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Inhibition of advanced glycation end products formation and inflammation in *C. elegans*: Studies of potential of *Lyngbya* sp. against expression of stress related genes and Live cell imaging

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ABSTRACT

Marine cyanobacteria *Lyngbya* sp. has been reported to produce many bioactive secondary metabolites which shows potent anti-oxidant, anti-inflammatory, anti-diabetic and anti-cancer activity. However, several potentials are yet to be unexplored. Hence in this study, ethanolic fraction (EF) of *Lyngbya* sp. was used to analyse its therapeutic potentials. The extract was collected and analyzed by Fourier-transform infrared spectroscopy and Gas chromatography–mass spectrometry. The anti-oxidant activity and Advanced Glycation End products (AGEs) inhibition by **EF** was explicitly studied in hyperglycaemic *C. elegans* as an animal model by live animal imaging and spectrofluorimetry assays. Live imaging and spectrofluorimetry analysis of hyperglycaemic *C. elegans* by high content screening revealed the decreased fluorescence intensity corresponding to AGE formation in **EF** treated animals than control. Likewise *C. elegans* treated with **EF** had also shown low internal glucose levels in 100 mmol/L and 200 mmol/L hyperglycemic worms, respectively than the hyperglycemic worms 100 mmol/L and 200 mmol/L, respectively. Moreover semi-quantitative RT-PCR studies showed the **EF** treated nematodes showed up-regulated *glod*− 4 and *daf-16* expressions, while a decrease in *daf-2* expression thus proving its role in AGE mitigation and increasing longevity in stress conditions. Anti-inflammatory study was also studied by using *daf*− 16 gene in *C. elegansTJ356 daf16::GFP* as an animal model. Thus, the ethanolic fraction of *Lyngbya* sp. extracts have the therapeutic potential as an inhibitor of AGE formation by modulating stress response gene expression and inflammation by controlling of *daf*− 16 gene.

1. Introduction

Diabetes mellitus, an endocrine metabolic disorder with characteristic complication occurs due to hyperglycemia, which predispose a person to chronic complications that may affect most of the parts of the body and it is considered as the sixth leading disease which causes death (Nash et al., 2001). Approximately 200 million people currently have type 2-diabetes (T2D) worldwide; the occurrence of T2D has been predicted to increase to 366 million by 2030 in the developing countries, mainly India and China. Hyperglycemia plays a pivotal role in the manifestation of diabetic complications by increasing protein glycation and the steady increase of advanced glycation end products (AGEs) in tissues are related with increased free radical generation that results in the bio-molecular damage in diabetes (Li et al., 2012). Protein glycation and AGE's accumulation play a key role in the developing complications of diabetes, inflammation, Alzheimer's disease, aging, renal failure and chronic diseases (Smit and Lutgers, 2004). So there is a need to identify the risk factor like AGE and to inhibit the action of AGEs formation, claiming as a promising therapeutic approach in treatment of diabetes and its related inflammatory complications.

Inflammation is responsible for causing various diseases such as asthma, cardiovascular diseases, cancer, diabetes, obesity, and bronchitis (Heo et al., 2010). Inflammatory processes are started in the beginning of the diabetic complication itself. Adipose cells, release of inflammatory cytokines and other mediators which is associated to insulin resistance in type-2 diabetes by activating NF-κß signalling pathway (Geraldes and King, 2010). NF-κβ can transcribe the other pro-inflammatory genes (TNF-α, IL-6, IL-1, COX-2 and iNOS) (Hegazy et al., 2015).

New therapeutics in the pharmaceuticals industries are being

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produced many bioactive compounds from marine microorganisms as effective drugs for the treatment of different diseases (Iwamoto et al., 2001). *Lyngbya* sp., the marine cyanobacteria, produces a variety of secondary metabolites that have been reported to be of therapeutic potential (Berry et al., 2002). Non-ribosomal polyketide synthase (NRPS) and polyketide synthetase of *Lyngbya* sp. are also reported as critical indicators of production of various secondary metabolites (Moore, 1996; Sharp et al., 2009). Streptozotocin (STZ)-induced diabetic rats were used as an animal model for testing efficacy of bioactive metabolites from any biological sources as the therapeutic agents. However, *C. elegans* is currently being used as an animal model, due to its ease in cultivation and maintenance. It was reported that high glucose accumulated in a nematode *C. elegans* could be surmount by increasing the glyoxalase-1-dependent detoxification of methylglyoxal (Schlotterer et al., 2009) where glyoxalase-1, is a chief enzyme that detoxifies the methylglyoxal (MG), a precursor for AGE formation (Morocos et al., 2008). Therefore, this study is aimed to understand the underlying mechanisms of preventing glucose induced toxicity by the bioactive compounds isolated from *Lyngbya* sp. and obtained results are clearly evidenced that altered expression of the stress responsive genes *glod-4*, *daf-16* and *daf-2* in the hyperglycaemic *C. elegans*. Moreover, the anti inflammatory effect of **EF** by in *C. elegans* was analyzed by the expression of DAF-16 regulation (similar to mammalian FOXO3a), which is a major factor to control the innate immunity in *C. elegans* during inflammation induced by infection (Zou et al., 2013; Miyata et al., 2008)

2. Methods

2.1. Algal sample collection, identification, extraction and separation of compounds

The cyanobacterial samples were collected from Gulf of Mannar, Rameshwaram, Tamil Nadu, India and samples were identified on the basis of morphological characters as described by Rippka et al. (1979) and it was already reported as *Lyngbya* sp. (Sameer et al., 2018). The freeze dried cyanobacterial samples (100 g) were lysed with liquid nitrogen, and further methanol/chloroform (ratio 1:2) was added to extract the bioactive compounds from the *Lyngbya* sp. since methanol is better solvent for the organic compound and kept at room temp (27 ± 1 °C) for overnight. The bioactive compounds were filtered using Whatman filter paper, and further were extracted using different solvents such as Hexane-Ethanol-Methanol (2:1:1) for phase separation to remove the non-polar components from the extract.

