Investigating the Effects of Traditional Medicines using *Caenorhabditis elegans* as a Model Organism

SMTP Research Paper

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Abstract

Despite the profound therapeutic advantages possessed by some medicinal plants, some of their constituents are extremely health hazardous. Reactive oxygen species are highly reactive by-products of aerobic metabolism predominantly generated during oxidative phosphorylation in the electron transport chain. They can damage macromolecules in cells such as nucleic acids and induce oxidative stress, potentially leading to many pathological and neurological conditions such as cancer and hypertension. Studies have shown that resistance to oxidative stress is crucial to staying healthy and minimises the adverse effects of ageing. This study aims to investigate the effects of different herbal products on the nematode *Caenorhabditis elegans*, their efficacy in reducing oxidative stress to restore its viability, as well as their DNA cleaving properties. *C. elegans* was chosen as a model organism due to its genotypic similarity to humans, which makes it a very useful model representation for human diseases. *Ganoderma lucidum*, *Angelica sinensis*, *Codonopsis pilosula* and *Panax notoginseng* showed signs of reduction of oxidative stress in the roundworms, of which *C. pilosula* was the most effective, whereas Daochi pill, Longdanxiegan pill and *Houttuynia cordata* exacerbated oxidative stress in *C. elegans*, of which Daochi pill was the most detrimental. Gel electrophoresis tests further confirmed the effects of these herbal products on the roundworms. While this research has warned the public against the consumption of certain herbal products, it has also shown the potential of some herbs in the biomedical field.

1. Introduction

In 2012, global sales of Chinese herbal medicine reached US$83 billion, a significant increase of more than 20% from 2011 (WHO, 2013), thus revealing the rapid build-up of a modern traditional medicine (TM) industry chain. Today, 70% to 95% of the populace in Asian and African countries still used traditional medicines for primary healthcare (WHO, 2011; Birhan et al., 2011; Sato, 2012), of which as high as up to 90% still believed that there were no adverse effects accompanied with the consumption of TM (Edziri et al., 2011; Nurolaini et al., 2014). Hence, it was imperative to explore common herbal recipes and products, not only for their cytotoxicity and genotoxicity levels and health threats like cancer-inducement, but also assess possible health benefits such as immunity enhancement and anti-aging properties.

In this study, seven herbal products were investigated, of which two are well-known traditional pharmaceutical recipes – Daochi pill and Longdanxiegan pill, as well as five herbs – *Houttuynia cordata*, *Ganoderma lucidum*, *Angelica sinensis*, *Codonopsis pilosula* and *Panax*

notoginseng. *H. cordata* had been found to contain the amides aristolactam I and aristolactum II (Chen et al., 2013), which were principal detoxication metabolites of aristolochic acid, a Type 1 carcinogenic compound. Aristolactam I induced apoptosis in renal tubular epithelial cells and secretes fibronectin and profibrotic transforming growth factor (Li et al., 2004), thus revealing aristolactam as a possible factor in the etiology of kidney damage and fibrosis (Zhang et al., 2015; Shang et al., 2008). Traditional pharmaceutical recipes such as the Daochi pill and Longdanxiegan pill had also been reported to contain precursors of this toxic compound, which induced DNA damage and resulted in genetic polymorphism (Garrick, 2012).

G. lucidum was well-known for containing a large number of bioactive molecules which enabled it to activate natural killer cells, increasing their activity and the body's ability to fight tumours and reduce the chances of metastasis (Nonaka et al., 2006; Chen et al., 2004), and thus rendering it as one of the few herbs known to be anti-cancer. *A. sinensis* had been found to be anti-atherosclerotic, anti-hypertensive and effective in reducing the size of cerebral infarction (Wu & Hsieh, 2011). *C. pilosula* had been suggested to aid memory acquisition and retention (Singh et al., 2004) and reduced the invasion and migration potential of tumour cells (Xin et al., 2012). *P. notoginseng* was known to promote cardiac angiogenesis and exhibited anti-myocardial and anti-atherosclerosis effects (Yang et al., 2014).

2. Objectives and Hypotheses

This study aimed to investigate the effects of traditional Chinese herbal products on the survival, development, locomotion and molecular genetics of *Caenorhabditis elegans*, through possible pathogenic, mutagenic, cytotoxic and genotoxic effects. It also aimed to discover potential health benefits of the herbal products such as high antioxidant levels.

It was hypothesised that Daochi pill, Longdanxiegan pill and *H. cordata* would have harmful effects on *C. elegans*, such as lowered survival rate, decreased metabolism, reduced longevity, abnormal behavioural characteristics and genotoxic instability. On the contrary, *Ganoderma lucidum*, *Angelica sinensis*, *Codonopsis pilosula* and *Panax notoginseng* would give rise to health-promoting effects not limited to immunity enhancement, anti-aging and inhibition of neurodegenerative conditions. The higher the concentration of herbal products exposed, the greater the amplification of its harmful or beneficial impacts.

