Investigating the effect of phytoextracts on oxidative stress in *Caenorhabditis elegans*

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ABSTRACT

Increased generation of reactive oxygen species (ROS) from various exogenous and endogenous sources can lead to oxidative stress when ROS levels exceed certain mechanisms of detoxification. The addition of antioxidant-rich phytoextracts to cells would be beneficial as the antioxidants would scavenge free radicals, ridding the body of excess ROS. In this study, hydrogen peroxide was used to induce oxidative stress in *Caenorhabditis elegans*, which is a good model organism for human diseases. By doing so, their survival rate was significantly reduced to 35.7%. However, addition of aqueous extracts of *Illicium verum* (star anise) and *Syzygium aromaticum* (cloves) elucidated protective effects on the *C. elegans,* raising the survival rate to 83.2% and 63.2%, respectively, helping to increase the resistance of the *C. elegans* towards hydrogen peroxide, thus reducing oxidative stress in *C. elegans*. A mixture of both aqueous extracts was not found to exhibit any synergistic effects towards protecting *C. elegans* against oxidative stress.

INTRODUCTION

Endogenous generation of reactive oxygen species (ROS) is an integral feature of normal cellular function (Singh, et al, 2004). Although beneficial at moderate levels as it is needed to synthesize cellular structures and to be used by the host defense system to fight pathogens (Droge, 2002), in excess, free radicals and oxidants give rise to oxidative stress. Such redox reactions produce a number of free radicals: these include, but are not limited to, the superoxide radical (O2-) and the hydroxyl radical (HO-). Oxidative stress arises from an imbalance between the formation of free radicals and the capacity of cells to clear them. Based on several studies (Halliwell, 2007; Young & Woodside, 2001), oxidative stress negatively affects several cellular structures, such as lipids, proteins, lipoproteins, membranes, and deoxyribonucleic acid (DNA) which can be part of the cause for the induction of several diseases, both chronic and degenerative, as well as speeding up body aging process.

To neutralise free radicals, antioxidants are used. There are two major groups of antioxidants in living cells: enzymatic antioxidants and non-enzymatic antioxidants. These groups are then divided further into several subgroups. The enzymatic antioxidants are divided into primary and secondary enzymatic defenses (Carocho & Ferreira, 2013). The primary enzymatic defenses are composed of three important enzymes that prevent the formation of and neutralize free radicals: glutathione peroxidase, which reduces peroxides by forming selenols and also eliminates peroxides; catalase, which converts hydrogen peroxide into water and oxygen; and superoxide dismutase, which converts superoxide anions into hydrogen peroxide as a substrate for subsequent catalase action (Rahman, 2007). The secondary enzymatic defence is composed of glutathione reductase and glucose-6-phosphate dehydrogenase which reduces glutathione, an antioxidant, from its oxidized form to its reduced form, thereby recycling itself to continue neutralizing more free radicals and regenerates nicotinamide adenine dinucleotide respectively (Aoyama, et al., 2012; Ratnam, et al., 2006). These two secondary enzymes only support the primary enzymatic defense antioxidants and do not neutralize free radicals directly.

Although our body produces antioxidants, it is inadequate. Hence, to further counteract the effect of oxidative stress, exogenous antioxidants need to be introduced by diet or even by supplementation. In Chinese cuisine, a myriad of herbs are utilised to aid in the preparation of foods. Many of the herbs contain therapeutic properties due to the presence of polyphenols. Hence, the two herbs we wish to investigate is Chinese star anise, *Illicium verum* , and cloves, *Syzygium aromaticum* as these herbs are often used interchangeably as cloves are a common star anise stand-in or pairing.

By investigating whether the phytoextracts of commonly used herbs in Singapore contain the necessary antioxidants to ameliorate the oxidative stress on *C. elegans*, not only could the herbs be utilised more in cooking to act as a natural source of antioxidants for people, but the herbs could also be developed as a novel, cheaper and more effective food preservative to help delay rancidity of food due to oxidation.

OBJECTIVES AND HYPOTHESIS

This study aims to investigate the effect of star anise and clove phytoextracts on the oxidative stress in *C. elegans* and whether a synergistic effect will be observed with a combination of extracts. We hypothesise that both phytoextracts are able to protect *C. elegans* from oxidative stress and they can work synergistically in reducing oxidative stress.

