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IN VITRO ANALYSIS OF *CORIANDRUM SATIVUM* ON HL-1 CELL LINE AGAINST FYCO1 AS A POTENTIAL THERAPEUTIC FOR CARDIOVASCULAR DISEASE

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ABSTRACT

Background: Autophagy is defined as the adaptation of cells to adverse situations such as hypoxia, food restriction, and starvation. Type II programmed cell death can be facilitated by autophagy in response to stressful situations and during cell growth. The induction of autophagy excessively in some circumstances, results in uncontrolled cellular breakdown and cell death. Autosis in the heart initiates cardiomyocyte death while also strongly facilitating cardiac damage. The gene FYCO1, which is part of the autophagic system, regulates cardiac stress adaptation. Upregulation of FYCO1 can result in overexpression of autophagy.

Objective: The goal of this study was to find a potent therapeutic target against CVDs caused by overexpressed autophagic flux in cardiomyocyte, by comparing the expression profile of the heart-specific gene FYCO1 to an extract of the plant *Coriandrum sativum*.

Results: The*in-vitro* studies, has proved that *Coriandrum sativum* has cryoprotective properties towards HL-1 cell lines in a dose manner up-to 50ug/ml, that are induced with LPS. The gene expression studies also reveal a considerable decline in the target FYCO1 expression from sixfold to one-fold due to application of *Coriandrum sativum*.

Conclusion: Therefore, FYCO1 as a target and the compounds of the herb *C. sativum*as a drug can be further investigated as a potential target for developing anti-autophagic therapeutics associated with CVDs.

Keywords: Autophagy, cardiomyocytes, FYCO1, *Coriandrum sativum*, HL-1cell lines.

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Introduction

Autophagy is a natural means of conserved degradation of cells that removes unnecessary or dysfunctional components. In most cells of the circulatory origin, such as cardiomyocytes, endothelial cells, and VSMCs, autophagy helps to maintain intracellular equilibrium (Ikeda, S. et al, 2017).A significant amount of preclinical research implies that autophagy plays a dual role in causing cardiovascular disease, by functioning in either helpful or detrimental ways depending on the setting. As a result of this, the autophagic mechanism in cardiomyocytes and other circulatory cell types has been hypothesized as a potential therapeutic target. FYCO1, a RAB effector protein that was recently discovered to be a heart-specific protein, is vital for autophagy. This protein is present within surface of autophagosomes, and are part of the autophagic machinery. Overexpression or a lack of FYCO1 has shown to prevent cells from adjusting to stressful conditions.

Several studies have found that numerous cardiovascular diseases are caused by deregulation of the cardiomyocyte autophagic mechanism. Autophagy dysfunction contributes to the development of certain CVDs (Bravo -San Pedro. et al, 2017). A deeper knowledge of the autophagic role in the cardio-vascular system possibly will lead to new curativeapproaches for regulation or prevention of CVDs. As a result, the function of autophagy in the circulatory system is currently being studied in depth.The main goal of cardiovascular translational research is to discover novel drug targets and test the proposed new therapeutics with clinical significance.

Coriandrum sativum was chosen for this study based on previous molecular docking studies. Docking experiments suggest that the compounds of the herb have a high affinity for the target FYCO1 gene. Although, molecular docking data cannot be readily used to translational research, *invitro* and *in vivo* evaluations are used. *In vitro* models are less expensive and more effective for determining the basic therapeutic characteristics of any compound (Cava, et.al., 2020). This study suggests *C. sativum* extract as a possible treatment with cryoprotective abilities on HL-1 cells, and its role in downregulating FYCO1 gene expression and suppressing autophagic activity.

Methodology

Methanol extract preparation of the herb

Fresh *Coriandrum sativum* leaves weighing 127g were harvested and sterilized. These were dried in the shade. The dried leaves were then crushed in a mortar, yielding 17.85 g of powdered leaves. This was then soaked in 80% methanol for 72h at room temperature. Whatman No 1 filter paper was used to separate the filter from the wet. This was repeated twice with the same mixture. The purified filtrate was stored in a dry oven at 40°C for alcohol evaporation. For later usage, the hydroalcoholic crude extract obtained was stored at 4° C.

Phytochemical Screening

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Test for Saponins- 2ml of extract was added to a test tube and vigorously shaken. 3ml of water was added to this. The presence of saponins was indicated by the formation of stable foam for 3-5 min.

Test for Tannins- 1 mL plant extract was added to a test tube, followed by 1 ml chloroform and 1 mL acetic anhydride. 1 mL concentrated H_2SO_4 was poured into the test tubes through the sidewalls. The presence of tannins was indicated by the bluish-green color.

Test for Flavonoids- To a test tube, 2mL of plant extract was added, along with a few drops of aqueous NaOH (2%) A few drops of dilute HCL changed the color to orange yellow, indicating flavonoids were present.

