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Research article

Effect of flaxseed's supplement on AMPKα2′s expression in non-diabetic and diabetic patients having follicular thyroid carcinoma on mitochondrial's ATP synthase for TPC-1 cell line ☆

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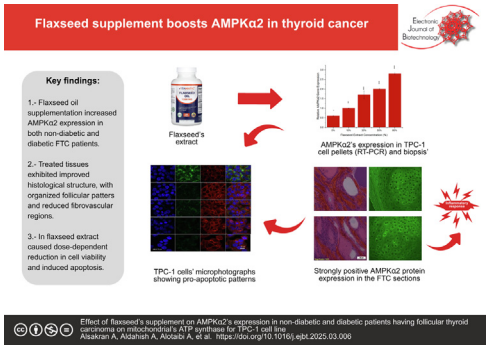
GRAPHICAL ABSTRACT

Effect of flaxseed's supplement on AMPKα2's expression in non-diabetic and diabetic patients having follicular thyroid carcinoma on mitochondrial's ATP synthase for TPC-1 cell line.



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ABSTRACT

Background: This study eipxamined the effects of flaxseed oil supplementation on the expression of adenosine monophosphate-activated protein kinase alpha2 (AMPKα2) in male patients with follicular thyroid cancer. The objective was to assess whether flaxseed oil could enhance AMPKα2 activity, improve

Abbreviations: AMPKα2, Adenosine monophosphate-activated protein kinase; ATP, Adenosine triphosphate; BMI, body mass index; cDNA, complementary deoxyribonucleic acid; CG, Chromatography; DAPI, 4′,6-diamidino-2-phenylindole; DNA, Deoxyribonucleic acid; FDG-PET, fluorodeoxyglucose – positron emission tomography; FTC, follicular thyroid cancer; G1, a low grade of cancer; G2, an intermediate grade of cancer; HbAlc, hemoglobin A1c test; IgG2a, immunoglobulin G2a antibodies; mTOR, mammalian/mechanistic target of rapamycin; MTT, commercial kit for cell viability and proliferation test; P-AMPK (Thr172), antibody detects endogenous AMPKα only when phosphorylated at threonine 172 (commercial antibody); PTC, papillary thyroid cancer; Real-Time PCR, real-time polymerase chain reaction; TPC-1, papillary thyroid carcinoma cell line.

☆ Audio abstract available in Supplementary material.

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Non-diabetic patients
RT-PCR
Tumor necrosis

histological features, and reduce tumor cell viability, offering a potential therapeutic benefit for diabetic and non-diabetic patients.

Results: A total of 303 patients, including 149 non-diabetic and 154 diabetic participants, received 1250 mg of flaxseed oil daily for one month. Flaxseed oil supplementation significantly improved tumor histology, with treated tissues showing organized follicular patterns and reduced fibrovascular cells compared to untreated tissues. Cell viability assays demonstrated a dose-dependent reduction in papillary thyroid carcinoma cell viability, with the strongest effects observed at flaxseed extract concentrations of 80, 50, and 30 μg/mL. Molecular analyses revealed increased AMPKα2 expression in both treated thyroid cancer tissues and cell line models, indicating enhanced protein activation. These findings suggest that flaxseed oil supplementation may inhibit tumor proliferation and improve histological organization in follicular thyroid cancer.

Conclusions: Flaxseed oil supplementation activates AMPKα2, reduces tumor cell viability, and improves histopathological features in follicular thyroid cancer tissues. These results highlight the potential of flaxseed oil as an adjunctive therapy to enhance cancer management strategies, particularly in diabetic and non-diabetic patients with follicular thyroid cancer.

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1. Introduction

Thyroid cancer affects about 1% of the global population, with rising incidences in recent years. Among thyroid cancers, follicular thyroid cancer (FTC) accounts for 10–15% of cases [1]. While FTC is often localized, it can act aggressively through hematogenous metastasis, increasing mortality risks [1]. Adenosine monophosphate-activated protein kinase (AMPK) plays a crucial role in cellular energy regulation during energy deficits, reinstating ATP levels [2]. AMPK acts as a negative regulator of the mammalian/mechanistic target of rapamycin (mTOR) pathway, which is vital in thyroid carcinogenesis [3]. Studies in mice highlight that mTOR cascades and protein kinase A are key to FTC progression, offering therapeutic potential [4,5].

AMPK deficiency has been linked to tumorigenesis and oncogenic transformation, emphasizing its role in metabolic adaptation [4,5]. In thyroid cells, AMPK regulates intracellular pathways involved in glucose and iodide uptake [3,6,7]. Structurally, AMPK is a heterotrimeric complex comprising regulatory γ- and β-subunits and a catalytic α-subunit [6]. Activation of AMPK prevents obesity and related metabolic disorders by promoting glucose utilization, mitochondrial biogenesis, and fatty acid oxidation [2,6,8]. Overexpression of mitochondrial ATP synthase, particularly the α-subunit, has been associated with cancer progression, making it a potential diagnostic biomarker and therapeutic target [7,8].