MATERIALS

Apparatus - Autoclave, Biological safety cabinet, Blender, Centrifuge, Incubator, Laminar hood, Microcentrifuge, Shaking incubator, UV-vis spectrophotometer

Materials - Agar, Bacto peptone, Cholesterol, DPPH solution, Hydrogen peroxide solution, *Illicium verum* fruits, LB medium, Methanol, *Syzygium aromaticum* flower buds, Na_2HPO_4, NaCl, $MgSO_4$, $CaCl_2$, KH_2PO_4

Organisms - *Caenorhabditis elegans* N2, *Escherichia coli* OP50 (Biosafety level 1)

METHODS

Preparation of LB (Luria-Bertani) broth

4g of LB powder was suspended in 100ml of deionised water. It was mixed thoroughly and heated with frequent agitation and boiled for one minute to completely dissolve the powder. The solution was then autoclaved at 121 degrees celsius for 15 minutes. Final pH was at 7.0 ± 0.2.

Growth of bacterial precultures

Escherichia coli OP50 was inoculated into 10 ml of LB broth and grown overnight at 30°C in a shaking incubator. The absorbance of the preculture at 600 nm was measured using a UV-vis spectrophotometer and was standardised at 0.8.

Preparation of extracts and NGM (Nematode Growth Medium) agar

25 g each of *Illicium verum* fruit and *Syzygium aromaticum* flower buds were blended separately in 100 ml of deionised water. The mixture was centrifuged at 7000 rpm for 10 min, the supernatant was collected and filter-sterilised. NGM agar was prepared and supplemented with 25% (v/v) extract or deionised water as the control. The composition of NGM was as follows: 1.5 g NaCl, 8.5 g agar, 1.25 g bacto peptone in 487.5 ml water. After autoclaving, the flask was cooled in a 55°C water bath for approximately 15 minutes. A solution of 0.5 ml cholesterol (5 mg/ml), 0.5 ml $MgSO_4$ (1 M), 0.5 ml $CaCl_2$ (1 M), 12.5 ml KH_2PO_4 solution pH 6.0 (1M) was added and mixed well, and the NGM agar was then poured into the petri plates.'

DPPH Antioxidant Assay

1.9 ml of methanol, 1 ml of DPPH solution, and either 0.1 ml of plant extract for the test setup, or 0.1 ml of deionised water for the negative control setup, were mixed together. For the respective blanks, 1.0 ml DPPH solution was replaced with 1.0 ml methanol. The setups were left in darkness for 20 minutes, and the absorbance readings are taken at 517 nm. The radical

scavenging activity was calculated as follows:
[(final absorbance of control – final absorbance of test) / final absorbance of control] x 100%

***C. elegans* Survival Rate and Body Bending assay**

A block of agar containing *C. elegans* N2 was placed on the centre of the NGM plate supplemented with phytoextracts (test setup) or sterile water (control setup) (previously added with *E. coli* OP50) and incubated at 25°C for 2 days. 100 µl 5mM hydrogen peroxide was then spread on the respective plates of incubated *C. elegans*. The plates were incubated for another 24 hours at 25°C. The percentage survival and number of body thrashes per minute of *C. elegans* were determined using manual counting.

In summary, five replicates of each of the following setups were prepared for each assay:
C. elegans treated with 200 µl *Illicium verum* only (control)
C. elegans treated with 200 µl *Syzygium aromaticum* only (control)
C. elegans treated with hydrogen peroxide solution only (test)
C. elegans treated with 200 µl *Illicium verum* and hydrogen peroxide solution (test)
C. elegans treated with 200 µl *Syzygium aromaticum* and hydrogen peroxide solution (test)
For the synergistic tests, the volume of each extract was 100 µl, followed by the mixing of both extracts.

RESULTS & DISCUSSION

DPPH Antioxidant Assay

The DPPH antioxidant assay was carried out to quantify the radical scavenging activities of both extracts. The radical scavenging activity of *Illicium verum* was determined to be 82.38% whereas that of *Syzygium aromaticum* was 49.48%. When the extracts were combined, the radical scavenging activity was 88.26% (Fig. 1).