Test for Sterols- A few drops of ethanol were added to 1ml of plant extract in a test tube, then 1ml of concentrated H_2SO_4 was poured through the walls of the test tube. Sterols were detected by the appearance of a violet-green color.

Test for Terpenoids- To 0.5mL plant extract, chloroform 2ml and 3ml of concentrated H_2SO_4 was carefully added forming a layer. The incidence of terpenoids was indicated by the formation of reddish-brown color formation at the interphase.

Test for Quinines- Few drops of plant extract were added to 2ml of concentrated HCL in a test tube. No formation of yellow precipitate, indicating absence of quinines.

Test for Glycosides- 5mL of plant extract was placed in a test tube with H_2SO_4 under layer to it. 2ml glacial acetic acid and a few drops of 2% ferric chloride were added to this. The incidence of glycosides was indicated by the formation of a brown ring on interphase.

Maintenance of HL-1 cell line

The HL-1cells were maintained in DMEM High Glucose media enhanced with 10% FBS along with the 1% antibiotic-antimycotic solution and 1% L-Glutamine (200mM) in 5% CO_2 and 18-20% O_2 atmosphere at a temperature of 37°C and maintained in the CO_2 incubator and sub cultured for two days.

Assay controls

To examine and limit the impact of environmental stimuli other than the test drug, the cells were divided into three controls. The controls are as follows:Medium control - The medium without HL-1 cells; Negative control – The medium with cells but without *C. sativum*; Positive control – The medium in which the cells were induced with 1ug/ml of LPS;

MTT Assay

A 200µl cell solution was seeded into a 96-well plate without the test drug, with a cell density of 20,000 cells per well. For roughly 24 h, the cells were allowed to grow. The test drug was prepared with the fresh DMEM media to achieve 1mg/ml concentration and further serially diluted in the media to obtain different working concentrations. The first set of cells (control) was supplemented with adequate amounts of the test substance alone. Another batch of cells (standard) was treated for 2 h with LPS-1ug/ml to induce autophagy, and then the test drug was given to the cells (treated) at various concentrations, as given in the table 1. The plate was kept for incubation at 37°C in a 5% CO_2 environment for 24 h. The spent media from the plate was separated after the

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incubation period, and MTT reagent with a final concentration of 0.5 mg/ml of total volume was added to the plate. To avoid light exposure, the plate was wrapped with aluminum foil. This was incubated for another three hours. The MTT reagent was drained, and 100µl of DMSO solubilization suspension was added. A spectrophotometer with a wavelength of 570nm was used to measure the absorbance.The formula for calculating percent (%) cell viability is as follows:

$$\% \text{ Cell viability} = \frac{\text{absorbance of treated cells}}{\text{absorbance of untreated cells}} \times 100$$

Table 1: The different concentrations of LPS and test compound (*C. sativum)* treated to the HL-1cell lines.

Sl.no	Test Compounds	Cell line	Concentration treated to cells
1	Untreated	HL-1	Blank
2	LPS	HL-1	1ug/ml
3	Blank	-	Only media without cells
4	*C.sativum*	HL-1	5(6.25, 12.5, 25, 50, 100µg/ml)
5	LPS+*C. sativum*	HL-1	LPS+5(6.25, 12.5, 25, 50, 100µg/ml)

ROS study expression study using Flow Cytometry

Cells were cultivated in a 6-well plate at a density of 0.5 x 106 cells/2 ml and incubated in a CO_2 incubator overnight at 37°C for 24 h. This was washed with 1ml 1X PBS after the spent medium was removed. The cells were stimulated with 1ug/ml of LPS for 2 h in the wells to be treated with the drug. The test drug was given to the cells at the concentrations indicated in the table. One well was left untreated, serving as a negative control, these were then incubated for 24 h. The medium from each well was removed and rinsed with 500µl PBS. After removing the excess PBS, 250µl of trypsin-EDTA solution was added and incubated at 37°C for 3-4 min. The cells were harvested into 12 x 75 mm polystyrene tubes after the culture media was poured back into the corresponding wells. The tubes were then centrifuged for 5 min at 300 x g at 25°C. The pellet was washed twice with PBS after the supernatant was decanted.The cells at a density of 1 x 106 cells/ml were suspended in H2DCFDA working solution and incubated at 37 °C for 30 min in the dark. The tubes were then centrifuged for 5 min at 150 x g. The supernatant was removed, and the cells were gently resuspended in 400µl pre-warmed DPBS. Flow cytometry was used to examine this, using excitation at 488 nm and detection at 535 nm using a laser of 488 nm.The control, standard, and treated cells were pelleted down, excess media was removed and then washed in PBS before being kept in trizol for cell lysis.

