Therapeutic Effect Of Luteolin Natural Extract Versus Its Nanoparticles On Tongue Squamous Cell Carcinoma Cell Line: In Vitro Study

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Presented by:

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List of Contents:

Administrative Information:

- **Title:**

  Therapeutic Effect of Luteolin Natural Extract Versus Its Nanoparticles on Tongue Squamous Cell Carcinoma Cell Line: In Vitro Study

- **Protocol Version:**

  Version (1)

- **Roles and Responsibilities:**

1-The Principle Supervisor:

- **Prof. Dr. Heba Ahmad Farag** (H.F.), Professor of Oral & Maxillofacial Pathology, Faculty of Oral & Dental Medicine, Cairo University.

2-The Assistant Supervisor:

- **Dr. Safa Fathy Abd-Elghany** (S.F.), Assistant Professor of Oral & Maxillofacial Pathology, Faculty of Oral & Dental Medicine, Cairo University.

3-The Investigators:

- **Dr. Esam Rashwan** (E.R.), Assistant Professor and Head of Confirmatory Diagnostic Unit, VACSERA-Egypt
- **Safaa Mohamed Mohamed Ibrahim Baz** (S.B.), (**The Principle Investigator**) Assistant Lecturer of Oral & Maxillofacial Pathology, Faculty of Oral & Dental Medicine, BUE.

Steering Committees:

1. Oral & Maxillofacial Pathology Department Board, Faculty of Dentistry, Cairo University.
2. Evidence Based Committee, Faculty of Dentistry, Cairo University.

3. Ethics Committee, Faculty of Dentistry, Cairo University.
4. Higher Education and Research Committee.
5. Faculty Board.

Assessment and Auditing:

The assessment and auditing of the study design was done by:

1. Oral & Maxillofacial Pathology Department Board, Faculty of Dentistry, Cairo University.
2. Evidence Based Committee, Faculty of Dentistry, Cairo University.

Research Ethics Approval:

This protocol will be reviewed by:

Ethical Committee, Faculty of Dentistry, Cairo University.

Access to Data:

All Investigators of the study will be given access to the data.

Dissemination Policy:

Study results will be published as a partial fulfillment of the requirements for the PhD degree in Oral & Maxillofacial Pathology.

Funding:

Self-funding.

1. Introduction:

1. 1. Background and Scientific Rationale:

Cancer still one of the most lethal diseases causing an expansive number of deaths globally. In 2015, the WHO stated that the number of individuals died from cancer all over the world was about 8.8 million and indicated that by 2030 this number will show a marked elevation exceeding 13 million [1]. Among malignancies, oral cancer is the sixth most common neoplasm around the world. Oral squamous cell carcinoma (OSCC) is the most widely recognized oral malignancy representing almost 90% of the oral cancers worldwide [2], with a variable global incidence which differs in each area according to the exposure to specific risk factors [3].

For treatment of OSCC, the primary therapeutic modalities are surgical intervention, radiotherapy, and chemotherapy [4,5]. Chemotherapeutic agents play a vital role in the treatment of malignancy by killing cancerous cells. Nevertheless, they cause cytotoxic impacts to the adjacent normal cells and leave behind tissue resistance which is considered to be serious obstructions encountered the chemotherapy treatment. Therefore, the scientific community has the responsibility to discover an alternative naturally occurring drug with minimal side effects and maximal efficacy in cancer therapy [6].

Among plant-derived compounds, flavonoids are accepted as natural chemotherapeutic agents [7] with many points of interest over the traditional chemotherapy, (e.g. better bioavailability, lower toxicological profile, as well as affordability) [8]. Luteolin, 3',4',5,7-tetrahydroxyflavone, is a naturally occurring flavonoid isolated from a wide variety of medicinal herbs, fruits, and vegetables (e.g. parsley, artichoke, celery, onion leaves, broccoli, carrots, green and sweet bell pepper, perilla leaves and chrysanthemum flowers) [9–12]. Luteolin has been appeared to display several significant biological properties, ranging from antioxidant and anti‑inflammatory effects to cancer chemo-therapeutic/chemoprevention activities [13].

A recent in vitro and in vivo study has demonstrated that luteolin and its nanoparticles have anticancer activity against cells of lung cancer and squamous cell carcinoma of head and neck [14].

Regarding the therapeutic effect of luteolin, a study on hepatic carcinoma cells has revealed a high level of apoptotic cells (reached to 58.18±2.11%) after 50 μM luteolin treatment for 24 hours [15].

To provide a magnificent enhancement in the treatment effectiveness and overcome the undesired current cancer therapy toxicity, the researchers have potentially turned to nanotechnology-based regimens incorporation with several promising qualities over the conventional chemotherapy [16]. Furthermore, the anticancer activity of nano-luteolin (nanoparticle delivery of luteolin) has suggested many advantages superior to luteolin and may have potential application in clinical settings [17].

1. 2. Objectives:

In vitro study to examine whether luteolin and nano-luteolin exert an inhibitory effect on tongue squamous cell carcinoma cell line by inducing apoptosis, and to assess if nano-luteolin has more efficient apoptotic activity than luteolin on tongue squamous cell carcinoma cell line.