Dietary flaxseeds may influence tumorigenesis by reducing systemic insulin levels and mitochondrial ATP synthase activity, which contribute to metastasis and cancer progression [9,10,11,12]. Flaxseed cultivation is widespread, with applications in fiber production, medicine, and oil extraction [13,14,15,16]. Over 400 million individuals worldwide are diabetic, with a rising prevalence [17,18,19,20,21,22]. Flaxseed's health benefits, including glucose regulation and potential anti-cancer properties, are documented, but its role in diabetic patients with thyroid cancer remains unclear [22,23,24,25,26]. Thus, this study investigates the effects of flaxseed oil supplementation (1250 mg daily) on AMPKα2 expression in non-diabetic and diabetic FTC patients and examines the impact of flaxseed extracts on mitochondrial ATP synthase activity in TPC-1 cells.

2. Materials and methods

2.1. Ethics statement

The current study, informed consent, and the relevant protocols were approved by the National Regulation Committee of Ethics, Princess Nourah Bint Abdulrahman University, KSA (study number H-01-R059, IRB LOG number 20-0287).

2.2. Human participants and informed consent

The written acquainted consents were taken from each patient before getting the samples from them. The study protocols complied with the requirements, relevant regulations, and guidelines of the Declaration of Helsinki.

2.3. Selecting and treating patients

Although formal power calculations were not conducted, the sample size was based on the availability of eligible patients and the need to ensure sufficient representation for subgroup analyses. A total of 303 patients having FTC participated in the current study subdivided into two groupings: 149 non-diabetic participants and 154 diabetic participants, diagnosed with follicular thyroid cancer. Thus, the eligibility criteria for this research entailed 4 participants' cohorts: first and second cohorts; 76 diabetic people and 74 non-diabetic people histologically known by core biopsy to have follicular thyroid carcinoma; untreated with flaxseed's oil supplement. In addition, in the third and fourth cohorts; 78 diabetic people and 75 non-diabetic people had taken flaxseed oil supplement (capsules form) –1250 mg daily from (SouKare Healthcare Tech, Dubai, UAE) and took the allocated daily dose together with meals for a month. The 1250 mg daily dosage of flaxseed oil for one month was chosen based on prior studies showing its effectiveness in activating AMPK and modulating metabolic pathways. This duration was considered sufficient to observe changes in gene expression and tumor histology, while remaining practical for patient adherence and safety. All the participants who never took soy meals and various hormone therapies, not having been on

antibiotics within the biopsy period (three days); had no known allergy to lactose, positive spices or wheat, and flaxseed; had enough tissue specimen derived from the middle biopsy to assess biomarkers. Patients were excluded if they had pre-existing conditions or were on medications that could interfere with the study outcomes, such as recent antibiotic use, hormone therapies, or soy-based diets. These criteria were applied to minimize confounding variables and ensure the reliability of the results.

2.4. Clinical biochemistry tests

Routine biochemical tests were conducted by Hitachi Automatic Biochemistry Analyzer (Hitachi High-technologies Corporation, Tokyo), and HbA1c was analyzed by high-performance liquid chromatography (Bio-Rad-D10).

2.5. Sample training for histological examination

Approximately 78 mm of thyroid tumors' biopsies were immersed at once in 10% neutral buffered formalin in categorized tubes for one hour; then, these constant biopsies were embedded in paraffin.

2.5.1. Immunohistochemistry (IHC)

The antibody detects endogenous AMPKα only when phosphorylated at threonine 172 (commercial antibody) P-AMPKα2 (Thr172) preferred antibodies were bought from Cell Signalling Technology (Beverly, MA, USA). Optimal staining conditions inclusive of epitope unmasking, antibody titer, and incubation and visualization methods had been established on conventional complete tissue sections [27]. Sections were immersed in 10 mM acid buffer, pH 6.0, and subjected to a microwave strain vessel for 15 min and then processed on a DAKO autostainer (Vectastatin® ABC kits) as per the manufacturer's protocol. Sections had been blocked through goat, horse, or rabbit serum containing 10% (v/v) avidin solution (Vector Labs) for 20 min followed by incubation with preferred antibody containing 10% (v/v) biotin solution (Vector Labs) for 1 h to reduce non-specific staining. Following incubation with the precise primary antibodies, sections were incubated with the biotinylated anti-rabbit antibody for 30 min and observed through Vectastain® Elite ABC reagent. Liquid diaminobenzidine (DAB; Dako) was used as a chromogenic agent for 5 min, and sections were counter-stained with Mayer's hematoxylin [28]. In between each immunostaining step, slides were washed briefly in Tris-buffered saline (TBS) buffer, pH 7.6.

2.6. Cell culture

TPC-1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA; ATCC number CRL-3245) and cultured in DMEM (Sigma-Aldrich, St. Louis, MO), high glucose (4.5 g/ mL; 25 mM) and supplemented with 10% FBS, penicillin (1 kU/ml) and streptomycin (0.1 mg/ml). Cells were incubated in 5% CO_2 and 95% humidified atmosphere at 37°C.

2.6.1. Lignan extraction from flaxseed

Flaxseeds, derived from the plant *Linum usitatissimum* (15 g), were purchased from an herbal market in Riyadh, Saudi Arabia. These plants were routinely pulverized, defatted with petroleum ether (T 55°C; 56 mL), and extracted with MeOH (90 mL). The extract was suspended in 4 mL of H_2O partitioned with EtOAc (5 × 4 mL) and then concentrated to 65.5 mg of EtOAc-soluble residue. Chromatography (CG) fractionation of the residue was subjected to a silica gel column and eluted with $CHCl_3$, and MeOH mixtures of growing polarity (0, 10, 30, 50, 80%), afforded four fractions. The crude extract and therefore the 4 extract fractions were dried and dissolved in phosphate-buffered saline (PBS) for the cellular tests (0, 10, 30, 50, 80 μg/ml) for 48 h. The experimental protocol complies with relevant institutional, national with international guidelines and legislation.