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Total RNA Extraction

The 1mg cell pellet was vortexed and incubated for 5 min at room temperature after being lysed in 500µl trizol reagent. Then 200µl of chloroform was added for phase separation, then the mixture was held at for incubation at room temperature. The mixture was centrifuged at 4°C for 15 min at 12,000rpm. The supernatant was transferred to new tubes containing 1µl of glycogen. These tubes were filled with an equal volume of IPA and incubated for 10 min before being centrifuged for 10 minat 12,000rpm at 4°C. The supernatant was collected and centrifuged at 7,500rpm for 5 min at 4°C after being rinsed with 75% ethanol. The pellet was then air dried, and dissolved in DEPC, and stored at -20°C.

c-DNA preparation

The first strand of cDNA was synthesized with 10µl of total RNA as template, and a master mix prepared with 4.0µl of 5X RT buffer, 2µl of random hexamer primers (10Xconcentration), 0.8µl of 25X dNTPs primers, and 1.0µl reverse transcriptase together with 2.2µl of milipore-water making the total volume 10µl into a PCR tube. The same was repeated for all the samples. The samples were amplified using a PCR program with the following reactions set for at 25°C for 10 min, followed by 1 h at 42°C, and 5 min at 85°C and a stored at 4°C.

Primer designing

The FASTA sequence of the target genes were taken from the GenBank database of NCBI. The primers were designed to be added to the template strand during PCR cycles. The primers were constructed using the eurofinsgenomicssoftware and comprised of a short forward sequence and a reverse sequence. The GADPH primer is an endogenous primer that are usually used for normalization, this was designed to be used as a PCR experiment control. The next set of primers were designed so as to target the gene FYCO1. The designed primer sequence along with their melting temperatures is given in the table 2.

Table 2: The short PCR primers designed for targeting housekeeping genes andtarget FYCO1 gene.

Sl.no	Oligo Name	Sequence 5' to 3'	MW (g/mol)	Tm (°C)
1	Human GADPH_forward	ACATCATCCCTGCCTCTAC	5643.66	56.67
2	Human GADPH_reverse	CTGCTTCACCACCTTCTTG	5665.65	53.67
3	Human FYCO1_forward	ATGAACACCAAAGTGACCAG	6128.05	55.25

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4	Human FYCO1_reverse	TCCACAGGTAAGCAGAAGAG	6184.08	57.30

cDNA template preparation

The following PCR templates were prepared to assess the housekeeping genes: For the first PCR reaction, 1µl cDNA, 5µl master mix (AURA cDNA synthesis kit), 1µl forward primer and 1µl reverse primer designed for GADPH, and 5µl distilled water were used in a total volume of 13µl for each, control, standard, and treated samples.To synthesis the target cDNA fragments, the FYCO1 primers listed in the table were used. Amplifications lasted 5 min at 94°C, followed by 45 cycles of 1minute at 95°C, 45s at 53.5°C, and 45 s at 72°C and a final extension step for 5min at 72°C. Following PCR, samples were run on a 1.5% agarose gel with ethidium bromide and a small aliquot (4µl) of the reaction in separate lanes.

Quantitative real time reverse transcription PCR (qRT-PCR)

Total RNA was extracted from the control, standard, and drug-treated HL-1 cell lines and reverse transcribed using a cDNA synthesis kit and custom-designed primers. The qRT-PCR was performed using 2µl cDNA (2:1 dilution of transcribed cDNA), SYBR Green I master mix, and designed PCR primers using an Applied Biosystems StepOne^{TM}. The following protocol was used to run the qRT-PCR program: Pre-incubation for one cycle was 20s at 95°C, followed by cycling stage with 40 amplification cycles at 95°C for 3 s, 30 s at 53.5°C, and a melt curve stage set at 95°C for 15 s and 60°C for 1 min. The function (RE = $2^{-\Delta\Delta Ct}$) was used to compute relative expression (RE) levels for statistical analysis, where (ΔΔCt) is the standardized variance in Ct values between the control sample and the sample treated with the test drug. The standard error of the mean (SEM) was derived from the standard deviation, and the mean of relative expression levels was calculated from the distinct RE values of the individual experiments.

Results

Phytochemical analysis of *C. sativum*

The crude extract was assessed for secondary metabolites like saponins, terpenoids, flavonoids, tannins, quinines, sterols and glycosides. The results obtained are mentioned in the table 3. Each phytochemical was screened using standard protocols, and the results obtained are as shown in the fig1.