2. PICO:

Population (P): Cultured tongue squamous cell carcinoma cell line.

Intervention (I):

- **I 1:** Application of luteolin
- **I 2:** Application of nano-luteolin

Control (C): No treatment.

Outcome(s) (O):

- **Primary O:** Apoptosis (Gene expression of Caspase 3).
- **Secondary O:** Cytotoxicity/Cell viability.

Outcome	Outcome name	Measuring Device	Measuring Unit
Primary	Apoptosis (Gene expression of Caspase 3)	RT-qPCR system [18].	fold-changes
Secondary	Cytotoxicity/Cell viability	MTT assay using an ELISA Reader [19].	Micromolar (μM)

Study design:

In vitro study

3. Research Question:

Do luteolin and nano-luteolin natural extracts exert an inhibitory effect on tongue squamous cell carcinoma cell lines by inducing apoptosis? Is nano-luteolin more efficient than luteolin in inducing apoptosis on tongue squamous cell carcinoma cell line?

4. Material and Methods:

4. 1. Collecting samples:

Tongue squamous cell carcinoma cell line will be purchased from veterinary serum & vaccine research institute (VACSERA), supplied with compatible nutrient media.

4. 2. Materials:

4. 2. 1. Intervention (I):

1. Luteolin (Sigma-Aldrich)
2. Nano-luteolin (Luteolin nanoparticles prepared using ultrasonication nanotechnology)

4. 2. 2. Check list for the used drug:

Item	Description
Name, common names and family name	Luteolin, 3′,4′,5,7-Tetrahydroxyflavone; Flavones, Flavonoids
Used product	Luteolin (Sigma-Aldrich)
Form	Powder
Color	Yellow
The type and concentration of solvent used	Ethanol (4.90 - 5.10 mg/ml)
Nano-luteolin	Luteolin nanoparticles prepared using ultrasonication nanotechnology

4. 3. Methods:

All the steps of the current experiment will take place in Confirmatory Diagnostic Unit, R and D sector, VACSERA-Egypt, by E.R. and S.B.

4. 3. 1. Sample preparation: (According to ATCC® protocol)

[Continuous Cell Line Cell Culture Protocol]

1. **Examination:**

   E.R. and S.B. will examine the culture prior to subcultivation using an inverted phase contrast microscope, and will check the general appearance of the culture.

2. **Cell harvesting:**

   When using 0.1% Trypsin solution for enzymatic dissociation:

   a) E.R. and S.B. will remove the old medium and discard it.
   b) We will rinse the cell monolayer gently.
   c) Then we will add the enzyme solution and incubate the culture until the cells are freleased.

3. **Cell counting:**

   To determine growth rates or set up cultures at known concentrations it is necessary to count the cell suspension.

   a) E.R. and S.B. will remove 0.5mL sample and place it in a tube for counting, with adding the vital stain Trypan blue. We will withdraw 20μL of the sample with a wide tip pipettor and carefully load a clean hemacytometer.
   b) We will count a viable cell count. We will calculate the number of viable cells/mL and the total cell number.

4. **Plating:**

   After making the appropriate dilutions, E.R. and S.B. will add the correct amount of cells to each culture vessel. Then we will add fresh medium to bring the cell culture vessel to its recommended working volume.

5. **Incubation:**

   a) E.R. and S.B. will leave caps on flasks slightly loosened and place them on a shelf in a 37°C, humidified CO_2 incubator*.
   b) We will examine culture daily and will change medium as needed.

* Thermo Scientific Heraeus, UK.

4. 3. 2. MTT assay: (According to Sigma-Aldrich protocol)

[IC50 Calculation and MTT Assay with ELISA reader* Protocol]

⇒ **IC50 calculation:**

- After plating and 24 hours incubation of the cell culture, E.R. and S.B. will remove the cell culture from incubator into laminar flow hood, and then apply to a 96-well plate (about 25,000-30,000 cells per well).
- E.R. and S.B. will add the drug with five serial concentrations (100, 10, 1, 0.1, and 0.01μg/mL) to calculate the IC_{50}% concentration in Micromolar (μM).

⇒ **MTT Assay** should include:

- Blank wells containing medium only.
- Untreated control cells.
- Test cells treated with either luteolin or nano-luteolin with IC_{50}% concentration.

1) After plating and 24 hours incubation of the cell culture, E.R. and S.B. will remove the cell culture from incubator into laminar flow hood, and then apply to a 96-well plate (about 25,000-30,000 cells per well).
2) E.R. and S.B. will reconstitute each vial of MTT to be used with 3 ml of medium or balanced salt solution without phenol red and serum. We will add the reconstituted MTT in an amount equal to 10% of the culture medium volume.
3) We will return cultures to incubator for 2-4 hours until purple precipitate is visible.
4) At the end of the incubation period, we will remove the cultures from incubator and dissolve the resulting purple formazan crystals by adding an amount of MTT Solubilization solution equal to the original culture medium volume.
5) Well mixing will improve dissolution.
6) We will measure absorbance spectrophotometrically at a wavelength of 450nm.

