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Evaluation of the Chemotactic and Genotoxic Effects of Selected Cycloartane-type Glycosides on *Caenorhabditis elegans*

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Abstract: *Astragalus* L., is the most populated plant genus on the planet. It has found use in folk medicine to treat many different forms of ailment. However, the toxicity studies of the genus and the chemicals isolated from it, are scarce and the toxicological profile is largely unknown. *Caenorhabditis elegans* has been used in multiple scientific disciplines. Its ease of use, genetic similarity to humans in many pathways, connectome and nervous system and resistance to environmental stress make it among the best organisms for toxicity testing. The present study used *C. elegans* chemotaxis responses, and micronucleus generation, to evaluate the potential neurotoxicity of some selected cycloartane-type glycosides; astragalosides IV **(1)** and VI **(2)**, astrasieversianin II **(3)**, baibutoside **(4),** and macrophyllosaponins B **(5)** and D **(6)**, in the short- and long-term domain, along with long-term genotoxicity testing. The results indicate long-term chemotaxis inhibition by compounds **1, 2, 3** and **5**, whereas only compound **3** inhibited chemotaxis in the short-term. Compound **1** was found to significantly increase micronucleus generation at the highest tested dose. The study demonstrates the usefulness of the Nematode Chemotaxis and *in vivo* Micronucleus Assays for the determination of the toxicity of natural products, while illuminating the points of consideration that future studies may be designed to address.

Keywords: *Caenorhabditis elegans;* chemotaxis; toxicity testing; experimental toxicology; micronucleus; cycloartane-type glycosides.

1. Introduction

Astragalus L. is a 3,000-membered genus of plant species, comprising the most populated genus of plant species currently known to humankind [1]. The genus is represented in Cyprus with 12 species and one subspecies, of which *Astragalus cyprius* Boiss. and *A. macrocarpus* ssp. *lefkarensis* Kirchhoff & Meikle are endemic [2]. Although used in the folkloric medicine of the Middle-East for a very long time and being a focus of many botanical and pharmacognostic studies, detailed toxicity analyses of *Astragalus* species in general, and *Astragalus* species endemic to Cyprus in particular, are not

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forthcoming. This genus has been in folk medicine usage all across the globe for a very long time for a wide variety of ailments [3–6].

Cycloastragenol, a cycloartane-type aglycone of the glycosides isolated from the *Astragalus* species, has been indicated to induce telomerase activation and subsequent telomere elongation [7]. As telomere elongation is one of the many factors participating in cellular proliferation, there is the possibility that *Astragalus* species may have tangible effects on the development and advancement of neoplasms [8,9]. Studies done on the immune response to *A. membranaceus* revealed that upon exposure to the extract, there were signs of marked age-reversal in the behaviour of the immune system, with the primary hallmarks of reduced CD8+ T cells and NK cells after half a year to a full year of exposure [10]. Moreover, some astragalosides have been found to have immunostimulatory and anti-inflammatory effects [11].

Caenorhabditis elegans (C. elegans) is a free-living, non-pathogenic nematode approximately 1 mm in length [12]. The typical nematode is an unsegmented vermiform organism that exhibits bilateral symmetry [13]. The organism features two genders: About one to three in a thousand are males, whereas the rest are hermaphrodites. As a result of their small size, they possess neither a circulatory nor a respiratory system. They do possess a gastrointestinal system suited especially to the consumption of bacteria. *C. elegans* has long been a staple model organism for the studies of organogenesis and developmental biology. Its small size makes it easy to handle, whereas the limited number of cells (959 in the hermaphrodite and 1,031 in the male) [14] makes the developmental tracing of cell lines easy. It is one of the smallest animals to have a nervous system [15] and the entirety of its connectome has been mapped, the first organism to have so [16]. Moreover, the eventual fate of every *C. elegans* cell has been elucidated [16].

The reproduction of *C. elegans* is also straightforward: The hermaphrodites can self-fertilise or use male sperm. Should the hermaphrodite self-fertilise, the number of the progeny is in the vicinity of 300, whereas fertilisation by a male may result in progeny up to 1,000. The hermaphrodite produces its sperm in the last larval stage, L4, after which it will only produce oocytes. If both hermaphroditic self-sperm and male sperm are present within the spermatheca, the oocytes will show preference for the male sperm [17]. At room temperature (20°C), the average lifespan of *C. elegans* is 14 to 21 days and the generation time is usually 4-5 days. The nematodes, as they grow, pass through an Embryonic (E) stage, where they grow in laid eggs, hatch and pass through four larval stages named L1, L2, L3 and L4, and finally an adult stage. In conditions of starvation or overcrowding, the larvae can alternatively enter a stage called Dauer Larva (L2d) stage, where they cannot consume bacteria, are thin and stress-resistant. Dauer Larvae can live for months before dying.

Although *C. elegans* is non-selective of diet and can consume a large variety of bacteria, yeasts and protista, the global standard in *C. elegans* cultivation is the use of monoxenic feeding such as Escherichia *coli* strain OP50, an uracil auxotroph, due to its inability to form dense colonies. This makes the nematodes much more easily observable. Other microorganisms are not preferred, not because they would cause injury to the nematodes themselves, but because the dense colonies they would produce could obscure the nematodes, making cultivation more difficult [18]. Although historically, *C. elegans* toxicity testing was confined to neurotoxicity testing primarily, the multiple advantages of the organism have resulted in a spill over of its use into other disciplines, such as cytotoxicity and genotoxicity [19–24].

Among these methods, the Chemotaxis Assay method is one of the oldest ones, with uses the chemosensory and chemoattractive characteristics of *C. elegans* to assess potential neurotoxicity. *C. elegans* has remarkable chemotactic properties and can respond to a variety of environmental cues related to the presence of nutrients, other nematodes, or predators [25]. The chemotaxis assay is well-cited in the literature, with descriptions of different methods of conducting the assay [26,27]. It has been used to establish the nematode's response to a variety of environmental cues such as sodium [28], food chemotaxis [29], and has even been used to develop models of associative learning [30]. It has also been used to develop cancer detection methods, in which the nematode was shown to demonstrate chemoattraction towards metabolites found in the urine of persons with breast cancer [31]. Therefore, the utility of this assay is evident and its implementation as a standalone test or as part of a wider battery

of toxicity tests can provide a wealth of information about the potential toxicity of a wide variety of tested compounds.

The micronucleus assay, which aims to determine the genotoxicity by way of counting the frequency of micronuclei generated during mitosis, is one of the commonly used genotoxicity assays and has been in use for nearly 40 years [32]. *C. elegans* has previously been studied as a model of DNA damage response [33,34]. Therefore, micronucleus formation in *C. elegans* cells can be used as a predictor of DNA damage in this organism.

