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New Study on the Effect of Scopoletin and Silver Nanoparticles of Fenugreek Extract as Pancreatic Cancer Inhibition Agents

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Abstract

Pancreatic cancer is a highly aggressive and lethal form of cancer with limited treatment options. Therefore, exploring novel therapeutic strategies is crucial to improve patient outcomes. Researchers are currently investigating the potential therapeutic effects of scopoletin and silver nanoparticles on pancreatic cancer patients, The researchers focused on pancreatic cancer and conducted experiments involving the synthesis and characterization of silver nanoparticles. They utilized fenugreek extract to create these nanoparticles and tested various concentrations of both the nanoparticles and scopoletin. The purpose was to evaluate the potential anti-cancer and anti-angiogenic effects of these substances. The aim of this research is to identify the most effective dose that can reduce the levels of cyclooxygenase-2 (COX-2) and lactate dehydrogenase (LDH), which are markers associated with cancer progression. The study was performed *in vitro*, The samples were categorized into three distinct groups. The control group, comprising 30 samples, consisted of healthy individuals of both genders, aged between 23 and 45 years. The second group consisted of 30 male and female patients, aged between 45 and 65 years, who were diagnosed with stage II pancreatic cancer. Lastly, the third group was composed of 30 male and female patients with stage III pancreatic cancer, aged between 52 and 79 years. In this study, it was discovered that patients with stage II and stage

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III pancreatic cancer experienced the most significant decrease in enzyme concentration when the nanoparticle solution was at a concentration of 2 ppm. The concentration values of the Cyclooxygenas-2 enzyme were 2.43±0.22 ng/ml, 3.04±0.31 ng/ml, and 1.59±0.06 ng/ml for stage II and stage III patients, respectively. Similarly, the concentration values of the Lactate dehydrogenase enzyme were 1.20±0.02 ng/ml, 1.14±0.02 ng/ml, and 0.85±0.18 ng/ml for the respective stages. Furthermore, the scopoletin compound solution at a concentration of 8 ppm showed a better reduction in COX-2 enzyme concentration compared to the control group. The concentration values of COX-2 enzyme at this concentration were 1.97±0.17 ng/ml, 1.72±0.10 ng/ml, and 1.22±0.07 ng/ml. The concentration values of the LDH enzyme were 0.93±0.07 ng/ml, 0.97±0.11 ng/ml, and 0.71±0.10 ng/ml for stage II and stage III patients. The results of this research demonstrate that scopoletin compounds and Ag-NPs derived from Fenugreek extract function as anti-cancer and anti-angiogenesis agents by reducing the levels of cyclooxygenase-2 (COX-2) and lactate dehydrogenase (LDH) enzymes in the blood serum of individuals with stage II and stage III pancreatic carcinoma in Iraq.

Keywords: Pancreatic cancer, Fenugreek, scopoletin, Ag-NPs.

دراسة جديدة حول تأثير السكوبوليتين والجسيمات النانوية الفضية لمستخلص الحلبة كعوامل تثبيط سرطان البنكرياس

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الخلاصة

سرطان البنكرياس هو نوع عدواني ومميت للغاية من أنواع السرطان مع خيارات علاجية محدودة. لذلك، استكشاف استراتيجيات علاجية جديدة مهم لتحسين نتائج المرضى. حاليًا يقوم الباحثون بدراسة تأثيرات scopoletin والجسيمات النانوية الفضية على مرضى سرطان البنكرياس. ركز الباحثون على سرطان البنكرياس وأجروا تجارب تشمل تخليق وتوصيف الجسيمات الفضية. لقد استخدموا مستخلص الحلبة لإنتاج هذه الجسيمات واختبروا تراكيز مختلفة من كل من الجسيمات النانوية والـ scopoletin. الهدف كان تقييم الآثار المحتملة المضادة للسرطان ومانعة للأوعية الدموية لهذه المواد. وتهدف هذه الدراسة إلى تحديد الجرعة الأكثر فعالية التي يمكن أن تقلل من مستويات السيكلوأوكسيجيناز-2 (COX-2)