* Bioline Technologies, India.

4. 3. 3. RT-PCR: (According to Bio-Rad Laboratories protocol)

[One-step Rotor-Gene Q Real-Time PCR System* Protocol]

1. Treatment of cell culture:
   After plating and 24 hours incubation of the cell culture, E.R. and S.B. will treat the flask with the IC_{50}% concentration of the drug for the treatment incubation time.
2. We will harvest cells, then lysis cells.
3. E.R. and S.B. will prepare all the required components (including SYBR® Green one-step kit, SYBR® Green Supermix, sequence-specific primers for Caspase, necessary enzymes and a buffer solution) except RNA on ice reaction set up.
4. We will mix the reaction set up thoroughly then dispense into the PCR tube.
5. Then we will add the template RNA to the PCR tube, seal and shake the tube.
6. The thermal cycling protocol will be programmed according to the steps which are summarized in the following table:

The first cycle	The second cycle	The next 35-40 cycles	
Reverse Transcription Reaction	Polymerase Activation and cDNA Denaturation	Amplification	
		Denaturation	Annealing/Extension
10 min. at 50ºC	1 min. at 95ºC	15 sec. at 95ºC	30 sec. at 60ºC

7. We will load the PCR tube onto RT-PCR instrument and allow the RT-qPCR to run.
8. E.R. and S.B. will then analyze the RT-PCR products by Melt-Curve Analysis using instrument default setting.

4. 4. Sample size:

No sample size could be applied on cell line experiments as we generate millions of naturally randomized cells.

*Qiagen Rotor-Gene®, USA.

4. 5. Randomization:

Randomization (with sequence generation and allocation concealment mechanism) is not applied on cell line experiments, as we generate millions of naturally randomized cells, where the amount of cells in each plate will be standardized and each of the 96-well plates will contain the same number of cells (about 25,000-30,000 cells/well).

4. 6. Blinding and assessment:

Blinding is not applied on cell line experiments.

4. 7. Statistical methodology:

Data will be analyzed using IBM SPSS advanced statistics (Statistical Package for Social Sciences), version 21 (SPSS Inc., Chicago, IL). Numerical data will be described as mean and standard deviation or median and range. Categorical data will be described as numbers and percentages.

5. Results:

5. 1. Outcome(s) (O):

- **Primary O:** Apoptosis (Gene expression of Caspase 3).
- **Secondary O:** Cytotoxicity/Cell viability.

5. 2. Testing the samples:

1) After treatment incubation period, E.R. and S.B. will measure the color intensity to estimate cell viability using an ELISA Reader.
2) After treatment incubation period, E.R. and S.B. will detect the fold-changes of Caspase 3 gene expression to estimate apoptosis using Rotor-Gene RT-PCR System.

6. Protocol amendments:

Any adjustments to the protocol which might affect the conduct of the study, patient's potential benefit or might impact patient safety, including any change in the design of the study, study objectives, methodology, sample sizes, or significant modification in the administrative aspects will require amendment to the protocol formally. Such amendments should be agreed upon by the Council of Oral & Maxillofacial Pathology department.

7. Search strategy:

7. 1. The PRISMA Statement

7. 1. 1. Structured summary:

Objective: To estimate the inhibitory effect of luteolin and nano-luteolin as a promoter of cancer cell death in tongue squamous cell carcinoma.

Data Sources: In vitro and in vivo articles using PubMed and Cochrane databases. An additional study was identified by contacting the Egyptian Universities Libraries Consortium website (www.eulc.edu.eg). Search terms included OSCC, TSCC, HNSCC, luteolin and nano-luteolin.

Study Selection: Only in vivo and in vitro studies of luteolin (luteolin 5,7,3',4'-tetrahydroxy-flavone) activity regarding apoptosis, cytotoxicity/cell viability on cell lines of oral squamous cell carcinoma (OSCC), tongue squamous cell carcinoma (TSCC) or head and neck squamous cell carcinoma (HNSCC) were included.

Data Extraction: Extraction of articles by the author using predefined data fields.

Data Synthesis: Out of all pooled analyses three articles met the inclusion criteria. All trials used luteolin. Apoptosis, cytotoxicity/cell viability were observed to be increased with different doses of luteolin (10 μM for 12 hours) (20-100 μM for 24, 48, 72 hours) (50 μM for 12, 24 hours), separately. Luteolin of 10 μM selectively decreased cell viability and

increased cytotoxicity and apoptosis on tongue squamous cell carcinoma cell line (SCC-25) while increased cell viability on human keratinocyte-derived cell line (HaCaT) versus standard-of-care. When luteolin was used in a dose-dependent and time-dependent pattern the apoptotic cells increased (71%) meanwhile the cell viability decreased (29%) and induced apoptosis selectively on tongue squamous cell carcinoma cell line (SCC-4) and oral cancer cell line (OC-2) versus control. Application of 50 μM of luteolin increased apoptosis of laryngeal HNSCC cell line (Hep-2 cells) versus control.