Table 3: Phytochemical analysis for methanolic extract of *C. sativum.*

Sl.no	Phytochemical compounds	Methanolic extract
1	Saponins	+
2	Tannins	+
3	Flavonoids	+

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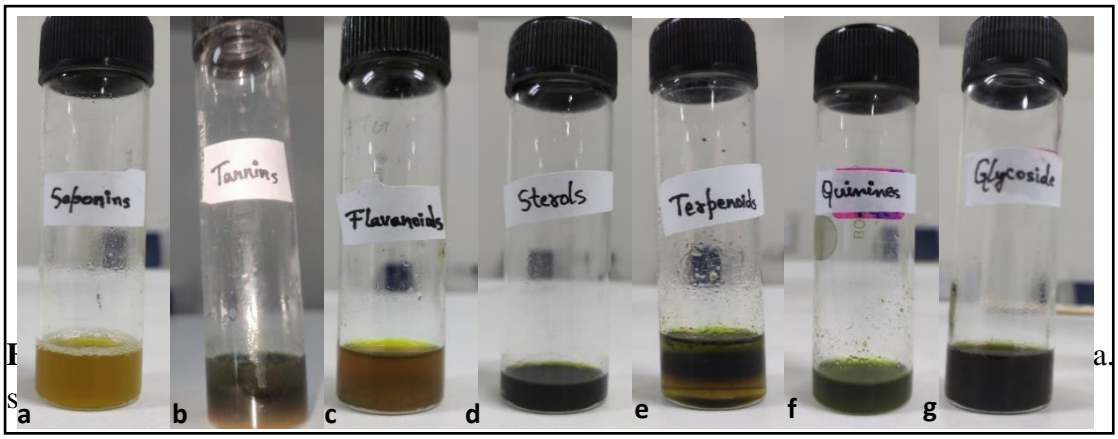
4	Sterols	+
5	Terpenoids	+
6	Quinines	-
7	Glycoside	+

+ = present, - = absent;



MTT cytotoxicity assay of *C. sativum* against HL-1 cell lines

According to statistical results from ELISA reader for cell cytotoxicity studies, the compound *C. sativum* against HL-1 cells has non-cytotoxic potential properties up to 50ug/ml concentration, with better than 90% cell viability. In the LPS-induced model(treated), the drug compound was able to gradually inhibit the harmful potential of LPS in a dose-dependent way till 50ug/ml, but was ineffective at 100ug/ml. The fig 2, depicts direct microscopic views of drug-treated cell lines with comparison to untreated cell lines. The observed absorbance readings and the calculations given in table 4 shows, were used to plot the bar graph as shown in fig 3.

