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Effect of flaxseed Lignans concentrate on oxidative stress in *Caenorhabditis elegans*

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Abstract
This experiment was carried out to determine the effect of the flaxseed lignans concentrate (LC) on the lifespan and oxidative status of *C. elegans* under oxidative stress. Lifespan was determined by counting the number of surviving nematodes daily under a Lumascope microscope camera after treatment with LC. The evaluated oxidative markers included lipofuscin, which was measured using a fluorescent microscope. In our study, 0.2 mg/ml LC was found to be the optimum concentration to increase the mean lifespan of *C. elegans*, The accumulation of the age marker lipofuscin, which increased with hydrogen peroxide exposure, was decreased with upon treatment with LC. The LC enhanced the lifespan C. elegans and reduced the accumulation of lipofuscin.

Keywords: Flaxseed Lignans, *Caenorhabditis elegans Linum usitatissimum* Linn

Introduction

Linum usitatissimum (Linn.), a member of family Linaceae common called as flaxseed or linseed has been the focus of increased during 90s in the field of diet and disease research due to the potential health benefits associated with some of α-linolenic acid (18:3; n-3), plant lignan secoisolariciresinol diglycoside (SDG) and other biologically active components (1). Further, Flaxseed is the richest source of plant lignans (2). SDG, an antioxidant isolated from flaxseed, is metabolized to secoisolariciresinol (SECO), enterodiol (ED), and enterolactone (EL) in the body [3]. the flaxseed lignan and its mammalian metabolites are known to have a number of potential health benefits, including decreased formation of breast, prostate and colon cancers attributed to (anti)-estrogenic and antioxidant properties [4, 5].

In many organisms ranging from invertebrates to humans, increased oxidative damage to lipids, proteins and DNA has been shown to correlate with increasing age [6, 7]. The nematode *C. elegans* is commonly used in the study of aging. Because these nematodes have relatively short life cycle, large production of progeny, ease of maintenance in the laboratory and their morphological simplicity [8]. The aging of C. elegans is characterized by a progressive decline in locomotion, decreased defecation and decreased pharyngeal pumping rate [9]. In addition, the intestinal cells of *C. elegans* accumulate an auto fluorescent aging pigment called lipofuscin throughout adulthood [10]. The modulation of endogenous defense by antioxidant supplementation is regarded as a promising strategy to delay aging.

Among the many available antioxidants, scientists had been tested vitamin E and its eight isomers, flvonoid-rich plant extracts like Catechin, Epicatechin, Epigallocatechin gallate, Myricetin and Quercetin. These molecules have been shown to modulate life span of *C. elegans*. The prominent flavonoid quercetin (100 to 200 μ) increases mean and median life span by about 6-18% and 19-21%, respectively [11, 12]. The increase in mean lifespan was attributed to the protective effect of antioxidants against oxidative stress. However, the role of flaxseed lignans concentrate (LC) in the aging process of *C. elegans* have not been extensively studied and this study was performed to further elucidate the effects of flaxseed lignans concentration on oxidative biomarkers in *C. elegans* after the induction of oxidative stress.

Materials and Methods

Bulk Extraction of Flaxseed lignans concentrate

The extraction of flaxseed lignans was carried out by using a method used by Naik *et al.* [23] with a scale up of sample quantity. Briefly, the flaxseed sample (10 kg) was washed and subjected for a dehulling process to obtain hull fraction of flaxseed using Kisan Krishi Yantra Udyog dehuller at Grain Science and Technology Department, CFTRI, Mysore, India. Flaxseeds hull fraction was taken, defatted by extracting with n-hexane. The lignans concentrate was prepared from defatted hull fraction of flaxseed. About 10 g of defatted hull fraction was ground and sieved, mixed with 400 ml of distilled water followed by 500 ml 2 M

aqueous sodium hydroxide. The contents were incubated for 1 h at 20 ºC using the shaking water bath. The hydrolysate was then acidified with dilute sulphuric acid to pH 3and then centrifuged at 5000 rpm for 10 min. The supernatant was centrifuged rapidly to a clear liquid phase and pooled together. The liquid phase (600ml) was mixed with 95% aq. Ethanol (900ml), left at room temperature for at least 10 min and total volume was divided into four equal volumes in centrifuge tubes) and again centrifuged at 10000 rpm for 5 min to precipitate. Water soluble polysaccharide and proteins were removed carefully. Ethanol extract was evaporated by using rotary evaporator at 40 ºC to obtain LC. The lyophilized LC was stored until further analysis.

