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Planarian toxicity fluorescent assay: A rapid and cheap pre-screening tool for potential skin irritants

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Abstract

Here we report a new planarian (*Dugesia lugubris*) fluorescent assay as a rapid and cheap pre-screening tool to predict strong skin irritants. Our aim was to provide a simple and cost-effective *in vivo* method that avoided use of higher vertebrates. Adapting previously reported methods for planaria mobility alongside an acute toxicity assay, different irritants at five concentrations (0.1%, 0.05%, 0.025%, 0.01% and 0.005% w/v) were tested but both methods failed to discriminate the irritation potential of the test compounds. Therefore, a new alternative fluorescence assay was developed, hypothesising that increasing damage from the irritant to the planarian outer protective membrane will increase accumulation of sodium fluorescein in the flatworm. Fourteen test chemicals were selected representing strong, moderate, mild and non-irritants. In general, results showed increasing sodium fluorescein accumulation within planaria following acute exposure to increasingly strong skin irritants; on exposure to the strong irritants, benzalkonium chloride, citronellal, methyl palmitate, 1-bromohexane and carvacrol, fluorescence within the planaria was significantly greater ($P<0.05$) than the negative controls and the common non-irritants PEG-400, dipropylene glycol and isopropyl alcohol; fluorescence values of planaria tested with negative controls and non-irritants were not significantly different. For all test compounds, fluorescence intensity of the planaria was compared with literature primary irritation index data and generated a statistically significant ($P<0.005$) Pearson correlation (r) of 0.87. Thus, the planarian fluorescent assay is a promising tool for rapid early testing of potential strong skin irritants, and non-irritants, and avoids use of higher vertebrate models.

Keywords: planaria; skin irritation; mobility; acute toxicity; fluorescence; invertebrate

1. Introduction

The design of formulations that contact human tissues requires toxicological testing and, in particular, topically applied formulations require skin irritation testing. Numerous methods have been used to evaluate the irritation potential of chemicals towards human tissues such as eyes, skin, nose or vagina. The classical Draize test used rabbits to assess the ocular and skin irritation of cosmetics and personal care products (Draize et al., 1944). Due to ethical as well as scientific concerns (Callens et al., 2001; Sharpe, 1985), alternative tests have been sought. For skin irritation testing, *in vitro* methods are available including the commercially available Episkin, Epiderm and Zenskin cell culture models (Ahn et al., 2010; Graham et al., 2018) although such tissue equivalents do not entirely recapitulate the in vivo tissue – for example lacking blood or lymph circulation or providing an incomplete tissue physiology. Numerous guidance documents exist for skin irritation testing, for example from the European Centre for the Validation of Alternative Methods (ECVAM) or the Organisation for Economic cooperation (OECD). Typically, the guidance specifies the skin equivalent to be used and its integrity testing, the numbers of replicates, duration of study etc., which requires specialist and relatively expensive laboratory services. Contrarily, planaria are readily available at a low cost and are easily cultured and maintained in a laboratory in artificial pond water (Gentile et al., 2011) and thus may offer a low cost *in vivo* alternative model to rapidly screen potential skin irritants prior to undertaking extensive regulatory studies.

Alternative *in vivo* tests have been previously sought, typically employing lower order models. For example, Adriaens and Remon. (1999) reported a slug mucosa irritation test (SMIT) to characterise toxicological and irritation properties of various pharmaceutical materials and formulations for ocular, nasal and vaginal drug delivery, as well as some consumer products (Callens et al., 2001; Dhondt et al., 2005; Lenior et al. 2011, Lenior et al. 2013). The SMIT has also been adapted by others; for example, Forbes et al. (2011) tested silicone elastomer gels for vaginal drug delivery and in a series of studies; we have previously used a SMIT to evaluate the irritation potential of ocular formulations (Khutoryanskaya et al., 2014; Al Khateb et al, 2016), mucoadhesive polymers for nasal drug delivery (Porfiryeva et al., 2019) and mucoadhesive nanoparticles for intravesical drug delivery (Kaldybekov et al., 2019). Other invertebrate *in vivo* models used in toxicological testing include *Brachionus calyciflorus* rotifers for screening the toxicity of various penetration enhancers on ciliated epithelium (Adriaens et al., 1997) and *Caenorhabditis elegans* nematodes (Hunt, 2017). As with our

present study, these models were proposed as a pre-screening tool prior to a time-consuming and relatively costly regulatory study.

Planaria are a freshwater-living flatworms commonly used as a model in developmental and regeneration research (Gentile et al., 2011b). As advanced invertebrates with a primitive brain having features similar to the vertebrate nervous system, planaria are used in neuropharmacology to predict the neurotoxicity of test substances (Hagstrom et al., 2016). They have a well-developed enzymatic system and so have been used to study organophosphorus pesticide toxicity (Hagstrom et al., 2018), the cytotoxic, genotoxic and mutagenic effects of metals (Pra et al., 2005) and for environmental toxicological studies (Li, 2008; Wu and Li, 2018). Importantly for the current work, planaria have a simple but well-characterised epidermal membrane (made of ciliated cells) that acts as the first point of contact between the worm and a foreign substance (Azimzadeh and Basquin, 2016).