2.2. Characterization of the ethanolic extract by FT-IR and GC-MS

Total three fractions were obtained from the phase separation. The ethanolic fraction was analyzed using FT-IR (Shimadzu, Japan) spectra to identify functional groups. In order to elucidate the nature of the components present in the ethanolic fraction **EF** it was further subjected to transesterification by acid catalysis (Ichihara and Fukubayashi, 2010) to convert it into volatile methyl ester which will aid to analyse the compound by GC-MS. Compounds in ethanolic fraction was collected in GC-MS vials, and equal volume of methanol (500 µL) and 15 µL of HCl were added to this vials which were further incubated at 100 °C for 120 mins for transesterification reaction. After incubation, using equal volume of hexane (500 µL) was added and subjected for GC-MS analysis of ethanolic extract was performed on Agilent Technologies, 6890, N series, Santa Clare, CA GC-MS system.

2.3. Biological activities of EF fraction

2.3.1. DPPH radical scavenging activity

Antioxidant activity of the **EF** was studied by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method (Girija et al., 2013). Briefly, 2 mL of DPPH solution (0.16 mM), prepared in methanol was added to 2 mL of **EF**. These mixtures were vortexed and incubated at room temperature 27 °C for 30 min. After incubation, absorbance was read at 517 nm using visible spectrophotometer (Eppendorf, Germany). Absorbance of the reaction mixture was represented as the antioxidant property and that was calculated using the following equation:

Percentage (%) inhibition of DPPH activity $= ([(A_0 - A_1 / (A_0)] \times 100\%$

The A_0 and A_1 are represented as the absorbance value of the control and test sample (ethanolic fraction). Ascorbic acid was used as a standard antioxidant for this assay. A curve of percentage of scavenging activity against sample concentration was required to calculate the IC_{50} value.

2.3.2. Analysis of total antioxidant activity of EF

Total anti-oxidant activity of ethanolic compounds was estimated by phosphomolybdenum assay (Prieto et al., 1999). In this assay, Mo (VI) oxidized into Mo (V) by the compounds and forms green phosphate complex at acidic pH. In this method, 0.3 mL of **EF** was added into a test tube and 3 mL of 28 mM sodium phosphate, 0.6 M sulphuric acid and 4 mM ammonium molybdate as reagent solution was further added into the reaction mixture. The tubes were incubated at 95 °C for 1 h and 30 min, cooled and the absorbance was read at 695 nm by using visible spectrophotometer (Eppendorf, Germany). Finally the total antioxidant activity of the **EF** compound was expressed as per microgram ascorbic acid equivalent to per mL of the compound.

2.3.3. Advanced Glycation End product (AGEs) inhibition assay

In order to check the AGE inhibition, by **EF**, BSA glycation inhibition assay was performed (Peng et al., 2008). Bovine serum albumin (100 mg/mL) and glucose (188 mg/mL) was dissolved in phosphate buffer (100 mM, pH 7.4) separately. Equal volume of BSA, glucose and **EF** (1 mL) was taken and 0.2 g/L of sodium azide was added to this solution to avoid bacterial contamination. Phosphate buffer was used as blank and phloroglucinol was used as positive control. These mixtures were incubated at 37 °C for 7 days. After incubation, samples were measured using spectrofluorimetry at emission of 410 nm and an excitation of 330 nm (Hoefer Scientific Instruments, San Francisco, USA). The percentage (%) of inhibition of AGE formation = [1 − (fluorescence of the experimental group/ fluorescence of the control group)] × 100%.

2.4. Anti-glycation activity of EF using C. elegans as an animal model

2.4.1. C. elegans culture conditions, age synchronization and seeding

The wild type nematodes, *C. elegans* (N2, Bristol) were cultivated on nematode growth medium (NGM) with agar. *C. elegans* were maintained by feeding live *E. coli* OP50 at 20 °C until the nematodes grow and to attain the gravid adults. Gravid nematodes were collected with M9 buffer from the plates and were transferred into 15 mL test tube. The supernatant consisting of M9 buffer was aspirated out and 5 mL of 20% alkaline hypochlorite solution (freshly prepared) was added into the tube. The tube was mixed gently for 5 min and then spun down at 8000 rpm for 1 min. Hypochlorite solution was removed from the tube without disturbing the pellet and which was washed thrice with M9 buffer and resuspended in fresh S-complete medium and incubated for overnight for hatching (L1 stage). Freshly prepared 100 mL of LB broth was inoculated with *E. coli* OP50 as feed to the nematodes and incubated for 24 h and then centrifuged at 8000 rpm for 10 min and the pellet of *E. coli* was collected and weighed. The pellet was resuspended in S-complete medium for further studies.

L1 stage of nematodes were selected and seeded in all the 24 wells and fed with *E. coli* OP50 in S-complete medium. Hyperglycemic condition for the nematodes was created in the medium by providing with glucose at final concentration of 100 mmol/L and 200 mmol/L. The nematodes were incubated overnight at 20 °C, for 24 h. On the second

day, 50 μL and 100 μL of **EF** was added to 100 mmol/L glucose plates and 100 μL and 200 μL of **EF** was added with 200 mmol/L glucose plates. The experimental setup and control was maintained in triplicates.

2.4.2. Live cell imaging of C. elegans by High Content Screening (HCS) analysis for AGE accumulation

On 9th day, live nematodes were analyzed by HCS (Perkin Elmer), for the fluorescence due to accumulation of AGE's in normal nematodes, hyperglycemic nematodes and treated with **EF**. The fluorescence intensity of accumulation AGEs was observed.

2.4.3. Quantitative analysis of AGEs accumulation by spectrofluorimetry

To quantify the accumulation of AGEs, the nematodes were collected after 9th day incubation and transferred into a tube with sterile water for preparing aqueous homogenates of the nematodes and subjected them to freeze-thaw cycles about 3 times. The cellular debris was separated by the centrifugation (at 8000 rpm) for 2 min and the supernatant was collected into new vials. Cell free supernatants were used for the analysis of fluorescence by the AGE's accumulation, using spectrofluorometer (Fluoromax-4 Spectrofluorometer HORIBA JOBIN YVON, Japan) at excitation 365 nm and emission 470 nm (Davis et al., 1982). All the experimental set up was maintained in triplicates.

2.4.4. Analysis of glucose in C.elegans

The total reducing sugar assay was performed to analyse the glucose level in all the experimental nematodes by DNSA method. The reducing sugar content in the initial medium of each group, and the residual medium of post incubation (at 9th day) of nematodes and the whole nematode extract was also analyzed. Nematodes from each experiment consisting of 25–30 adult nematodes were used for this assay. The estimated glucose in nematodes at all experimental conditions was plotted as a graph.

