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

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Abstract
In continuation with our previous study, this experiment was carried out to determine the effect of the transferosome (TRF) loaded with flaxseed lignans concentrate on the lifespan and oxidative status of *C. elegans* under induction of oxidative stress by H_2O_2. Transfersomal formulation prepared using bio surfactant sophorolipid and transferosomes were tagged with FITC to confirm the penetration through nematode cuticle hypothesis was tested. Lifespan was determined by counting the number of surviving nematodes daily under a Lumascope microscope after treatment with TRF. The evaluated aging oxidative markers included lipofuscin, which was measured using a fluorescent microscope. In our study, 0.2 mg/ml TRF was found to be the optimum concentration to increase the mean lifespan of *C. elegans*, t he accumulation of the age marker lipofuscin, which increased with hydrogen peroxide exposure, was de creased with upon treatment with TRF. The TRF enhanced the lifespan *C. elegans* and reduced the accu ulation of lipofuscin trend remains matches with the non-encapsulated flaxseed lignans concentrate. Further, study confirms that possibility of penetration of FITC tagged transfersomes via cuticle of nematode was confirmed by imaging.

Keywords: Lignans concentrate, *Caenorhabditis elegans Linum usitatisimum* L.

Introduction
Flax (*Linum usitatisimum* L.) is grown as either an oil crop or as a fibre crop, with fibre (linen) derived from the stem of fibre varieties and oil from the seed of linseed varieties [1, 2]. While lignans are found in many plants, flaxseed is the richest source of SDG (0.7 and 1.9% in ground flax) [3]. SECO and SDG may possess chemo preventive properties in animals and humans [4] including the potential to prevent hormone sensitive cancers (e.g. breast, prostate and colon cancer), hypercholesterolemic atherosclerosis and diabetes [5]. Because flaxseed lignans are the richest source of precursors for the mammalian lignans most studies use whole defatted flaxseed or SDG to assess the role of the mammalian lignans.

The free radical theory of aging, first suggested by Harman in 1956 [6], proposes that the accumulation of molecular damage provoked by reactive oxygen species (ROS) is a major factor in aging. This theory has been supported by a number of studies in several organisms showing a correlation between the aging process and oxidative stress as well as delayed aging by exogenous treatment with antioxidants [7, 8]. Recently, A new vesicular derivative, the "transferosomes", has paved the way to minimize the defective transdermal permeation of a number of low and high molecular weight drugs, [9] which has been found to be one of the major advancement in vesicle research. Transferosomes are a special type of liposomes, consisting of phosphatidylcholine and an edge activator. They are soft malleable vesicles tailored for enhanced delivery of active agents [10]. Are drawing attention as a platform for targeted drug delivery [11, 12].

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 [13]. The aging of C. elegans is characterized by a progressive decline in locomotion, decreased defecation and decreased pharyngeal pumping rate [14]. In addition, the intestinal cells of *C. elegans* accumulate an auto fluorescent aging pigment called lipofuscin throughout adulthood [15]. The modulation of endogenous defense by antioxidant supplementation is regarded as a promising strategy to delay aging. The increase in mean lifespan was attributed to the protective effect of antioxidants against oxidative stress. However, the role of transfersome loaded with flaxseed lignans concentrate in the aging process of *C. elegans* have not been extensively studied and this study was performed to further elucidate the effects of transfersome loaded with flaxseed lignans concentrate 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.* [28] 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.

Transfersome preparation

The LC-loaded Transfersomes were prepared as mentioned by [16] with slight modification. The phosphatidyl choline and surfactant/ edge activators were added to the beaker (100ml) and dissolved in chloroform: ethanol (1:1) and kept overnight for evaporation of solvent at room temperature. The film deposited was hydrated with pH 5.5 phosphate buffer containing LC. Then beaker subjected to rotation at 150 rpm for 1 h. The LC-loaded transferosomal vesicles formed by above process were kept for 2 h at 25 °C for swelling and were sonicated using bath Sonicator for 20 min.

Preparation of FITC tagged transfersomes

Ethanol solution of FITC (1 mg/ mL) in a proportion of 1:100 v/v was added during preparation of transfersomes. [17] 50 µL of this solution is then mixed with 200 µL of M9 buffer. After 2 hours live animals are transferred to the plates. The animals are transferred to a plate devoid of the dye for at least 10 min to remove free FITC before viewing under the microscope.

Nematode growth medium (NGM) with TRF infusion

The optimal dose of TRF 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 TRF. NGM containing 0.2mg/ml TRF was then prepared according to a previous study with minor modifications [19]. The TRF 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 TRF and treated accordingly

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

Oxidative stress induction with H_2O_2

C. elegans nematodes were treated with H_2O_2 for two hours at the L4 stage [20]. 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 [21]. 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

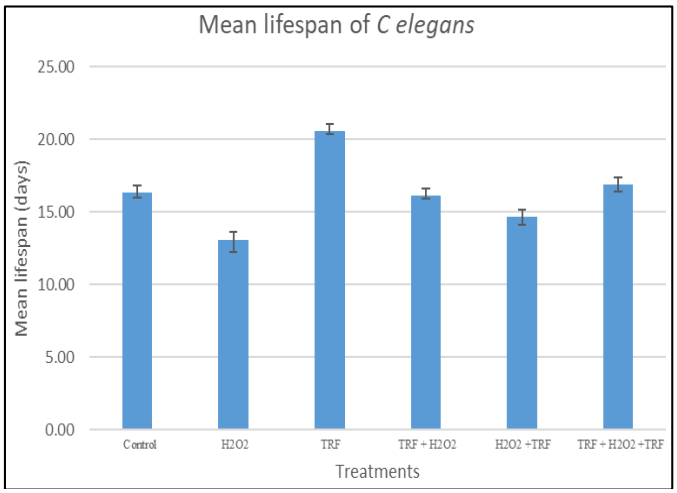

Fig 1: Mean lifespan of C. elegans treated with TRF. TRF +H_2O_2 +TRF and TRF +H_2O_2 restored the mean lifespan of the H_2O_2-treated worms to that of the control group. Each bars point is the Mean ±S.D. of three replicates from three independent experiments.

