Organic solvents can influence acetylcholine neurotransmission in *Caenorhabditis elegans*

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KEY WORDS

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ABSTRACT

Background: Identification of novel drugs by bio-prospecting natural products like various parts of the plants, or other extracts and drug discovery requires differential fractionation with various organic solvents followed by their concentration through evaporation under nitrogen gas, which is a standard practice.
Purpose: Determination of contribution of vehicle control of organic solvents (chloroform, ethanol, ethyl acetate and n-hexane) processed in the similar manner in the modulation of acetylcholine(ACh) neurotransmission in *Caenorhabditis elegans*, Aldicarb induced paralysis assay.
Methods: The organic solvents concentrated as described in background was used to identify their contribution in ACh modulation through ACh esterase inhibitor, Aldicarb, treatment of *C. elegans*, which leads to time dependent paralysis of the worms.
Results: The vehicle, organic solvents, control itself bestows modulation of acetylcholine release as Aldicarb resistance in *C. elegans*.
Conclusion: Given the exorbitant cost and time taken for drug discovery, identification of efficacy of bioactive molecules fractionated through organic solvents and concentrated under nitrogen gas should have appropriate vehicle control as described above to avoid the rate of false positives. This is universally applicable whether the drug is chemically synthesized or purified from natural products.

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Introduction

Bioprospecting of natural products, identification of single bioactive molecules from the natural products is a very expensive, time consuming, but one of the most successful model of drug discovery for various diseases as seen in the discovery of anticancer drugs Taxol and Camptothecin from their respective tree barks [1]. *Caenorhabditis elegans* is an excellent model for preclinical drug discovery [2]. *C. elegans* has been used for identification of various drugs [2] and antimicrobials from Actinomycetes [3].

C. elegans has significantly contributed to the understanding of synaptic structure and function. The historical chemical (Ethyl methyl sulfonate-EMS) genetic mutagenesis by the founding father of the *C. elegans* system, Sydney Brenner [4], followed by screening for the simple, elegant, un-co-ordinated (*unc*) movement phenotype to identify the specific genes that play a role in neurotransmission, has unravelled the mechanism of neurotransmission and its components. Further, aldicarb sensitive/resistant mutants have shed light on acetylcholine neurotransmission and decipher the synaptic structure and function at the neuromuscular junction (acetylcholine is the major neurotransmitter system for movement in *C. elegans*) [5]. This has led to the deciphering of the same in the higher organisms including humans.

C. elegans is a very good model for preclinical drug discovery, especially neurodegenerative diseases like Alzheimer disease [6–7] for which the current treatment is inhibition of the excitatory neurotransmitter, acetylcholine, degrading enzyme, acetylcholine esterase. One of the very simple, yet powerful assay for identification of acetylcholine release modulation at the neuromuscular junction of *C. elegans* is the utilization of acetylcholine esterase (AChE) inhibitor, aldicarb [5,8,7]. Because of the AChE inhibition, acetylcholine accumulates at the neuromuscular junction (NMJ) leading to continuous activation of the ionotropic ACh receptors in the muscle leading to paralysis. This acute Aldicarb induced paralysis (AIP) follows a time course up to 120 minutes. The compound treatment or gene mutants which accelerates AIP are stimulating

and increasing ACh release (aldicarb hypersensitive- al^h), while the ones which slows down AIP is modulating/reducing ACh release (aldicarb resistance- al^r).

During, any bio-prospecting or drug discovery process the extracts/compounds are differentially fractionated with organic solvents, ethanol, n-hexane, chloroform and ethyl acetate for enrichment and to obtain pure bioactive molecule eventually, followed by concentration through evaporation of organic solvents under Nitrogen gas. During this process, only the almost dry compound(s) will be present. For example, the antimalarial drug, artemisinin, is purified using ethanol and/or n-hexane[9]. Similarly, forskolin, the cAMP activating compound extraction from the roots of *Coleus forskohlii* involves organic solvent extraction[10]. The Anticancer drug, taxol yield could be increased with organic solvents, like dibutylphthalate extraction[11]. All these emphasize the importance of organic solvent extraction to obtain drugs. While these are for increasing the yield of the established drugs, bioprospecting for new drug discovery is completely different. Bioassay is the only predictor of the active molecule.

Here, we address the significant (false) positive contribution of the organic solvents utilized for extraction and concentration of various compounds in the aldicarb assays of the *C. elegans* system as a proof of concept so that caution could be exercised given the enormous cost and the time taken for drug discovery.

Methods

Wild type N2 worms, Aldicarb (Sigma Aldrich), Chloroform, n-hexane and ethyl acetate are from Merck (analytical grade-EMPLURA). Ethanol (99.9% pure) is from Changshu Hongsheng Fine Chemicals,China.

Solvent

4 ml of each solvent (Ethyl acetate, hexane, Chloroform and ethanol) was evaporated under Nitrogen gas in glass tubes in BiotageTurboVap –LV- Concentrator, and the dried remnants was dissolved in 700ul of 85% aqueous ethanol. 40 ul of this was mixed with 160ul of M9 buffer and spread on the OP50 lawn containing NGM plates (35 mm) dried for 30 minutes and used.

Solvent treatment

Synchronous population of wildtype N2 worms grown by standard conditions (Brenner, 1974) at 20°C, obtained through timed egg laying, were exposed to these solvents concentrated and reconstituted in 85% ethanol, starting from embryo stage and the aldicarb assay was carried out on day 3 counting L4 as day0.