2.4.5. Semi-quantitative RT-PCR analysis

The expression of stress responsive genes *glod-4*, *daf-16* and *daf-2* were studied in **EF** treated (50 μL) and untreated hyperglycaemic (100 mmol/L) *C. elegans*. The total RNA was extracted from these groups of *C. elegans* on the 9th day using the Trizol method (Gandhimathi et al., 2015). The extracted RNA was DNAase treated and checked for its purity and integrity on agarose gel by electrophoresis and 3 μg of RNA was used for cDNA synthesis using oligo dT primers (Table 1). The mRNA expression of the *glod-4, daf-16* & *daf-2* were measured by semi-quantitative PCR, with β- *actin* as internal control.

The expression of *glod-4*, *daf-16*, *daf-2* and β- *actin* was studied at the exponential phase of 32 PCR cycles, at annealing temperature of 58 °C. The amplified products were run on 2% agarose gel electrophoresis. Further, the difference in the expression levels of the three genes as given by the intensities of band was analyzed by the densitometry software ImageJ.

2.5. Anti-inflammatory activity of EF

2.5.1. Nitric oxide radical scavenging assay of EF

To check the *in vitro* anti inflammatory activity, nitric oxide scavenging potential of **EF** was assayed according to Tsai et al. (2007). The quantities of nitrate or nitrite can be determined by using Griess reagent (Marcocci et al., 1994). **EF** (60 μL) was mixed with sodium nitroprusside (10 mM) in PBS added into 96-wells plate and further incubated at 28 °C under light condition for 2 h. The nitrite contents were measured after the addition of Griess reagent. Ascorbic acid was used as standard and the NO-scavenging effect of compound was calculated by the given formula:

$$[(A_0 - A_1)/A_0] \times 100\%$$

The A_0 and A_1 was the absorbance of the control and sample reaction.

2.5.2. Study of anti-inflammatory effect of EF in C. elegans

C. elegans TJ356 daf16:: GFP were seeded into a 6 well plate and exposed to 100 μL of overnight grown culture of *Pseudomaonas aeruginosa*, to cause inflammation in nematodes. Subsequently, the nematodes were treated with **EF**. After 24 h, worms were collected and subjected to RNA isolation to quantify the *daf*-16 gene expression which is related to human FOXO-3a gene. The *daf*-16 expression was also validated by high content screening of the localization of *daf*-16 tagged GFP.

3. Results and discussion

3.1. Extraction and separation and characterization of the EF using FT-IR analysis and GC-MS analysis

The isolated sample was identified as *Lyngbya* sp. as already reported by Sameer et al. (2018). The dried sample of *Lyngbya* sp. was used to extract the compounds and was further separated by solvent phase separation with hexanes-ethanol-methanol gradient. Here ethanol was used as solvent because it can dissolve many organic compound and the ethanolic fraction showed the maximum yield (dry weight-20 mg/mL) of the compound when compared with other solvents. The **EF** was further analyzed by FT-IR analysis and revealed the different functional group at different wave numbers (ESI Fig. 1). A compound in ethanolic fraction had given functional groups (ArO-H bonded, RCH_2CH_3, trans RCH = CHR, C-C in ring, $-CH_3$ group, Ar2NH, RCO-OH). GC-MS analysis showed the chemical composition of the ethanolic compound (Fig. 1). Ethanolic fraction showed five peaks in the GC-MS chromatogram. These nine compounds were identified and characterized on the basis of GC retention time (Table 2) with their corresponding mass numbers (*m/z*). Interestingly, the biological function of the compound 1-tetradecane, 1-octadecane and 1-hexadecane was already reported by Guo et al. (2008) and it's showed anti-microbial activity. Phenol, 2,4-bis (1,1-dimethylethyl) is reported for its anti-pathogenic activity which can use to treat drug resistance infections (Padmavathi et al., 2014). Heptadecane showed potential anti-inflammatory activity by modulating the NF-κB related gene or NF-κB dependent gene-related (Kim et al., 2013). 1-docosene compounds showed potent cell-cytotoxicity activity against cancer cells (Figueiredo et al., 2014). Cycloeicosane was also reported potent anti-microbial, anti-cancer and anti-oxidant activity (Madkour et al., 2017).

Table 1
Primers used for the amplification of stress related genes.

Genes	Forward primer	Reverse primer
glod -4	5′-TCTTCAAAGTGGCGAATCGTG – 3	5′-TCTTGCTCCATCGTCCGTTAT – 3
daf- 2	5′-GGAGCTTCGGAGTTGTTCTCTA – 3′	5′-CCAGCACATTTTCATCACCTTA – 3′
daf-16	5′-TCAACATCCAACTCATCTCCAC – 3′	5′-GATCGGGAAGTGTCAGATTTTC – 3′,
ß-actin	5'-ATCGTCCTCGACTCTGGAGATG – 3'	5'-TCACGTCCAGCCAAGTCAAG – 3'

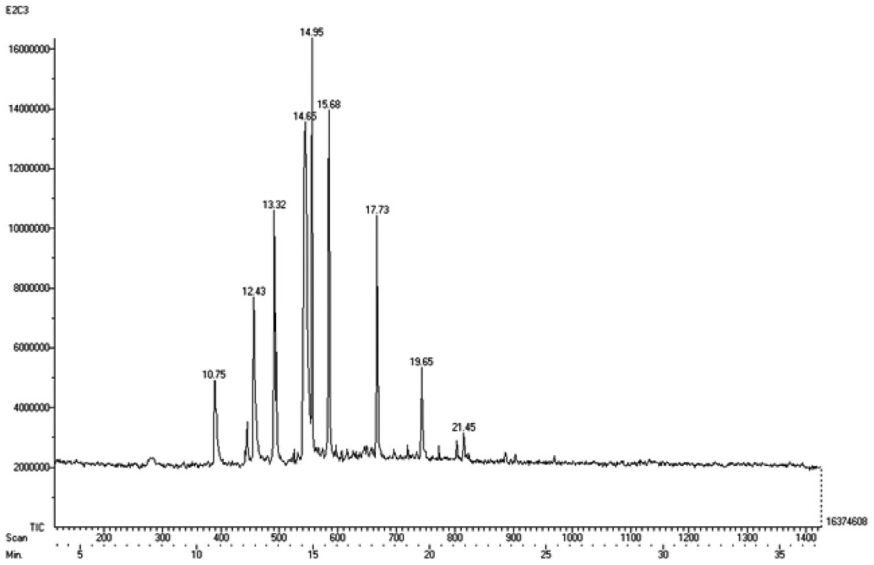

Fig. 1. GC-MS chromatogram of **EF** fraction of *Lyngbya* sp.