The Globally Harmonised System (GHS) aims to consolidate global differences by classifying hazardous materials according to their health, environmental and physical hazards (Winder et al., 2005). For skin irritants, the GHS system draws on human experience, structure-activity models or the Primary Irritation Index (PII) caused by a chemical, derived from *in vivo* studies following OECD guidelines. The PII test applies 0.5mL or 0.5g of test substance to intact animal skin for up to 4 hours. For each animal, the dermal response scores (sum of the scores for erythema formation and oedema formation) at 24, 48, and 72 hours post exposure are recorded to generate a mean irritation score per time point (Bagley et al., 1996; Marzuki et al., 2019). PII scores < 1.5 are considered as non-irritant, PII $\geq 1.5 < 2.3$ corresponds to mild irritants, PII $\geq 2.3 < 4.0$ show moderate irritants whereas PII values ≥ 4 are seen with strong irritants.

The purpose of this study was to develop a rapid and cheap pre-screening tool to reduce the use of complex cell culture, organ and animal models. Here, we have used *Dugesia lugubris* as a model to predict human skin irritation of test substances by measuring the uptake of a fluorescent marker (sodium fluorescein) into the flatworms following exposure to various irritants; our hypothesis is that increasingly toxic substances will disrupt the barrier function of the planarian epidermis hence leading to greater accumulation of sodium fluorescein inside the worm.

2. Materials and methods

2.1. Chemicals and reagents

Benzalkonium chloride (BKC), glycerol, parafluoro aniline (PFA), polyethylene glycol-400 (PEG-400), carvacrol, isopropyl alcohol, decanol, tri-isobutyl phosphate, terpinyl acetate, sodium fluorescein and agarose were purchased from Sigma-Aldrich (UK). Benzyl alcohol, citronellal, linalyl acetate, 1-bromohexane and methyl palmitate were purchased from Fischer Scientific, UK. Instant ocean salt was from Aquarium Systems (UK).

Table 1. Test articles with CAS number, GHS classification and in order of PII values

Test article	**CAS number**	**GHS classification**	**PII***
Polyethylene glycol-400 (PEG-400)	25322-68-3	Not classified (Non-Irritant)	0.0
Dipropylene glycol	25265-71-8	Not classified (Non-Irritant)	0.33
Isopropyl alcohol	67-63-0	Not classified (Non-Irritant)	0.78
Benzyl alcohol	100-51-6	Category 3 (Mild Irritant)	1.56
Terpinyl acetate	80-26-2	Category 3 (Mild Irritant)	2.0
Tri-isobutyl phosphate	126-71-6	Category 3 (Mild Irritant)	2.0
Decanol	112-30-1	Category 2 (Moderate irritant)	3.33
Parafluoro aniline (PFA)	371-40-4	Category 2 (Moderate irritant)	3.5
Linalyl acetate	499-75-2	Category 2 (Moderate irritant)	3.67
Citronellal	106-23-0	Category 1B (Strong irritant)	4.0
1-Bromohexane	111-25-1	Category H315 (Strong irritant)	4.0
Carvacrol	115-95-7	Category 1B (Strong irritant)	4.2
Methyl palmitate	112-39-0	Category H315 (Strong irritant)	4.56
Benzalkonium chloride (BKC)	63449-41-2	Category 1B (Strong irritant)	6.54

*PII < 1.5 = Non irritant, PII ≥ 1.5 < 2.3 = mild irritants, PII ≥ 2.3 < 4.0 = moderate irritants, PII ≥4 as strong irritants.

2.2. Test organisms

Planaria (*Dugesia lugubris*) were purchased from Blades Biological Ltd (Kent, UK). The animals were maintained in artificial pond water (APW) (0.5 g of instant ocean salt in 1 L of Milli-Q water), prepared by the method of Cebrià and Newmark (2005) at room temperature. Animals were fed raw chicken (cut into small pieces), at a quantity sufficient to feed the planaria once a week. The pond water was changed every 48 hours.

2.3. Mobility assay

Planarian mobility was assessed using the method previously described by Mei-Hui-Li (2012). Five concentrations of each test substance were prepared (0.1, 0.05, 0.025, 0.01 and 0.005 % w/v) by dissolving the test substance in APW. Where the irritants were not directly soluble, these compounds were first dissolved in dimethylsulphoxide (DMSO) before adding to APW

followed by vigorous stirring until a clear solution was obtained. For these, the final volume of DMSO was maintained at 1% (v/v) to avoid irritation from the solvent itself (Pagán et al., 2009). An individual planarian was placed into a glass Petri dish containing 15 mL of the test solution or into APW or APW with 1% (v/v) DMSO as a control. The petri dish was placed over 1 cm grid graph paper and a video recorder was used from above. After 5 minutes' equilibration, planarian mobility was recorded as the number of times they crossed a grid line over the next 5 minutes. For each solution, mobility was assessed for 3 planaria and data are represented as the mean ± SD.