3. Materials and Methods

3.1 Preparation of NGM (Nematode Growth Medium) agar

3.0 g NaCl and 2.5 g bacto peptone were mixed with 472 ml deionised water. 47.2 ml aliquots of the mixture were added with 1.7 g agar each. After autoclaving at 15 psi for 15 min, 0.1 ml of cholesterol (5 mg/ml), 0.1 ml of $MgSO_4$ solution (1 M), 0.1 ml of $CaCl_2$ solution (1 M), 2.5 ml of KH_2PO_4 solution pH 6.0 (1 M) were added.

3.2 Preparation of herb extracts and agar plates

20 g herb was blended in 100 ml deionised water, autoclaved at 121°C for 15 min and centrifuged at 7232 × *g* for 15 min. The supernatant was then collected. 50 ml NGM agar was supplemented with 50 ml herb extract (as the test) or 50 ml deionised water (as the control). This mixture was then poured onto agar plates and incubated at 25°C for two days.

3.3 Cultivation and growth of *C. elegans*

Escherichia coli OP50 was inoculated and grown in 10 ml of Luria-Bertani broth overnight at 36.0°C in a shaking incubator. The absorbance of the bacterial culture at 600 nm was standardised at 0.8. 250 µl of *E. coli* OP50 liquid culture was added to each stock plate and grown overnight at 36.5°C. This served as a food source for the roundworms. A block of agar containing *Caenorhabditis elegans* wild type strain N2 was cut and placed in the centre of the plate. After three days, *C. elegans* and *E. coli* OP50 were collected in M9 buffer and filtered through a sterile 8 µm membrane filter. *C. elegans* remained on the membrane and were suspended in M9 buffer. 250 µl of M9 buffer containing the worms was micropipetted to the centre of each NGM plate, then incubated at 24.5°C.

3.4 Lethality test

Serial three-fold dilutions were performed on each herb extract. 50 ml of herb extract and each 50 ml diluted mixture was added with 50 ml NGM agar to form NGM mixtures with herbal concentrations of 2.5%, 5.0%, 7.5% and 10.0%. This mixture was poured onto agar plates and incubated overnight at 24.5°C for 2 days. In the control set-up, 50 ml deionised water replaced the 50 ml herb mixture. The percentage survival of the *C. elegans* was calculated at the 24 h mark. Worms were considered dead if they stopped moving.

3.5 Antioxidant DPPH assay

To each herb extract, an equal volume of deionised water was added to reduce its concentration by half. In the test set-ups, 0.1 ml of diluted herb extract was mixed with 1.9 ml of methanol and 1.0 ml of DPPH solution. In the control set-up, 0.1 ml of diluted herb extract was replaced with 0.1 ml of deionised water. For their respective blanks, 1.0 ml DPPH was replaced with 1.0 ml methanol. Five replicates of the test and control set-ups were prepared. The initial absorbance reading was measured at 517 nm against the respective blanks and the mixtures were left in darkness for 20 min before the final absorbance readings were taken. The % radical scavenging activity (RSA) was calculated as follows:

$$RSA\ (\%) = \frac{final\ absorbance\ of\ control - final\ absorbance\ of\ test}{final\ absorbance\ of\ control} \times 100\%$$

3.6 Oxidative stress, locomotion, thrashing and omega bends assays

50 µl of M9 buffer containing the worms was micropipetted to the centre of each petri dish. In the positive control set-ups, 50 µl of 1.75 mM hydrogen peroxide was added. Five replicates were prepared. An equal volume of deionised water replaced the hydrogen peroxide for the negative control. The percentage survival of worms was determined after 4 h. Worms were considered dead if they stopped moving. The software WormLab was utilised to determine the movement speed and body bending angles of the worms. The number of body bends greater than 60°, as well as omega bends observed at 2 min intervals were recorded.

3.7 Isolation of genomic DNA of *C. elegans*

This was modified in accordance to the method described by Qiagen (2014). A *Gentra Puregene Tissue Kit* from Qiagen (Hilden, Germany) was used to extract DNA from the worms.

After two days, the *C. elegans* from 10.0% herb concentration NGM agar plates were washed in M9 buffer, centrifuged at 741 × *g* for 3 min at 20°C and filter-sterilised through a sterile 8 µm membrane filter. The mixture of *C. elegans* and M9 buffer was centrifuged at 741 × *g* for 1.5 min at 5°C. Sonification was performed on each mixture for 20 min at 25°C at regular intervals of 2 min by using an omni-ruptor. 4.5 ml cell lysis solution was dispensed into each homogenate. The tubes were then vortexed at high speed for 30 s and heated at 65.3°C for 75 min. 22.5 µl Puregene Proteinase K was micropipetted into each mixture, gently inverted for 30 times, then incubated at 55.3°C for 5 h until all tissue had been completely lysed. During the incubation, the tubes were periodically inverted 30 times at 10 min intervals. 22.5 µl RNase A solution was then dispensed into each solution, mixed by 25 continuous inversions, vortexed at high speed for 1 min, then incubated at 39.1°C for 90 min. The tubes were then incubated for 10 min on ice. 1 ml protein precipitation solution was added into each tube and vortexed vigorously for 45 s at high speed. Each tube was then centrifuged for 5 min at 10 864 × *g* at 5°C. Avoiding the pellet, the supernatant was collected and poured into a new tube dropwise. Five repeats were conducted for this process of cold ice treatment and centrifugation.