Table 2
Retention time, mass (*m/z*) and the compound present in **EF** analyzed by GC-MS analysis.

Retention Time	Mass (*m/z*)	Compound	% of each compound
10.75	196.19	1-tetradecane	7.5
12.43	206.16	Phenol, 2,4-bis (1,1-dimethylethyl)	7.3
13.32	224.25	1-hexadecane	11.1
14.65	240.25	Heptadecane	29.5
14.95	238.23	8-heptadecane	18.3
15.68	252.26	1-octadecane	15.2
17.73	290.27	Cycloeicosane	5.4
19.65	308.31	1-docosene	4.2
21.45	336	Cyclotetracosane	1.6

3.2. *In vitro* biological activities of EF of Lyngbya sp

3.2.1. Anti-oxidant activities of EF

The biological potential of **EF** was analyzed by performing anti-oxidant activity using DPPH where ascorbic acid (vitamin C) was used as a standard antioxidant. The anti-oxidant is generally scavenging the radicals in per-oxidation and oxygen containing compounds or ability of metal chelating, that can prevent the hydrogen peroxide induced cell damage (Li et al., 2011) and they might be further involved in development of disease progression like diabetes and cancer *etc.* **EF** showed great potential against DPPH and comparatively it had shown low IC_{50} value (25.89 ± 0.21 μg/mL), ascorbic acid (46.8 ± 0.02 μg/mL). Total anti-oxidant activity of **EF** was analyzed by using phosphomolybdnenum assay, a quantitative method to investigate the reduction reaction rate among anti-oxidant, oxidant and molybdenum ligand. It measures the reduction degree of Mo (VI) to Mo (V) and gives direct estimation of reducing capacity of anti-oxidant. Here, total antioxidant activity of 100 μg of the **EF** was equivalent to 192 ± 1.41 μg of ascorbic acid, thus **EF** had comparably better antioxidant capacity than ascorbic acid. Thus, **EF** derived from *Lyngbya* sp. is causing potential anti-oxidant activity and it can be used as very good source of natural anti-oxidant which may protect the radicals mediated cellular damage. Likewise, other cyanobacteria including *Anabaena, Cyanothece, Prochlorothrix and Synechococcus* had been reported to shown good anti-oxidant properties where the protein extracts of cyanobacteria was used (Kelman et al., 2009). A notable example is the anti-oxidant activity of phenolic compounds released during degradation of coir pith by *Oscillatoria annae* which was evidenced in Albino rat and cyanobacterial extract on swiss mice (Prabha et al., 2009; Navanietha Krishana Raj et al., 2012).

3.2.2. Anti-glycation assay for AGE inhibition by EF

Moreover, the BSA reducing sugar system as an *in vitro* model was used to study the non-enzymatic glycation where the spontaneous glycation might mediate the protein modification when it is exposed to reducing sugars and it would fluoresce at emission (410 nm) and excitation (330 nm). The intensity of fluorescence developed by the glycation is the characteristic feature of AGEs. The AGEs formation is a crucial biochemical abnormality which is responsible for the diabetes mellitus and inflammation (Basta et al., 2004). The **EF** exhibited a pronounced AGE inhibitory activity when compared with pholoroglucinol as standard. The IC_{50} value (16.42 ± 0.28 μg/mL) of **EF** was lesser than the pholoroglucinol (52.57 ± 0 μg/mL) which might be due to the reactive carbonyl scavenging activity of the **EF** similar to the effect of phlorotannins as reported by Liu and Gu (2012).

3.3. *In vivo* biological activities of EF of Lyngbya sp

3.3.1. Studies of AGE inhibition by EF in C. elegans

Based on the *in vitro* results obtained from AGE inhibition assay, *in vivo* studies using *C. elegans* at whole animal levels were performed for live cell imaging to substantiate the inhibition of AGE formation. Interestingly, *C. elegans* induced with hyperglycemia (100 mmol/L and 200 mmol/L of glucose) showed pronounced fluorescence in the emission range of 400–450 nm. Whereas, the hyperglycemic age synchronized adult nematodes (100 mmol/L and 200 mmol/L of glucose) treated with **EF**, had shown prominent decrease in fluorescence intensity, which was equivalent to the normal nematodes and that was further confirmed with live cell imaging of *C. elegans* by HCS (Fig. 2 A). The emission is in the range of 400–500 nm, which is corresponding to the fluorescence range of AGE products (Shakambari et al., 2015).

The effect of **EF** on AGE accumulation was also quantified by spectrofluorimetry at excitation 360 nm and emission at 420 nm, on 9th day of growth using cell free aqueous extract of whole nematodes and was found that in *C. elegans* induced with 100 mmol/L glucose, the nematodes treated with **EF** (50 μL and 100 μL) showed 61.1% and 65.9% reduction in fluorescence respectively, when compared to the untreated nematodes. Whereas, for hyperglycemic nematodes induced with 200 mmol/L glucose, treated with **EF** (50 μL and 100 μL) had shown 58.8% and 59.1% reduction in fluorescence (Fig. 2 B).