2.4. Acute toxicity assay

The toxicity of the test substances to planarian was assessed by the method previously described by Mei-Hui-Li (2012) with some modifications. Five concentrations of each test substance were prepared (0.1, 0.05, 0.025, 0.01 and 0.005 % w/v). For each concentration, five animals were added to a Petri dish containing 25 mL of the test solution and each study was conducted in triplicate. Acute toxicity was assessed over 96 hours with planaria inspected every 24 hours; those without detectable movement were assumed dead and removed from the test solution. Again, APW or APW with 1% (v/v) DMSO was used as a control.

2.5. Fluorescence intensity (FI) test

The protocol was informed by the mobility and toxicity studies and so test substances at 0.1% (w/v) were employed with planaria exposure of 1 min followed by washing with APW for a further 1 min. The planaria were then placed in a 0.1% (w/v) solution of sodium fluorescein in APW for 1 min. Finally, the planaria were washed with APW (15 mL) for 1 min to remove excess sodium fluorescein adsorbed to the outer worm surface. The test animal was then immobilised by embedding it in 2% agarose solution following the protocol of Shen et al. (2018) with minor modifications. In brief, a planarian was transferred onto a microscopic slide (VWR, UK), after which few drops of agarose solution were carefully added to cover the whole animal. The slide was immediately placed on ice leading to gelling of the agarose solution, immobilising the test animal. Fluorescence images of individual planaria were collected with a Leica MZ10F stereomicroscope (Leica Microsystems, UK) with Leica DFC3000G digital camera, 1.6× magnification with 160 ms exposure time (gain 2.6×), Gamma = 0.7 and wavelength=519 nm (excitation wavelength). The negative controls were planaria treated only with sodium fluorescein in APW for water-soluble test compounds and sodium fluorescein in

1% DMSO solution (v/v) in APW for the poorly water-soluble test compounds. To quantify sodium fluorescein inside a planarian, the fluorescence of the whole animal was measured using ImageJ (version 1.8.0_112) software and the value obtained normalised by dividing by the area (cm^2) of the individual planarian. All experiments were conducted in triplicate.

2.6. Statistical analysis

Statistical significance for the fluorescence intensity test was determined using one-way analysis of variance (ANOVA), followed by Bonferroni correction using Graphpad Prism software (version 7.0). To correlate the experimental fluorescence intensity values with literature data for skin irritants, a Pearson correlation test was performed.

3. Results and discussion

3.1. Mobility assay

A planarian mobility assay has been previously used to assess neurotoxicity of several substances (Hagstrom et al., 2015) and hence we extrapolated the approach as a tool to assess skin irritants. For this purpose, a range of compounds was selected, spanning known non-, mild-, moderate- and strong-irritants along with control groups in APW alone (for water soluble compounds) or APW with 1% (v/v) DMSO (for poorly water-soluble compounds).

Locomotion was plotted as a function of the concentration of irritant (Figure 1). Planaria movement was invariant with increasing concentrations of the non-irritant PEG-400. Conversely, exposure to the strong irritant carvacrol stopped planaria mobility entirely and at the lowest tested concentration (0.005% w/v). The response to the mild irritant benzyl alcohol indicated some dose-response behaviour, but the locomotion inhibition was more pronounced and at lower concentrations for the other test materials. Indeed, the profiles for benzalkonium chloride (strong irritant) and linalyl acetate (moderate irritant) were indistinguishable over the selected concentration range, and the profiles for tri-isobutyl phosphate (mild) and parafluoro aniline (moderate) were contradictory. Given these confounding results, the mobility test appears unreliable for determining irritation from different classes of irritant and so was not developed further. However, given the sensitivity of planarian mobility to these agents, this approach could potentially identify chemicals that are non-irritant.

3.2. Acute toxicity assay

Acute toxicity was assessed every 24 hours for up to 96 hours of exposure to non-irritants (dipropylene glycol and PEG-400), a mild irritant (tri-isobutyl phosphate), a moderate irritant (linalyl acetate) and a strong irritant (carvacrol). Again, APW alone was used as a control for test compounds soluble in water while APW with 1% (v/v) DMSO was used as a control for those poorly soluble in water and the data was normalised against these; for both controls, all planaria survived the test. As with the mobility assay, the planaria showed no adverse effects on exposure to the non-irritants (dipropylene glycol and PEG-400) whereas exposure to the strong irritant carvacrol was lethal at the lowest dose (0.005% w/v). However, the data for the mild (tri-isobutyl phosphate) and moderate (linalyl acetate) irritants were confounding with planaria not surviving low dose exposure to the mild irritant but were more robust on exposure to higher concentrations of the moderate irritant. It was notable that the effects of exposure to the irritants did not change beyond the first 24-hour exposure period.