Table 1
The primer sequences were used in the study.

	Forward	Reverse
AMPKα2	AGACCAGCTTGCAGTGGCTTATCA	AGAGGTGGCATCCTTTCTGGATGA
β-actin	GGCATAGAGGTCTTTACGGATGTC	TATTGGCAACGAGCGGTTCC

Table 2
Clinical biochemistry information for the patients and baseline profiles.

Parameter	Cohort 1 Nondiabetic	Cohort 2 Diabetic	*p-value*
No. of participating patients	149	154	0.67
Median age (years)	42 ± 4.3	53 ± 9.4	0.08
Body weight (kg)	81 ± 1.8	69 ± 11.6	0.15
BMI (kg/m^2)[a]	37.3 ± 2.6	22.4 ± 5.3	0.05
HbA1c (%)[b]	3.9 ± 0.3	7.2 ± 0.5	0.04
Fasting plasma glucose (mmol/L)	5 ± 0.4	8.3 ± 0.2	0.03
Total cholesterol (mmol/L)	4.9 ± 1.5	3.9 ± 0.6	0.21
Triacylglycerol (mmol/L)	1.9 ± 0.7	3.7 ± 0.5	0.06
Aspartate transaminase (U/L)	19.2 ± 1.7	27.2 ± 3.2	0.12
Alanine transaminase (U/L)	19.4 ± 4.3	34.6 ± 4.3	0.09
Histological type			
PTC[c]	22	29	0.73
FTC[d]	113	103	0.65
Hurthle cell	14	21	0.58
Anaplastic	0	1	0.82

All patients underwent a 12-h fasting period prior to blood collection for biochemical testing.
[a] Body Mass Index.
[b] Hemoglobin A1c test.
[c] Papillary thyroid cancer.
[d] Follicular thyroid cancer.

2.6.2. Cell viability and mobile proliferation assays

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) cellular viability assay is based on the conversion of MTT to violet-colored formazan crystals via mitochondrial dehydrogenases. For the MTT assay, the cells were seeded in 96-well plates at a density of 1 × 104 cells/well. The cells were allowed to attach and proliferate for 24 h, after which they were subjected to cellular tests. Then, the cells are incubated with 0.1 mg/mL MTT at 37°C for 4 h and lysed in dimethyl sulfoxide for 10 min to dissolve the formazan crystals. The absorbance in each well was measured at 570 nm employing a SpectraMax i3 spectrophotometer (Molecular Devices, LLC, Sunnyvale, CA, USA), and results were expressed as cellular viability relative to the manipulated cells.

2.6.3. Cell immunofluorescence and morphological examination using confocal microscopy

4% paraformaldehyde was used to treat TPC-1 cells for 10 min at a temperature, then washed using cold phosphate-buffered saline (PBS) for two times and stained using an anti-ATP synthase subunit antibody [29]. Then, cells were incubated overnight using (1 μg/ml) anti-ATP synthase (subunit mouse mAb) at 4°C and washed in PBS twice. Later, immunostaining was conducted for 1 h in darkness using an ancillary antibody against immunoglobulin G2a antibodies (IgG2a) mouse conjugated for cells' staining using red-fluorescent Alexa 594-phalloidin labeled phalloidin (1 μl/ml) by Molecular Probes, Sigma to see actin networks' integrity at higher resolution. Staining of the nuclei was done using 1 μl/ml 4'-6-dia midino-2-phenylindole (DAPI) for 5 min. Then, after final washing, the cells were examined and mounted using the Zeiss LSM700 (Switzerland) confocal microscope.

2.7. RNA isolation and RT-PCR

Real-time PCR was employed as previously described [30]. Total RNA was isolated from the biopsies using RNeasy kit (Qiagen). The complementary deoxyribonucleic acid (cDNA) was synthesized

Table 3
Correlation between flaxseed oil supplement (clinic-pathological) in capsule configuration (1250 mg daily) for a month and histological information.