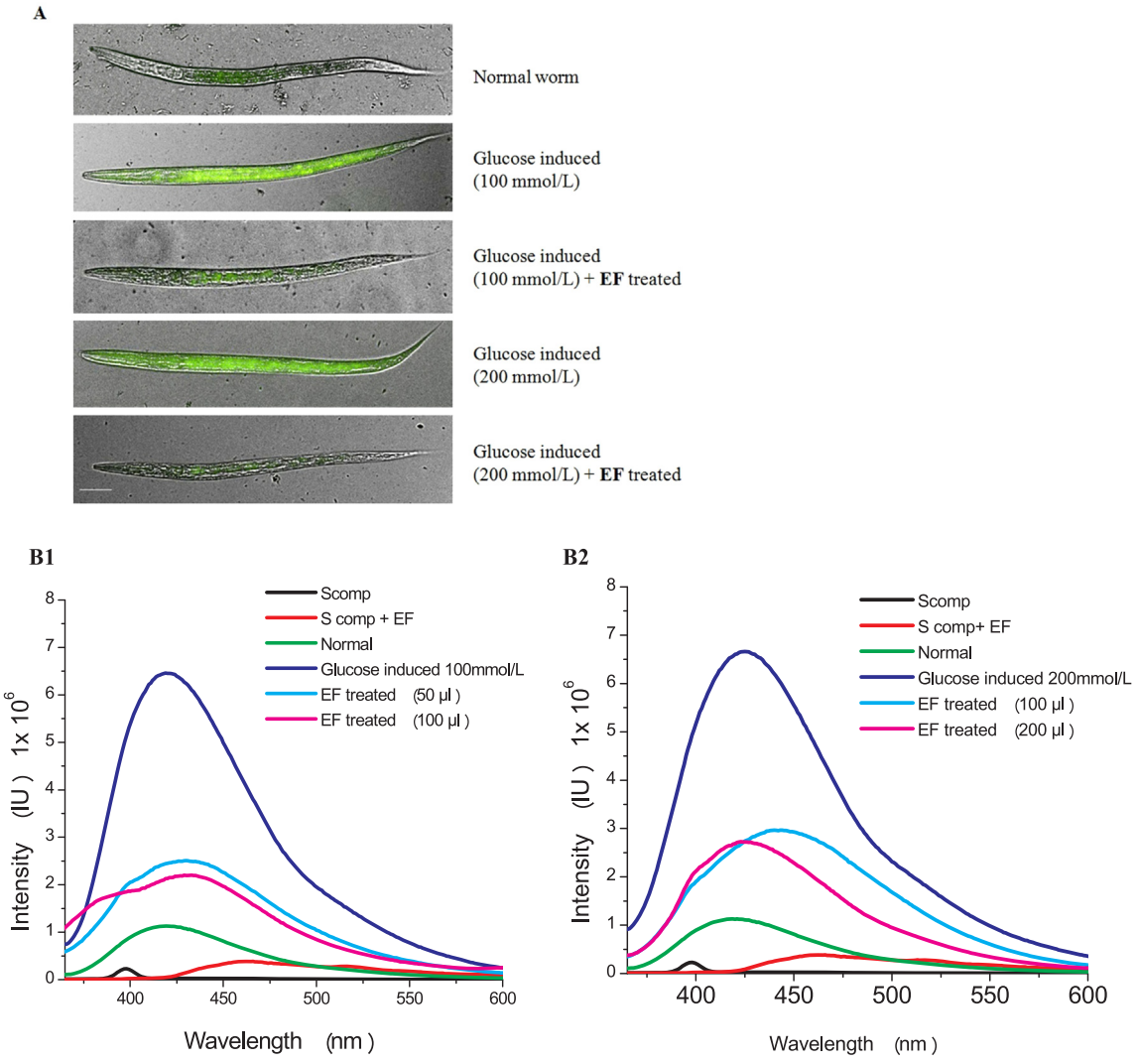

Fig. 2. (A) Live Cell Imaging of *C. elegans* using HCS, (B) Plot of fluorescence intensity for quantitative spectrofluorimetric estimation of AGEs accumulation in normal, glucose induced and treatment with **EF** of *C. elegans*.

Generally, the presence of lipofuscin (fluorescent pigment) granules in *C. elegans* are the indication of aging and glucose toxicity induced stress in *C. elegans*, also exhibit the auto fluorescence at similar excitation and emission pair (Yin, 1996). However, the accumulation of AGEs would fluoresce at the same 340 nm/430 nm excitation and emission. Hence, the findings of our study illustrated that the peak obtained at the emission spectra was high in nematodes induced with high glucose. Obviously, the induced nematodes treated with the **EF** were more or less same with the normal nematodes. Similar reports have been documented for the anti-glycative ability of microalgae, which were analyzed for the fluorescent product AGEs formation at excitation 330 nm and emission 410 nm (Shakambari et al., 2015). Thus, the inhibition of AGEs formation assay suggested that the **EF** can reduce the hyperglycemic conditions and its associated complications in *C. elegans* distinctly.

3.3.2. *Quantification of glucose in C.elegans*

In order to substantiate the inhibition of AGEs formation in hyperglycaemic condition in *C. elegans* DNSA analysis was performed to estimate the reducing sugars from the induced and un induced nematodes and the media where the nematodes grown. Anti-glycative activity was also proved by the estimation of the reducing sugars content of the induced, treated and normal nematodes on 9th day of growth (Fig. 3 A & B). The internal sugar concentration of the normal nematodes (1.95 ± 0.05 mmol/L) was significantly lower than the untreated hyperglycemic nematodes induced with 100 mmol/L and 200 mmol/L (5.66 ± 0.60 mmol/L and 9.23 ± 0.89 mo the normal mol/L) and almost as equal to the normal nematodes. This emphasises the role of **EF** in restoring the normalcy in hyperglycemia induced nematodes. However the internal sugar concentration of the hyperglycemic nematodes treated with **EF** (50 µL) was 2.00 ± 0.05 mmol/L and 1.74 ± 0.04 mmol/L in 100 mmol/L and 200 mmol/L induced nematodes respectively. Similarly, the internal sugar concentration of the hyperglycemic nematodes treated with **EF** (100 µL) was 1.78 ± 0.18 mmol/L and 1.89 ± 0.0.06 mmol/L in 100 mmol/L and 200 mmol/L induced nematodes respectively. Thus results are clearly witnessing that the **EF** has reduced the glucose level of *C. elegans*. The anti-diabetic activity of some cyanobacterial extract obtained from *Oscillatoria annae* (Varalakshmi et al., 2012) and *Spirulina sp.* (Layam and Reddy, 2006) using animal model was also reported earlier. Therefore, based on the prominent results of the above experiments compounds in **EF** of *Lyngbya* sp. would be one of the promising candidates to inhibit AGEs formation in the diabetic condition and this study may provide a new avenue to carry out further experiments with higher animals in order to reduce the hyperglycaemic condition.