The above results illustrate a generic issue of seeking a lower order animal model to screen irritants for human use. Clearly the planarian membrane is extremely simple and fragile compared to, for example, human skin with its robust outer stratum corneum barrier. Whilst tri-isobutyl phosphate is a mild irritant on human skin, as an organophosphorus compound it is used in herbicides and fungicides and is listed by the European Chemicals Agency (ECHA) as “acutely harmful to aquatic organisms” (Eto, 1997; Hendriks et al., 1994). Indeed, an LC_{50} (96h) of 18-22 mg/L is reported for fish and an EC_{50} (48h) of 24 mg/L for aquatic invertebrates (as are planaria), equivalent to 0.0024% w/v and in a similar range to the results shown here in Figure 2. Linalyl acetate is a naturally occurring phytochemical and a principle component of lavender essential oil (Batool et al., 2020). Toxicity data towards marine invertebrates is limited as the compound is volatile (though our experiments were conducted under occlusion) and there is potential for hydrolysis of the ester to liberate some linalool. Notwithstanding these issues, an EC_{50} of 59 mg/L has been reported towards aquatic invertebrates (Daphnia) (Silver Registration Dossier ECHA @ www.echa.europa.eu) indicating that linalyl acetate would be expected to be less harmful to our test animal than tri-isobutyl phosphate, in accordance with the trend in Figure 2.

As with the mobility assay, the planaria acute toxicity assay was unsuitable to predict human skin irritation of our test compounds, other than to potentially identify chemicals that are non-irritant.

3.3. Fluorescence study

The above results clearly demonstrate that both concentration and exposure time to irritants impact the viability of planaria (Hagstrom et al., 2015). Thus, a method was required that is simple, rapid and discriminating and hence short-term exposure (1 min) to low concentrations (0.1% w/v) of irritant followed by 1 min exposure to sodium fluorescein was selected. It is known that sodium fluorescein can penetrate damaged tissue and has been used to assess the extent of injury to human vaginal and eye tissues (Ayehunie et al., 2011; Morrison et al., 2017). Here, we assume that irritation to planaria causes damage to its outer membrane and that such damage will allow sodium fluorescein to enter the animal, with concentrations related to severity of damage from the irritant. To normalise the results, total fluorescence is expressed as per cm^2 of the planaria surface area. The protocol used to evaluate penetration of fluorescein into planaria is schematically shown in Figure 3 and Figure 4 shows exemplar fluorescent images.

Fourteen test substances were evaluated, five strong irritants and three from each class of moderate-, mild- or non-irritants, alongside controls of 1 minute exposure to APW with or without DMSO, and an untreated planarium to determine autofluorescence. Autofluorescence was negligible and fluorescence uptake into the control planaria was minimal following short term exposure to the dye; uptake of 4 a.u./cm^2 from APW with DMSO indicates no substantive damage to the outer membrane. Following exposure to the non-irritants, fluorescence was not signficantly different to that of the control animals, with greatest intensity seen for PEG-400 exposure at 5.0 ± 2.3 a.u./cm^2. Data for the mild-irritants was also not signficiantly different to that of the controls, with benzyl alcohol causing fluorescence of 4.6 ± 3.9 a.u./cm^2 which rose to 10.0 ± 5.8 a.u./cm^2 with tri-isobutyl phosphate. The increasing trend in fluorescence intensity continiued with the moderate irritants, ranging from decanol (9.5 ± 3.2 a.u./cm^2) to linalyl acetate (20.0 ± 3.0 a.u./cm^2). It is notable that in our acute toxicity assay (Figure 2), linalyl acetate appeared less harmful to the planaira than expected from its GHS classification or PII value (Table 1) but in the fluorescence assay is shown to be a moderate irritant close to the borderline with the strong irritant classification. As a strong irritant, citronellal (18.0 ± 6.2 a.u./cm^2) gave similar fluorescence to linalyl acetate, whilst methyl palmitate and bromohexane

showed similar F.I's, (24.8 ± 4.1 a.u./cm^2 and 22.6 ± 6 a.u./cm^2 respectively). Both benzalkonium chloride (53.0 ± 11.2 a.u./cm^2) and carvacrol (48.0 ± 12.5 a.u./cm^2) caused catastrophic damage to the membrane resulting in significantly higher fluorescence intensities than all other tests. The result show that all the strong irritants gave significantly greater fluorescence intensities (P at least <0.05) than the non irritants and controls.

3.3 Correlation between human Primary Irritation Index (PII) and planaria Fluorescence Intensity (FI)

Despite the dissimilar membrane structures, we sought to correlate the membrane damage caused to planaria by the irritants with literature data that has been used in predictions of human skin irritation. The Primary Irritation Index (PII) is from a patch test on albino rabbit skin and is a composite score of the number and severity of erythema / oedema to a test substance (Chakrabarti et al., 2018). Figure 6 shows test substances whose PII values were available in the literature (n=12) plotted against our experimental fluorescence intensities (per cm^2); due to catastrophic membrane damage (Figure 4), fluorescence values following treatment with BKC and carvacrol do not represent uptake of dye through a membrane and so were excluded. Clearly our fluorescence intensities within the planaria increase with increasing values of the primary irritation index. The Pearson's correlation (r) value (0.87) shows a statistically significant ($p<0.005$) positive correlation. This correlation suggests that this assay can serve as a rapid pre-screening tool to identify the likely category or irritation potential of compounds towards human skin. Though further study is merited using a broader library of compounds, the speed and simplicity of the assay provides an attractive alternative to tests using higher order vertebrates.