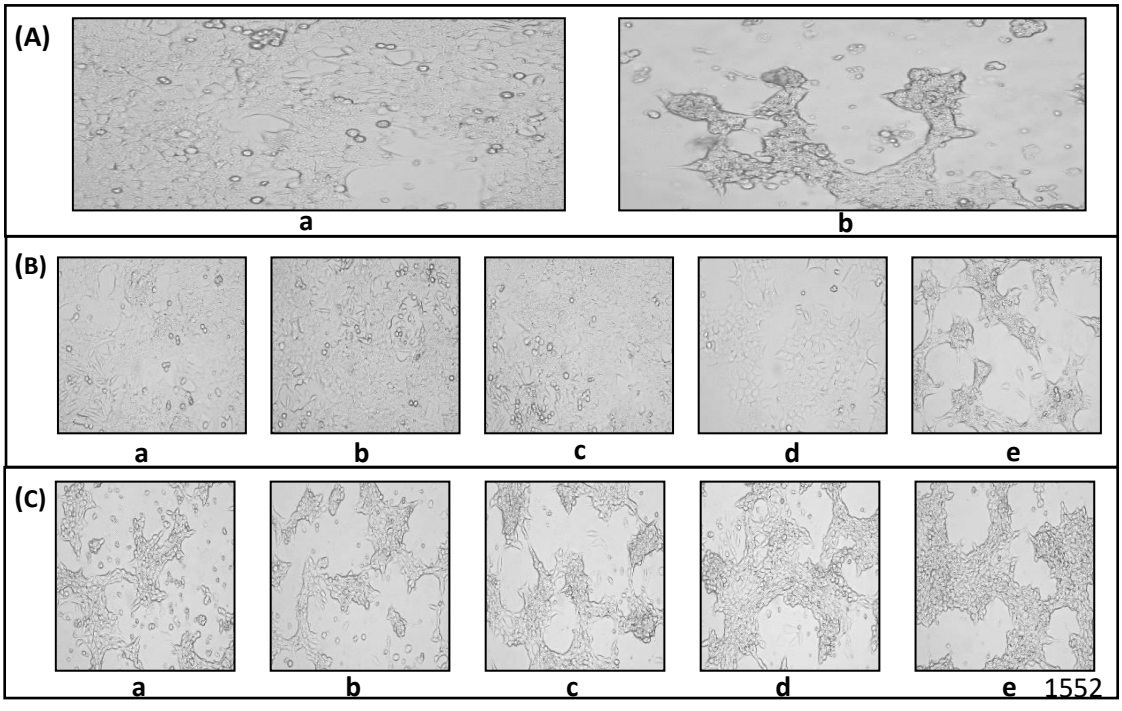


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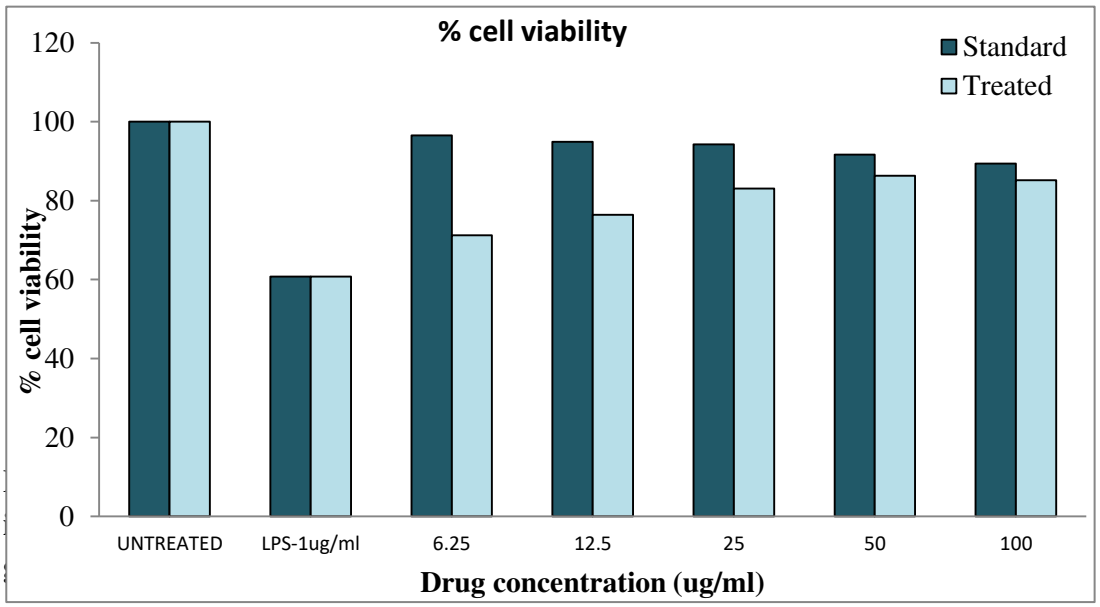
Fig 2: The direct microscopic views at 10X of the HL-1 cell lines after the experimental treatments. A. (a) An image of untreated HL-1 cells with, **(b)** The comparison of the HL-1 cells treated with 1ug/ml of LPS. **B.** The images of HL-1 cells after treatment with different concentration of *C. sativum*- the concentrations being **(a)**6.25ug/ml, **(b)**12.5ug/ml, **(c)**25ug/ml, **(d)**50ug/ml and **(e)**100ug/ml. **C.** The images of HL-1 cells treated with LPS and *C. sativum* together at different concentrations of **(a)**6.25ug/ml, **(b)**12.5ug/ml, **(c)**25ug/ml, **(d)**50ug/ml and **(e)**100ug/ml.

Table 4: The % cell viability values after the incubation period of 24h, for *C. sativum* and LPS induced HL-1cells treated with *C. sativum*.

Sl.no	Culture conditions	*C. sativum*	Treated
1	Untreated	100	100
2	LPS-1ug/ml	60.78	60.78
3	6.25 ug/ml	96.51	71.24
4	12.5 ug/ml	94.93	76.41
5	25 ug/ml	94.22	83.06
6	50 ug/ml	91.66	86.27
7	100 ug/ml	89.43	85.18



3: A comparative bar graph for MTT assay. The graph depicts the % cell viability of HL-1 cells to the drug concentration.

H2DCFDA expression study of *C. sativum* against HL-1 cells

In comparison to LPS-induced cells, the test drug *C. sativum* significantly inhibited DCF expression when applied to the cells. LPS alone caused DCF intensity of 73.71%, however LPS combined with the drug compound *C. sativum* was successful

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in downregulating DCF expression by 50.29%. The overlay of the results shown in the table 5 is plotted as a bar graph as in the fig 4. This conclusively confirms that the drug compound's cytoprotective effectiveness via an oxidative stress-induced apoptotic mechanism as determined by H2DCFDA staining by flow-cytometry.Cells in the M1 and M2 phases are distinguished by H2DCFDA histograms of gated HL-1 singlets. The fig 5 depicts the histograms plotted for % cells expressing DCF intensity, with different cell culture conditions. (M1 represents a negative expression/region, while M2 represents a positive expression/region.) The analysis software (Cell Quest Software, Version 6.0) was used to refine the gating of the M1 and M2 phases.