The aim of the present study was therefore to evaluate the chemotactic and genotoxic effects of *Astragalus* metabolites on *C. elegans* by way of the Nematode Chemotaxis Assay and *in vivo* micronucleus assay, respectively.

2. Materials and Methods

2.1. Materials

Caenorhabditis elegans Bristol N2 Ancestral WT Strain and *Escherichia coli* OP50 were obtained from the Caenorhabditis Genetics Centre (CGC) of the University of Missouri. Butanone was obtained from Merck Millipore (Supelco 106014) as chemoattractant. Triton X-100, HEPES, Sodium Dodecyl Sulphate, DTT, sucrose, sodium, potassium, calcium and magnesium chlorides were all purchased from Sigma Aldrich/Merck Millipore (Germany).

2.1.1.Tested Compounds

The compounds tested in the study were obtained from the personal library of Prof. Dr. İhsan Çalış, isolated in previously published studies [35–37] (Figure 1). The structures of all the isolated compounds were elucidated by means of spectroscopic (NMR: 1D and 2D-NMR: ^{1}H-NMR, ^{13}C-NMR, COSY, HSQC, HMBC) methods and comparison with literature data. Different column chromatography techniques were used for the isolation and purification of the tested compounds. Each of the isolated compounds was elucidated by comparison of their NMR spectroscopic data with published values in the literature. The final concentrations in assays were selected to provide a logarithmic scale based on powers of 10. A further limitation on concentration was the liquefaction of long-term assay plate agars when exposed to larger concentrations of macrophyllosaponins (Compounds **5** and **6**) than tested in the present study.

2.2. Methods

The micronucleus method presented in the study were developed through modification from existing literature for alkaline comet assay in *C. elegans* [21]. Chemotaxis assays were similarly adapted from existing literature with minor modifications [27].

2.2.1. Preparation of Cultures and Nematode Isolation

The cultures were prepared in accordance with Wormbook Guidelines as detailed in their online publication entitled “Maintenance of *C. elegans*” [31]. Nematodes were isolated from growth cultures by washing with M9 buffer, then centrifuging at 200g for 5 minutes to precipitate the nematodes. The nematode pellet was then resuspended using a fresh M9 buffer. The centrifugation and M9 washing steps were repeated thrice to ensure all bacteria were removed. After the last wash, the nematodes were age-synchronised with NaOH/NaOCl, and a new culture was generated from the resultant eggs. After the nematodes developed to the young adult stage, they were washed off the plates, centrifuged and resuspended with M9 thrice. After the last wash, the nematode density was adjusted with M9 to 20 nematodes/50 µL.

2.2.2.Long-term (agar) Exposure Chemotaxis Assays

2.2.2.1.Preparation of Agar Test Cultures

Test plates were prepared by dissolving pre-determined masses of test compounds in absolute ethanol and mixing into liquid NGM agar formulation at a ratio of 1 mL ethanolic test solution: 10 mL NGM agar formulation. Four different final concentrations were used: 1000 µg/L, 100 µg/L, 10µg/L and 1µg/L. The mixture was then poured as a thin layer into 90mm Petri dishes and kept at elevated temperature (~50°C) to drive ethanol out of the agar. The test plates were then seeded with *E. coli* OP50 via an inoculation loop and spread all over the surface of the agar via cell spreader, followed by incubation at 35°C overnight. 55µL of nematode suspension was transferred to test plates with freshly grown OP50 lawn. Nematode numbers were visually confirmed under microscope (n=21 to n=24) and extraneous nematodes were removed with a sterile HIPS inoculation loop to ensure each new colony had exactly 20 hermaphroditic nematodes. The nematodes were allowed to propagate through the seeded NGM plates for 24 hours, after which they were removed via sterile inoculation loops. The plates were then incubated at 25°C for 4 days, at which time the nematodes were washed off the plates with the aforementioned centrifuge/M9 method, and resuspended to a final volume of 2mL.

2.2.2.2.Chemotaxis Assays

Chemotaxis plates were prepared by pouring Chemotaxis agar into 90mm petri dishes in a thin layer and allowing them to lose excess moisture by evaporation for 48 hours while lidded. The plates were divided into 4 equal quadrants by drawing lines on the bottom with a marker. Two opposite quadrants were named the Chemoattractant (CA) Quadrant and the M9 Control (MC) quadrants, for M9 Buffer+0.5% butanone v/v and only M9 Buffer, respectively. The remaining 2 quadrants were named Nematode quadrants, and served as the inoculation point of nematodes into the agar.

At the beginning of the assays, 50 µL of freshly resuspended nematode suspension were pipetted into the middle of the two Nematode Quadrants and rested at 25°C for the suspension to soak into the agar. After the soaking, 50µL of M9 solution was added into the MC quadrant, while 50µL of M9+0.5% butanone was added to the CA quadrant. At the same time, a timer was started and the nematodes in the CA and MC quadrants were counted at 120 minutes. The main metric of chemotaxis determination is the Chemotaxis Index (CI), usually calculated as the number of nematodes in the CA quadrant minus the number of nematodes in the MC quadrant, divided by the number of nematodes in both quadrants.

$$CI = \frac{CA - MC}{CA + MC}$$

This method of measurement circumvents the problems that might arise with the counting of immotile nematodes, but adequate numbers of nematodes must be ensured in both the CA and MC quadrants to improve reliability. Negative control assays were run by utilising M9 buffer alone for both the MC and CA quadrants without exposure to any test chemicals. Positive controls were run by adding 50µL of M9 solution into the MC quadrant and 50µL of M9+0.5% butanone into the CA quadrants without exposure to any test chemicals. Each experiment was conducted in triplicate. At the end of the determined period, the nematodes were immobilized with the addition of 100 µL of chloroform to the centre of the assay plate.

2.2.3.Short Term (M9) Exposure Chemotaxis Assays

Age-synchronised nematodes produced at the last step in nematode isolation were centrifuged once again and then resuspended in M9 mixed with test compounds in predetermined concentrations (1000 µg/L, 100 µg/L, 10µg/L and 1µg/L). The nematodes were incubated at 25°C for 2 hours, after which they were centrifuged and washed with M9 thrice, with the final resuspension adjusting the

volume to 2mL. Freshly resuspended nematodes were then transferred onto M9 Chemotaxis plates, prepared in the same manner as Agar Chemotaxis Plates with M9 Buffer+0.5% butanone v/v and only M9 Buffer, in the CA and MC quadrants, respectively. The counting was performed at 60 minutes. The same metrics, namely CI and Total Nematodes, were obtained from these assays. Negative control assays were run by utilising M9 buffer alone for both the MC and CA quadrants without exposure to any test chemicals. Positive controls were run by adding 50µL of M9 solution into the MC quadrant and 50µL of M9+0.5% butanone into the CA quadrants without exposure to any test chemicals. Each experiment was conducted in triplicate. At the end of the determined period, the nematodes were immobilized with the addition of 100 µL of chloroform to the centre of the assay plate.