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ولاكتات ديهيدروجيناز(LDH) ، وهما علامتان مرتبطتان بتقدم السرطان. تمت الدراسة في المختبر, وتم تصنيف العينات إلى ثلاث مجموعات متميزة. المجموعة الأولى، تتألف من 30 عينة، وتضم أفرادًا أصحاء من كلا الجنسين، تتراوح أعمارهم بين 23 و 45 عامًا. المجموعة الثانية تتألف من 30 مريضًا ذكورًا وإناثًا، تتراوح أعمارهم بين 45 و 65 عامًا، تم تشخيصهم بمرض سرطان البنكرياس مرحلة II. أما المجموعة الثالثة فتضم 30 مريضًا ذكورًا وإناثًا بمرض سرطان البنكرياس مرحلة III، تتراوح أعمارهم بين 52 و 79 عامًا. في هذه الدراسة، تبين أن المرضى الذين يعانون من سرطان البنكرياس مرحلة IIومرحلة III شهدوا انخفاضًا كبيرًا في تركيز الإنزيمات عندما كانت محلول الجسيمات النانوية بتركيز 2 جزء في المليون. وبلغت قيم تراكيز إنزيم السيكلوأوكسيجيناز - 2-ما يلي: 2.43 ± 0.22 نانوغرام/مل، 3.04 ± 0.31 نانوغرام/مل، و1.59 ± 0.06 نانوغرام/مل لمرضى المرحلتين II و III على التوالي. وكذلك بلغت قيم تراكيز إنزيم لاكتات ديهيدروجيناز ما يلي: 1.20 ± 0.02 نانوغرام/مل، 1.14 ± 0.02 نانوغرام/مل، و0.85 ± 0.18 نانوغرام/مل للمراحل المعنية. وعلاوة على ذلك، أظهر محلول مركب scopoletin بتركيز 8 جزء في المليون تقليلًا أفضل في تركيز إنزيم COX-2 مقارنة بالمجموعة الضابطة. وبلغت قيم تراكيز إنزيم COX-2 عند هذا التركيز ما يلي: 1.97 ± 0.17 نانوغرام/مل، 1.72 ± 0.10 نانوغرام/مل، و1.22 ± 0.07 نانوغرام/مل. وبلغت قيم تراكيز إنزيم LDH ما يلي: 0.93 ± 0.07 نانوغرام/مل، 0.97 ± 0.11 نانوغرام/مل، و0.71 ± 0.10 نانوغرام/مل لمرضى المرحلتين II و III. تظهر نتائج هذا البحث أن مركبات scopoletinو Ag-NPsالمصنعه من مستخلص الحلبة تعمل كعوامل مضادة للسرطان ومثبطة للأوعية الدموية عن طريق تقليل مستويات إنزيمات السيكلوأوكسيجيناز-2 (COX-2) و لاكتات ديهيدروجيناز (LDH) في مصل الدم للأفراد الذين يعانون من سرطان البنكرياس مرحلة II ومرحلة III في العراق.

الكلمات المفتاحية: سرطان البنكرياس، الحلبة، scopoletin، Ag-NPs.

Introduction

Pancreatic cancer has a higher death rate compared to other common types of cancer because it is typically diagnosed at an advanced stage and is challenging to cure[1]. The average survival time for individuals with pancreatic cancer is only six months, and the five-year survival rate is less than 5%. Due to the invasive nature and complex chemoresistance mechanisms of pancreatic malignancies, as well as the significant role of hypoxia in pancreatic adenocarcinoma. There is an urgent need to discover molecular markers to help implement effective treatment approaches and to predict cases early on. Early recognition of pancreatic cancer is difficult due to the retroperitoneal location of the pancreas and the absence of specific serological markers [2],[3]. Enzyme Cyclooxygenase (COX-2) has the EC 1.14.99.1

