixture of 95% O_2/5% CO_2, the pH of this solution was set at 7.0. The preparations were stimulated indirectly with supramaximal stimuli (4 x threshold, 0.1 Hz, 0.2 ms) delivered from a Grass S48 stimulator to the nerve through bipolar electrodes. Isometric twitch tension was recorded with a force displacement transducer (Load Cell BG-10 GM) coupled to a physiographic (Gould, Model RS 3400) via a Gould universal amplifier. The preparations were allowed to stabilize for at least 20 min before addition of solvents listed in Table 1, the obtained extracts (200 µg/mL) of *C. sylvestris* solubilized with selected solvents: BthTX-I (40 µg/mL), mixture of BthTX-I plus selected extract, or Tyrode solution alone (control).

Statistical analysis

Each pharmacological protocol was repeated at least three times. The results were expressed as the mean ± S.E.M., as appropriate. Student's t-test was used for statistical comparison of the data. A value of $p<0.05$ was considered to indicate significance.

RESULTS

The obtained extracts (HE, DME, EAE and ME) were assayed according to their solubilities as shown in Table 2. The inefficient solvents, in this preliminary test, in which the extracts were

Extracts from *C. sylvestris*	**Solvent**	**Final concentration of solvent**	**Solubility results**	**Interference**
Hexane (HE)	Soy lecithin (Calbiochem®)	1 mg/mL	Soluble	Yes (•)
	Dimethyl sulphoxide (DMSO, Sigma®)	10 µL/mL	Soluble	No (•) and Yes (♦)
	DMSO	10 µL/mL	Soluble (DME); Partially soluble (EAE)	Partial (•)(♦) NA
Dichloromethane (DME) and Ethyl acetate (EAE)	Polyethylene glycol (PEG 400, Synth®),	3 µL/mL	Insoluble (DME); Soluble (EAE)	NA No (•)(♦)
	Silicone oil (Audaz®)	100 µL/mL	Insoluble (*)	NA
	Mineral oil (Audaz®)	100 µL/mL	Partially soluble (*)	NA
	Coco fatty acid diethanolamine (Audaz®)	100 µL/mL	Partially soluble (*)	NA
	Triethanolamine (Merck®)	100 µL/mL	Insoluble (*)	NA
	Triethanolamine lauryl sulphate (Audaz®)	100 µL/mL	Partially soluble (*)	NA
	Acetic acid (Ecibra®)	50% v/v	Partially soluble (*)	NA
	Sulphuric acid (Synth®)	50% v/v	Insoluble (*)	NA
	Coco amido propyl betaine (Audaz®)	100 µL/mL	Soluble	Yes (•)
	Polyoxyethylene-20-sorbitan monolaurato - Tween 20 (Audaz®)	20 µL/mL	Soluble	Yes (•)
	PEG 400	3 µL/mL	Soluble	No (•)(♦)
Methanol (ME)	Polyoxyethylene-80-sorbitan mono oleate – Tween 80 (Audaz®)	100 µL/mL	Insoluble (*)	NA
	Sodium lauryl sulphate (Synth®)	0.05% p/v	Soluble	Yes (•)
	Propylene glycol (Reagent®)	100 µL/mL	Soluble	Yes (•)

Table 2. Solvent tested for solubilizing extracts from *Casearia sylvestris* and interference on the neuromuscular preparation (*) discarded solvent. (•) solvent alone. (♦) solved extract. NA, not assayed.

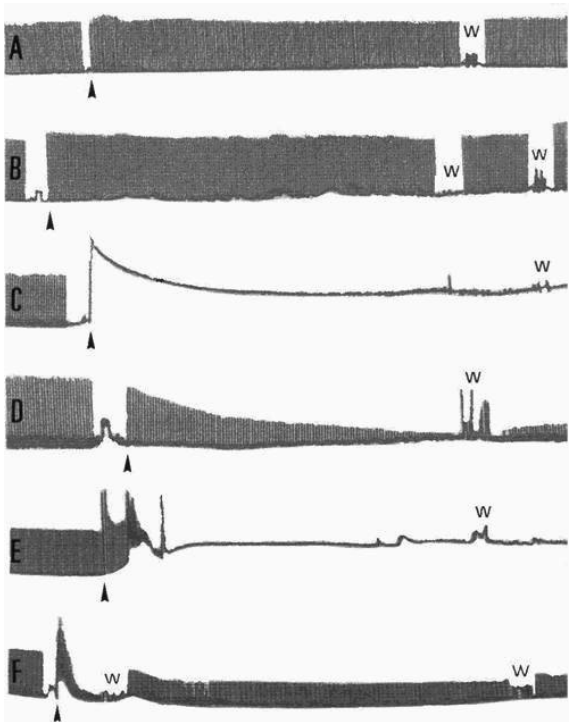

Figure 1. Screenings on mouse phrenic nerve-diaphragm preparations with solubilizing agents: Dimethyl sulphoxide, DMSO (**A**), Polyethylene glycol, PEG 400 (**B**); coco amido propyl betaine (**C**), Tween 20 (**D**), sodium lauryl sulphate (**E**) and propylene glycol (**F**). Note that DMSO (**A**) and PEG 400 presented the better response (PEG > DMSO) along the preparation and during all the observation time (120 min). After washing (W), preparations were kept in nutritional solution only.