Covariate	Cohort 1 (non-treated)			Cohort 2 (Treated)		
	# of Nondiabetic patients	# of Diabetic patients	*p* value	# of Nondiabetic patients	# of Diabetic patients	*p* value
# of patients	74	76	NA	75	78	NA
FDG-PET 1						
Positive in metastases (Uptake)	48	58	$X^2 = 1.8$ $p > 0.345$	35	43	$X^2 = 0.6$ $p > 0.31$
Negative (No uptake)	26	18	$X^2 = 1.8$ $p > 0.21$	39	35	$\chi 2 = 0.6$ $p > 0.37$
Tumoral necrosis						
Focal	53	34	$X^2 = 11.1$ $p < 0.009$	47	45	$X^2 = 0.21$ $p > 0.29$
Extensive	21	42	$X^2 = 11.1$ $p < 0.008$	28	33	$X^2 = 0.21$ $p > 0.17$
Tumoral size (cm)						
≤ 3	29	36	$X^2 = 0.7$ $p > 0.19$	26	34	$X^2 = 0.9$ $p > 0.13$
> 3	45	40	$X^2 = 0.7$ $p > 0.15$	49	44	$X^2 = 0.9$ $p > 0.12$
Extrathyroidal extension						
Absent	34	28	$X^2 = 1.4$ $p > 0.24$	39	47	$X^2 = 0.21$ $p > 0.26$
Present	40	48	$X^2 = 1.4$ $p > 0.22$	36	31	$X^2 = 0.21$ $p > 0.25$
Vascular invasion						
Absent	32	21	$X^2 = 3.3$ $p > 0.19$	43	39	$X^2 = 0.5$ $p > 0.39$
Present	42	55	$X^2 = 3.3$ $p > 0.18$	32	39	$X^2 = 0.5$ $p > 0.24$
Capsular invasion						
Absent	29	23	$X^2 = 0.9$ $p > 0.21$	41	43	$X^2 = 0.0$ $p > 0.29$
Present	45	53	$X^2 = 0.9$ $p > 0.23$	34	35	$X^2 = 0.0$ $p > 0.34$
Lymph node metastasis						
Absent	23	15	$X^2 = 1.9$ $p > 0.32$	40	46	$X^2 = 0.2$ $p > 0.35$
Present	51	61	$X^2 = 1.9$ $p > 0.13$	35	32	$X^2 = 0.2$ $p > 0.26$
Histological degree						
G1 3	40	51	$\chi 2 = 2.1$ $p > 0.14$	32	34	$X^2 = 0.0$ $p > 0.27$
G2 4	34	25	$X^2 = 2.1$ $p > 0.26$	43	44	$X^2 = 0.0$ $p > 0.24$

FDG-PET 1: Fluorodeoxyglucose – Positron emission tomography, G1: a low grade of cancer, G2: an intermediate grade of cancer.

utilizing Omni script reverse transcriptase and random hexamers according to the manufacturers guidelines. Real-time RCR was completed using SYBR green (Bio-Rad). The Time PCR reaction for the AMPKα2 mRNA levels had been normalized to β-actin levels (Table 1).

2.8. Statistical analysis

The graphs were generated using GraphPad Prism and OriginPro software, and significant differences between groups were analyzed using the Waller-Duncan Posthoc test with SAS version 9.4 software (Cary, NC). Effect sizes were calculated to determine the magnitude of the differences, and 95% confidence intervals were used to assess the reliability and clinical significance of the findings.

3. Results

3.1. Identification of baseline variables and clinicopathological characteristics

Out of 423 diabetic and non-diabetic cancer patients who volunteered, 120 did not meet the standards of inclusion criteria, after the preliminary biopsy, due to the very fact that their preliminary tumor became benign and that they had taken other medications. The baseline profiles of eligible 303 candidates who had been entered into and finished this randomized trial of preoperative nutritional intervention were presented in Table 2, the Anthropometric data. All patients undertook a 12-h fasting before blood collection for biochemical tests. Fasting was either self-administered or managed during the hospital stay. No significant differences were observed in any of the baseline variables ($p > 0.345$, in all cases). The traits of the patients are also summarized in Table 3. In a treated cohort of diabetes, tumor necrosis was significantly associated ($p < 0.05$) with flaxseeds treatment in diabetic patients as compared to non-diabetics.

3.2. Immunohistochemistry and histopathology

AMPKα2's protein expression distribution and tissue structure in the FTC are recorded in Fig. 1 and Fig. 2. Eosin-stained tissue and hematoxylin's micrographs of the thyroid tissue obtained from non-diabetic and diabetic patients (untreated using flaxseed supplement), having been diagnosed as having FTC underwent inspection using brightfield microscopy (left panels). Eosin-stained tissue and hematoxylin indicated regions that had irregular follicle structures, fibrovascular cores, proliferation dysregulation, and intranuclear inclusion having patterns of cells' asymmetry, filled with red blood cells while cells' nuclei in the neoplastic areas indicated a peculiar morphology (Fig. 1a and Fig. 1b respectively). On the other hand, as shown, Fig. 2a and Fig. 2b indicated FTC tissues' regions derived from non-diabetic and diabetic patients (untreated using flaxseed supplement), respectively, having improved follicle structures in comparison to Fig. 1a and Fig. 1b, respectively. In addition to the disorganized tissue's structure, cells' nuclei in the neoplastic