4. Conclusion

Our studies demonstrate planaria mobility or acute toxicity testing poorly discriminate between categories of skin irritants but may demonstrate materials that are either non-irritating or act as very strong skin irritants. The planarian fluorescence assay is more discriminating with a direct correlation between fluorescence uptake into worms following short term exposure to irritants and literature reported irritation defined by their primary irritation index values. The fluorescence assay offers a rapid *in vivo* screening tool employing a model that is readily available and easy to maintain and which could act as a pre-screening method to inform subsequent sophisticated and costly assessments of skin irritants. Potentially this assay could

be further extended to test irritation properties of various chemicals towards ocular, nasal and vaginal mucosa.

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Captions to Figures

Figure 1. Effect of test compound concentrations on planaria locomotion. Cumulative movement = Number of times a planarian crossed the 1cm gridline during the 5-minute study. Data are represented as mean ± standard deviation (n = 3). The dashed/dotted lines are for the guidance of the eye only.

Figure 2. Acute toxicity study showing the effects of selected irritants on planaria survival. Data are represented as mean ± standard deviation (n = 15).

Figure 3. Illustrative diagram depicting the planaria fluorescence assay: (a) planarian in a solution of test substance (0.1 % w/v) for 1 min; (b) planarian washed in fresh APW for 1 min; (c) planarian in a solution of sodium fluorescein (0.1 % w/v) for 1 min; (d) planarian washed in fresh APW for 1 min to remove surface absorbed dye; (e) planarian placed on microscopy slide and covered with agarose sol; (f) slide placed on ice for 5-10 mins to allow agarose to solidify; (g) fluorescence assessed microscopically

Figure. 4. Exemplar fluorescent images of auto fluorescence (a), negative control without and with DMSO in sodium fluorescein solution (b and c) and after planaria being exposed to PEG-400 (d), dipropylene glycol (e), isopropyl alcohol (f), terpinyl acetate (g), tri-isobutyl phosphate (h), benzyl alcohol (i), linalyl acetate (j), decanol (k), para-fluoroaniline (l), citronellal (m), carvacrol (showing disintegration of lower part of the planaria body) (n), benzalkonium chloride (also showing evidence for catastrophic membrane damage) (o), 1-bromohexane (p), and methyl palmitate (q). Scale bar is 1mm

Figure 5. Fluorescence intensity (per cm^2) of individual planaria exposed to different test substances. Data are expressed as mean ± standard deviation (n = 3). Statistically significant differences are given *as: **** represents $p<0.0001$, *** $p=0.0005$, while ** and * $p<0.05$ and ns = not significant*.

Figure 6. Correlation between the Primary Irritation Index of compounds (PII) and the Fluorescence Intensity (FI) values obtained in this study. Pearson's correlation value $r = 0.87$ ($p<0.005$).

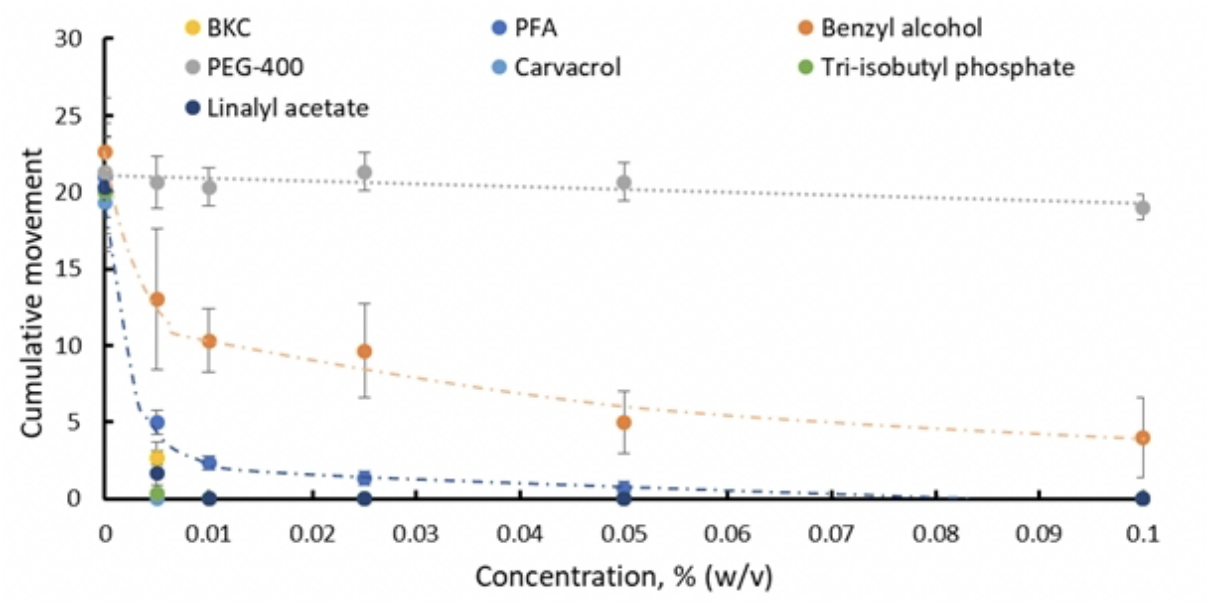
BKC
PFA
Benzyl alcohol
PEG-400
Carvacrol
Tri-isobutyl phosphate
Linalyl acetate
Cumulative movement
30
25
20
15
10
5
0
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09
0.1
Concentration, % (w/v)