The supernatant collected was then mixed with 4.5 ml isopropanol. Each tube was inverted continuously for 60 times, vortexed for 55 s at high speed, then centrifuged for 3 min at 10 846 × *g*. The supernatant was decanted and each tube was drained on absorbent filter paper, then allowed to air dry for 10 min. The residue in the tubes was then mixed 4.5 ml of 70 % ethanol, inverted 50 times and vortexed at high speed for 90 sec, then centrifuged for 3 min at 10 846 × *g*. The supernatant was decanted off and each tube was drained on absorbent filter paper, then allowed to air dry for 10 min. Next, 50 µl of DNA Hydration Solution was micropipetted into each solution and vortexed for 15 s at medium speed. The tubes were then incubated at 65.3°C for 3 h, then placed in a shaking incubator overnight at 35.0°C.

The extracted DNA was stored in the freezer at –20°C for future downstream usage.

3.8 Examination of DNA fragments using agarose gel electrophoresis

1.0% agarose gel solution was microwaved, poured into a gel tray and cooled. The gel was then placed within an electrophoresis chamber filled with TAE buffer solution. 20 µl of DNA sample was mixed with 2.25 µl loading dye and 1.5 µl Sybr Green in a microfuge tube. To prepare the DNA ladder, 5 µl of 1 kb DNA replaced the 20 µl DNA sample. The tubes were then centrifuged at 12 600 × *g* for 25 s. 20 µl of mixture was micropipetted into each well. The gel was allowed to run at 120 V for 45 min. When viewed under UV radiation, DNA bands fluoresced. A photo was taken to record the position of the bands for genotoxic analysis.

3.9 Data Analysis

Using the computer software GraphPad Prism 8.0, the Mann-Whitney U test (for 2 set-ups) or Kruskal-Wallis test (for 3 or more set-ups) were conducted to account for significant differences in the assays conducted on the *C. elegans* in both the control and test set-ups.

4. Results and Discussion

4.1 Lethality test

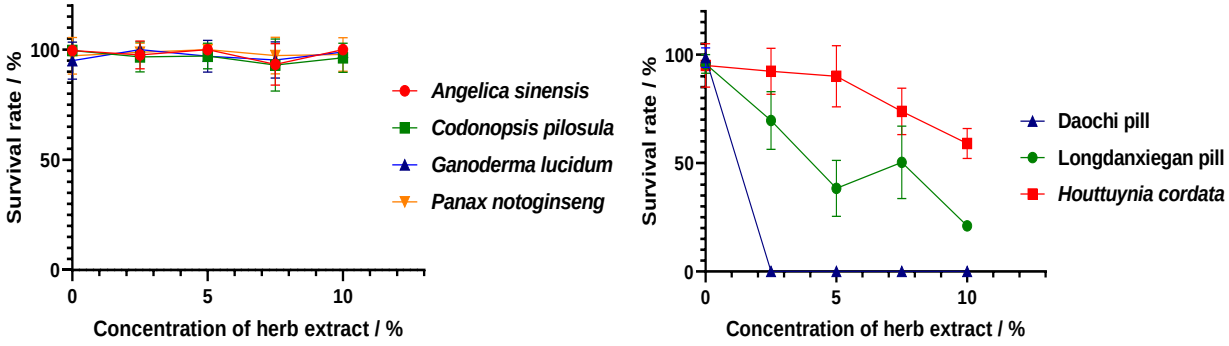

Fig. 4.1.1: Survival rate of the *C. elegans* when exposed to **(a)** *G. lucidum*, p = 0.3467, *A. sinensis*, p = 0.2051, *C. pilosula*, p = 0.3467, *P. notoginseng*, p = 0.7952 and **(b)** Daochi pill, p = 0.0004, Longdanxiegan pill, p < 0.0001, *H. cordata*, p = 0.0054 at the 24 h mark at 2.5%, 5.0%, 7.5% and 10.0% herbal extract concentrations. *: p < 0.05, **: p < 0.01.

As shown in Fig. 4.1.1a, *G. lucidum*, *A. sinensis*, *C. pilosula* and *P. notoginseng* exhibited neutral effects on the *C. elegans* as only a marginal disparity that is not significant existed between the survival rates of the worms with increasing herbal extract concentration.

As shown in Fig. 4.1.1b, Daochi pill, Longdanxiegan pill and *H. cordata* demonstrated inhibitory effects on the growth of the nematodes as they stimulated significant decreases in survival rate. At 24 h mark, the survival rate of *C. elegans* decreased by 98.8%, 74.8% and 36.0% respectively as herbal extract concentration increased from 0% to 10.0%.