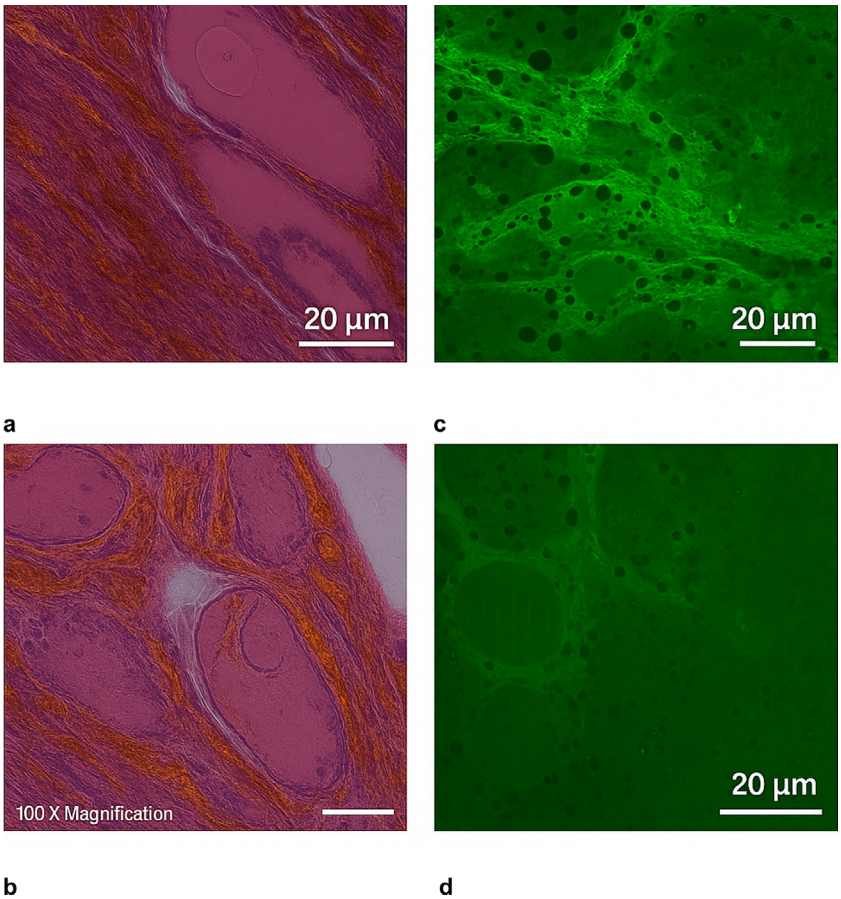

Fig. 1. AMPKα2's activity in the thyroid cancer specimens obtained from non-diabetic and diabetic patients and diagnosed using untreated flaxseed supplements and FTC, respectively. Images (a and b) indicate histopathological alterations for eosin-stained and hematoxylin sections, greatly disorganized follicle structure, fibrovascular cores having patterns of cells' asymmetry, proliferation dysregulation with many red blood cells. Images (c and d) indicate weakly positive AMPKα2's protein expression in the FTC sections obtained from non-diabetic and diabetic patients respectively.

regions restored normal morphological nuclei's appearance, having lower proliferation dysregulation levels as well as less red blood cells' levels.

Images were taken using the confocal laser scanning microscope for FTC tissues obtained from non-diabetic and diabetic patients having FTC and untreated with flaxseed supplement (Fig. 1c and Fig. 1d, respectively). AMPKα2's protein distribution (right panels and green in color) turned different in the irregular ones having weak AMPKα2's expression endogenous distribution in the neoplastic areas. At the same time, FTC sections obtained from non-diabetic and diabetic patients having FTC underwent treatment with flaxseed supplement (1250 mg daily) indicating a strong positive AMPKα2's expression endogenous distribution. AMPKα2's protein expression was localized to the vesicles just near to the apical cell wall at the untreated patients' neoplastic areas (non-diabetic and diabetic) having thyroid carcinoma tissue (Fig. 2c and Fig. 2d, respectively). Besides, AMPKα2's protein expression proportions using the AMPKα2 immunohistochemistry for cohort 2 and cohort 1 were shown in Table 4. Nondiabetic patients having FTC in group 2 indicated the greatest positive level of AMPKα2's expression at 82.66%, while diabetic participants having FTC in group 1 indicated the least positive proportion at 27.63% AMPKα2's expression.

3.3. Cell morphological study using confocal microscopy

Flaxseed's extract effect on the TPC-1 cell lines was examined using the inverted phase contrast microscopy. In Fig. 3, morphological changes occurring in the tumor cell lines are indicated at concentrations of 80, 50, 30, and 10 μg/mL. DAPI staining indicates changed nuclear shape, or/and neighboring nuclei's DNA fragments and non-treated control cells had healthy shape having a round nucleus. Red-fluorescent Alexa 594-phalloidin staining indicates the flaxseed extract treatment effect on actin networks' integrity at higher resolution for treated cells in comparison to untreated cells with concentrations of 80, 50, 30, and 10 μg/mL. Mitochondrial ATP synthase immunostaining showed the impact of flaxseed extract treatment on the inhibition of mitochondrial ATP synthase in the TPC-1 cells undergoing treatment using different concentrations.

3.4. Cell viability and cell proliferation assays

The cell viability data showed the effect of flaxseed extract on TPC-1 cell lines, observed using a hemocytometer with an inverted microscope and by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. As shown in Fig. 4, it was noted that the results of the cell viability showed a significant ($^{**}p < 0.001$) increase in the dead cell numbers when treated with flaxseed extract at concentrations (50 and 80 μg/mL). Moreover, TPC-1 treated with flaxseed extract at concentrations 10 and 30 μg/mL showed a significant ($^{*}p < 0.05$ and $^{**}p < 0.01$) decrease in the cell viability with a gradual increase in the concentrations of flaxseed extract.

3.5. AMPKα2's expression in TPC-1 cell pellets (RT-PCR) and biopsies' specimens

To examine the flaxseed's extract effect on AMPKα2's expression, the study analyzed the expression's levels at different concen-