Figure 1

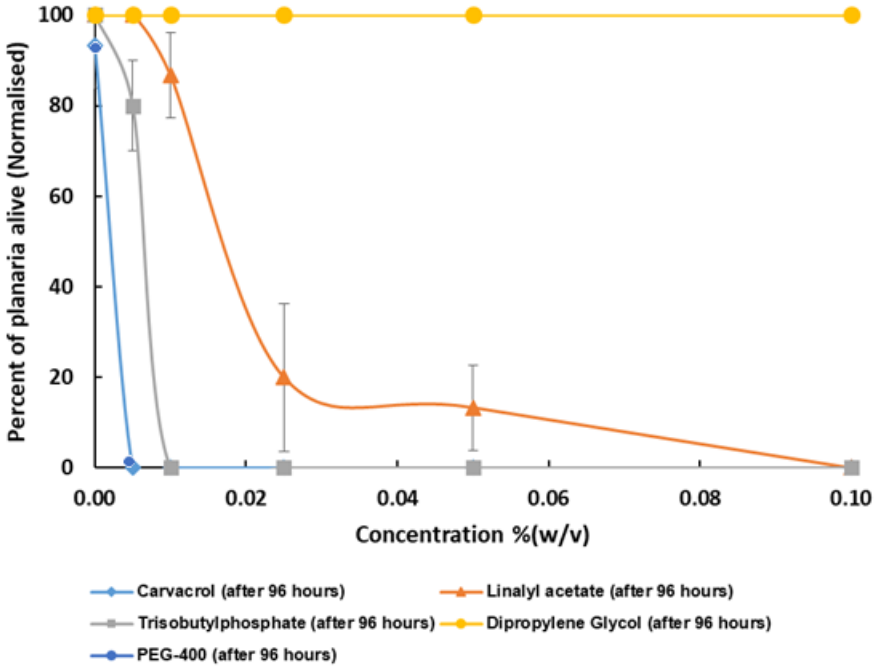
Percent of planaria alive (Normalised)
100
80
60
40
20
0
0.00
0.02
0.04
0.06
0.08
0.10
Concentration %(w/v)
Carvacrol (after 96 hours)
Linalyl acetate (after 96 hours)
Trisobutylphosphate (after 96 hours)
Dipropylene Glycol (after 96 hours)
PEG-400 (after 96 hours)