Limitations: Data of results of the included studies is insufficient.

Conclusions: Luteolin between 10 to 100 μM appears to decrease viability and increase cytotoxicity and apoptosis selectively to OSCC, TSCC and HNSCC cell lines.

7. 1. 2. Eligibility criteria:

7. 1. 2. 1. Inclusion criteria:

- Squamous cell carcinoma cell lines (OSCC, TSCC and HNSCC).
- Luteolin 5,7,3',4'-tetrahydroxy-flavone (MeSH term).
- Application of luteolin as a chemotherapeutic drug.
- Detection of luteolin activity in apoptosis or cytotoxicity/cell viability.

7. 1. 2. 2. Exclusion criteria:

- Any cancer cell line other than OSCC, TSCC and HNSCC cell lines.
- Luteolin glycosides.
- Any use of luteolin other than chemotherapy.
- Detection of luteolin activities other than apoptosis or cytotoxicity/cell viability.

7. 1. 2. 3. Report characteristics:

- No year limitation.
- No language limitation.

7. 1. 3. Information sources:

Studies were identified by searching electronic databases and scanning reference lists of articles. No limits were applied for language or date of search. This search was first started on 6 May 2017 applied to PubMed and Cochrane electronic databases. The last search was run on 15 June 2017. In addition, the hand search was done through: searching in the department of oral and maxillofacial pathology of Cairo University, checking reference lists of the included articles, searching in the Egyptian Universities Libraries Consortium website www.eulc.edu.eg as well as contacting the authors of the included articles to acquire missing results and there is no reply till now. A limited update literature search was performed from the date of inception till now.

7. 1. 4. Synonyms of PICO items:

The following search terms and synonyms were used to search all databases:

Index Terms	
PICO items	**Synonyms**
Tongue squamous cell carcinoma cell line	Tongue squamous cell carcinom* Tongue squamous cell tumour* Tongue squamous cell tumor* Tongue squamous cell neoplasm* Tongue squamous cell malignan* Tongue squamous cell cancer* TSCC Tongue SCC Oral tongue squamous cell carcinom* Oral squamous cell carcinom* Oral squamous cell tumour* Oral squamous cell tumor* Oral squamous cell neoplasm* Oral squamous cell malignan* Oral squamous cell cancer*

	Oral cavity squamous cell carcinom* OSCC OSCC cell OSCC cell line OSCC cancer Head and neck squamous cell carcinom* Head and neck squamous cell tumour* Head and neck squamous cell tumor* Head and neck squamous cell neoplasm* Head and neck squamous cell malignan* Head and neck squamous cell cancer* HNSCC SCCHN
Luteolin	Luteolin
Nano-luteolin	Nano-luteolin Nano luteolin Nanoluteolin Luteolin nanoparticles Luteolin nanotechnology

7. 1. 5. Databases:

Search Strategy in PubMed and Cochrane, was run on 6 May 2017 till now:

	Index Term	PubMed	Cochrane
#1	"Tongue Neoplasms" [MeSH]	9190	56
#2	tongue squamous cell carcinom*	781	95
#3	tongue squamous cell tumour*	1	9
#4	tongue squamous cell tumor*	7	53
#5	tongue squamous cell neoplasm*	36	54
#6	tongue squamous cell malignan*	3	5
#7	tongue squamous cell cancer*	16	70
#8	tscc	289	6
#9	tongue scc	827	11
#10	oral tongue squamous cell carcinom*	202	54
#11	oral squamous cell carcinom*	7720	816
#12	oral squamous cell tumour*	8	98
#13	oral squamous cell tumor*	42	324
#14	oral squamous cell neoplasm*	228	504
#15	oral squamous cell malignan*	10	75