4.2 Oxidative stress assay

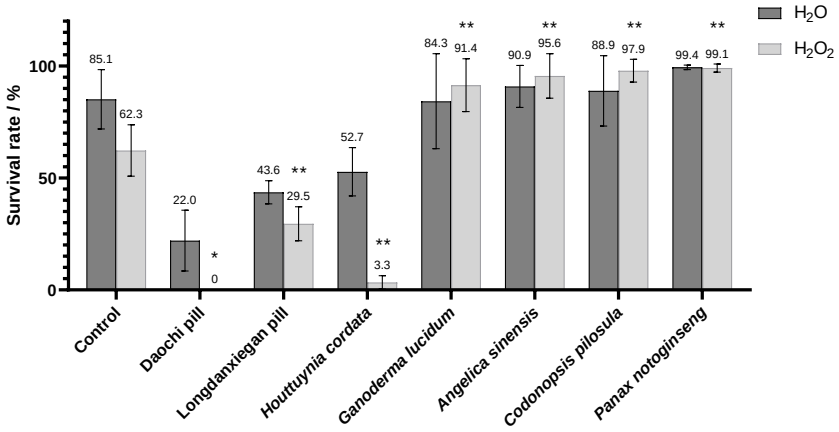

Fig. 4.2.1: Survival rates of the *C. elegans* after exposure to hydrogen peroxide (for test set-ups) or sterile water (for control set-ups) for 4 h. *: $p < 0.05$, **: $p < 0.01$.

As shown in Fig. 4.2.1, when the *C. elegans* were exposed to oxidative stress-inducing agent hydrogen peroxide, four herbs protected them from oxidative stress, as apparent from the significant increases in survival rates of the worms as compared to those in the control set-up, where the survival rate is merely 62.3%. They were *G. lucidum* (91.4%), *A. sinensis* (95.6%), *C. pilosula* (97.9%) and *P. notoginseng* (99.1%). As these four herbs exhibited ameliorative effects on the growth of the worms under heavy oxidative stress, this suggested that these herbs possibly contained antioxidants which reacted with the free radicals produced by the worms, hence suggesting health beneficial effects that accompanied them.

On the contrary, Daochi pill, Longdanxiegan pill and *H. cordata* extracts exacerbated oxidative stress in the nematodes as the survival rates of the *C. elegans* decreased way below that of the control, from 62.3% to 0%, 3.3% and 29.5% respectively. As such, it could be inferred that not only do these herbal extracts not protect the worms from heavy oxidative stress, but they also contained toxic substances which catalysed the death of the roundworms.

4.3 Locomotion assay

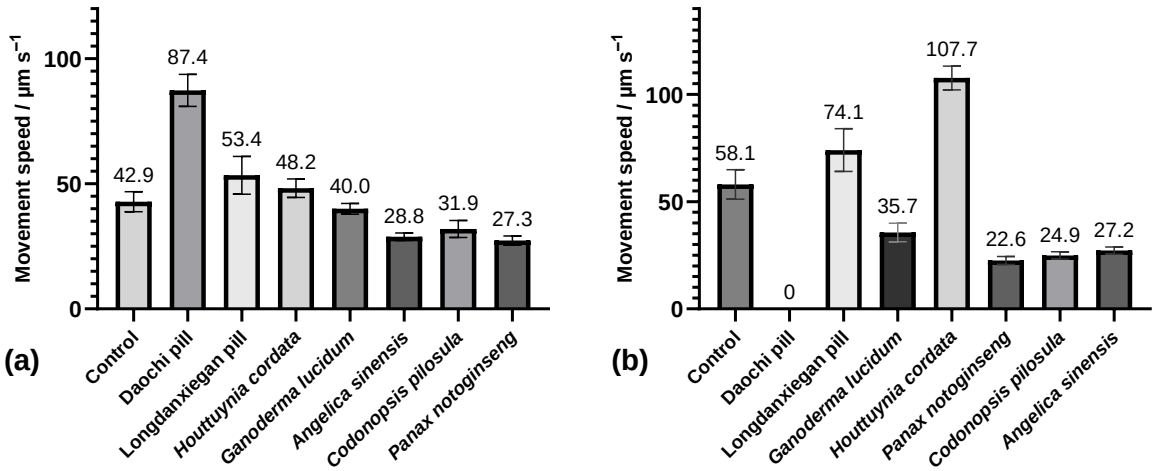

Fig. 4.3.1: Movement speed of *C. elegans* after 4 h **(a)** in absence of hydrogen peroxide (replaced with sterile water) **(b)** when exposed to hydrogen peroxide.

Fig. 4.3.1a showed that in the absence of hydrogen peroxide, the worms experienced significant increases in movement speed when exposed to Daochi pill, Longdanxiegan pill and *H. cordata*, from 42.9 μm s^{-1} to 87.4 μm s^{-1}, 53.4 μm s^{-1} and 48.2 μm s^{-1} respectively. On the contrary, the nematodes exposed to Daochi pill, *G. lucidum*, *A. sinensis*, *C. pilosula* and *P. notoginseng* experienced significant decreases in locomotion speed from 42.9 μm s^{-1} to 0 μm s^{-1}, 40.0 μm s^{-1}, 28.8 μm s^{-1}, 31.9 μm s^{-1} and 27.3 μm s^{-1} respectively. An explanation for the immobility of the *C. elegans* after 4 h is that all the worms were already dead and stopped moving. Fig. 4.3.1b showed that in the presence of hydrogen peroxide, these effects are even more significant as evident from the greater magnitude in change of movement speed.