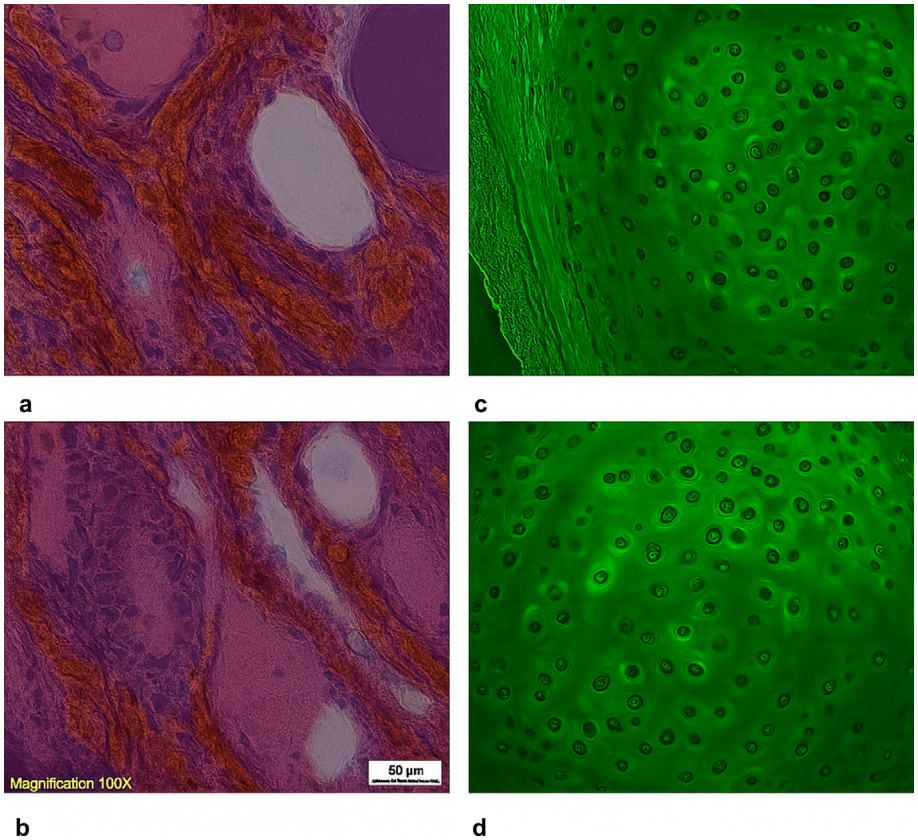

Fig. 2. AMPKα2's activity in the thyroid cancer specimens obtained from non-diabetic and diabetic patients and diagnosed using FTC with treatment from flaxseed's supplement (capsules form), 1250 mg daily for a month. Images (a and b) indicate moderate histopathological changes in eosin-stained and hematoxylin sections, an improved follicle structure, lowered proliferation dysregulation, and instigated tumor necrosis with less red blood cell levels. Images (c and d) indicate strongly positive AMPKα2 protein expression in the FTC sections obtained from non-diabetic and diabetic patients respectively, due to flaxseed's supplement treatment.

trations (80, 50, 30,10 μg/ mL) of flaxseed extract in TPC-1 cells. The findings obtained by RT-PCR assay indicated substantial AMPKα2's upregulation (***$p < 0.001$) in Fig. 5 for TPC-1 cells after treatment with 80, 50, and 30 μg/ mL concentrations of flaxseed extract, then using 10 μg/ mL concentration (**$p < 0.01$) in comparison to TPC-1 cells (untreated) (0 μg/ mL). Relative AMPKα2's gene expression on TPC-1 cancer cells indicated proportionality to flaxseed's variable concentrations (Fig. 5).

The findings of AMPKα2's mRNA expression obtained from RT-PCR test data show substantial AMPKα2's activation (***$p < 0.001$) for non-diabetic participants having FTC with treatment from flaxseed's oil supplement (capsules form), 1250 mg daily and took the allocated daily dose, with food for a month in comparison to non-diabetic participants having FTC, untreated with flaxseed's oil supplement. In contrast, AMPKα2's expression was substantially inhibited in the untreated diabetic participants with FTC in comparison to diabetic participants having FTC, treated with flaxseed oil supplement (capsules form), 1250 mg daily with food for a month. The data obtained for AMPKα2's expression underwent normalization using β-actin as the housekeeping gene. The varying AMPKα2's expression in the FTC biopsies (obtained from non-diabetic and diabetic patients) was treated and untreated with flaxseed's oil supplement (capsules form) as indicated by (Fig. 6).

4. Discussion

Flaxseed supplements exhibit strong anti-proliferative effects on cancer cell lines by activating AMPK, inhibiting mTOR activity, and inducing cell cycle arrest [3,9,10,11,13,16,17,27]. A weak association exists between thyroid cancer and diabetes, with mechanisms involving elevated thyrotropin levels [24,31]. Flaxseed's omega-3 fatty acids are crucial for normal thyroid function and should be considered in hyperthyroid diets [6,7,8,9,10,11,12,13,14,15]. Elucidating the molecular mechanisms of flaxseed's anti-cancer effects provides valuable insights for its potential use in clinical trials, especially for diabetic thyroid cancer patients [11,12,13].

Our findings demonstrate significant activation of AMPKα2 expression in treated non-diabetic and diabetic FTC patients, contrasting with suppressed AMPKα2 expression in untreated cohorts.

Table 4
FTC's sections indicating positive cells at 64% in group 1 and 36.6% in group 2 using AMPKα2 immunohistochemistry information.