#16	oral squamous cell cancer*	112	615
#17	oral cavity squamous cell carcinom*	420	278
#18	oscc	4492	51
#19	oscc cell	4448	51
#20	oscc cell line	1066	4
#21	oscc cancer	4171	32
#22	head and neck squamous cell carcinom*	7849	1923
#23	head and neck squamous cell tumour*	9	166
#24	head and neck squamous cell tumor*	6	616
#25	head and neck squamous cell neoplasm*	377	1029
#26	head and neck squamous cell malignan*	3	84
#27	head and neck squamous cell cancer*	497	1178
#28	hnscc	5763	303
#29	scchn	1355	277
#30	#1 or #2 or #3 or #4 or #5 or #6 or #7 or #8 or #9 or #10 or #11 or #12 or #13 or #14 or #15 or #16 or #17 or #18 or #19 or #20 or #21 or #22 or #23 or #24 or #25 or #26 or #27 or #28 or #29 (tongue squamous cell carcinom*) OR (tongue squamous cell tumour*) OR (tongue squamous cell tumor*) OR (tongue squamous cell neoplasm*) OR (tongue squamous cell malignan*) OR (tongue squamous cell cancer*) OR (tscc) OR (tongue scc) OR (oral tongue squamous cell carcinom*) OR (oral squamous cell carcinom*) OR (oral squamous cell tumour*) OR (oral squamous cell tumor*) OR (oral squamous cell neoplasm*) OR (oral squamous cell malignan*) OR (oral squamous cell cancer*) OR (oral cavity squamous cell carcinom*) OR (oscc) OR (oscc cell) OR (oscc cell line) OR (oscc cancer) OR (head and neck squamous cell carcinom*) OR (head and neck squamous cell tumour*) OR (head and neck squamous cell tumor*) OR (head and neck squamous cell neoplasm*) OR (head and neck squamous cell malignan*) OR (head and neck squamous cell cancer*) OR (hnscc) OR (scchn)	20055	2633
#31	#1 or #30 ("Tongue Neoplasms" [Mesh]) OR (tongue squamous cell carcinom*) OR (tongue squamous cell tumour*) OR (tongue squamous cell tumor*) OR (tongue squamous cell neoplasm*) OR (tongue squamous cell malignan*) OR (tongue squamous cell cancer*) OR (tscc) OR (tongue scc) OR (oral tongue squamous cell carcinom*) OR (oral squamous cell carcinom*) OR (oral squamous cell tumour*) OR (oral squamous cell tumor*) OR (oral squamous cell neoplasm*) OR (oral squamous cell malignan*) OR (oral squamous cell cancer*) OR (oral cavity squamous cell carcinom*) OR (oscc) OR (oscc cell) OR (oscc cell line) OR (oscc cancer) OR (head and neck squamous cell carcinom*) OR (head and neck squamous cell tumour*) OR (head and neck squamous cell tumor*) OR (head and neck squamous cell neoplasm*) OR (head and neck squamous cell malignan*) OR (head and neck squamous cell cancer*) OR (hnscc) OR (scchn)	27867	2660
#32	"Luteolin"[Mesh]	1263	2