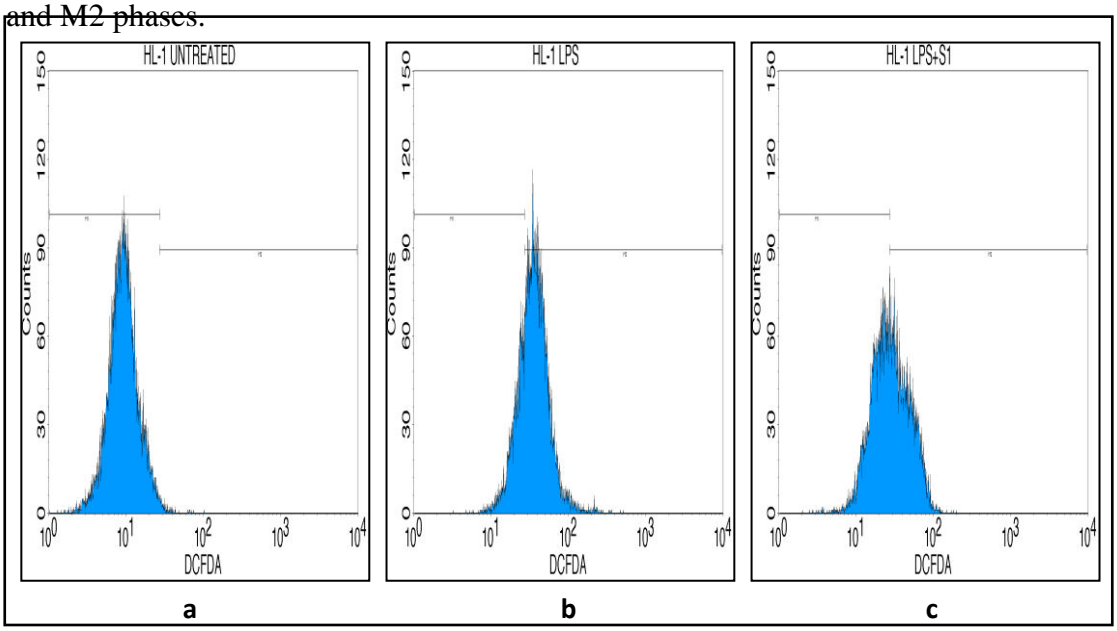

Fig 4: H2DCFDA expression study of cells using a histogram of M1 and M2 phase. (a)HL-1 cell lines untreated (Control) **(b)** HL-1 cell lines treated with 1ug-LPS **(c)** HL-1 cell lines treated with LPS and *C. sativum.*

Table 5: The % HL-1 cells that expressed DCF intensity in different culture conditions.

Culture condition	Precent (%) cells that expressed DCF intensity (M2 region)
Untreated	0.93
LPS-1ug/ml	73.71
LPS+ *C. sativum* (50ug/ml)	50.29

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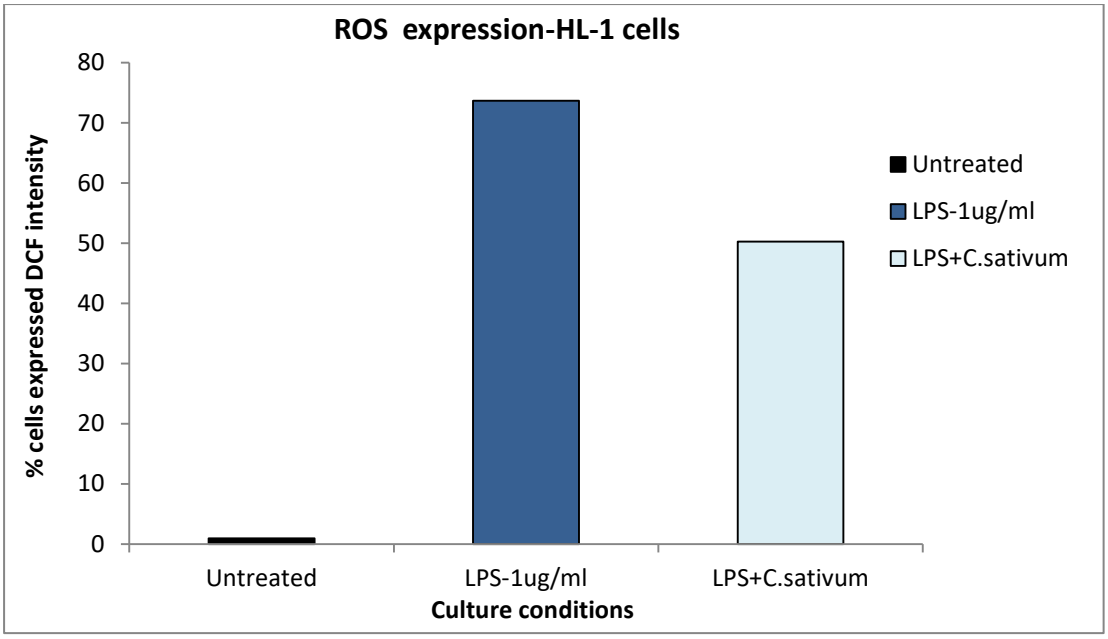