Aldicarb assay

Half an hour before use 1mM aldicarb (Sigma Aldrich) in 70% ethanol was spread on fresh nematode growth medium (NGM) plates containing OP50 lawn with and without the solvent (Saharia *et al.,* 2012). Around 30–50, 3 day old worms, counting L4 stage as Day 0, were transferred to these plates and paralysis was followed at 0, 1hr , 2hr and 3hr time points. The assay was repeated three times. Cumulative percentage paralyzed worms (N = 130) at 2 hrs was compared between control and various treatments. By 3 hrs all the worms got paralyzed in control and organic solvents. Statistical analysis was carried out using SigmaPlot 10.

Results

As organic solvents are widely used during the bioprospecting of natural products for novel drug discovery, it becomes imperative to exercise extreme caution due to the long duration in years (more than a decade) and exhorbitant cost. In one such scenario, to our surprise, we find that the organic solvents that are used for differential fractionation for enrichment of bioactive molecules followed by evaporation under the inert nitrogen gas, per se, after evaporation in the same manner and the dried remnants dissolved in 85% ethanol, generally used vehicle control could bring about significant changes in the aldicarb sensitivity profile in *C. elegans*.

When treated from embryo stage onwards, ethyl acetate, chloroform, ethanol and n-hexane induced Aldicarb resistance, al^r, the worms are not motile (moving from one point to other), but wriggle their body (Fig. 1), instead of complete paralysis even after 2hrs of aldicarb treatment. While only the control showed ~30% paralysis after 1 h, all the worms got paralyzed both in control and organic solvents by 3hrs in the presence of aldicarb. In addition, the Aldicarb resistance (Fig. 2) brought about in the organic solvents was statistically significant.

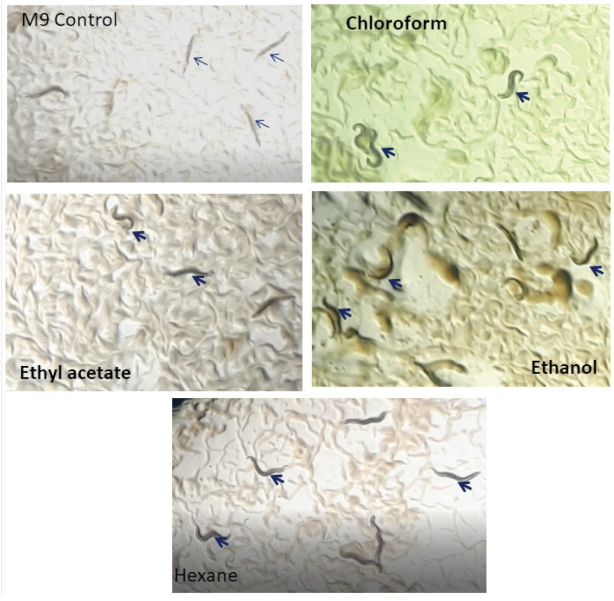

Fig. 1: Differential Aldicarb resistance upon organic solvent treatment

Control – M9 buffer; Chloroform, ethyl acetate, n-hexane and ethanol are the respective solvent treated worms as they appear when placed in Aldicarb. Thin arrows in control indicate the paralysed worms while partially paralysed (which could wriggle and drag, but cannot move fast from one place to another) are indicated by thick arrows at 2hrs of aldicarb treatment.

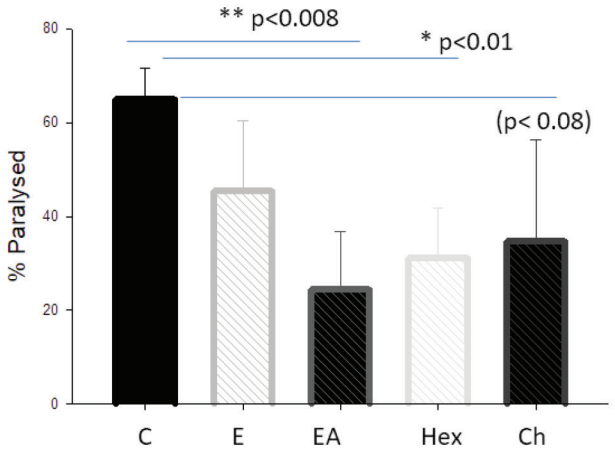

Fig. 2. Organic solvents induced Aldicarb resistance
The Y –axis is Percentage of worms paralyzed at 2hrs. C- Control (M9 buffer); E- Ethanol; EA- Ethyl acetate; Hex- n-hexane; Ch- Chloroform; The total number of worms for each treatment is 130.

Discussion

Organic solvent extraction is one of the time tested method of obtaining bioactive molecules from crude phyto-compounds, other natural products and chemically synthesized entities. Some of the plant derived drugs like Taxol and artemisinin yield could be significantly enhanced through extraction with n-hexane[9] and dibutylphthalate[10], respectively. Further, organic solvents are widely used in the recovery and biotransformation of antibiotics like erythromycin[12].

Differential fractionation with organic solvents like n-hexane, ethanol, chloroform and ethyl acetate is very common in medicinal chemistry, bioprospecting for active biomolecules in the mega cocktail of natural products (Fain *et al*., 2017). The false positive effects of processed organic solvents which acts as vehicle control in the aldicarb assays (Fig. 1 and Fig. 2) of *C.elegans* (Saharia et al., 2012) is implicating the necessity for extreme caution in all drug screening bioassays, given the enormous cost and the time frame of more than a decade and a half for drug discovery.

Further, deciphering the mechanism of such vehicle induced aldicarb resistance, especially, wriggling in the same place and not rigid paralysis could provide fundamental insights into neurotransmission, especially of acetylcholine.

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Authorship contribution

VJD carried out all the *C.elegans* experiments. RL did the selection of various organic solvents and their concentration. JRS designed, supervised, analyzed the data and wrote the manuscript.

Ethical statement

Ethical approval is not required.

Conflict of interest

None

ICMJE Guidelines-This article complies with International Committee of Medical Journal Editor's uniform requirements for manuscript.

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