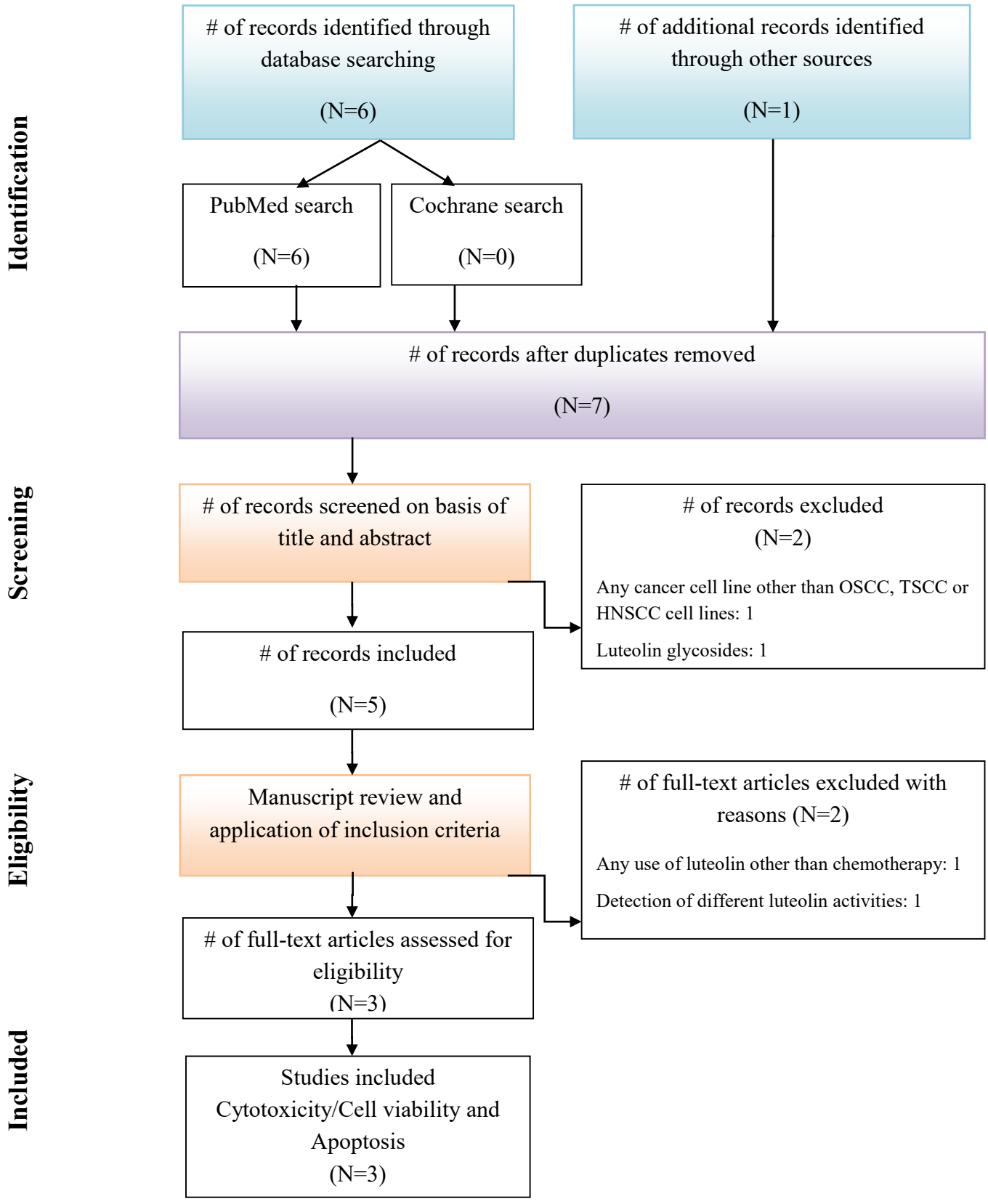
#33	Luteolin	3224	18
#34	#32 or #33 ("Luteolin"[Mesh]) or (Luteolin)	3224	18
#35	nano-luteolin	1	0
#36	nano luteolin	11	0
#37	Nanoluteolin	0	0
#38	luteolin nanoparticles	19	0
#39	luteolin nanotechnology	8	0
#40	#35 or #36 or #37 or #38 or #39 (nano-luteolin) OR (nano luteolin) OR (nanoluteolin) OR (luteolin nanoparticles) OR (luteolin nanotechnology)	31	0
#41	#34 and #40 ("Luteolin"[Mesh]) OR (luteolin) AND (nano-luteolin) OR (nano luteolin) OR (nanoluteolin) OR (luteolin nanoparticles) OR (luteolin nanotechnology)	31	0
#42	#31 and #34 ("Tongue Neoplasms" [Mesh]) OR (tongue squamous cell carcinom*) OR (tongue squamous cell tumour*) OR (tongue squamous cell tumor*) OR (tongue squamous cell neoplasm*) OR (tongue squamous cell malignan*) OR (tongue squamous cell cancer*) OR (tscc) OR (tongue scc) OR (oral tongue squamous cell carcinom*) OR (oral squamous cell carcinom*) OR (oral squamous cell tumour*) OR (oral squamous cell tumor*) OR (oral squamous cell neoplasm*) OR (oral squamous cell malignan*) OR (oral squamous cell cancer*) OR (oral cavity squamous cell carcinom*) OR (oscc) OR (oscc cell) OR (oscc cell line) OR (oscc cancer) OR (head and neck squamous cell carcinom*) OR (head and neck squamous cell tumour*) OR (head and neck squamous cell tumor*) OR (head and neck squamous cell neoplasm*) OR (head and neck squamous cell malignan*) OR (head and neck squamous cell cancer*) OR (hnscc) OR (scchn) AND ("Luteolin"[Mesh]) or (Luteolin)	6	0
#43	#31 and #40 ("Tongue Neoplasms" [Mesh]) OR (tongue squamous cell carcinom*) OR (tongue squamous cell tumour*) OR (tongue squamous cell tumor*) OR (tongue squamous cell neoplasm*) OR (tongue squamous cell malignan*) OR (tongue squamous cell cancer*) OR (tscc) OR (tongue scc) OR (oral tongue squamous cell carcinom*) OR (oral squamous cell carcinom*) OR (oral squamous cell tumour*) OR (oral squamous cell tumor*) OR (oral squamous cell neoplasm*) OR (oral squamous cell malignan*) OR (oral squamous cell cancer*) OR (oral cavity squamous cell carcinom*) OR (oscc) OR (oscc cell) OR (oscc cell line) OR (oscc cancer) OR (head and neck squamous cell carcinom*) OR (head and neck squamous cell tumour*) OR (head and neck squamous cell tumor*) OR (head and neck squamous cell neoplasm*) OR (head and neck squamous cell malignan*) OR (head and neck squamous cell cancer*) OR (hnscc) OR (scchn) AND (nano-luteolin) OR (nano luteolin) OR (nanoluteolin) OR (luteolin nanoparticles) OR (luteolin nanotechnology)	1	0

#44	#31 and #34 and #40 ("Tongue Neoplasms" [Mesh]) OR (tongue squamous cell carcinom*) OR (tongue squamous cell tumour*) OR (tongue squamous cell tumor*) OR (tongue squamous cell neoplasm*) OR (tongue squamous cell malignan*) OR (tongue squamous cell cancer*) OR (tscc) OR (tongue scc) OR (oral tongue squamous cell carcinom*) OR (oral squamous cell carcinom*) OR (oral squamous cell tumour*) OR (oral squamous cell tumor*) OR (oral squamous cell neoplasm*) OR (oral squamous cell malignan*) OR (oral squamous cell cancer*) OR (oral cavity squamous cell carcinom*) OR (oscc) OR (oscc cell) OR (oscc cell line) OR (oscc cancer) OR (head and neck squamous cell carcinom*) OR (head and neck squamous cell tumour*) OR (head and neck squamous cell tumor*) OR (head and neck squamous cell neoplasm*) OR (head and neck squamous cell malignan*) OR (head and neck squamous cell cancer*) OR (hnscc) OR (scchn) AND ("Luteolin"[Mesh]) or (Luteolin) AND (nano-luteolin) OR (nano luteolin) OR (nanoluteolin) OR (luteolin nanoparticles) OR (luteolin nanotechnology)	1	0