Low movement speed was attributed to desirable environmental conditions, such as the absence of toxic compounds, whereas high movement speed indicated undesirable living conditions and behavioural adaptations to seek regions of safety (Zhao et al., 2003) as they were agitated by external factors. Excessive reactive oxygen species levels might be attributed to pathogenic response to homeostatic disruptions like metabolic damage and disturbances (Li et al., 2017), and thus suggesting that the three herbs had adverse impacts on the worms.

4.4 Thrashing assay

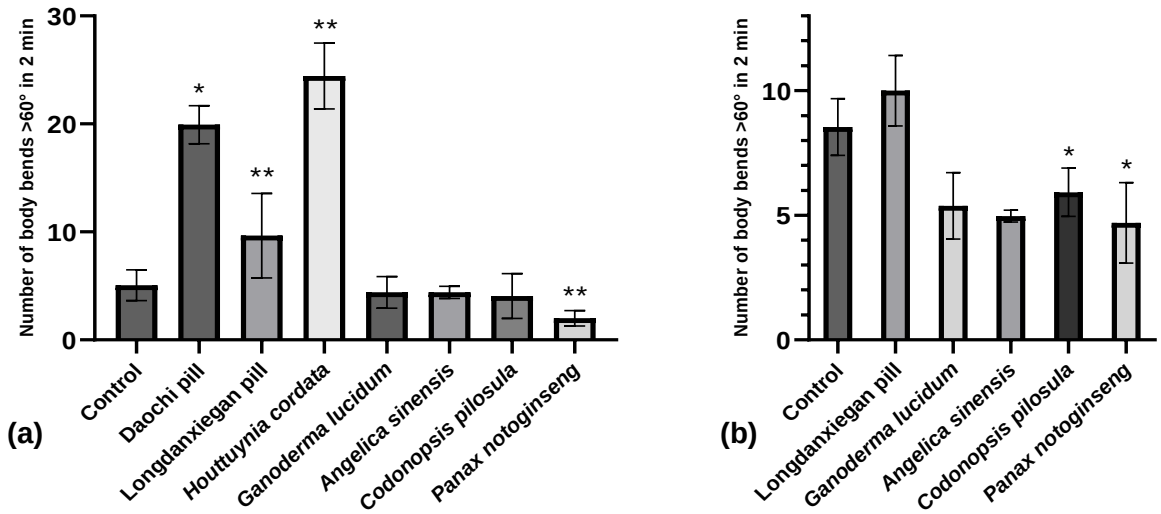

Fig. 4.4.1: Frequency of body bends greater than 60° observed in the *C. elegans* **(a)** in presence of sterile water **(b)** in presence of hydrogen peroxide. *: $p < 0.05$, **: $p < 0.01$.

Fig. 4.4.1a revealed that *P. notoginseng* significantly reduced the number of body bends, hence suggesting its health beneficial effects while Longdanxiegan pill, Daochi pill and *H. cordata* significantly increased the number of body bends larger than 60° observed in the *C. elegans*, hence suggesting their health detrimental effects. As shown in Fig. 4.4.1b, *C. pilosula* and *G. lucidum* exerted positive effects on the nematodes as observed from the significant decreases in the number of body bends larger than 60°.

The amplitude of thrashing was correlated to the wavelength of the bending waves. An assumption had been made that the viscosity of the different agar media was roughly constant, thus ruling out the possibility of varying bending amplitudes being attributed to the mechanical needs of gait adaptation in response to the external load imposed by the physical environment. As such, a shorter wavelength implied greater muscle power needed to generate adequate force (thrust) to generate propulsive power to push the worm against its high-resistance environment. As deduced earlier, the frequency of high-amplitude thrashing increased with the aforementioned trends in movement speed. This is consistent with the following equation, which relates the velocity of the worm, V, and that of its undulatory motion to the resistance coefficient, C, and angle of bending, θ: $\frac{V}{V_{und}} = (C-1)(sin^2\theta)$ (Zhen & Samuel, 2015).