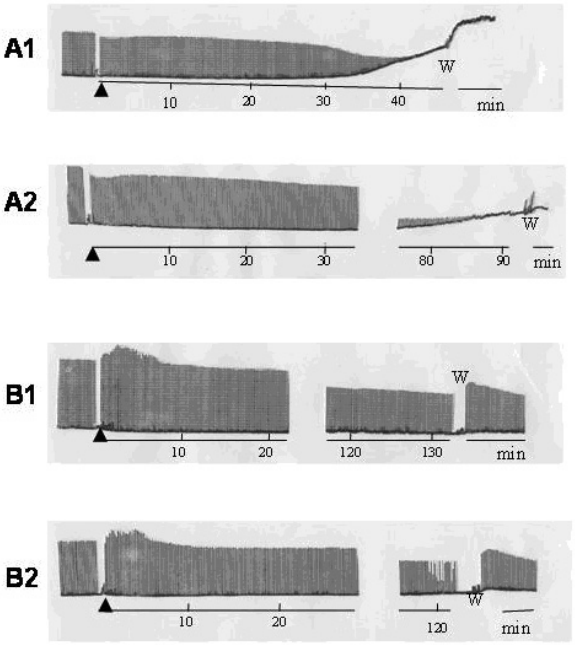

Figure 2. Assays on mouse phrenic nerve-diaphragm preparations with (1) extracts obtained from *Casearia sylvestris* leaves (screenings) and (2) extracts preincubated with BthTX-I (n=6). Arrows: addition time – (**A1**) hexane extract, HE; (**A2**) HE + BthTX-I; (**B1**) dichloromethane, DME; (**B2**) DME + BthTX-I. Note that HE was not able to avoid the neuromuscular blockade induced by BthTX-I, as shown in B1, while a partial protection of DME is viewed in B2. W, washing.

insoluble or partially soluble were discarded (8 of them). Soy lecitin, DMSO, PEG 400, coco amido propyl betaine, Tween 20, sodium lauril sulphate and propylene glycol were selected for further biological assays.

Figure 1 shows the solvents profile under the myographic parameter. Seven solvents were assayed on isolated mouse neuromuscular preparations: soy lecithin (not shown and discarded since it produced a lot of bubbles when added to aerated bath disabling technically the experiment), DMSO (A), PEG 400 (B); coco amido propyl betaine (C), Tween 20 (D), sodium lauryl sulphate (E) and propylene glycol (F). Note that DMSO (A) and PEG 400 presented the better response (PEG > DMSO, practically 3-fold considering the dose used as solvent, 3 µL and 10 µL, respectively) on the preparation and during all the observation time (120 min). Coco amido propyl betaine (C) caused immediate contracture and neuromuscular blockade and by this reason it was rejected; Tween 20 (D) produced progressive muscle paralysis does not showing to be ideal as solubilizer agent in these conditions. Similarly, sodium lauryl sulphate (E) was not appropriate for solubilizing the extracts, since it caused alone, contracture and blockade. Propylene glycol (F) also was discarded although, after washing the preparation the twitch tension was partially recovered.

As shown above, the solubilizing agents selected were DMSO (10 µL/mL) (for HE and DME) and PEG 400 (3 µL/mL) (for EAE and ME). Figs. 2 and 3 show the obtained extract (200 µg/mL) solubilized in their respective solvents (always as screening) or in mixture with 40 µg/mL BthTX-I (n = 6 for each protocol). The HE solubilized with DMSO caused an irreversible neuromuscular blockade (Fig. 2, A1) and also was unable to protect against the paralysis induced by BthTX-I (Fig. 2, A2). The DME solubilized with DMSO produced an initial facil-

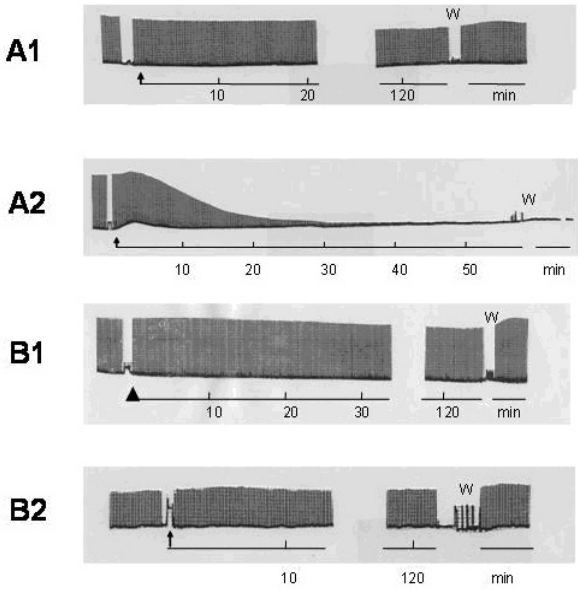

Figure 3. Assays with (1) extracts obtained from *Casearia sylvestris* leaves (screening) and (2) extracts preincubated with BthTX-I (n=6), on mouse phrenic nerve-diaphragm preparations. Register represents the average number of experiments performed. Arrows: addition time – (**A1**) ethyl acetate extract, EAE; (**A2**) EAE + BthTX-I; (**B1**) methanolic extract, ME; (**B2**) ME + BthTX-I. Note that EAE did not avoid the characteristic blockade by BthTX-I, as shown in B1. However, a total protection of ME against the neuromuscular blockade is viewed in B2. W, washing.