commission code[4]. Cyclooxygenase is a key enzyme in the biochemical pathway leading to the synthesis of prostaglandins. Inflammatory circumstances induce the expression of cyclooxygenase-2 [5][6]. Many human cancers, including colon, stomach, breast, lung, and hepatocellular carcinomas, are linked to cyclooxygenase-2 overexpression. Moreover, cyclooxygenase-2 is not expressed in normal cells but is extensively expressed in tumours and inflammatory diseases. Active COX-2 can also cause metastasis, angiogenesis, cell proliferation, and the inhibition of cell death, which are all significant phases in the development of tumours[7], [8]. Pancreatic, breast, colorectal, stomach, and lung carcinomas exhibit elevated levels of cyclooxygenase-2. Thus, COX-2 is an important target for the development of novel anticancer medicines [9]. In a study provided by Prima et al., 2017 the results showed that COX-2 has a significant function in the formation and progression of strong tumours, and COX-2 inhibition may hinder the development of a variety of strong malignancies. Thus, it may be beneficial to improve clinical outcomes in patients with malignant tumours, by disrupting the action of COX-2 in cancer cells[10].

Lactate dehydrogenase (LDH) is a crucial enzyme in the anaerobic metabolic route; hence, it is regarded as one of the metabolic enzymes that feed cancer cells [11][12]. It belongs to the oxidoreductase class, with the EC enzyme commission number 1.1.1.27[13]. The blood enzyme lactate dehydrogenase (LDH) is involved in the conversion of pyruvate into lactate and is essential for maintaining glycolysis, which is intimately linked to the development and spread of cancer. Since it is required for tumour maintenance, LDH has been proposed as an indicator of tumour burden and aggressiveness. Also, there is proof that blood LDH levels are elevated in a variety of tumours and have been associated with many cancer patients' prognoses, including those with pancreatic cancer [14]. LDH levels in the blood are linked to tumour expression and bad outcomes [2]. One of the hallmarks of cancer is dysregulated metabolism. ATP is largely produced by oxidative phosphorylation under normal physiological conditions. Despite the presence of oxygen, cancers frequently experience a drastic shift toward glycolysis. The Warburg effect is a phenomenon that necessitates lactate dehydrogenase-A activity (LDHA). At the final stage of glycolysis, pyruvate is converted to lactate by LDHA, which is frequently overexpressed in cancer. Because LDHA blockage causes cancer cells to undergo

apoptosis, LDHA inhibitors represent a promising treatment alternative[15]. The foundation of contemporary medicine plants, which have been utilized for therapeutic purposes since the dawn of human history. The majority of compounds derived from plants or their synthetic derivatives are employed in the production of chemotherapy medications used to treat cancer[16] [17][18].

Widespread in plants, the phenolic substance coumarin scopoletin (7-hydroxy-6-methoxy coumarin) contains coumarin derivatives that are superior in many plant compounds with diverse therapeutic effects [19]. It is also present and extracted from the *Trigonella* plant [20]. The melting point of scopoletin, an amorphous light yellow powder, is 202–204 °C, with a boiling temperature of 413.5 °C, and the chemical formula $C_{10}H_8O_4$ with a molecular weight of 192.17 g/mol. Scopoletin is hardly soluble in aqueous buffers and barely soluble in water[20]. Many pharmacological effects of scopoletin include an anti-cancer effect. [21]. Scopoletin has been shown to inhibit the growth of cancer cells[22]. It has anti-cancer properties; research shows that it dramatically reduces cancer cells' ability to invade healthy tissue [23]. Due to its outstanding pharmacological properties, A therapeutic plant known as *Trigonella* has long received attention from traditional healers around the world [24][16]. The method of employing plant extracts to change metal ions into nanoparticles is known as phytoremediation. It has become common practice in recent years to investigate plant extracts for use in the creation of nanoparticles[25]. As reducing, capping, and stabilizing agents, plant extracts are used. An interdisciplinary scientific discipline called nanotechnology seeks to develop atomic, molecular and supramolecular materials with improved properties [26]. It has become common practice in recent years to investigate plant extracts for use in the creation of nanoparticles. As reducing, capping, and stabilizing agents, plant extracts are used [27].