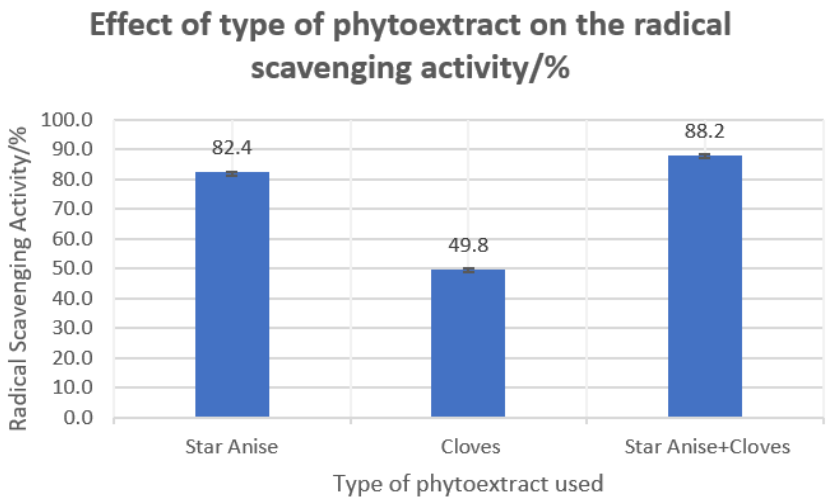

Fig. 1: Graph showing radical scavenging activity/% in the DPPH test. Higher change in absorbance at 517 nm illustrates stronger antioxidant activity.

Survival Rate Assay

The survival rates of the *C. elegans* were manually counted under a light microscope at 4x objective magnification with 10x eyepiece magnification. 3 readings per plate were recorded, each taken from a different part of it. The plates were separated into test and control plates with test plates having hydrogen peroxide and control plates having the hydrogen peroxide replaced with sterile water. Only 35.7% of *C. elegans* placed in hydrogen peroxide solution with sterile water survived. Meanwhile, 63.2% and 83.2% of *C. elegans* placed in hydrogen peroxide solution with *Syzygium aromaticum* and *Illicium verum* extracts respectively survived. In the hydrogen peroxide solution containing both *S. aromaticum* and *I. verum* extracts, 71.1% of the *C. elegans* survived (Fig. 4.1). In the control setups, 93.6%, 85.6%, 86.9% and 91.1% of *C. elegans* in sterile water, *S. aromaticum, I. verum* and both *S. aromaticum* and *I. verum* solutions survived (Fig. 4.2).

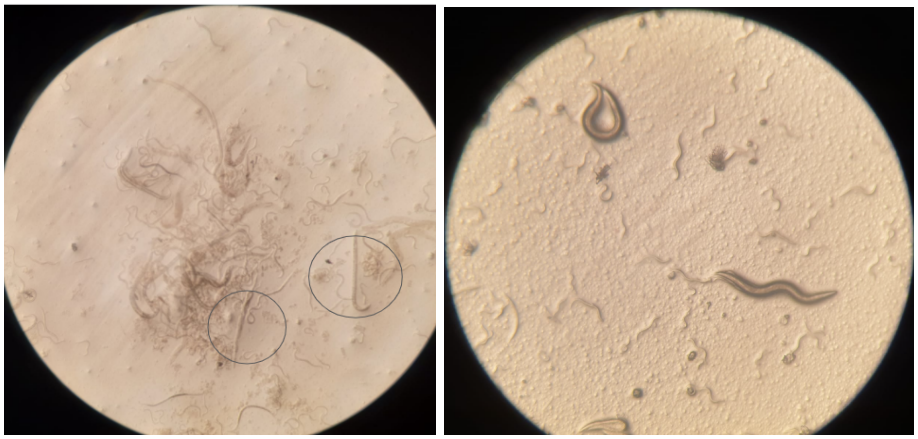

Fig. 2: Plate containing hydrogen peroxide treated *C. elegans* with *S. aromaticum* under hydrogen peroxide. *C.elegans* similar to the circled ones are rod-like and immobile are counted as dead. (Left picture)

Fig. 3: Plate containing untreated *C. elegans* with sterile water. *C. elegans* are not rod-like and respond to outside stimulants, suggesting they are alive. (Right picture)