itation (increase of the muscular force amplitude) followed by a slow decrease of muscular response (Fig. 2, B1). A similar response was obtained when incubated with the toxin, showing that this extract protected only partially (50%) against the blockade-induced by BthTX-I (Fig. 2, B2), considering the amplitude measured apart from the initial facilitation. The EAE was solubilized with PEG 400 (since DMSO was less efficient than the first, see Table 2) and caused no change in the isolated preparation (Fig. 3, A1). However, when BthTX-I was incubated, the extract was unable to avoid the neuromuscular toxin blockade (Fig. 3, A2). The ME was solubilized in PEG 400 and caused no change in the isolated preparation as shown in Fig. 3, B1. The same pattern was kept when BthTX-I was added (Fig. 3, B2), showing the efficient protection (100%, $p > 0.05$ compared to Tyrode control or $p < 0.05$ compared to myotoxin alone) of the methanolic extract against BthTX-I neurotoxicity.

DISCUSSION

In this study four extracts from leaves of *Casearia sylvestris* were obtained in order to separate the several constituents present in the plant, such as terpenes and chlorophils (hexane extract); chlorophils and components with low polarity (dichloromethane extract); low chlorophil content components (ethyl acetate extract); flavonoids with high polarity and phenolic acids (methanolic extract). Such diversity related to polarity makes difficult the miscibility of these compounds with saline or nutritive solutions required in certain isolated preparations. Thus, an important step is the solubilization of these extracts before their use in animals.

This work focused the choice of solubilizing agents for vegetal extracts, since solubility is often a limiting factor when testing new compounds in animal experiments [2]. Many pharmacological assays with plants considered as medicinals have been carried out and this aspect is underestimated or simply not considered by authors. To find an agent able to solubilize extracts from plants is advantageous since it is expected to improve their pharmacological potential, but it must cause no interference on the basal response of isolated preparations [10] or even on the human organism. We demonstrated the efficacy of polyethylene glycol (PEG 400) on both conditions. Also the DMSO showed good efficiency as a solubilizing agent, but less efficient than PEG 400 in the neuromuscular junction. In fact, DMSO and PEG 400 are known as very good solvents, which are able to dissolve most compounds [11-14]. DMSO is an amphipathic molecule with a highly polar domain and two apolar groups, making it soluble in both aqueous and organic media [13].

PEGs are polymers of ethylene oxide and water and their ethers. They vary in consistency from liquid to solid, depending on the molecular weight, indicated by a number following the name. They are used as surfactants, dispersing agents, solvents, ointment and suppository bases, vehicles, and tablet excipients [15,16]. Their various applications are widely reported in the literature, for example, as substrate by molecular biologists to fuse plasma membranes of cells suspended in aqueous medium [17], to repair the cut ends of an invertebrate-myelinated central nervous system axon in the earthworm; as solubilizing agents [2], to solubilize plasmid DNA in selected polar organic solvents without using cationic polymers and surfactants. PEGs are expected to have a wide range of applications for gene therapy including DNA vaccine [18], and more recently in the nanotechnology area, in-

cluding nanomedicine [11,19], being considered an important compound in biomedical and biomaterial areas.

Bothropstoxin-I which causes an irreversible neuromuscular blockade [7-9] was used for evaluating the potential medicinal effects of these different *C. sylvestris* extracts. Clearly, only methanol extract was efficient against myotoxin paralysis at least in this animal model. These results open the following questions: how many plant extracts of different polarities are evaluated regarding their own effects on the organism? In addition, how many solubilizing agents can be aggressive against the organism? If these questions could not be answered, how can we interpret data? How can we attribute some medicinal value to a given plant?

Such questions were observed in this study. For example, some solubilizing agents, except PEG 400 and DMSO, and/or plant extracts alone produced neuromuscular blockade as BthTX-I does. However, it was also demonstrated that, when PEG is activated with tresyl chloride (in a PEGylation process), it can be covalently attached to amino groups present in proteins [20], confering pharmacological benefits and increasing their potential biomedical and biotechnological applications [21]. In this study, a possible covalent interaction between PEG and BthTX-I was discarded, since in protocols carried out with PEG:BthTX-I mixtures (25 µL:40 µg/mL), the characteristic neuromuscular blockade of BthTX-I was maintained (data not shown).

These data only reinforces the medicinal potential of methanol extract of *C. sylvestris* against a neuroblocker as BthTX-I-type and the possibility to carry out this animal experimental model using PEG 400 and DMSO as solubilinzing agents. Because PEG 400 solubilization was 3-fold higher than DMSO and causes no interference on the experimental model, we emphasize its use as solubilizing agent.

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