3.3.3. *Effect of EF in glod-4, daf-2 & daf-16 gene expression in C.elegans*

Insulin, a common therapeutic drug administered for lowering the

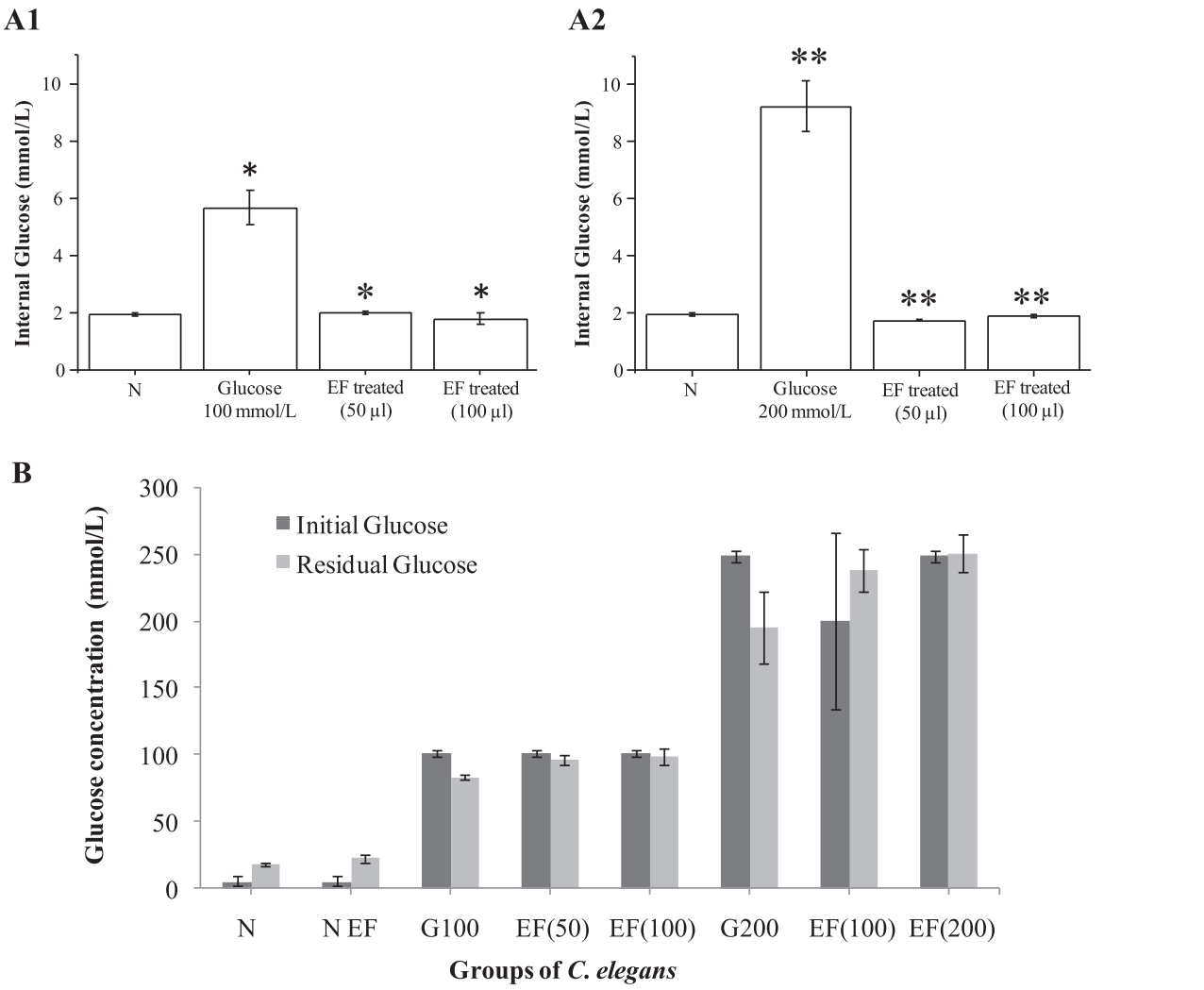

Fig. 3. (A) *C. elegans* induced with 100 mmol/l glucose & *C. elegans* induced with 200 mmol/l glucose & (B) DNSA analysis of reducing sugars in initial and residual medium, of *C. elegans* at 9th day growth.

glucose levels in a diabetic condition which shows dependence on glyoxalase-1 activity that is significantly expressed in diabetes and Alzheimer's disease, where insulin had demonstrated to affect the cognitive skills (Mendler et al., 2015). Hence the study of expression of gene coding for its homologue in *C.elegans*, *glod*-4 (glyoxylase-1), is particularly interesting in this study. The regulation of lifespan and stress resistance traits in *C. elegans* regulated by the well characterized insulin like growth factor 1(IGF-1) under stress conditions and the receptor of IGF-1 is homologue to *daf-2* which activates the phosphatidylinositol-3-OH kinase (PI(3)K)/3- phosphoinositide-dependent kinase-1 (PDK1)/Akt signal transduction pathway. Further this signal could inhibit the entry of *daf-16* gene transcription factor into the nucleus (Morris et al., 1996; Paradis and Ruvkun, 1998; Paradis et al., 1999)

In the present study, *C. elegans* cultured under hyperglycaemic conditions, *glod*-4 expression was 0.78 ± 0.13 with respect to *C. elegans* cultured in normal conditions (Fig. 4A). However, treatment with **EF** up-regulate the *glod*-4 expressions in the treated hyperglycaemic *C. elegans* to 1.41 ± 0.33 which is a significant increase ($p < 0.05$) when compared to the untreated hyperglycaemic nematodes. Methylglyoxal (MG) is the main precursor for the formation of AGEs. MG not only responsible for the diabetic complications, but also plays a vital role in the development of the obesity (Matafome et al., 2013), cancer (Thornalley and Rabbani, 2011), neurodegenerative disease (Srikanth et al., 2013) and atherosclerosis (Hanssen et al., 2014). The glyoxalase enzyme involved in the detoxification of MG. So, it can be concluded as, the **EF** is responsible for reduction in methylglyoxal (MG) and the AGE accumulation, and hence the expression of *glod-4* was increased where the treatment was given to the hyperglycaemic nematodes.