7. 1. 6. Study selection:

The search started with 7 articles. The retrieved records were screened on the basis of title and abstract and sequentially two out of them were excluded according to the exclusion criteria. (The used cell lines were human breast cancer adenocarcinoma, human colon colorectal carcinoma and human hepatic carcinoma cell lines, not OSCC, TSCC or HNSCC cell lines (n=1), and luteolin glycosides were used with different molecular formula than luteolin 5,7,3',4'-tetrahydroxy-flavone (n=1)). Manuscript review and application of inclusion criteria of the remaining five articles with full text publication were done. This resulted in Reasonable exclusion of two additional articles. (Luteolin was used in chemoprevention not as a chemotherapeutic drug (n=1), and luteolin was used to detect different outcomes other than apoptosis or cytotoxicity/cell viability (n=1)). The three remaining full-text articles met the eligibility criteria and included to extract data out of them. For note, each stage of these steps was carried out only by the author.

PRISMA flow diagram of study selection:



7. 1. 7. Data collection process:

Information was extracted from each included study and refined accordingly. The authors of the included studies were contacted for information that is unclearly reported. However, there is no answer till now.

7. 1. 8. Data items:

Information were extracted from each included study on: (1) characteristics of trial population (including number of groups), and the trial's inclusion and exclusion criteria; (2) type of intervention (including type, dose, duration versus control, standard-of-care or versus no treatment), (3) type of outcome measure (including apoptosis and cytotoxicity/cell viability).

7. 2. Summary of finding tables (SOFT):

7. 2. 1. Demographics of included studies:

No. of study	Study 1st Author (Year/ country)	Name of journal	Title of the study	Type of samples	Control	Type of study	Source
1	K.C. Tjioe, France 2016	Nutrition And Cancer	Luteolin Impacts on the DNA Damage Pathway in Oral Squamous Cell Carcinoma	1- Tongue squamous cell carcinoma cell line (SCC-25 cells) 2- Human keratinocyte-derived cell line (HaCaT)	DMSO	In vitro	PubMed
2	S. F. Yang, Taiwan 2008	J Dent Res	Luteolin Induces Apoptosis in Oral Squamous Cancer Cells	1- Tongue squamous cell carcinoma cell line (SCC-4 cells) 2-Oral cancer cell line (OC-2 cells)	DMSO	In vitro and In vivo (Animal)	PubMed
3	H. Zhang, China 2014	Eur Arch Otorhinolaryngol	Luteolin induces apoptosis by activating Fas signaling pathway at the receptor level in laryngeal squamous cell line Hep.2 cells	- Laryngeal HNSCC cell line (Hep-2 cells)	DMSO	In vitro	PubMed

7. 2. 2. Material and Methods:

No. of study	Cell line types	Culture media	Drug	MTT assay						Crystal violet staining					Western blot					DAPI staining				Flowcytometric analysis				RT-PCR			
				Groups	Control	Standard-of-	Dose	Duration	Material	Groups	Control	Dose	Duration	Material	Control	Dose	Duration	Antibodies	Material	Control	Dose	Duration	Material	Dose	Duration	Protein expression	Material	Housekeeping	Primer	Antibodies	Material
1	1-SCC-25 2-HaCaT	DMEM/F12 10% FBS 1% penicillin and streptomycin 400ng/ml of hydro-cortisone 15mM N-2 HEPES	Luteolin	NA	DMSO	 CP AG1478	5, 10,20 μM 5, 10 mm 10 μM	72, 24, 48, 72 h	Colorimetric assay 1-(4,5-dimethyl-thiazol-2-yl)-3 5-diphenyl formazan Thiazolyl blue formazan 25ml of MTT reagent	6-well plate (25,000 cells/well)	DMSO	10 μM	72 h	Glutaral-dehyde 2%, PBS, Crystal violet 0.05%, Acetic acid 1%	Nil	10 μM	30 min	p-JNK p-EGFR p-AKT p-p38 p-ERK1/2 p-ATM p-ATR p- H2AX p-CHK2 p-BRCA1 ERK1/2 JNK1/2 ERK2 CHK2	TNT buffer, MicroBCA kit (30 μg/well) NuPAGE electrophoresis Nitrocellulose membranes	Not used				Not used				Not used			
2	1-SCC-4 2-OC-2	DMEM/F12 10% FBS 1% penicillin and streptomycin 400ng/ml of hydro-cortisone 2 mM glutamine	Luteolin	24-well plate	DMSO	 Paclita	5, 10 μM 0-100 μM 0.3	24, 48, 72 h 24 h 24, 48,	0.5 mg/mL MTT reagent, Blue formazan crystals	Not used					β-actin	0-100 μM	NA	Bcl-2 Bax Caspase9 Caspase 3 PARP	NA	Nil	0-100 μM	48 h	4',6-Diamidino-2-phenylindole (DAPI) (1 μg/ml)	0-100 μM	24 h	NA	NA	Not used			
3	Hep-2 cells	DMEM/F12 penicillin and streptomycin	Luteolin	NA	DMSO	No	20-100 μM	NA	3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)	Not used					β-actin	0, 30, 50 μM	6, 12, 24 h	anti-c-FLIPS/L anti-Fas per-oxidase-conjugated goat anti-rabbit 2ry antibody	SDS/PAGE (12 % w/v) Nitrocellulose membranes non-fat milk (5 %) TBST	DMSO	0, 30, 50 μM	24 h	4',6-Diamidino-2-phenylindole(DAPI)	50 uM	6, 12, 24 h	Caspase3, Caspase 8, Caspase 9	Annexin V-FITC Apoptosis Detection Kit Becton–Dickinson CELL Quest software	β-actin	8-12	c-FLIP Fas IgG peroxidase-conjugated 2ry antibody	Aliquots of PCR products agarose gel (1.5 %) electrophoresis ethidium bromide