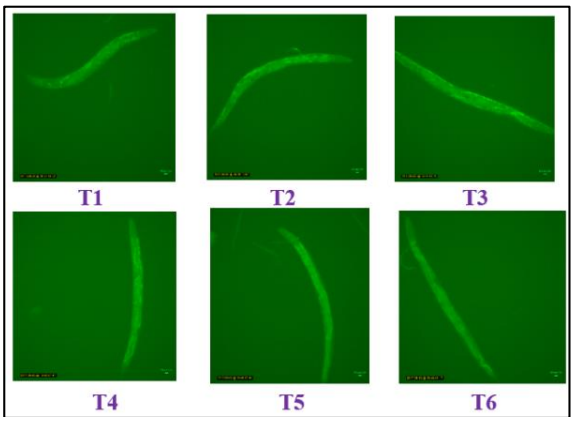

Fig 2: Autofluorescence of lipofuscin granules in the intestines of *C. elegans*. Fluorescent images were taken using green excitation light (450-490 nm). Accumulation of lipofuscin granules in the (T1) control group, (T2) H_2O_2-treated group, (T3) TRF group, (T4) TRF +H_2O_2 group, (T5) H_2O_2 group+ TRF and (T6) TRF + H_2O_2 group + TRF.

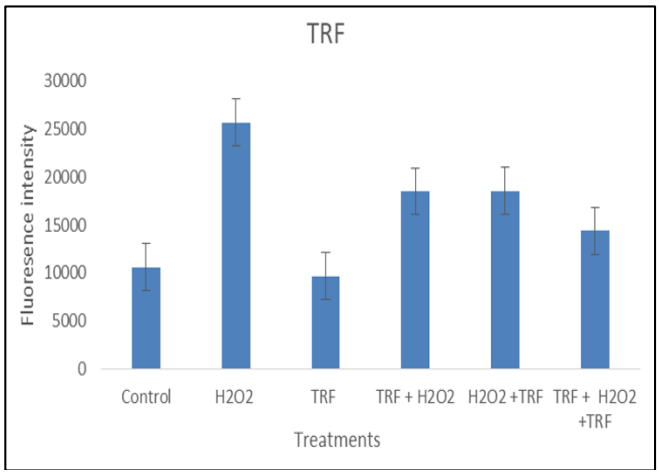

Fig 3: Effect of TRF on accumulation of lipofuscin, the fluorescence intensity of the lipofuscin was measured. Each bars point is the Mean ±S.D. of three replicates from three independent experiments.

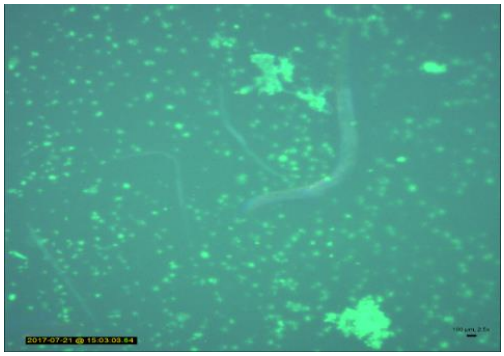

Fig 4: Incubation of worms containing formulation with Fluorescein isothiocyanate.

Fig 5: Image depicting the presence of transfersome inside the nematode might be through skin and pharynx.

Lifespan study H_2O_2-induced oxidative stress decreased the mean lifespan of *C. elegans*, whereas the TRF treatment alone increased the mean lifespan relative to the control (Figure 1). The TRF 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 TRF treatment after H_2O_2 induction.

In our study, 0.2 mg/ml TRF 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 [22]. 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 [23]. Vitamin E has generally been found to increase the lifespan of *C. elegans* by slowing development, decreasing fecundity and delaying reproduction [24]. 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 [25]. 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 [26]. 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 [27]. The TRF treatments were able to reduce lipofuscin accumulation in nematodes under oxidative stress. Although the pre-treatment with TRF inhibited the accumulation of lipofuscin, the post-treatment with TRF reflected the function of TRF as not only a chain-breaking antioxidant that prevents the propagation of free radical damage (figure-2 and figure-3). Trend remains same for both TRF as well as LC (unpublished data) but we have noticed that decreased mean lifespan of *C. elegans*, relatively increased lipofuscin content. Reason might be due to poor encapsulation efficiency of transfersome or quantity of LC loaded is not sufficient serve the purpose. Even though transferosome facilitate internalisation of LC at faster rate, quantity of LC is also an important factor for beneficial effect. Images clearly indicates presence of FITC tagged TRF in pharynx and surprisingly we found that presence of TRF inside body apart from pharynx and we suspect that might be entered through skin of worm (Figure 4-5).

Conclusion

In conclusion, treatment with TRF 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 TRFconferred better protection against oxidative DNA damage than either pre- or posttreatment with TRF alone. Further, study confirms that possibility of penetration of FITC tagged transfersomes via skin of nematodes.

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