C. elegans were grown under hyperglycemic conditions, to analyse *daf-2* and *daf-16* gene expressions and found that 1.26 ± 0.11 and 0.79 ± 0.17 fold relative expression of *daf-2* and *daf-16* in *C. elegans* cultured under normal conditions and the p value < 0.05 was noticed (Fig. 4 B). However, treatment with **EF** had reduced *daf-2* expression (0.82 ± 0.09 and p value < 0.05) in hyperglycemic *C. elegans* while, a noticeable increase in *daf-16* (0.90 ± 0.12) gene expression was observed in hyperglycemic *C. elegans* treated with **EF**. The *daf-16* is a transcription factor which functions in the insulin/IGF-1-mediated signalling (IIS) pathway that regulates dauer stage formation, longevity of life span, metabolism of fat, response to stress, and innate immunity (Sun et al., 2017). Thus, the reduction or loss of function of *daf-2* may bring out a longer lifespan in *C.elegans* (Kenyon et al., 1993). Therefore, *daf-2* expression in the hyperglycaemic nematodes treated with **EF** evidenced that the animals were recovered from the effects of AGE accumulation and thereby lifespan has been increased.

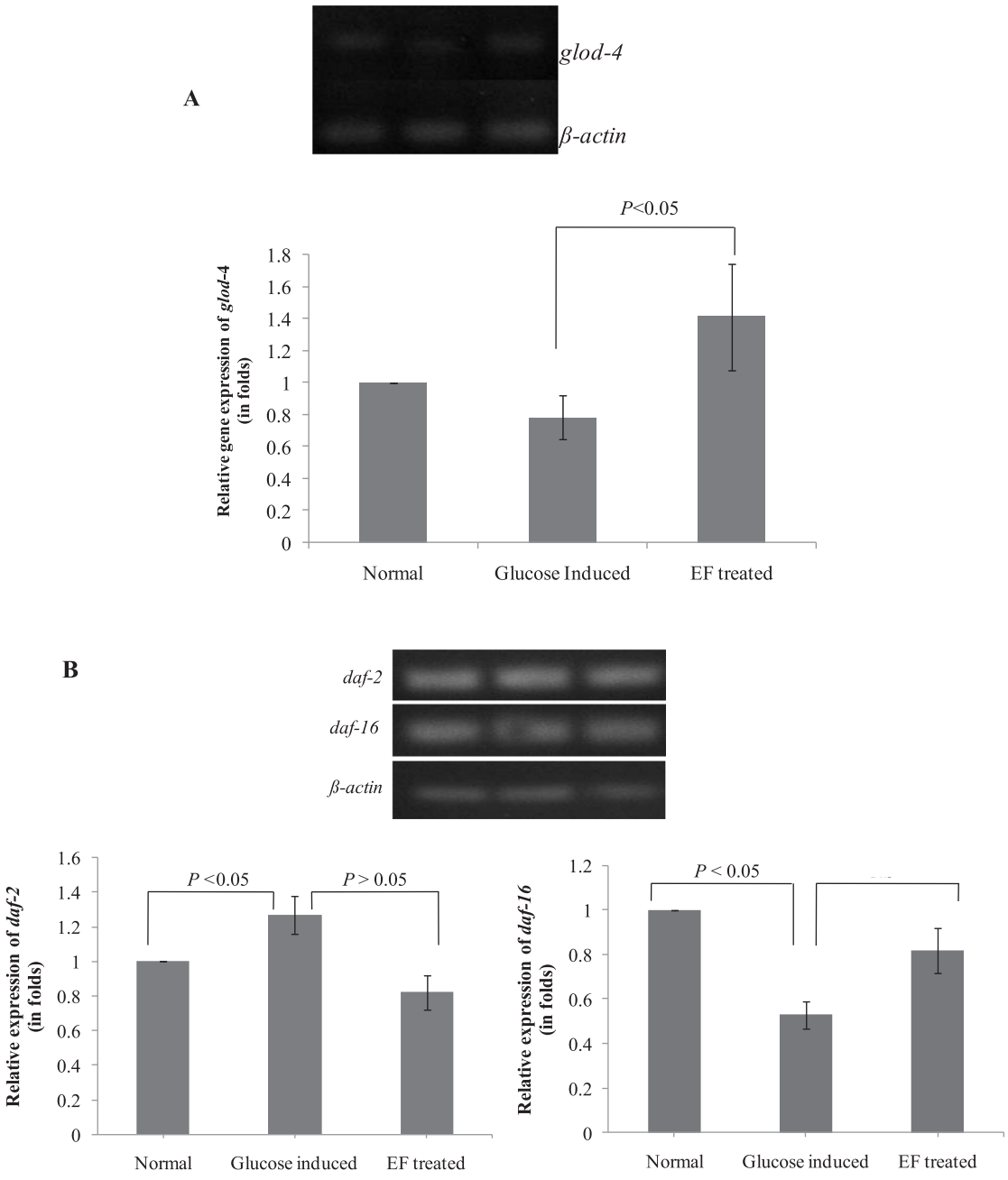

Fig. 4. (A) Relative expression of *glod-4* gene in *C. elegans* in different conditions like normal, hyperglycaemic nematodes and treated with **EF** by semi-quantitative RT-PCR analysis (B) Relative expression of *daf-2* and *daf-16* gene in *C.elegans* in normal, glucose induced hyperglycemic and hyperglycemic worms treated with **EF**.

3.4. Anti-inflammatory activity of EF

3.4.1. Effect of EF in nitric oxide scavenging assay

Nitric oxide, from different cells, plays key function in cell signalling and inflammation. Inducible nitric oxide synthase (iNOS) is responsible for sustained nitric oxide production. NO functions as host defence toxic molecule to infectious agents and may induce adverse reactions that could cause damages to other tissues of the host itself (Coleman, 2001). The regulation of NO production serves as an important therapeutic target against inflammatory diseases. In this study, **EF** of interest exhibited noticeable nitric oxide scavenging activity (IC_{50} value 117.98 ± 0.41 μg/mL) to prevent the induced inflammatory response. COX-2 and iNOS expression is activated during AGE's formation (Huang et al., 2009), thus, in this study we found that **EF** inhibited to COX-2 expression and it's affected the NO production.