Aim of the study

The purpose of the study is to compare the anti-cancer and anti-angiogenic properties of the fenugreek extract's silver nanoparticles and the scopoletin molecule. The study also attempts to determine the nanocomposite and scopoletin solution concentrations that best reduce COX-2 and LDH enzyme concentrations.

Materials and method

Blood samples from pancreatic cancer patients were collected from January 2022 to June 2022. (Teaching Oncology Hospital and Teaching Hospital for Gastroenterology and Hepatology). and they have been separated into the three groups listed below:

G1- Thirty samples from a healthy, age-ranged (23-45) control group.

G2- 30 samples from stage II patients, 45 to 65 years old, of both sexes, were used.

G3- 30 samples from patients in stage III, aged 52 to 79 years, of both sexes.

Specimen collection and preparation

Disposable plastic syringes containing 10 millilitres (ml) were used to draw eight millilitres (ml) of venous blood. Each person and the control group had their blood drawn, and the samples were placed in ordinary plastic tubes without any anticoagulant. After that, blood was allowed to coagulate at 37°C for 20 to 30 minutes. Serum was collected, separated into tiny Eppendorf tubes, and stored at -20°C until analysis after 10 minutes of centrifugation at 3000 rpm.

Chemicals

Silver Nitrate with a purity of 99.0%, Thomas Bacer Company from India. The product was provided by Scopoletin from the UK, and Sigma- Aldrich. Importing Cyclo-oxygenase-2 kit from China, a product of Fine Test. Lactate dehydrogenase kit from England Randox Laboratories. *Trigonilla foenum –creacum* plant imported from markets of Baghdad Iraq Country (Al Razi Medical Herbal Center).

Synthesis of fenugreek extract and silver nanoparticles from fenugreek extract.

Silver nanoparticles were prepared by fenugreek extract and they were also diagnosed using several techniques according to what was reported by Nihad and Nijoud's 2023 research [28].

Preparation of solutions

The concentrations of scopoletin and silver nanoparticles were determined in this study by dissolving (0.005mg) of each ingredient in (100 ml) of water to form a stock standard solution. Working solutions of varied concentrations were prepared by diluting stock solutions in water (2ppm,4ppm,8ppm and 10ppm). Just before usage, stock standard solutions and diluted solutions are created.

Measurement of serum COX-2 level

Principle:

The sandwich enzyme-linked immune-sorbent assay method was used in this kit. The capture antibody was coated on 96-well plates before usage. The detecting antibody was an antibody conjugated with biotin. The wells were washed with wash buffer before adding the standards, test samples, and biotin-conjugated detection antibodies. After adding HRP-Streptavidin, a wash buffer was used to get rid of the unbound conjugates. TMB substrates were used to see the enzymatic reaction of HRP. HRP helped turn TMB into a blue substance, which turned yellow when an acidic stop solution was added. The amount you wish to capture on the plate influences the quantity of yellow you need to use. With a microplate reader, you can determine the quantity of the target by reading the O.D. absorbance at 45nm, which is [29].

After preparing concentrations of the scopoletin solution, as well as for a silver nanoparticles solution (2,5,8, and 10 ppm) samples are transmitted and placed in the measurement plate within the special measurement group Enzyme of Cyclo-oxygenase-2. After taking 50 micro-litre of serum and putting it into the well add 50 micro-litre of each concentration and mix with serum. The steps are complemented by the method of work to measure ELISA as in reference [29].

Serum LDH concentration determination

Principle:

This kit makes use of a method called a "Double Antibody Sandwich." The Double Theory A target analyte having more than two possible epitopes that can be recognized simultaneously by both the pre-coated capture antibody and the detection antibody forms the basis of an antibody sandwich [30].

After preparing concentrations of the scopoletin solution, as well as for a silver nanoparticles solution (2,5,8, and 10 ppm) samples are transmitted and placed in the measurement plate within the special measurement group Enzyme of Lactate dehydrogenase. After taking 50 micro-litres of serum and putting it into the wells add 50 micro-litres of each concentration and mix with serum. The steps are complemented by the method of work to measure ELISA as in reference 30.