4.5 Omega bends assay

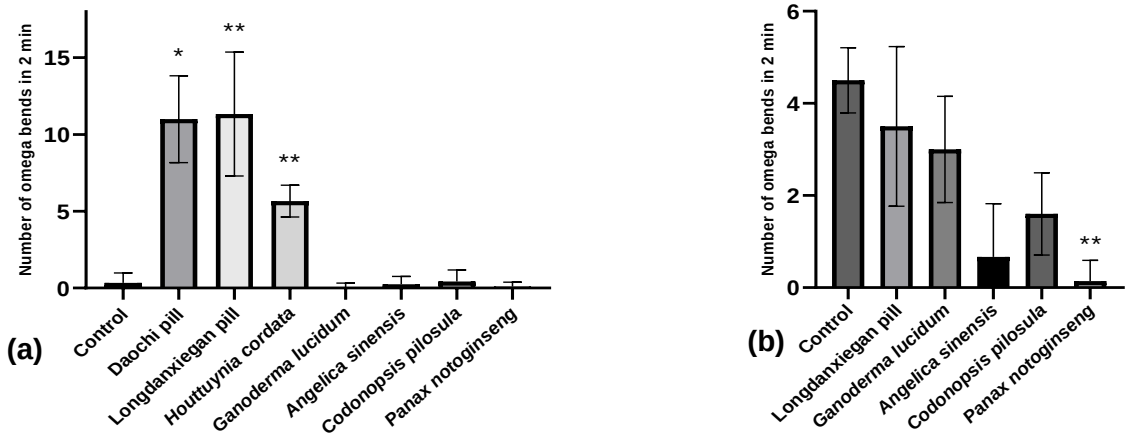

Fig. 4.5.1: Frequency of omega bends observed in the *C. elegans* **(a)** in absence of hydrogen peroxide **(b)** in presence of hydrogen peroxide. *: $p < 0.05$, **: $p < 0.01$.

Fig. 4.5.1a showed that Daochi pill, Longdanxiegan pill and *H. cordata* intensified the oxidative stress experienced by the worms as significantly great numbers of omega bends were observed. Fig. 4.5.1b depicted that *P. notoginseng* was effective in reducing oxidative stress in the worms as the number of omega bends observed showed significant decreases.

Omega bends, in contradistinction to common bends smaller than 90°, performs a function of reorientation and locomotory reversal (Herman, 2012). Mechanosensory neurones present in the anterior of the worms were capable of sensing environmental changes, allowing it to consistently reassess priorities that its movement path had to address such as to navigate to a food-rich region (Angstman, Franz, & Schmitz, 2016). Reversals and hence omega bends were behavioural parameters representing signs of avoidance of undesirable factors as part of a programmed defence mechanism (Zhao et al., 2003). Hence, a high frequency of omega bends indicated the presence of toxic compounds in the NGM which influenced the *C. elegans* to continuously calibrate their direction in an effort to crawl towards a region of safety.

4.6 Antioxidant DPPH assay

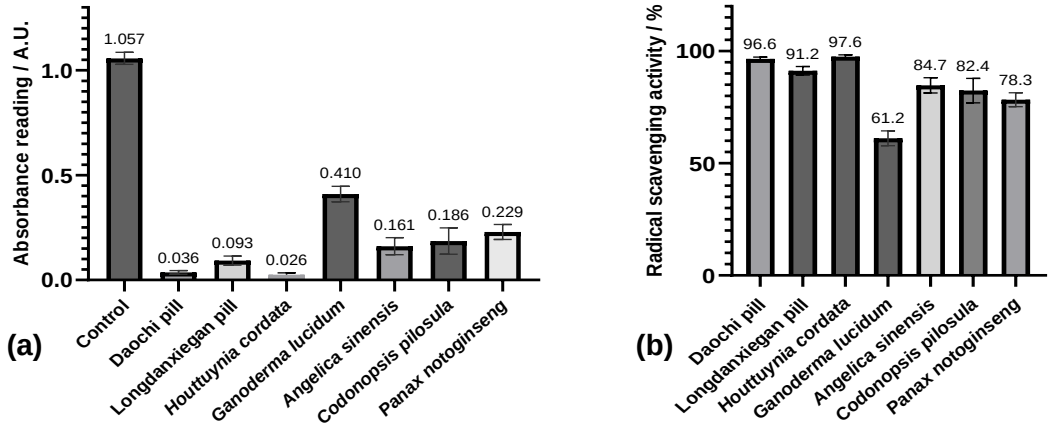

Fig. 4.6.1: (a) Absorbance readings of the herbal extracts against their respective blanks. **(b)** RSA levels of the herbal extracts. *: $p < 0.05$, **: $p < 0.01$.

As shown in Fig. 4.6.1, all the herbal products examined demonstrated high RSA levels, including *G. lucidum* (61.2%), *A. sinensis* (84.7%), *C. pilosula* (82.4%) and *P. notoginseng* (78.3%), hence suggesting that these herbs had high antioxidant activities and were effective in removing reactive DPPH radicals. A possible explanation could be these herbs contained significant concentrations of potent antioxidant moieties such as herbal fibres which effectively bound to and hence removed heavy metal ions such as Fe^{2+} or Cu^{2+} ions which catalysed the formation of harmful reactive oxygen species (Mehrandish, Rahimian, & Shahriary, 2019).

The three processed herbal products, Daochi pill (96.6%), Longdanxiegan pill (91.2%), and *H. cordata* (97.6%) exhibited remarkably high RSA levels, contradicting the results discussed earlier. RSA had a positive correlation to the movement speed and survival rate of the *C. elegans*, where protection from oxidative damage and lesion of motor neurones would secure longer lifespans and increased motility (Truong et al., 2015).

One possible explanation could be these store-bought herbal products had protracted periods of shelf life, when compared to raw herbs which are seasonally sold and not intended for long-term storage. Therein lies the possibility that antioxidative preservatives, which have the ability to inhibit oxidative reactions, could have been introduced into the herbal products during the manufacturing processes. These included natural antioxidants such as ascorbic acid and tocopherols or common synthetic antioxidants like propyl gallate, butylhydroquinone and butylated hydroxytoluene (Iverson, 1995). Therefore, this explained the disproportionally high antioxidant activity demonstrated by the herbal products when compared to the raw herbs.