Worm culture

The N2 strain of *C. elegans* were transferred on to agar plates pre seeded with a lawn of E. coli (strain OP50) and cultured for 3 days at 20°C until the worms reached adulthood then washed off with cold K-medium (53 mM NaCl, 32 mM KCl). The worms were pelleted by centrifugation (3,000 g, 5 min), washed twice with cold K-medium, and suspended in K-medium to obtain 50-60 nematodes per 10 µL [13].

Nematode growth medium (NGM) with LC infusion

The optimal dose of LC for the treatment of C. elegans, 0.2mg/ml, was ascertained based on the highest rate of nematode survival after exposure to various concentrations of FLC. NGM containing 0.2mg/ml LC was then prepared according to a previous study with minor modifications [14]. The LC solution was then added aseptically in 96 well plate with OP50 optical density @550nm adjusted to 1.0.

The nematodes were divided into six groups with LC and treated accordingly

T1. Control
T2. H_2O_2 induction
T3. LC treatment,
T4. LC treatment pre-H_2O_2 induction (LC + H_2O_2)
T5. LC treatment post-H_2O_2 induction (H_2O_2 +LC)
T6. LC treatment pre and post-H_2O_2 induction (LC + H_2O_2 +LC).

Oxidative stress induction with H_2O_2

C. elegans nematodes were treated with H_2O_2 at the L4 stage (15). The dose of H_2O_2 used to induce oxidative stress in *C. elegans* was predetermined to be 1.2 mM because this dose resulted in greater than 90% nematode survival compared with the control. The use of lower doses of H_2O_2 did not affect nematode survival, and higher doses decreased nematode survival to less than 90%.

Lifespan study of *C. elegans*

The lifespan study was conducted based on a previously described study [16]. Three replicates containing 50 nematodes each were prepared for each treatment group and assayed simultaneously. The nematodes were counted daily to score live or dead based on movement. Nematodes that were recognized as dead, i.e., with a straight body and no response upon probing, were considered dead.

Determination of lipofuscin content

The presence of lipofuscin was observed using a Lumascope microscope camera (Etaluma, USA). The nematodes from each group were washed from the petri dish and mounted on an agarose pad (2% agarose in M9 buffer containing 0.1% sodium azide). The nematodes were then observed with green excitation at 360–490 nm. Quantification of lipofuscin using florescent microplate reader at excitation 360-370 nm and emission 420-460nm.

Statistical analysis

All the experiments were repeated at least three times with three replications for each treatments. Therefore, the data represent the means and standard errors (Mean ± SD) and were calculated using Microsoft Excel 2010 software.

Result and Discussion

Table 1: Mean lifespan of *C. elegans* treated with different doses of LC.

LC Concentration (µg/ml)	Mean life span
Control	19.27±0.53
100	20.44±0.67
200	23.05±0.38
300	21.88±0.38
400	21±0.57
500	21.11±0.38

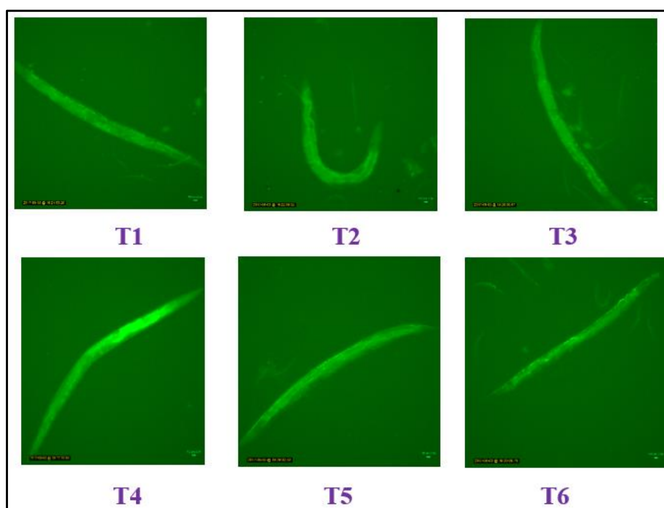

Fig 1: Auto fluorescence of lipofuscin granules in the intestines of *C. elegans*. Fluorescent images were taken using green excitation light. Accumulation of lipofuscin granules in the (T1) control group, (T2) H_2O_2-treated group, (T3) LC group, (T4) LC +H_2O_2 group, (T5) H_2O_2 group+ LC and (T6) LC + H_2O_2 group + LC.