Fig 5: The ROS expression of HL-1 cells. A bar graph plotted with % cells that expressed DCF intensity against different cell culture conditions.

c-DNA synthesis

The RNA isolated from the cell pellets were used to synthesis template c-DNA using random primers. The c-DNA was analyzed for the house keeping genes, using the designed forward and reverse PCR primer of Human_GADPH. This was then run in an agarose gel, bands of 200basepairs were obtained as shown in the fig **6**(A)**.** The c-DNA was later checked for the target gene using the forward and reverse primer for FYCO1, which on agarose gel showed bands of approximately 170-180 base pair, as shown in fig6(B)**.**

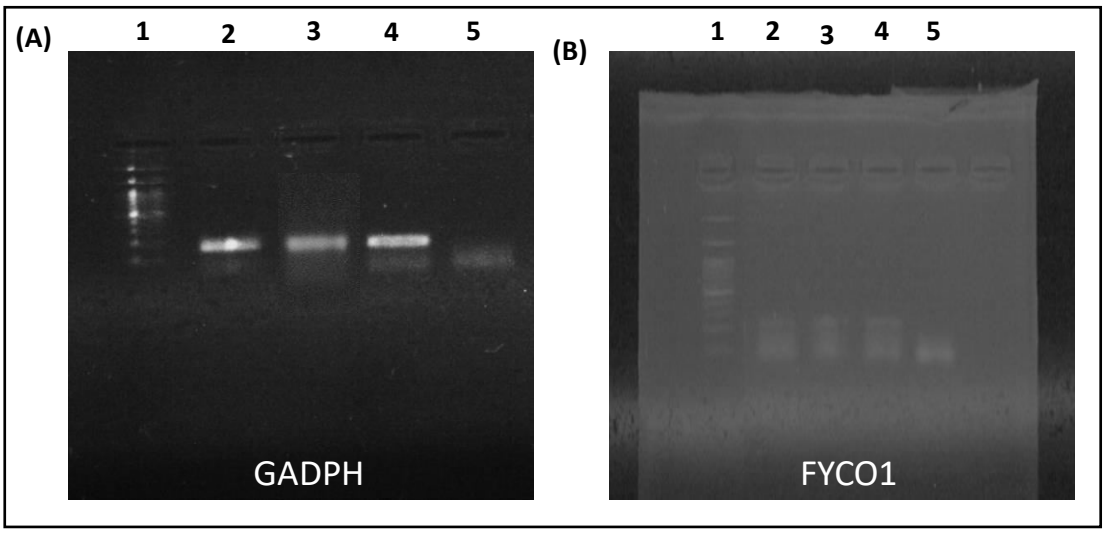

Fig 6: The agarose gel images of synthesized c-DNA analyzed using designed primers. (A). c-DNA analyzed with GADPH primer where lane1 is the ladder, 2 is the control sample, 3 is the standard and 4 is the sample treated with drug, lane 5 is the negative control without template **(B)** c-DNA analyzed for target gene with primer

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where the ladder is on lane 1, the control sample is on lane 2, the standard is on lane 3, and the sample treated with drug is on lane 4, and the negative control without template is on lane 5.

Analysis of FYCO1 gene expression using RT-PCR

A program to execute two genes was set up in the quantitative real-time reverse transcriptase PCR. The target gene's expression level is determined by comparing the two genes. Because it is found in almost every mammalian cell, the GADPH gene was employed as a housekeeping gene. The CT values obtained and the standard deviation were compared to analyze the expression level of FYCO1. The CT values obtained, as well as their duplicates, are listed in the table 6. The values were used to create a bar graph as shown in fig 7. This standard deviation shows that the expression of FYCO1 from six-fold has steadily decreased in LPS induced cells when treated with the test drug *C. sativum* to one-fold expression.

Table 6: The CT values, duplicates and the RQ values obtained by running the cDNA on qRT-PCR.

Target gene	CT1	CT2	Mean	RQ	Standard deviation
FYCO1-Control	28.81	27.98	28.395		
GADPH-Control	22.1	22.26	22.18	1	0
FYCO1-Standard	25.91	26.48	26.195		
GADPH-Standard	23.68	23.93	23.805	14.17228	6.71276
FYCO1-Treated	27.98	28.58	28.28		
GADPH-Treated	23.21	23.08	23.145	2.1140361	1.354701