AMPKα2 Protein expression in FTC sections	Cohort 1 (Non-treated)		Cohort 2 (Treated with flaxseed Supplement, 1250 mg/day for 30 d)	
	Nondiabetic patients with FTC	Diabetic patients with FTC	Nondiabetic patients with FTC	Diabetic patients with FTC
Positive	33/74 (44.60%)	21/76 (27.63%)	62/75 (82.66%)	56/78 (71.79%)
Negative	41/74 (55.40)	55/76 (72.37%)	13/75 (17.30%)	22/78 (28.20%)

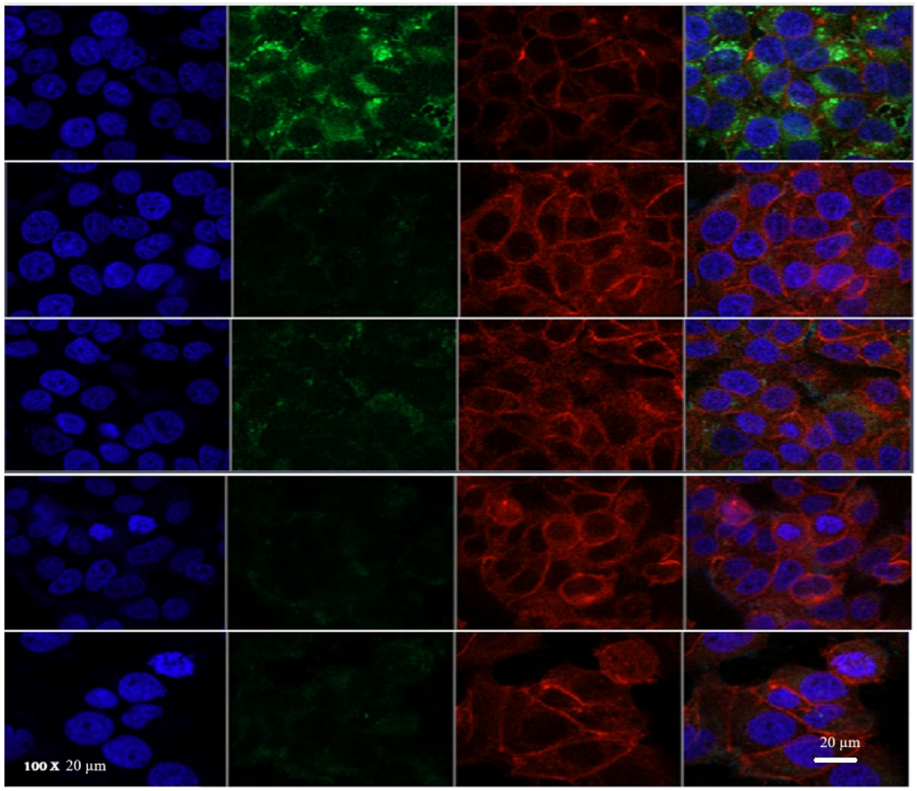

Fig. 3. TPC-1 cells' microphotographs showing pro-apoptotic patterns: nuclei's abnormal shape, inhibited mitochondrial ATP synthase, and disorganized actin networks in all the treated cells using flaxseed's extract treatment 80, 50, 30, 10 μg/mL using a microscope (confocal).

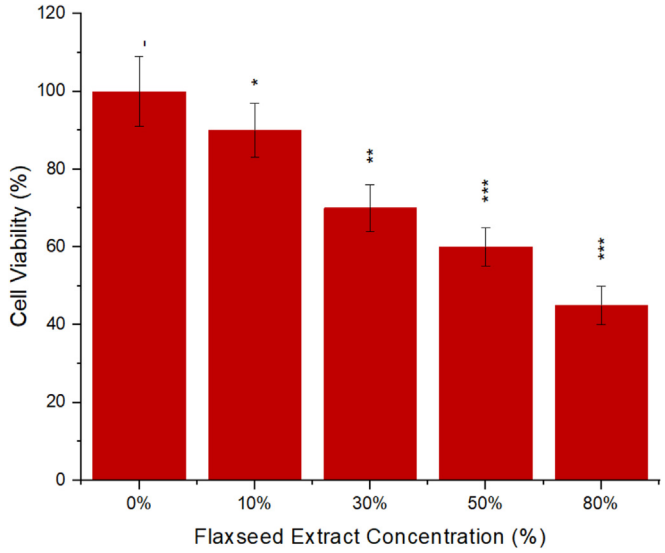

Fig. 4. Cell viability data by MTT assay exhibits a reduction in viability with variable concentrations of flaxseed extract-treated TPC-1 cancer cells represented by bar diagram. Results are expressed as the mean ± SD (n = 3). Values of cell viability bearing different superscript letters are significantly different according to the Waller-Duncan test (at * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$).

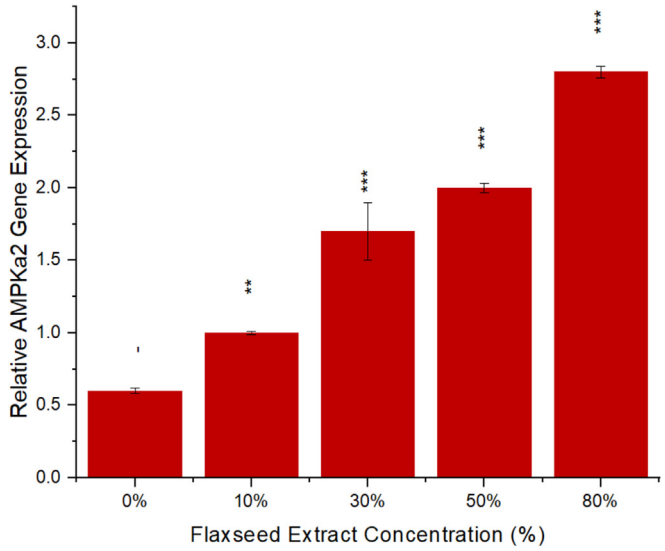

Fig. 5. RT-PCR's quantification products particularly for AMPKα2 mRNA expression within the TPC-1 cancer cells indicated AMPKα2's upregulation expression with increased variable concentrations of flaxseed's extract is shown by 80, 50, 30, 10, and 0 respectively that exhibited flaxseed's extract in four concentrations. Data were represented using bar diagram with mean ± SD (n = 3), collected for every grading cohort. Cell viability values having superscript were substantially different according to the Waller-Duncan test (at *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$).