Figure 2

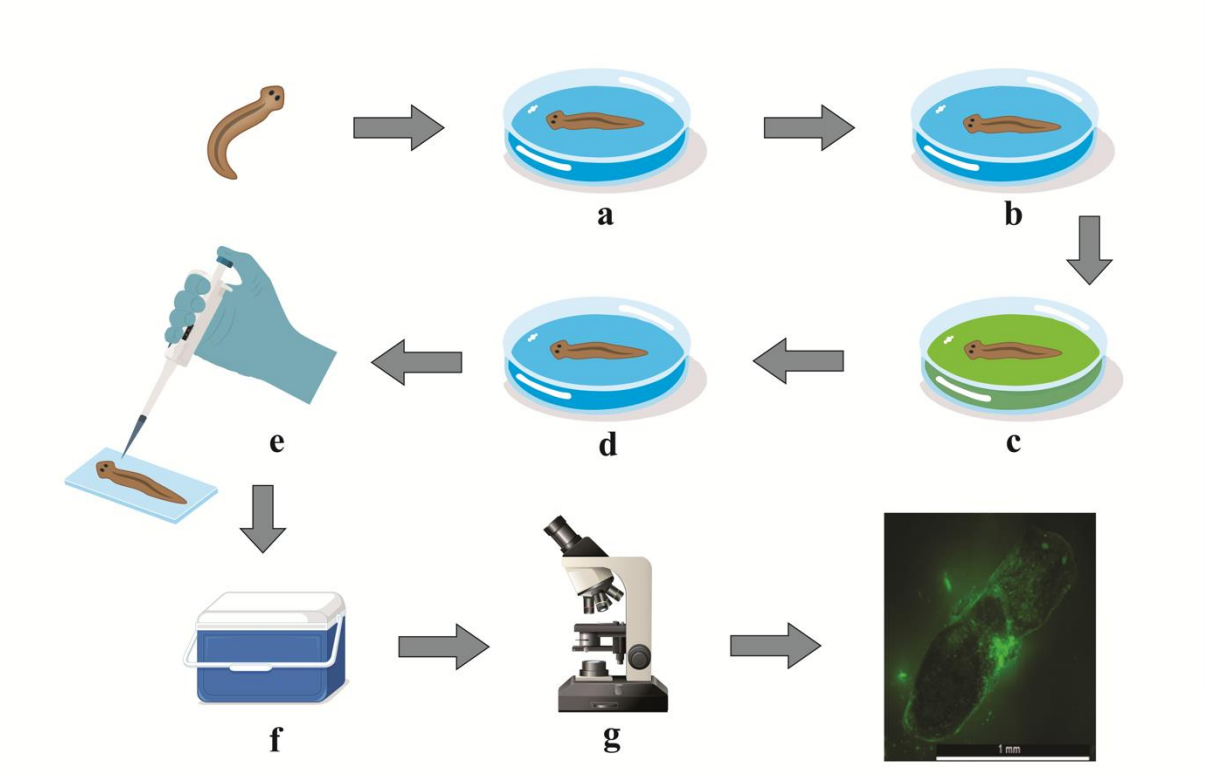

Figure 3

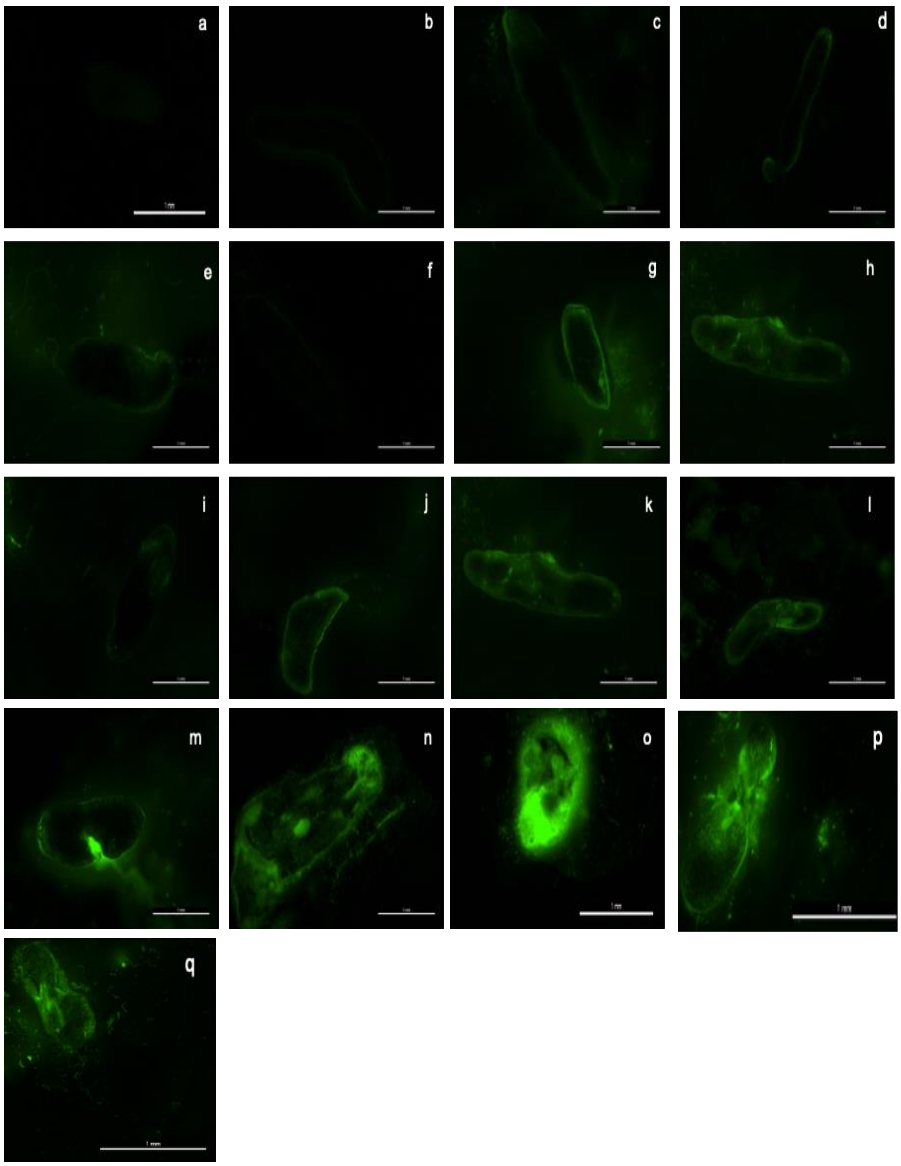

Figure 4

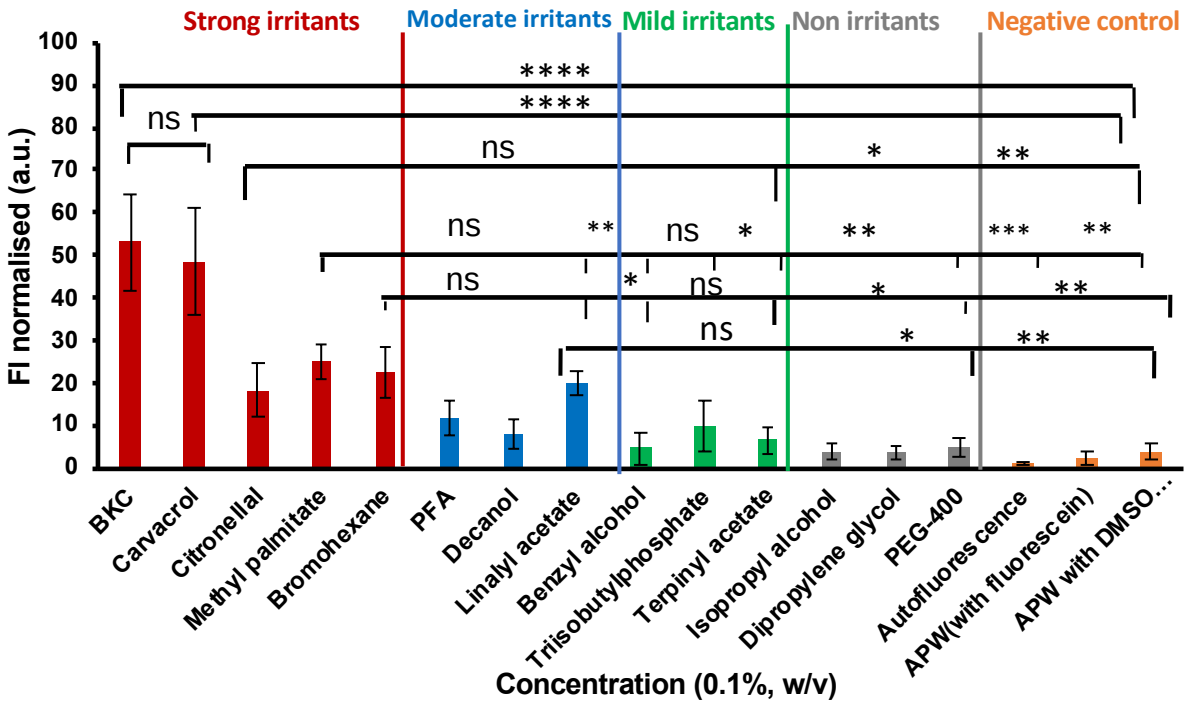
Strong irritants
Moderate irritants
Mild irritants
Non irritants
Negative control
FI normalised (a.u.)
100
90
80
70
60
50
40
30
20
10
0
****
****
ns
ns
*
**
ns
**
ns
*
**
***
**
ns
*