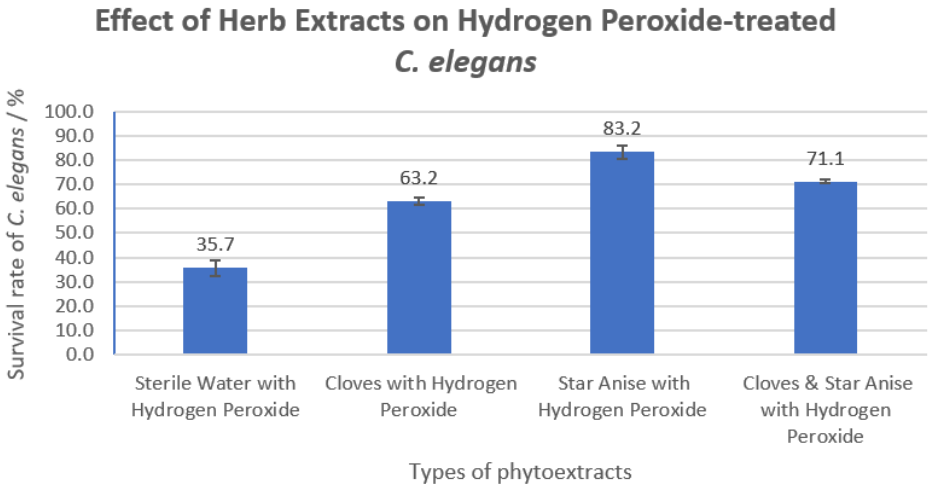

Fig. 4.1: Graph showing the effect of herb extracts on hydrogen peroxide-treated *C. elegans.*

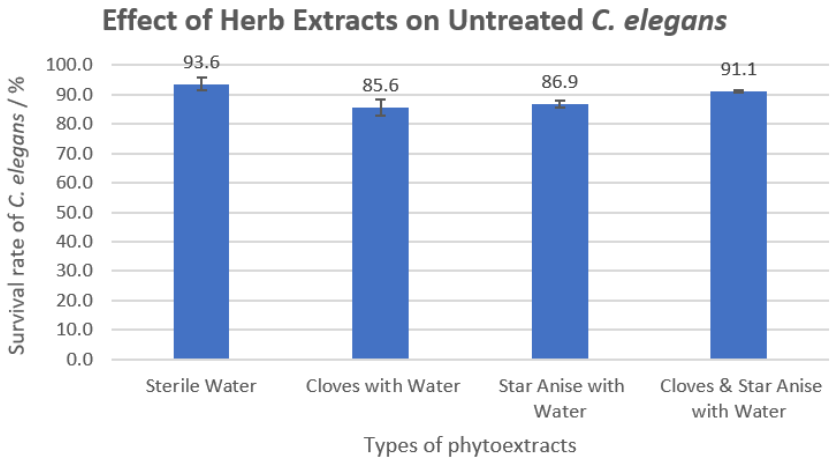

Fig. 4.2: Graph showing the effect of herb extracts on untreated *C. elegans.*

Body Bending Assay

The number of body bends of *Caenorhabditis elegans* in set-ups shown in were assessed. The number of body bends of one worm in one minute was manually counted using a counter and timer under a light microscope at 4x objective magnification and 10x eyepiece magnification. 2 readings per plate were recorded, each taken from a different worm on the plate. The results were then collected and tabulated. In solutions containing hydrogen peroxide, the average number of body bends per worm were at 54.0, 41.5, 97.0 and 58.0 in sterile water, *Illicium verum, Syzygium aromaticum* and both *I. verum* and *S. aromaticum* solutions respectively (Fig. 5.1). In solutions containing sterile water, the average number of body bends per worm were at 19.0, 17.0, 17.5 and 14.0 in sterile water, *I. verum, S. aromaticum* and both *I. verum* and *S. aromaticum* solutions respectively (Fig. 5.2).