2.2.4.Direct Chemotaxis Assays

Direct chemotaxis assays evaluated any possible direct chemotactic effects of test compounds on the nematodes. In this method, the isolated nematodes were transferred to Chemotaxis Assay Plates that had been treated with the test compounds in the CA quadrant in the same predetermined concentrations 6 hours before the commencement of the experiment. 50 µL of M9 buffer was added to both the CA and MC quadrants. The results were read at 30, 60, 90, and 120 minutes to establish potential chemoattraction. Each experiment was conducted in triplicate. At the end of the determined period, the nematodes were immobilized with the addition of 100 µL of chloroform to the centre of the assay plate.

2.2.5.In vivo Micronucleus Assays

Isolated and age-synchronised nematodes grown on agar spiked with tested chemicals in the same concentrations were dissociated at 4°C with Dissociation Buffer (0.5 mL of Triton X-100, 2 mM HEPES, 0.25g of Sodium Dodecyl Sulphate, 20 mM DTT and 3g of sucrose in 100 mL distilled water) for 5 minutes and washed and centrifuged thrice at 2500g with Egg Buffer (2.5 mM HEPES, 11.8 mM NaCl, 4.8 mM KCl, 0.2 mM $CaCl_2$ and 0.2 mM $MgCl_2$ in 100 mL distilled water). Homogenisation was carried out by mechanical disruption combined with 10mg/mL papain for 10 minutes at a temperature of 4°C. After the last wash, enzymatic degradation was stopped by centrifugation at 2500g, discarding the supernatant and adding fresh Foetal Bovine Serum (FBS). After 5 minutes in FBS, the cells were centrifuged again at 2500g for 5 minutes, the supernatant discarded and Dulbecco's Phosphate Buffered Saline (dPBS) added. The PBS washes were also repeated thrice. The cells were mixed with equal volume of 0.65% low-melting agar (LMA) and spread onto microscope slides pre-coated with 0.65% high-melting agar (HMA), and covered with pre-cleaned slide covers. After the agar was allowed to set for 30-60 minutes at 4°C, the slide covers were removed at the slides stained with 5% Giemsa in distilled water for 30 minutes. Each experiment was conducted in triplicate.

2.3.Observation

All visualisation and counting were performed using an Olympus SZ61 light microscope at 1.5x objective magnification for the chemotaxis assays. The microscope was powered in transillumination mode with contrast enhancement. *In vivo* micronucleus assays were visualised using Olympus CX21 upright transilluminating microscope at 100x objective magnification using an oil immersion objective. A blue light filter on the transilluminating light source was utilised to shift observation light towards bluer colours to enhance visualisation. The equipment lacked a microscope camera; therefore, all photography was conducted by placing a mobile phone with its camera lens the focal point of the eyepiece. All micronucleus counting was conducted via visual observation.

2.4.Statistical Analyses

All statistical analyses were performed through the use of PASW/SPSS v26.0 by IBM. Levene's test was utilised to establish homogeneity of variances prior to ANOVA, one-way ANOVA and Dunnett's post-hoc tests were utilised to compare against the positive control (chemotaxis assays) and

the negative control (micronucleus assays). Statistical significance was accepted at $p<0.05$ level. All experiments were run in triplicate (n=3) to ensure statistical reliability.