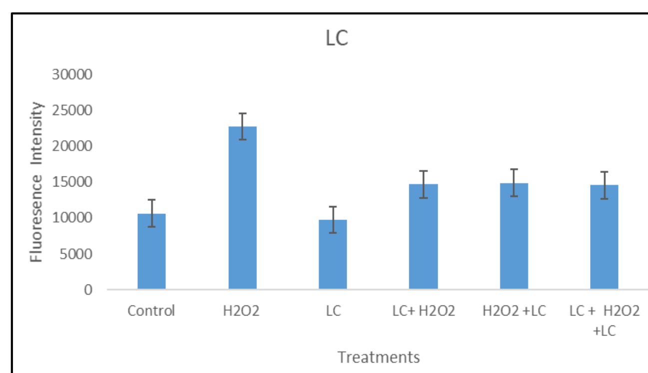

Fig 2: Effect of LC on accumulation of lipofuscin, the fluorescence intensity of the lipofuscin was measured. Each bars point is the Mean ±S.D. of three replicates from three independent experiments.

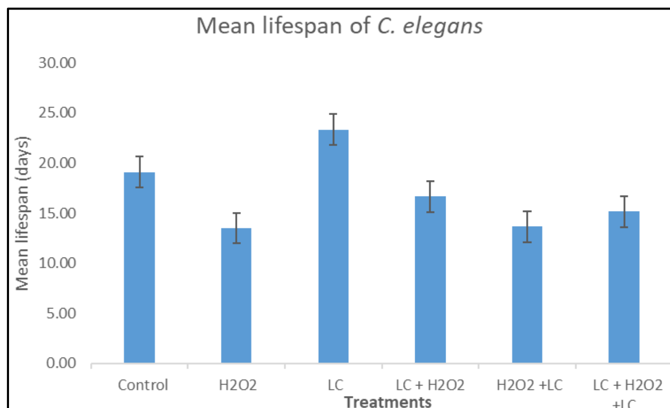

Fig 3: Mean lifespan of C. elegans treated with LC. LC +H_2O_2 +LC and LC +H_2O_2 restored the mean lifespan of the H_2O_2 -treated worms to that of the H_2O_2 group. Each bars point is the Mean ±S.D. of three replicates from three independent experiments.

Lifespan study H_2O_2-induced oxidative stress decreased the mean lifespan of *C. elegans*, whereas the LC treatment alone increased the mean lifespan relative to the control (Figure 3). The LC treatments before and both before and after H_2O_2 induction also increased the lifespan of the nematodes relative to nematodes treated with H_2O_2 alone. However, the mean lifespan of *C. elegans* was not affected by LC treatment after H_2O_2 induction.

In our study, 0.2 mg/ml LC was found to be the optimum concentration to increase the mean lifespan of C. elegans (Table-1). Our preliminary results show that induction of oxidative stress by 1.2 mM H_2O_2 for 2 h was non-lethal, with a survival rate higher than 90% relative to the control. This concentration of H_2O_2 significantly decreased the sinusoidal body movement of the nematodes as observed under a light microscope. The mean lifespan of the nematodes was reduced following oxidative stress induced by H_2O_2, which mediates aging signals [17]. The H_2O_2 treatment may have interfered with the cellular function of *C. elegans*, thereby leading to a shortened lifespan by increasing intracellular ROS levels [18]. Vitamin E has generally been found to increase the lifespan of *C. elegans* by slowing development, decreasing fecundity and delaying reproduction [19]. The prominent flavonoid quercetin (100 to 200 μ) increases mean and median life span by about 6–18% and 19–21%, respectively. Additionally, nematodes treated with quercetin showed a higher (5 to 18%) maximum life span than controls [11, 12].

As in other multicellular organisms, the aging of *C. elegans* is characterized by the accumulation of age associated auto fluorescent lipofuscin in the intestine [12]. Lipofuscin is an age pigment that is present in intra lysosomal granules and primarily composed of cross-linked protein residues and lipid peroxidation residues formed as a result of iron-catalysed oxidative processes [20]. In this study, lipofuscin accumulation was measured as a marker of aging and as a measure of oxidative damage to lipids in *C. elegans*. Previously, H_2O_2 was shown to increase lipofuscin accumulation in an *in vitro* study [21]. In line with this finding, we found in the present study that accumulation of lipofuscin was increased following induction with H_2O_2. The enhanced formation of lipofuscin granules may be the result of increased cellular free radicals, which accumulate with age [22]. The LC treatments were able to reduce lipofuscin accumulation in nematodes under oxidative stress. Although the pre-treatment with LC inhibited the accumulation of lipofuscin, the post-treatment with LC reflected the function of LC as not only a chain-breaking antioxidant that prevents the propagation of free radical damage (figure-1 and figure-2).

Conclusion

In conclusion, treatment with LC restored the mean lifespan of C. elegans under oxidative stress and reduced the accumulation of lipofuscin. In addition, a combination of pre- and post-treatments with LC conferred better protection against oxidative DNA damage than either pre- or posttreatment with LC alone.

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