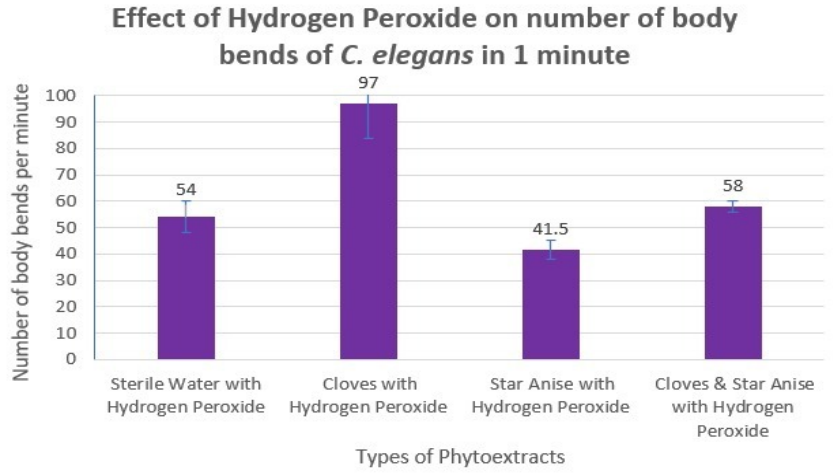

Fig. 5.1: Graph showing the effect of herb extracts on body bends of treated *C. elegans.*

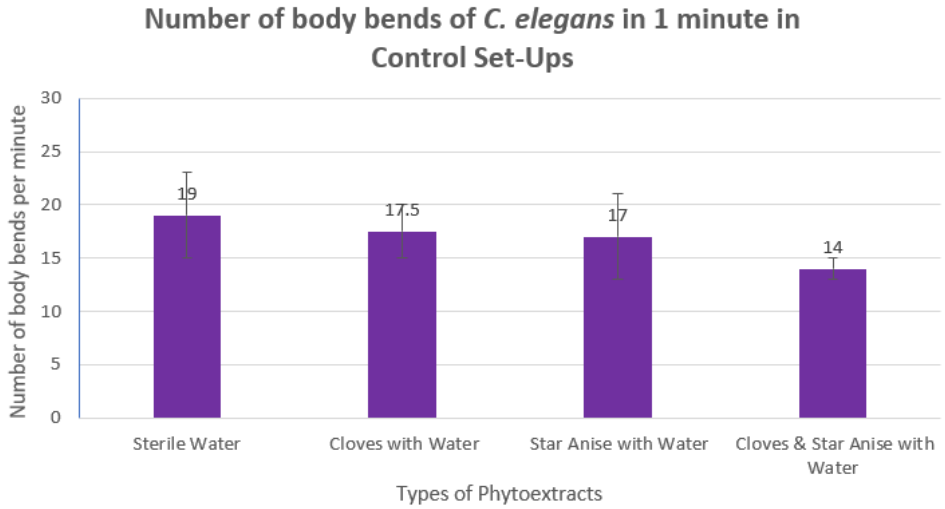

Fig. 5.2: Graph showing the effect of herb extracts on body bends of untreated *C. elegans.*

Discussion

Hydrogen peroxide is a reactive oxygen species itself, so the decrease in survival rates and increased body bends per minute of *Caenorhabditis elegans* after treatment with hydrogen peroxide can be attributed to the action of ROS which causes oxidative stress, directly leading to cell death (Wijeratne et al., 2005). *C. elegans* were also utilised due to their gene similarity to mammalian genes as well as how they are often used as a model organism to study metabolic diseases at a molecular level.

The DPPH assay illustrates how *Illicium verum* exudes strong antioxidant properties, given that they were able to reduce oxidative stress on *Caenorhabditis elegans* and prolonged their lifespan in a hydrogen peroxide solution. This could be attributed to how *I. verum* increases the activity of superoxide dismutase and glutathione peroxidase; a 2018 study (Yu, et al., 2018) showed that the supplementation of *I. verum* oil to the diet of laying hens indicated higher capacity in clearing out the reactive oxygen species by increasing the activities of superoxide dismutase and glutathione peroxidase and reduced malondialdehyde content. The enhanced antioxidant status is likely due to trans-anethole, estragole, limonene, linalool, and cis-anethole contained in star anise (Dzamic et al., 2009; Padmashree, et al., 2007).