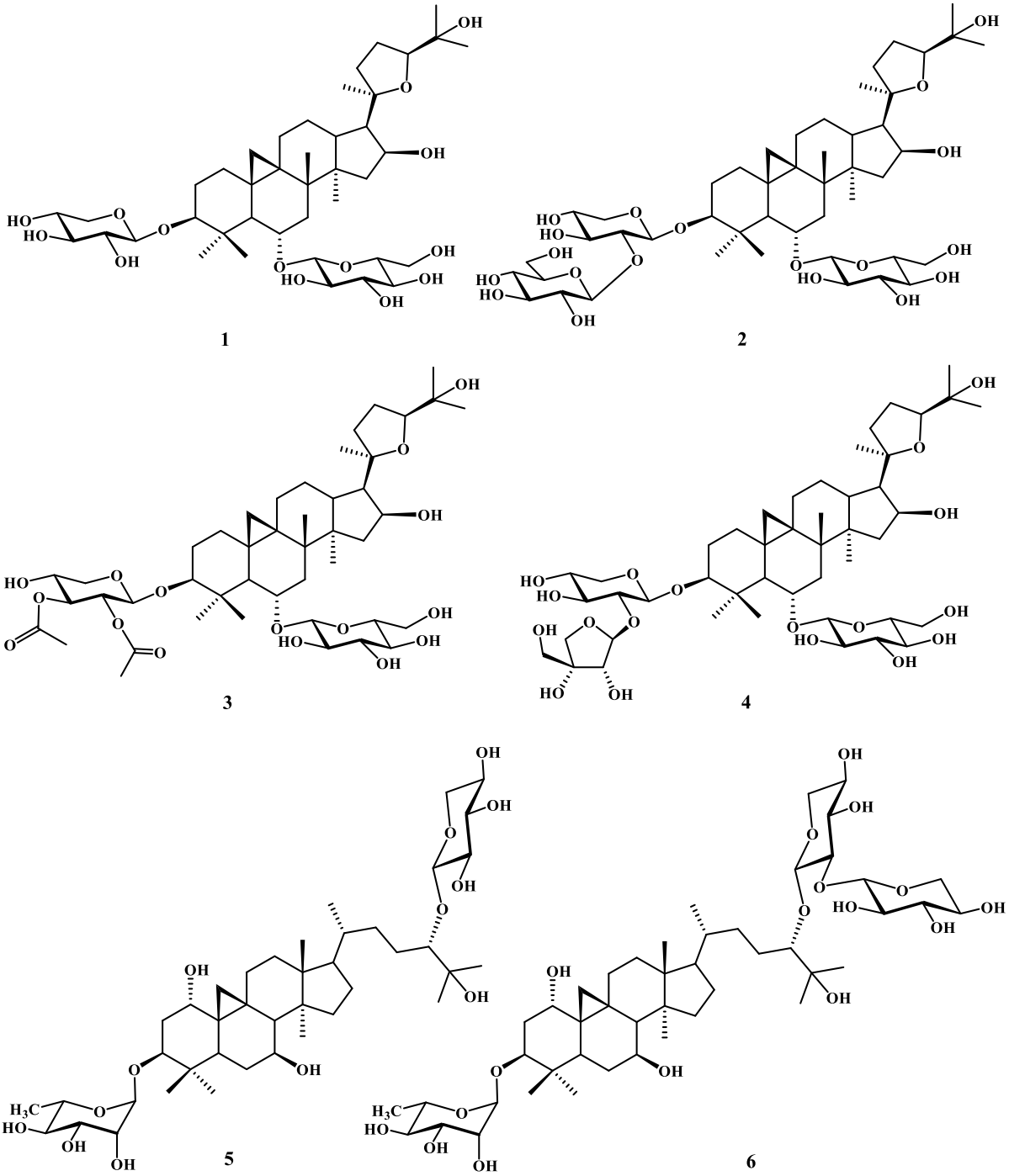

Figure 1. Structural formulae of glycosides used in the study **1:** Astragaloside IV, **2:** Astragaloside VI, **3:** Astrasieversianin II, **4:** Baibutoside, **5:** Macrophyllosaponin B, **6:** Macrophyllosaponin D

3. Results and Discussions

Levene's test of homogeneity of variances yielded p>0.05 for both the chemotaxis assays, and the micronucleus assays. The results obtained are tabulated in Table 1. Among the direct chemoattraction assays, no compound possessed a direct chemoattractive effect on the nematodes (p>0.05).

Table 1. Butanone chemotaxis indices of the tested compounds (Mean±SD)

Test Compound	**Concentration (µg/L)**	**M9 Exposure CI (60 minutes)**	***p*-value PC**	**Agar Exposure CI (120 minutes)**	***p*-value PC**
Negative Control (NC)	N/A	-0.0278±0.12108	**0.000**	0.1778±0.16777	**<0.05***
Positive Control (PC)	N/A	0.6854±0.09401	**N/A**	0.8232±0.03620	**N/A**
Astragaloside IV (**1**)	1	0.5602±0.14137	**>0.05**	N/D[a]	**0.000[a]**
	10	0.6131±0.14736	**>0.05**	N/D[a]	**0.000[a]**
	100	0.6602±0.19637	**>0.05**	N/D[a]	**0.000[a]**
	1,000	0.6528±0.12064	**>0.05**	N/D[a]	**0.000[a]**
Astragaloside VI (**2**)	1	0.4913±0.05892	**>0.05**	N/D[a]	**0.000[a]**
	10	0.3888±0.07856	**>0.05**	N/D[a]	**0.000[a]**
	100	0.5272±0.07834	**>0.05**	N/D[a]	**0.000[a]**
	1,000	0.6519±0.21569	**>0.05**	N/D[a]	**0.000[a]**
Astrasieversianin II (**3**)	1	0.5333±0.17638	**>0.05**	N/D[a]	**0.000[a]**
	10	0.6124±0.18562	**>0.05**	N/D[a]	**0.000[a]**
	100	0.5551±0.21379	**>0.05**	N/D[a]	**0.000[a]**
	1,000	0.1666±0.2357	**<0.05***	N/D[a]	**0.000[a]**
Baibutoside (**4**)	1	0.8078±0.13526	**>0.05**	0.8190±0.14569	**>0.05**
	10	0.791±0.16365	**>0.05**	0.8570±0.07426	**>0.05**
	100	0.6937±0.08975	**>0.05**	0.8272±0.04277	**>0.05**
	1,000	0.8067±0.09814	**>0.05**	0.8342±0.18694	**>0.05**
Macrophyllosaponin B (**5**)	1	0.9091±0.01298	**>0.05**	N/D[a]	**0.000[a]**
	10	0.4884±0.11739	**>0.05**	N/D[a]	**0.000[a]**
	100	0.5000±0.00000	**>0.05**	N/D[a]	**0.000[a]**
	1,000	0.3809±0.06734	**>0.05**	N/D[a]	**0.000[a]**
Macrophyllosaponin D (**6**)	1	0.8205±0.09299	**>0.05**	0.9063±0.11970	**>0.05**
	10	0.4287±0.17371	**>0.05**	0.7897±0.04181	**>0.05**
	100	0.4968±0.09662	**>0.05**	0.8712±0.03939	**>0.05**
	1,000	0.6333±0.04714	**>0.05**	0.7658±0.08939	**>0.05**

CI: Chemotaxis Index
a: No nematode movement observed. Motility only on agitation. Below 95% survival rate.
N/A: Not applicable
N/D: Not detected