ns
*
**
ns
*
**
BKC
Carvacrol
Citronellal
Methyl palmitate
Bromohexane
PFA
Decanol
Linalyl acetate
Benzyl alcohol
Trisobutylphosphate
Terpinyl acetate
Isopropyl alcohol
Dipropylene glycol
PEG-400
Autofluores cence
APW(with fluoresc ein)
APW with DMSO…
Concentration (0.1%, w/v)

Figure 5

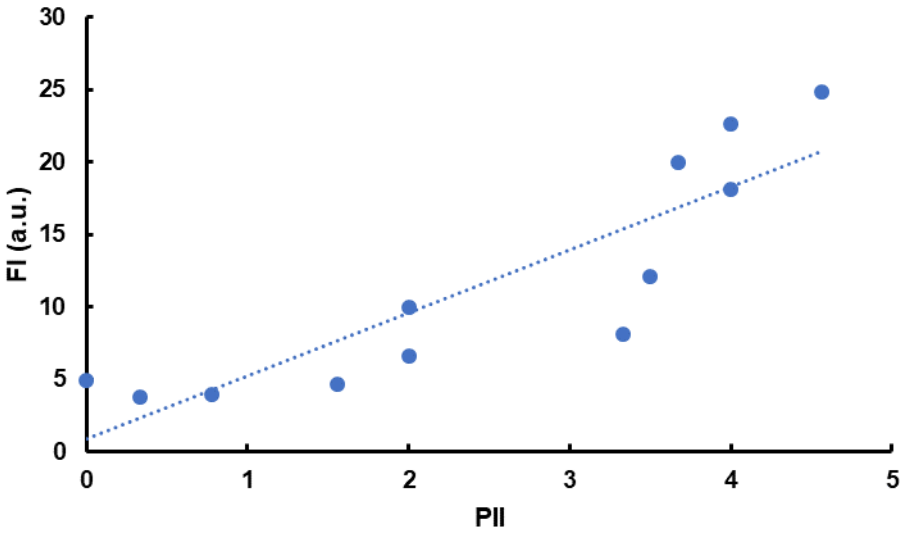

Figure 6