Thus, it could mean that Daochi pill, Longdanxiegan pill and *H. cordata* unequivocally contained chemical compounds deleterious to the growth and survival of *C. elegans*, to the extent where the ameliorative effects posed by the high concentrations of antioxidant compounds were overwhelmed by the toxic effect exerted by these harmful compounds on the *C. elegans*, hence leading to the significantly low survival rates of the roundworms.

4.7 Gel electrophoresis test

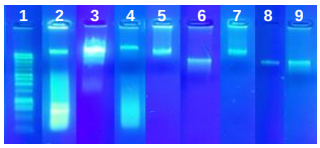

Fig. 4.7.1: Results of DNA migration on agarose gel matrix.

Lane 1 – 1 kb DNA ladder
Lane 2 – Daochi pill
Lane 3 – Longdanxiegan pill
Lane 4 – *Houttuynia cordata*
Lane 5 – *Ganoderma lucidum*
Lane 6 – *Angelica sinensis*
Lane 7 – *Codonopsis pilosula*
Lane 8 – *Panax notoginseng*
Lane 9 – Control

As shown in Fig. 4.7.1, the bands and smears of genetic material seen in lanes 2 to 4 depicted genetic fragmentation when the worms were exposed to Daochi pill, Longdanxiegan pill and *H. cordata*, implying that these herbs contained toxic compounds which resulted in the DNA cleavage. This suggested that these herbal products could have possibly induced genomic damage in the worms, hence resulting in genotoxic instability. On the contrary, as evident from the single bands of DNA observed in lanes 5 to 9, it can be deduced that *G. lucidum*, *A. sinensis*, *C. pilosula* and *P. notoginseng* conferred genetic stability to the worms.

5. Conclusion and Future Work

Throughout the course of this study, our results have displayed that Daochi pill, Longdanxiegan pill and *H. cordata* exhibited health hazardous impacts on the roundworms as not only do they inhibit the growth of the nematodes, but they also significantly exacerbated oxidative stress in the worms. On the contrary, *G. lucidum*, *A. sinensis*, *C. pinosula* and *P. notoginseng* have health beneficial effects on the *C. elegans* due to the absence of inhibitory effects on the growth of the nematodes and their formidable ability in significantly reducing oxidative stress in the worms. Further agarose gel electrophoresis tests conducted confirmed the positive and negative effects of the respective herbs on the genetic stability of the worms.

One of the major limitations in our study was the inability to consistently standardise the lifespans, sizes and quantity of the *C. elegans* present throughout all the different set-ups.

An application of the results of our research is its potential use in the pharmaceutical industry. Our study had demonstrated that the use of *G. lucidum*, *A. sinensis*, *C. pilosula*, *P. notoginseng* reduced oxidative stress in organisms that have remarkable similarities to the human genome. This can aid in the solution to many pertinent health issues pervading today's society, such as atherosclerosis and neurodegenerative diseases like Alzheimer's disease and Parkinson's disease which humanity has yet to find a cure hitherto. In addition, our results strongly warns the public against the consumption of these traditional medicines – Daochi pill, Longdanxiegan pill and *H. cordata*, which had shown to exhibit health hazardous effects.

A possible extension to our research is to perform combinatorial testing by mixing the herbs together to test for synergistic or antagonistic effect. Another prominent assay, widely known as the *in vitro* micronucleus test, as well as high performance liquid chromatography could be conducted to screen for potential cytotoxic effects and teratogenicity that can be induced by the toxic substances, such as aristolochic acid, a health hazardous carcinogenic compound that could be found in trace amounts in the traditional pharmaceutical recipes.

6. References

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Appendix

Lethality test (individual graphs)