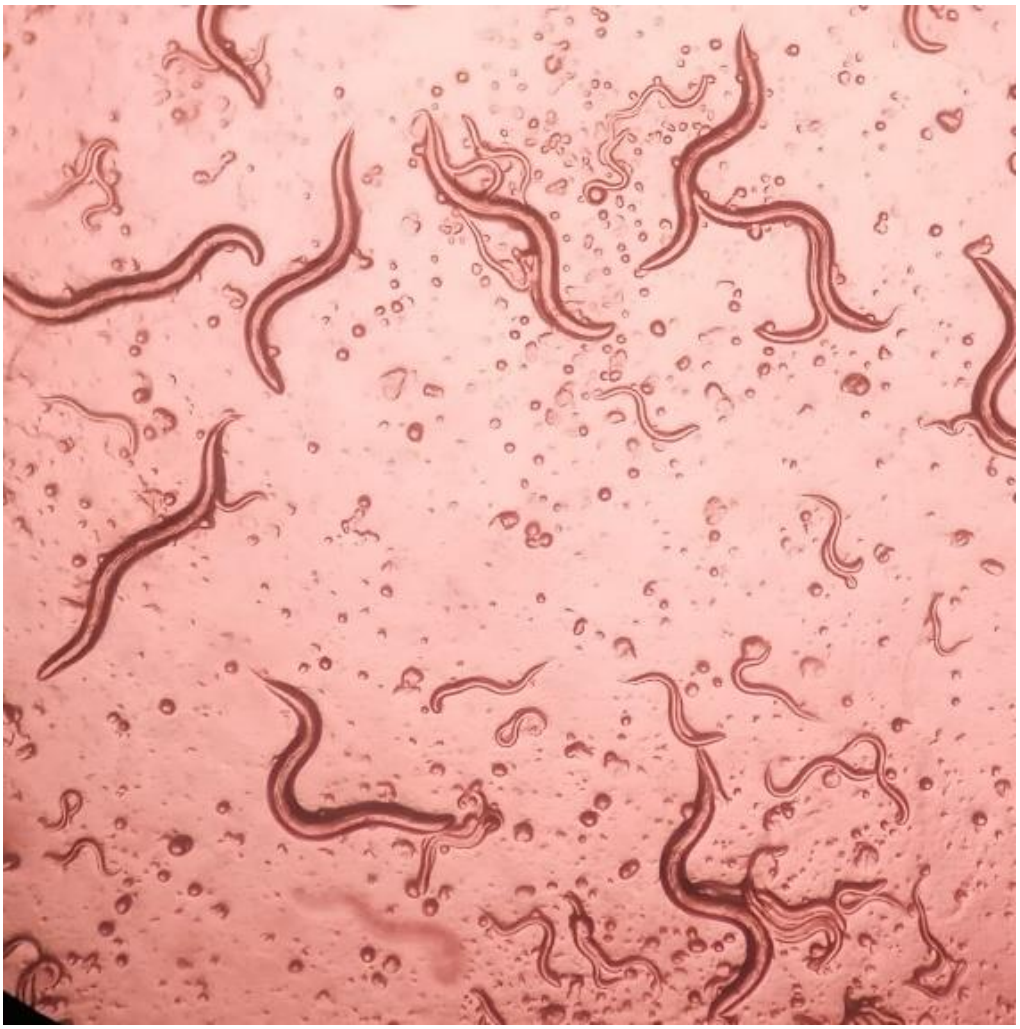

Figure 2. Representative Nematode Field in the logarithmic growth stage (round objects dispersed among the nematodes are eggs in the final stages of development, in the E stage of growth)

3.1. Short-term (M9) Exposure Results

There exists a statistically significant difference in the CI scores of nematodes exposed to compound **3** at a concentration of 1000 µg/L. Other compounds tested did not induce statistically significant reductions in the CI scores. Pearson's Correlation Analysis was also run to elucidate the presence of any correlations between the concentrations and the CI scores obtained during the study. No correlations were found with significant statistical result ($p>0.05$). The results of short-term (M9) exposure chemotaxis are demonstrated in Figure 3.

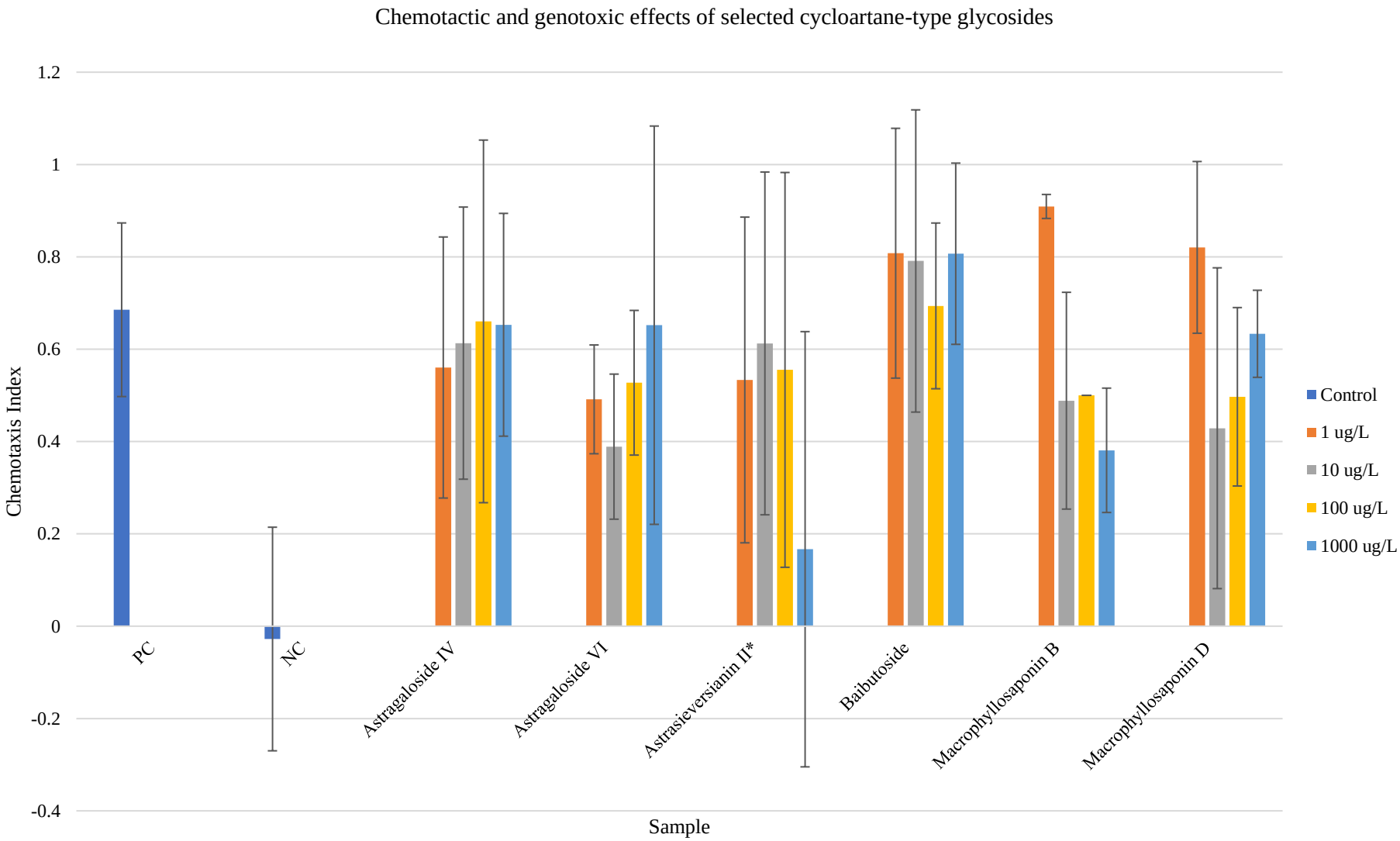

Figure 3. Short-term (M9 Exposure) Chemotaxis Indices (Mean±SD) (PC: Positive Control, NC: Negative Control) (*Dunnet's Post-hoc significant result [$p<0.05$])

3.2. Long-term (Agar Exposure) Results

Agar exposure results have yielded outcomes with respect to long-term toxicity (Figure 4). The method was calibrated by assessing the statistical significance between NC and PC, and verified with p=0.003. The nematodes obtained from compounds **1**, **2**, **3**, and **5** cultures did not display any form of chemotaxis on the assay plates, rather remaining in the original quadrants into which they were originally placed. Among the two compounds in which chemotaxis was observed, neither compound **4** nor compound **6** elicited any retardation of chemotaxis.