7. 2. 3. Results:

No. of the study	Drug	Apoptosis										
		Antibodies				Duration					Dose	Effect
		p-JNK	Caspase-3	Caspase-9	cFLIP$_L$	6 h	12 h	24 h	48 h	72 h		
1	Luteolin	p-JNK	_	_	_	_	_	_	_	72 h	10 µM	0.34 fold
2	Luteolin	_	Caspase-3	_	_	_	_	_	48 h	_	60-100 µM	32 kDa
	Luteolin	_	_	Caspase-9	_	_	_	_	48 h	_	60-100 µM	47 kDa
3	Luteolin	_	_	_	cFLIP$_L$	6 h	_	_	_	_	30 µM	0.65 fold
	Luteolin	_	_	_	cFLIP$_L$	6 h	_	_	_	_	50 µM	0.40 fold
	Luteolin	_	_	_	cFLIP$_L$	_	12 h	_	_	_	30 µM	0.38 fold
	Luteolin	_	_	_	cFLIP$_L$	_	12 h	_	_	_	50 µM	0.19 fold
	Luteolin	_	_	_	cFLIP$_L$	_	_	24 h	_	_	30 µM	0.15 fold
	Luteolin	_	_	_	cFLIP$_L$	_	_	24 h	_	_	50 µM	0.13 fold

No. of the study	Drug	Cytotoxicity/Cell viability (%)						
		Duration					Dose	Effect
		6 h	12 h	24 h	48 h	72 h		
1	Luteolin	_	_	_	_	72 h	20 µM	83.33%
2	Luteolin	_	_	24 h	_	_	60 µM	0.12 *10^4 %
	Luteolin	_	_	24 h	_	_	80 µM	0.15*10^4 %
	Luteolin	_	_	24 h	_	_	100 µM	0.17*10^4 %
	Luteolin	_	_	_	48 h	_	60 µM	0.63*10^4 %
	Luteolin	_	_	_	48 h	_	80 µM	1.08*10^4 %
	Luteolin	_	_	_	48 h		100 µM	1.60*10^4 %
	Luteolin	_	_	_	_	72 h	60 µM	1.17*10^4 %
	Luteolin	_	_	_	_	72 h	80 µM	1.75*10^4 %
	Luteolin	_	_	_	_	72 h	100 µM	2.35*10^4 %
3	Luteolin	6 h	_	_	_	_	50 µM	4.33 %
	Luteolin	_	12 h	_	_	_	50 µM	4.66 %
	Luteolin	_	_	24 h	_	_	50 µM	16.00 %

7. 2. 4. Table of excluded studies:

No. of study	Study 1st Author (Country, Year)	Journal	Title of study	Reason of exclusions	Source
1	L. Gohar, Egypt 2016	International Journal of Pharmacognosy and Phytochemistry	Phytochemical and Biological Studies of Certain Plants Possessing Cytotoxic Activity	Human breast cancer adenocarcinoma, Human colon colorectal carcinoma and Human hepatic carcinoma cell lines. **NOT** OSCC, TSCC or HNSCC cell lines	www.eulc.edu.eg
2	T. Matsuta, Japan 2011	In Vivo	Biological Activity of Luteolin Glycosides and Tricin from Sasa senanensis Rehder	Application of Luteolin glycosides. **NOT** the molecular formula Luteolin 5,7,3',4'-tetrahydroxy-flavone	PubMed
3	D. Majumdar, USA 2014	Cancer Prev Res	Luteolin nanoparticle in chemoprevention – in vitro and in vivo anticancer activity	Application of luteolin in chemopevention. **NOT** as a chemotherapeutic drug	PubMed
4	R. B. Selvi, India 2015	Oncotarget	Inhibition of p300 lysine acetyltransferase activity by luteolin reduces tumor growth in head and neck squamous cell carcinoma (HNSCC) xenograft mouse model	Detection of p300 KAT/p300 acetyl-transferase and cancer progression activity. **NOT** detecting apoptosis or cytotoxicity/cell viability.	PubMed

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