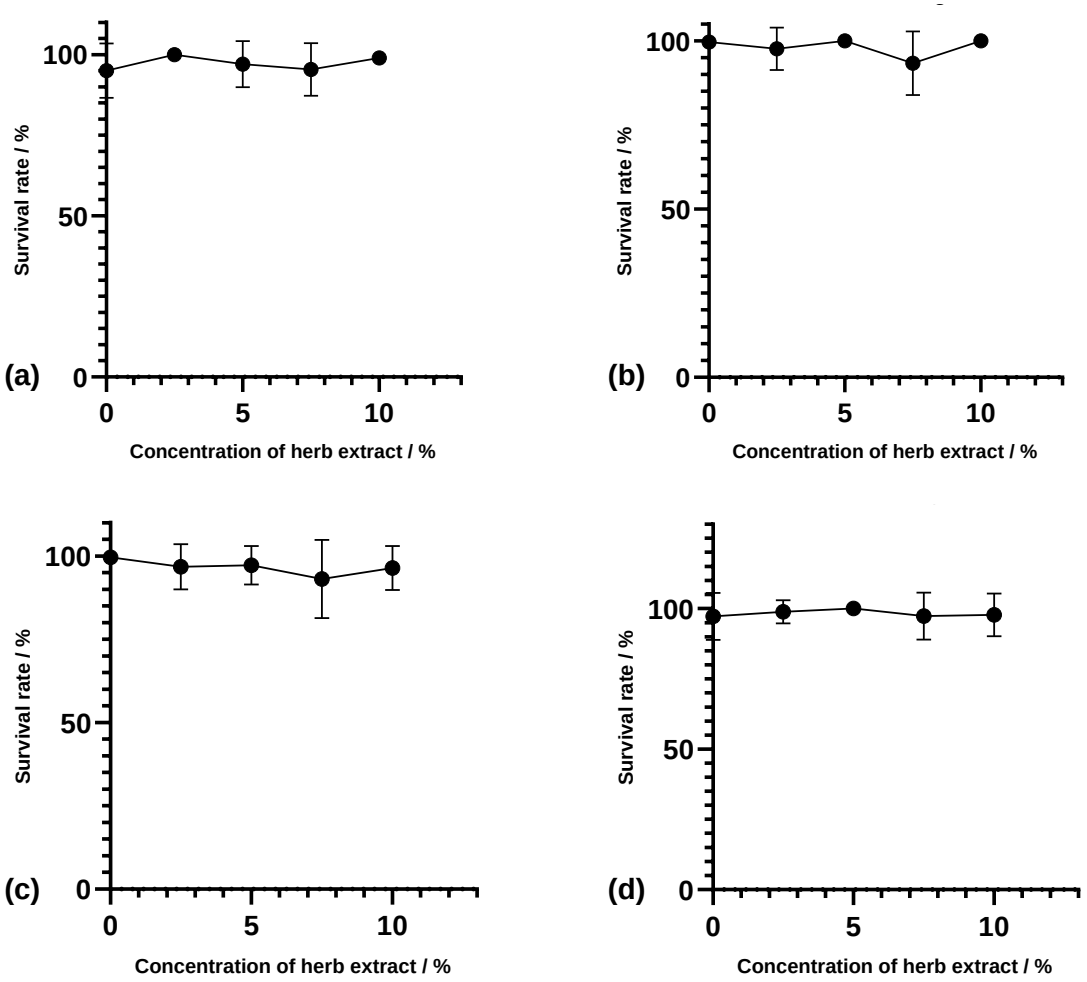

Fig. A.1: Survival rate of the *C. elegans* when exposed to **(a)** *G. lucidum*, p = 0.3467 **(b)** *A. sinensis*, p = 0.2051 **(c)** *C. pilosula*, p = 0.3467 **(d)** *P. notoginseng*, p = 0.7952 at the 24 h mark at 2.5%, 5.0%, 7.5% and 10.0% herbal extract concentrations.

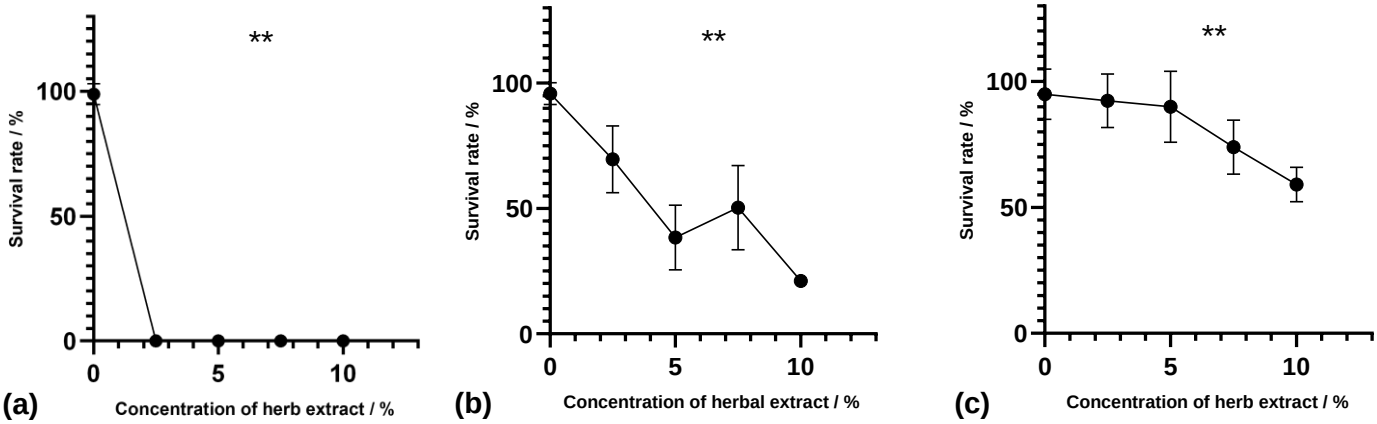

Fig. A.2: Survival rate of the *C. elegans* when exposed to **(a)** Daochi pill, p = 0.0004 **(b)** Longdanxiegan pill, p < 0.0001 **(c)** *H. cordata*, p = 0.0054 at the 24 h mark at 2.5%, 5.0%, 7.5% and 10.0% herbal extract concentrations.