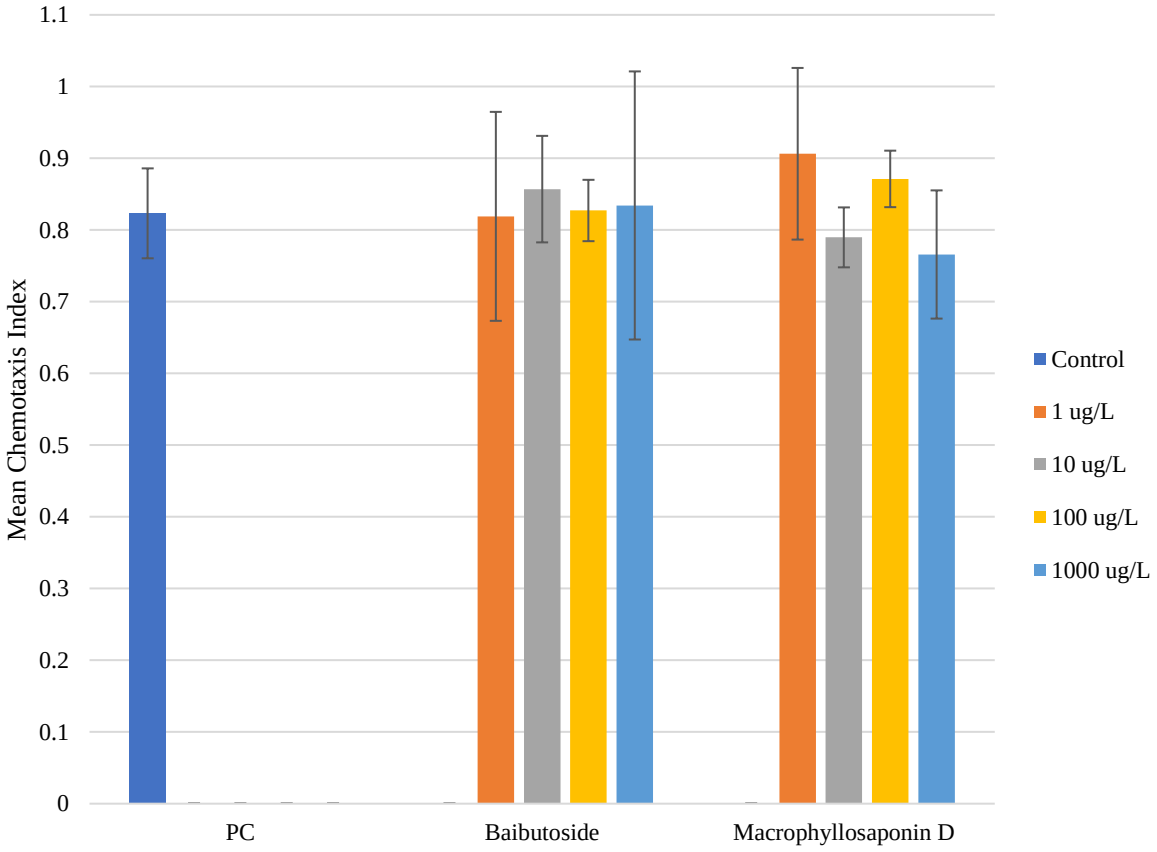

Figure 4. Long-term (Agar Exposure) Chemotaxis Indices (Mean±SD) in Agar Exposure Chemotaxis (PC: Positive Control)

2.1. In vivo Micronucleus Assay Results

The *in vivo* micronucleus assay was utilised to determine genotoxicity of the tested compounds. Example cells obtained with micronucleus assay are given in Figure 5. The results obtained as result of these assays are demonstrated in Figure 6. For each sample, the number of cells per sample was determined by the selection of 20 random regions from each sample slide and counting the number of eligible cells in each field. The number of fields necessary to count were then extrapolated from the average number of cells in each field to reach 10,000 total counted cells per sample. Micronucleated cells were then counted in the pre-determined number of fields. There existed statistically significant increase in the micronucleus frequency in nematodes exposed to compound **1** at a concentration of 1000 µg/L, as well as the positive control (PC). The other chemicals did not elicit a statistically significant increase in micronucleus frequency in any of the concentrations tested ($p<0.05$).

Figure 5. Intact, non-micronucleate cell (left) and damaged, micronucleate cell with contact micronucleus (right)

This study is fundamentally pioneering in nature. The authors are in the process of implementing, and refining the measurement of chemotaxis index and associated parameters, as well as established genotoxicity tests as reliable and robust methods of determining neural and genotoxic effects, respectively, of tested compounds on *C. elegans*. In addition, while the testing of compounds on *C. elegans* with the chemotaxis assay is not a new methodology, to the authors' best knowledge, this has not been done in an extensive level on plant glycosides or *Astragalus* spp.

Long-term agar-exposure chemotaxis could not be performed for compounds **1**, **2**, **3** and **5**. These four compounds failed to achieve a 95% nematode survival rate with respect to both the negative and positive controls that was a prerequisite established at the beginning of the study. Agar-exposure chemotaxis assays were run nevertheless, but the nematodes, despite being alive and mobilising when agitated with an inoculation loop, rapidly ceased all motility in the absence of agitation. Another observation made was that at the time of nematode isolation, despite the presence of a modest number of nematodes on the exposure plate and still ample OP50 supply, a large number of nematodes in each plate were still in the larval stage, and some in L2d or Dauer stage, despite the nematodes being age-synchronised before inoculation into the plate and given ample time to develop into young adults. Therefore, these four compounds appear to have interfered with *C. elegans* biology in such a manner that stress is induced, retarding nematode development, and inducing development into Dauer larvae. Agar-exposure plates with compounds **4** and **5** had negligible numbers of larvae (=<50) and no deceased nematodes were observed in 5 randomly selected areas in either sample.

Compounds **5** and **6** differ by the presence of one more hexose moiety on the aglycone, with compound **5** having two and compound **6** having three (Figure 1). The toxicity of either compound is inadequately known, and as far as the authors can know, there have been few reliable toxicity studies conducted on either compound. Compound **5** is known to have haemolytic activity, indicating the presence of some form of immune-mediated cytotoxic activity [38]. Neither compound induced a short-term reduction in CI or increase in micronucleus frequency. Therefore, the effects that arose in *C. elegans* are likely a form of toxicity that occurs over long periods of time and is non-genotoxic in nature.

A similar relationship exists between compounds **1** and **2** with compound **2** having one more hexose in comparison (Figure 1). However, both compounds were observed to be toxic to *C. elegans* in all concentrations tested over long periods of time, therefore comparative toxicity evaluation is currently not possible between the two compounds in the chronic domain. Compound **1** is already known to be involved in neuroprotection in the PC12 cell line [39], therefore is already known to have neuroactive effects.