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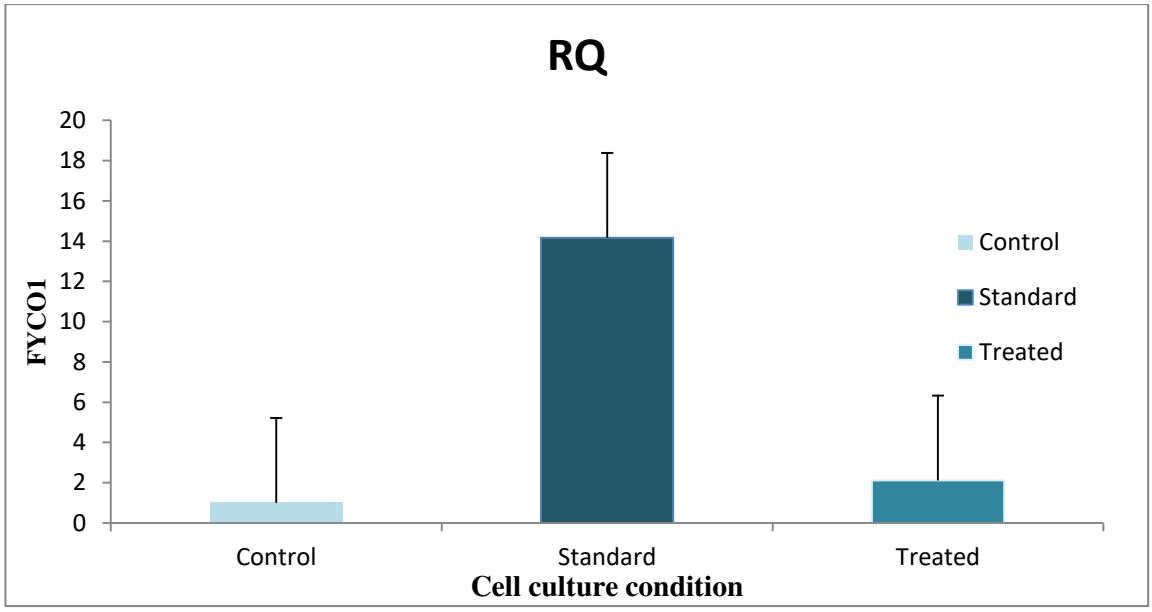

Fig 7: The FYCO1 gene expression of the synthesized c-DNA. The bar graph was plotted with RQ values of cells under different culture conditions against FYCO1 expression.

Discussion

The detailed analysis of the study revealed that *Coriandrum sativum* could be exploited as a possible target therapeutics for autophagy upregulation. When cells are handled in varied culture conditions, the in vitro tests clearly reveal a difference in the cell viability. The figure reveals that *C. sativum* exhibits moderate cytotoxicity against HL-1 cells, with 89.43 % cell viability at the highest dose of 100ug/ml during a 24-h incubation period at 37°C. The drug compound was non-toxic to HL-1 cells until it reached a concentration of 50ug/ml, which was then considered as the optimum concentration. According to the ROS study, when C. sativum was combined with LPS on cell lines, the percentage of cells producing DCF intensity gradually decreased from 73.71% to a positive range of 50.29%. The relative quantification values for the genes FYCO1 and GADPH of the c-DNA synthesized were obtained using the qRT-PCR program. The expression of the target FYCO1 gene progressively decreased from six folds to one-fold, when the cell cultures were treated with *C. sativum* in the presence of LPS. These results indicates that *C. sativum* contains compounds that actively suppress excessive autophagy.

Previous molecular docking studies suggested that compounds from the herb *C. sativum* exhibited a high binding affinity to the gene FYCO1 as a target. FYCO1 has recently been discovered to be a heart-specific protein that aids cells in coping with stressful situations. In animal models, overexpression or downregulation of FYCO1 has an effect on the autophagic activity (Kuhn, C., et al, 2021). The data obtained from this study can be used to develop therapeutics that can target excessive autophagy The adaptive and maladaptive roles of autophagy present a unique difficulty in this regard. The goal of this investigation was to check if *C. sativum*

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could be used as a drug to reduce overexpressed autophagy by downregulating the expression of FYCO1. This study also found that when *C. sativum* extract is used in its whole, it is not hazardous to cell lines. Further research is needed to investigate the molecular mechanism of action behind the cytoprotective capacity of *C. sativum* against HL-1 cells in *in vitro* conditions. This research can be utilized to further explore individual *C. sativum* compounds and develop therapeutics to combat autophagic associated cardiovascular diseases.

Conclusion

In summation, *Coriandrum sativum* can be used to develop an effective therapeutic that inhibits excessive autophagic levels. According to the results of the studies, *C. sativum* has cryoprotective properties against cardiomyocyte cells at dose levels up to 50ug/ml. The use of *C. sativum* on cells cultured with LPS lowered the expression fold of the target gene FYCO1 from six to one, thereby reducing autophagic activity.This work can also be used to investigate the molecular mechanisms and pathways that are associated to the potential capabilities of *C. sativum* in autophagy downregulation. This can also be used in *in vivo* cardiomyocyte models, where specific cardiovascular research causes undesired autophagy as a result of remodeling studies. This research could pave the way for further *in vivo* studies and translational research towards developing effective therapeutics for cardiovascular diseases based on *C. sativum* compounds.

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