Statistical analysis

The current study's results can be expressed using the means standard deviation. The t-test was also used to highlight the significance of a difference between two groups with different mean values when $P \leq 0.05$ indicated a statistically significant difference between the two groups. The office application (Excel 2010) was utilized to calculate all of the results values for all of the study's groups.

Results and Discussion

Scopoletin and Ag-NPs of Trigonella extract's effects on COX-2 and LDH in sera from PC stage II and stage III patients.

Table 1: COX-2 concentration in serum of three studied categories

GROUPS	**NO**	**COX-2 (NG/ML)** WITHOUT ADDED **MEAN±SD**	**P**	**COX-2 (NG/ML)** WITH SCOPOLETIN **MEAN±SD**	**P**	**COX-2 (NG/ML)** WITH NAPS OF TRIGONELLA **MEAN±SD**	**P**
G1	**30**	1.61±0.80		1.22±0.07		1.59±0.06	
G2	**30**	2.50±0.41	1.3×10^{-6}	1.97±0.17	3.4×10^{-29}	2.43±0.22	1.5×10^{-27}
G3	**30**	3.57±0.84	5.5×10^{-13}	1.72±0.10	5.8×10^{-29}	3.04±0.31	1.2×10^{-32}
			5×10^{-8}		2.9×10^{-8}		4.6×10^{-12}

Table (1) compares the levels (mean±SD) of Cyclooxygenase-2 in the blood of patients with stage II and III pancreatic cancer to control groups both with and without the addition of a solution of concentrated scopoletin (8 ppm) and a solution of concentrated AgNPs of *Trigonella* extract (2ppm).

Table 2: LDH concentration in serum of three studied categories

GROUPS	NO	LDH (NG/ML) WITHOUT ADDED MEAN±SD	P	LDH (NG/ML) WITH SCOPOLETIN MEAN±SD	P	LDH (NG/ML) WITH NAPS OF TRIGONELLA MEAN±SD	P
G1	**30**	0.97±0.13		0.71±0.10		0.85±0.18	
G2	**30**	1.42±0.18	1.7×10^{-15}	0.93±0.10	1.6×10^{-11}	1.20±0.02	1.6×10^{-14}
G3	**30**	1.32±0.26	1.7×10^{-8}	0.97±0.11	1.3×10^{-13}	1.14±0.02	1.6×10^{-14}
			0.09		0.14		8.5×10^{-14}

Table (2) gives data on(mean±SD) the concentration of Lactate dehydrogenase in the blood of patients with stage II and stage III pancreatic cancer compared to control groups, without and with a concentrated solution of scopoletin (8ppm) and a concentrated solution AgNPs of *Trigonella* extract (2ppm).

According to the data presented in tables (1) and (2), there was a statistically significant rise ($P\leq0.05$) in the value of COX-2 and LDH concentration in the patient groups G2 and G3 when compared with the control group G1. without any addition and after adding solutions of silver nanoparticles of fenugreek extract with concentration (2ppm) and scopoletin with (8ppm), it also observed that the values of COX-2 and LDH concentration after adding the solution of scopoletin with concentrated (8ppm) reduced a more than compared to the values of COX-2 and LDH concentration after adding ANPs solution with concentrated (2ppm) and without any addition in all study groups.

These results are in agreement with the results of previous studies, where many studies found that COX-2 and LDH are elevated in pancreatic cancer. Additional investigations demonstrated that the inhibition of pancreatic cancer development is caused by the lowering of COX-2 and

LDH by pharmacological compounds administered in vivo or synthetic compounds applied in vitro [31] and [32] respectively.

Previous reports have shown that COX-2 is the acritical mediator of tumour angiogenesis[33]. Numerous trials have shown that COX-2 promotes tumour angiogenesis, and it has been shown that COX-2 inhibitors significantly reduce the risk of cancer, and this may be due to the inhibition of angiogenesis[34].

Angiogenesis has been linked to COX-2, an enzyme involved in inflammation and cancer, indicating that drugs that target COX-2 and prostanoid-related signalling cascades may be efficient antiangiogenic treatments [35].