In addition, certain astragalosides have cytokine modifying effects, resulting in anti-inflammatory and anti-cancer effects being manifested [40]. Astragalosides have also demonstrated anti-trypanosomal and to a degree, mammalian cytotoxic effects [37]. It is therefore entirely possible, that astragalosides have potent toxicity towards *C. elegans*. Similar anti-trypanosomal activity has been demonstrated by compound **4** as well [37]. However, the results indicate that on *C. elegans* exposed to the latter, there is no reduction in chemotaxis or increase in micronuclei, therefore, if such an effect even exists, it is neither chemotactic nor genotoxic.

Compound **3** is yet another *Astragalus* metabolite that is underrepresented in the toxicity literature, with limited information available on its toxic effects. Extracts containing it have been found to be hepatoprotective against alcohol-induced liver damage [41], however, how this relates to its other possible effects is as of yet highly unclear due to the scarcity of studies. The present study demonstrated what appears to be a genotoxic effect at high concentrations, thus indicating that this compound may also be potentially genotoxic to humans as well, though this will require follow-up studies using human cell lines for verification. Compound **3** was found not to induce genotoxicity over an entire embryogenesis cycle. It was, however, determined to reduce chemotaxis in the present study at 1000 µg/L(1µg/mL).

There exist structural differences between the macrophyllosaponins (Compounds **5** and **6**) and the other compounds discussed herein. One such difference is that the "hydroxylation pattern", that is, the sites of hydroxylation on the skeleton of the aglycone, feature hydroxyl groups on C1, C3 and C7 on macrophyllosaponins, but on C3, C6 and C16 on other compounds. In addition, the aliphatic side chain on C17 in macrophyllosaponins is formed by a furan skeleton in the other compounds. It is therefore expected that the toxicity profile of macrophyllosaponins is fundamentally dissimilar to other cycloartanic glycosides. Further studies focusing especially on the metabolomics of these compounds will help elucidate if such differences truly exist.

The deductions that can be made from the micronucleus assay, especially with respect to *C. elegans* need to be made in light of the fact that the mitotic cell divisions that underlie the formation of micronuclei only occur in the somatic cells during embryogenesis, therefore only the exposure occurring during embryogenesis can be detected via this assay. Therefore, the micronucleus assay results obtained from adult nematodes are indicative of not short-term exposure in adults, but short- or long-term toxicity occurring due to exposure during embryogenesis. Therefore, it is more appropriate to use micronucleus assay as an indicator of developmental genotoxicity, rather than adult genotoxicity. The time limitation of micronucleus assay is also an advantage, as during the period of embryogenesis, there needs to be a rapid succession of sequential cell divisions, so that the totality of the somatic cells comprising the totality of the nematode can be produced. This, in turn means that a large number of cell divisions must occur in rapid succession, therefore only a very limited time is available to repair the DNA damage incurred during this rapid development period. This makes the micronucleus assay during this particular period a very sensitive assay to DNA damage, making it a useful genotoxicity assay for this organism.

This intricacy is also reflected in the study results, with compound **1** being more toxic during embryogenesis and compound **3** being more toxic in adulthood. As the long-term exposure chemotaxis assays and the micronucleus assay share a common nematode cultivation methodology, the long-term chemotaxis assays and the micronucleus assays can be directly used together to draw more detailed conclusions. The first and foremost deduction is that despite the observation of genotoxic effects in nematodes exposed to 1000 µg/L of compound **1**, these did not reflect a comparable reduction in chemotaxis. Similarly, nematodes that did not feature increased micronucleus frequencies lost chemotactic capabilities after long-term exposure to lower doses of compound **1** and all doses of compounds **2**, **3**, and **5**. Moreover, compounds **4** and **6** did not induce any reduction in chemotaxis or any increase in micronucleus frequencies. These results point to the mechanisms responsible for increased micronucleus frequencies and the mechanisms responsible for reduced chemotaxis being largely independent of each other.

The absence of concurrent genotoxic damage in compounds **1**,**2** and **5** with long-term chemotaxis loss, combined with the genotoxic damage in only the highest concentration of compound **1** indicates that the loss of chemotaxis is possibly due to the effects of the metabolites of these compounds, rather than themselves. This hypothesis is corroborated by the absence of chemotaxis loss in nematodes exposed to these chemicals for 2 hours in short-term chemotaxis assays, which is more than enough for the nematodes to absorb the chemical being tested and demonstrate loss of chemotaxis, as demonstrated by compound **3**. Therefore, the authors surmise that compounds **1**,**2** and **5** are metabolized into more toxic compounds, which are responsible for the loss of chemotaxis on long-term exposure. A similar situation is observed in compound **3**, which demonstrates short-term chemotaxis inhibition only in the highest concentration tested but causes an inhibition of chemotaxis in all concentrations tested in long-term chemotaxis.