3.4.2. Effects of EF on inflammation induced C. elegans

Innate immunity consists of various defence processes against microbial infection. When pathogens attacked on the worms, they started to synthesize different anti-microbial peptides and neuropeptides to resists the infection of pathogens (Ewbank and Zugasti, 2011; O'Rourke et al., 2006; Schulenburg et al., 2004). In *C. elegans*, various signalling components were involved in innate immunity including DAF-16 (Zou et al., 2013).

In this study, 100 μL of overnight grown culture of *Pseudomonas aeroginosa* was sufficient to cause inflammation in *C. elegans TJ356 daf16:: GFP*. Subsequently, DAF-16 expression was dispersed throughout the *C. elegans* and intensity of fluorescence was higher in worms treated with **EF** than to inflammation induced worms (Fig. 5A). The worms were monitored that they were recovered from the induced inflammation when it was treated with the **EF** compound. Live cell

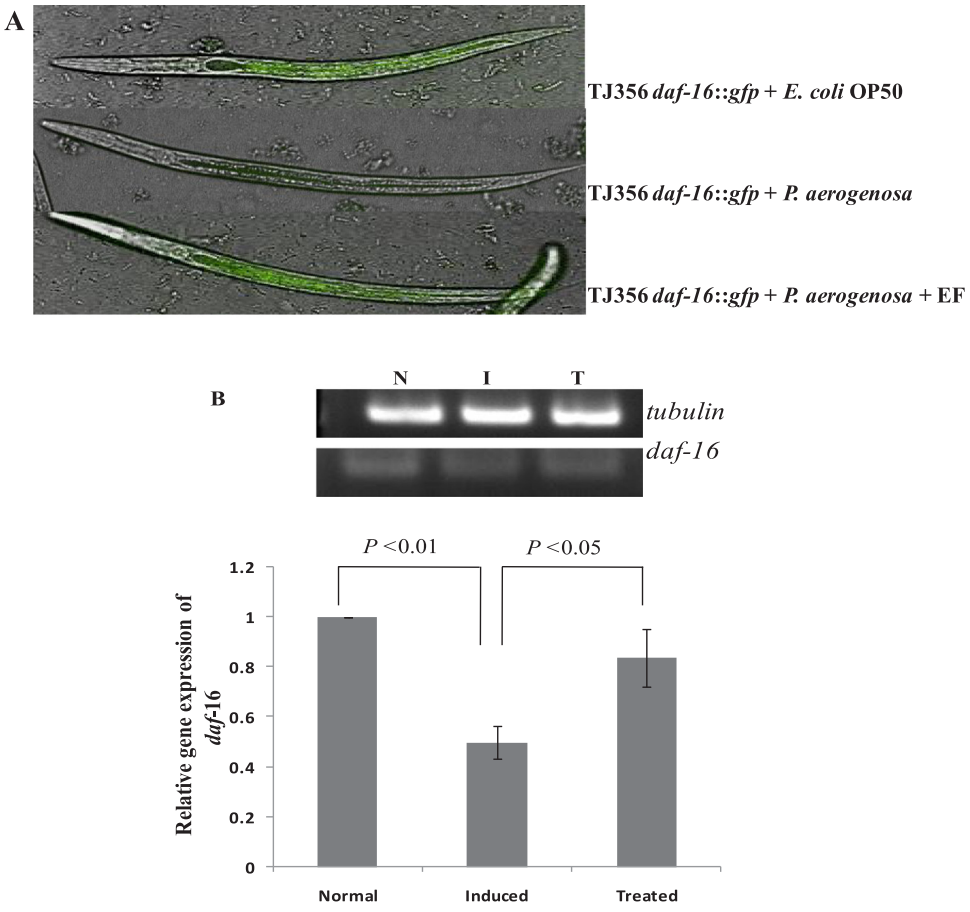

Fig. 5. (A) High content screening (HCS) analysis of *C. elegans* under different conditions. DAF-16 is dispersed throughout the *C. elegans* and intensity of fluorescence of the worms was higher in nematodes treated with **EF** comparison to inflammatory worms. (B) Relative gene expression of the *daf-16* in different conditions like normal, *P. aeroginosa* infected worms and **EF** treated worms (*p < 0.05 (significant & ** p < 0.01 most significant).

imaging was performed to elucidate the inflammatory response using *C. elegans* as animal models. In *C. elegans TJ356 daf16:: GFP*, DAF-16::GFP was expressed in whole body (almost in all tissues and nucleus) during normal conditions. When worms were infected with *P. aeroginosa* DAF-16 was not expressed in all tissues, because during stress conditions *daf*-16 accumulated only in the nucleus. The *daf*-16 gene expression of *C. elegans* were cultured with *P. aeroginosa* to detected that inflammation induced worms had 0.84 ± 0.11fold relative expression in EF treated worms while only infected worms were showed down regulation (0.50 ± 0.06 fold) of the gene (Fig. 5B). Generally, invertebrates have only one FOXO gene which is *daf*-16 in *C. elegans* (Albert et al., 1981). FOXO3a genes are expressed in peripheral lymphoid organs where it plays vital role in lymphoid haemostasis (Lin et al., 2004). FOXO3a inhibits NF-κß activity and regulates helper T cells activation and tolerance. FOXO3a deficient mice develop autoimmune diseases by over-expression of NF-κß, cytokines and hyper-activation of T cells [54]. Hence this analysis clearly evidenced that **EF** had induced the expression of *daf*-16 in inflammatory worms.

4. Conclusions

Conclusively, this study evidences the presence of novel bioactive compounds in **EF** of *Lyngbya* sp., and its biological potential with AGEs inhibition and anti-inflammatory activities in *C. elegans* for the first time. The bioactive metabolites in **EF** showed pronounced inhibition against AGEs formation and modulated expression levels of stress responsive genes *glod-4, daf-2* and *daf-16* in hyperglycemic *C. elegans* at whole animal levels and also exerted potent anti-inflammatory activity by interfering with *daf-16* expression in *P. aerogenosa* challenged *C. elegans*. Thus, it is apparent that these bioactive metabolites could serve as a promising natural therapeutic molecule for alleviating the complications of diabetes mellitus and inflammatory diseases.

Conflict of interest

The authors declare that they have no conflict of interest in the publication.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bcab.2018.11.020.

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