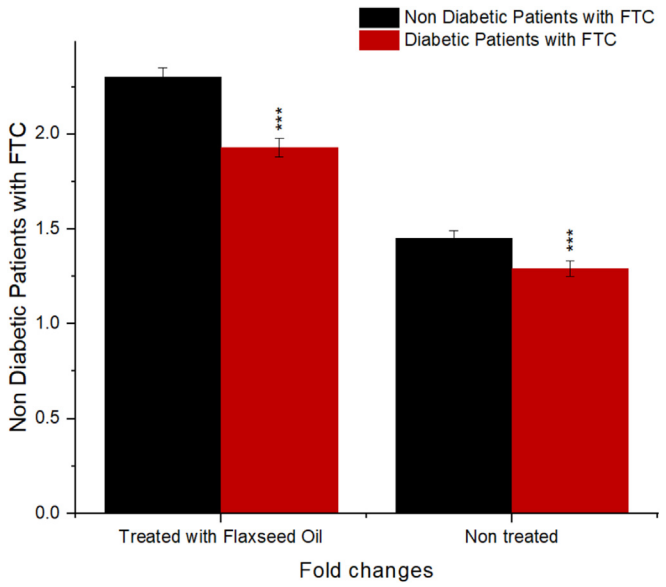

Fig. 6. The impact of flaxseeds' oil supplement (capsules form), 1250 mg daily for a month on AMPKα2's activation in the FTC biopsies obtained from non-diabetic and diabetic patients compared to the FTC biopsies obtained from non-diabetic and untreated diabetic patients. The data are represented by a bar graph with mean ± SD (n = 3) as collected from every grading cohort. Cell viability values having superscript were substantially different in the Waller-Duncan test (at *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$).

This aligns with earlier studies showing reduced AMPKα2 activity in breast cancer samples [32]. AMPKα2 also mediates cell apoptosis via the AMPKα2-p53 signaling axis [33]. Flaxseed extracts induced nuclear shape changes, actin network disorganization, DNA fragmentation, and decreased cell viability in TPC-1 cells, with the highest cell death observed at concentrations of 80, 50, and 30 μg/mL. These results underscore AMPK's role in responding to cellular energy deficits and inhibiting ATP-consuming pathways [2,7,8,29].

Previous research supports flaxseed's ability to inhibit cancer cell growth, induce apoptosis, and activate AMPK, thereby reducing tumor metastasis and proliferation [13,34,35,36]. Flaxseed supplementation also improved glycemic control, reduced inflammation, and mitigated cardiac dysfunction in type 2 diabetes patients [11,12,15,17]. Consistent with these findings, our study shows that flaxseed oil supplementation significantly upregulates AMPKα2 expression in FTC biopsies and TPC-1 cells, demonstrating its therapeutic potential. While chronic hyperglycemia in thyroid disorders may lead to metabolic syndrome, its direct link to thyroid cancer requires further investigation [9].

Flaxseed's anti-cancer effects have been observed in hormone-related cancers, such as breast cancer. Its potential role in thyroid cancer prevention and survivorship warrants further human studies to validate these findings. Future research should explore the detailed mechanisms of flaxseed's action and its integration into clinical treatments for FTC, especially in diabetic populations [11]. While the study provides valuable insights, several limitations must be acknowledged, including the short duration of flaxseed supplementation, potential confounding factors, variability in patient response, and the lack of long-term follow-up data. Future research should address these limitations by incorporating a broader range of patient profiles and extending the supplementation period to evaluate sustained effects. Although the results suggest a link between flaxseed, AMPKα2, and mitochondrial ATP synthase, the exact molecular pathways remain speculative. Further studies are needed to clarify these interactions and underlying signaling pathways.

5. Conclusions

Flaxseed extract treatment enhances AMPK activity by inhibiting tumor cells' metastasis and proliferation in thyroid cancerous tissues. From the findings of this study, the flaxseed extract has healing abilities as mitochondrial ATP synthase inhibitors can contribute to anticancer efficacy for metabolic tablets like flaxseed extract.

CRediT authorship contribution statement

Amena Alsakran: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Afaf Aldahish:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Amani Alotaibi:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Dalal Alshaya:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Elham Alzahrani:** Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Mohammad Alshugeer:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Manal Alduwish:** Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Dalia Domiaty:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Fatimah Albani:** Writing – review & editing, Writing – original draft, Supervision, Software, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Wedad Al-Qahtani:** Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Informed consent

Written informed consent for publication was obtained from all the participants. Each patient agreed to publish their medical details.

Ethical approval

The study was approved by a written letter from King Fahad Medical City according to ICH GCP H-01-R059 guidelines, IRB LOG number 20-0287, dated on August 27, 2020.

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Declaration of competing interest

The authors declare that this research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

Supplementary material

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Data availability

Data will be made available on request.

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