For *Syzygium aromaticum*, its change in absorbance was lower than *I. verum* but still decent. The survival rate also reflects this trend. The antioxidant properties is justified by secondary

sources such as a study by Shyamala et al. (2001) which showed that an enhancement in the concentration of cholesterol, phospholipids and triglycerides in the serum, liver and kidneys of hyperlipiedmic rats due to lipid peroxidation evoked by a high fat diet, after the consumption *S. aromaticum* extracts. According to Gülçin et al. (2012), *S. aromaticum* has the ability to maintain nutritional quality, lower or prevent lipid oxidation in pharmaceutical products and food, retard the formation of toxic oxidation products and increase the shelf life of food and pharmaceuticals. This is because of the presence of eugenol (76.8%) that gives *S. aromaticum* its enhanced antioxidant status (Jirovetz, 2006).

Looking at the results it could suggest a correlation between radical scavenging ability of the two herbs and the ability ro restore survival of *C. elegans* with hydrogen peroxide, proving the validity of our results.

Nevertheless, although *S. aromaticum* proved to be effective against oxidative stress in the survival rate assay, but in the body bending assay, it seemed to not be as effective as *I. verum* when hydrogen peroxide was added. In the plate with *S. aromaticum* and hydrogen peroxide, the mean number of body bends of *C. elegans* in 1 minute is approximately 97, which is double of that in the plate with *I. verum* and hydrogen peroxide. This trend is also similar to the DPPH assay, where the radical scavenging activity of *S. aromaticum* was 49.48%, whereas that of *I. verum* was 82.38%, suggesting that *S. aromaticum* has a lower antioxidant activity than *I. verum*, potentially helping to explain why *S. aromaticum* was less effective than *I. verum*.

Furthermore, in Fig. 5.1, an anomaly was noticed as the number of body bends for the test plate with *S. aromaticum* dwas lower than the test plate with sterile water. This could potentially be attributed to random errors associated with the body bending assay; for example, the timing between the addition of hydrogen peroxide and the counting of body bending may have slightly differed between the different set-ups and difficulty in standardising stage of growth of *C. elegans* to name a few. The error bar for *S. aromaticum* with hydrogen peroxide is larger than the error bars of the other set-ups, further supporting the fact that there are discrepancies in the values. Nevertheless, the results can still be utilised for proving the general trend that *I. verum* is more efficient at reducing oxidative stress.

CONCLUSION

In this study, oxidative stress was induced on *Caenorhabditis elegans* via exposure to hydrogen peroxide resulting in significant decreases in survival rates and increases in body bends. However, the above mentioned malignant impacts originating from oxidative stress were greatly reduced with the addition of aqueous extract of *Illicium verum* and, to a smaller extent, *Syzygium aromaticum*, which can be attributed to the various antioxidant compounds present in both phytoextracts. This is supported by the results from the DPPH assay showing the strong free-radical scavenging properties of both phytoextracts. Nevertheless, the effectiveness of *S. aromaticum* is in question since when tested in body bending assays, instead of reducing the body bends, it instead increased it from 54.0 (sterile water) to 97.0 (cloves) body bends per minute (Fig. 5.1). Although a synergistic effect was found in DPPH assay, for physical tests with the *C.elegans* such as the survival rate assay, the mixture often did worse than *I. verum* (Fig 4.1). Hence, overall the synergistic effect is not very pronounced.

Therefore, from the data collected, *I. verum* and *S. aromaticum* can be considered to be included in the production of more foods as a natural alternative to synthetic antioxidants. Furthermore, both phytoextracts can also be considered as natural food preservatives as their high antioxidant activities correlate with higher rates of retarding autoxidation which delays the appearance of such undesirable qualities as rancidity in foods.

Limitations of the study were the difficulty to standardise the antioxidant content in the phytoextracts each time a fresh batch of extract was prepared as well as an inability to pinpoint the exact antioxidant that protects *C. elegans* from hydrogen peroxide, thus leading to varying concentrations of antioxidants and varying ability to protect *C. elegans* against oxidative stress.

Further research could be conducted using other inducers of oxidative stress apart from those used in the present study such as utilising ethanol or UV exposure, and also investigate whether other solvents could be used to more effectively extract antioxidants from the phytoextracts such as non-organic solvents to allow for a more accurate gauge of the antioxidant properties of the phytoextracts as well as determine the exact antioxidant at play in the various herbs to gain a better understanding of the results.

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