Miladiyah et al. produced trihydroxy xanthone, a novel xanthone derivative. The derivative chemical was the most effective against the colorectal cancer (WiDR) cell line (IC50 = 37.79 M; SI = 66.40). The molecular docking analysis of the chemical Trihydroxy xanthone revealed that it interacts with telomerase, COX-2, and cyclin-dependent kinase (CDK). It was determined that the normal VERO cell line was not affected by this chemical. Trihydroxy xanthone formed hydrogen bonds with the Arg120, Tyr355, and Tyr385 of COX-2, inhibiting COX-2's activity, which kills cancer cells and inhibits the growth of tumours [9]. We expect that our compound under study (Scopoletin) interferes with the enzyme (COX-2) via hydrogen bonds, so we agree with the study of Miladiyah *et al.,* which suggested that the compound of Trihydroxy xanthone similar to our compound (Scopoletin) confirmed the interaction between Trihydroxy xanthone and the (COX-2) enzyme.

A previous study showed that silver nanoparticles prepared by Plant extracts can inhibit colon cancer, through their ability to inhibit COX-2 which is important in the development and growth of cancer by stimulating the formation of blood vessels and supplying the tumour with food and oxygen, where cancer colon patient with silver nanoparticles at 1µg/ml. According to cytotoxicity and colony formation assay results, the biogenic AgNPs exhibited antiproliferative action on colon cancer cells. Dispersed and agglomerated AgNPs ingested into the cell via endocytosis were captured in the vacuoles. Colon cancer cells treated with AgNPs endure

apoptosis by causing damage to the mitochondrial membrane and nuclear membrane, leading to an increase in the production of reactive oxygen species and DNA degradation, respectively. In addition, AgNPs downregulate antiapoptotic genes and upregulate apoptotic genes, causing colon cancer cells to undergo apoptosis. The COX-2 and NF-kB signalling pathways are crucial for tumour progression and metastasis[36].

A prior study found that Gallofaven(GF), As anti-colorectal cancer inhibited LDH-A, one of the five isoforms of the lactate dehydrogenase family. According to the findings, GF, which occupies the Nicotinamide adenine dinucleotide NADH site of LDH-A, strongly inhibited the enzyme's interaction with single-stranded DNA (ssDNA) in vitro. GF decreased Ribonucleic acid (RNA) synthesis in colorectal cancer cell line (SW620) cells cultivated in the absence of glucose after a short time of exposure, allowing the effects of GF due to an impairment of LDH-A enzymatic activity to be dismissed. As a result, GF's anticancer activity on normal glycolysis neoplastic cells was most likely mediated not only by the suppression of aerobic glycolysis but also by the inhibition of RNA synthesis [37]. The Galloflaven compound has a similar chemical structure to the scopoletin compound under study in terms of functional groups that contribute to the effectiveness of the compound, so we can imagine that our compound under study (scopoletin) can work with the same inhibition mechanism as the galloflavin compound for the LDH enzyme. Silver nanoparticles prepared by green synthesis (hydrothermal) from *Trigonella* extract. It has anti-cancer effects, as many studies reported on the anticancer effect of silver nanoparticles in general[38]. In our current study, we found the effect of silver nanoparticles on the enzyme lactate dehydrogenase, where the concentration of the enzyme LDH decreased in the serum of patients, and this effect can be explained by the interaction between the silver nanoparticles with the thiol group of amino acids (such as cysteine) in the structure of the enzyme[40]. Perhaps the interference between the enzyme and the silver nanoparticles, when they are bound to the active site of the enzyme, leads to inhibition of the enzyme action[40].

Conclusion

In conclusion, it was found that the scopoletin compound and the nanocomposite have a great ability to inhibit pancreatic cancer through the ability of the studied compounds to reduce the concentration of the enzyme Cyclooxygenase-2, as well as to reduce the concentration of the enzyme lactate dehydrogenase. We stress the need to conduct *in vivo* research and clinical trials showing that scoplatin and the nanocomposite are effective against pancreatic cancer, especially in stage II and III pancreatic cancer.

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