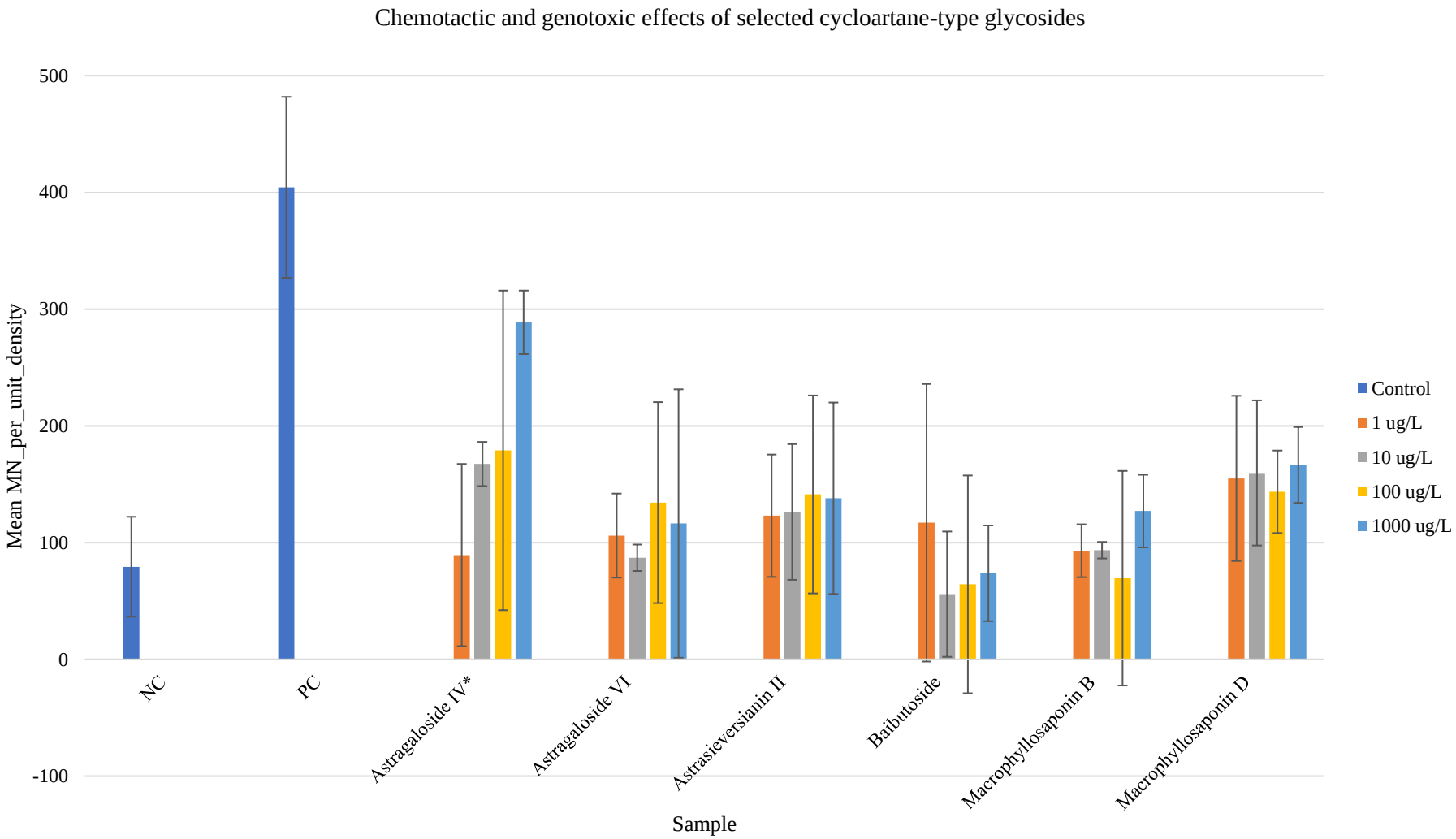

Figure 6. Micronuclear Density per 10,000 cells (NC: Negative Control, PC: Positive Control) (*Dunnet's Post-hoc significant result [$p<0.05$])

Micronucleus generation is a direct mechanism of action of aneugenic genotoxins, which induce sister chromatid non-disjunction, which results in asymmetric division of genetic material during the cascade of rapid mitotic divisions during embryogenesis. The genetic code, as well as the mechanisms that maintain it, are considered universally conserved among eukaryotes. The centromere, the locus on the chromosome that is used in sister chromatid disjunction, is also strongly functionally conserved in eukaryotes. Therefore, the genotoxic effects of compound **1** as observed in C. elegans can be directly extrapolated to humans, suggesting that compound **1** is also genotoxic to humans at a concentration of 1000 µg/L.

Metabolism is an inherent part of the mechanisms of toxification in many organisms, including humans. While human cell lines have been used in the past to elucidate the toxicity of these compounds [37,38,40], human cell lines typically lack the capability to account of the metabolism of many such compounds. An integrated model such as *C. elegans* provides the benefit of incorporating metabolic pathways into the experimental results. Compound **1**, for example, in light of the anti-cancer activity on human cell lines of many astragalosides [38] and itself specifically [42] was found to induce the formation of micronuclei in *C. elegans*.

The results of the present study indicate the utility of both the Chemotaxis Assay and the *in vivo* Micronucleus Assay in the determination of toxic insult to *C. elegans* and underlie the usefulness of both the organism and the utilised methods in the determination of toxicity of natural products. The micronucleus assay was selected over the other 3 major genotoxicity tests due to the ease of its implementation. The ease and straightforward nature, therefore the simplicity, of the micronucleus assay is advantageous over the other 3 in terms of the potential unknowns and confounding factors. It was therefore selected as the assay of genotoxicity testing for the study.

Chemotaxis assay, as discussed and employed in the present study, can act as a generalised proxy test that can provide organism-level response to potential toxicants. Its primary advantage over all other methods is that it provides a measurement of the general well-being of the organism, independently of the mechanism of how that well-being may be disrupted. Chemotaxis is an essential part of the nematode's behaviour, with 5% of its genes and 52 of its genes and the majority of its nervous system being dedicated to chemotaxis, with 32 neurons being dedicated to chemosensation alone [21]. Therefore, any disruption of chemotaxis is strongly indicative of the normal neurology of the nematode being also disrupted, which allows chemotaxis assaying to act as a proxy for neurotoxicity, a long-acting and strongly dose-dependent, potentially irreversible form of toxicity.

Another limitation was that the assays were conducted on isolated, pure chemicals, rather than the whole extract. Pure substances were preferred over the combined extract for two reasons: The first is that the determination of toxicity of the individual components allows for not only their individual risk profiles, but also provides valuable information on the potential toxicity of extracts containing these compounds in varying concentrations. The other reason is that should a raw extract be determined to be toxic in any concentration, the individual components still need to be assessed in terms of their toxicity. By assaying the individual components for toxicity, the toxic profile of the raw extract may be estimated based on the concentrations of the individual chemical components.

On the other hand, the testing of individual components does not provide information on the synergistic, additive or antagonistic effects the components might have on each other, therefore, raw extract assays shall provide this information. Raw extracts therefore need to be tested in a similar manner.

3. Conclusion

The results of the study indicate that chemotaxis determination as a toxicity testing tool is potentially a very useful and powerful utility. There is a large volume of unknowns in the biochemical mechanisms of these compounds, therefore, chemotaxis determination with *C. elegans* will add to the growing wealth of information about these compounds and possibly add towards the knowledge base concerning *C. elegans*, ultimately aiding in the proliferation of this organism as an experimental toxicity testbed. It is for this reason that the elaboration of chemotaxis in *C. elegans* upon exposure to cycloartenol-type glycosides from *Astragalus* must be continued in the future. More detailed studies to determine the exact characteristics of the toxicity observed in this study are necessary, along with the

addition of parallel and complementary data, such as concurrent further genotoxicity testing. Such endeavours are already underway through the efforts of the authors, and will add to the compendium of techniques and knowledge concerning both *C. elegans* and *Astragalus*. Micronucleus assays are also tremendously useful in the determination of potential toxicity to *C. elegans*, however, their limitation to the embryogenetic stage of nematode growth poses an obstacle, which will be supplemented in the future, through the efforts of the authors, by further studies employing the other major genotoxicity assays, namely the Sister Chromatid Exchange Assay, the Chromosomal Aberration Assay, and the Alkaline Comet Assay. The same analyses shall also be conducted on raw extracts as well, which will also provide valuable input to the toxicity of the chemical species in the present study.

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