Apoptotic and Anti-Proliferative Effects of Licorice Extract (Licochalcone A) and Paclitaxel Chemotherapy on Human Oral Squamous Cell Carcinoma Cell Line

(In Vitro Study)

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Presented by

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List of Contents:

Administrative Information:

1- Title:

Apoptotic and Anti-Proliferative Effects of Licorice Extract (Licochalcone A) and Paclitaxel Chemotherapy on Human Oral Squamous Cell Carcinoma Cell Line.

2- Protocol Version:

Version (1)

3- Roles and Responsibilities:

The Principle Supervisor:

Prof.Dr. SawsanNaguib Abdel-Bari, Professor of Oral and Maxillofacial Pathology, Faculty of Dentistry, Cairo University

The Assistant Supervisor:

Ass. Prof. Dr. Nermine Raouf Amin, Ass.Professor of Oral and Maxillofacial Pathology, Faculty of Dentistry, Cairo University

The Principle Investigator:

Mohamed Osama Mostafa, Assistant Lecturer, Oral Pathology, Faculty of Dentistry, Beni-Suef University.

Investigator of cell viability assay and fluorescence microscopy:

Dr. L. Rashed, Professor of Medical Biochemistry, Faculty of Medicine, Cairo University.

Investigator of cell culturing technique:

Dr. E.Rashwan, Head of the Confirmatory Diagnostic Unit (VACSERA-EGYPT).

Steering Committees:

1 -Oral & Maxillofacial Pathology Department Board, Faculty of Dentistry, Cairo University.

2 -Research Plan Committee, Faculty of Dentistry, Cairo University.

3 -Evidence Based Committee, Faculty of Dentistry, Cairo University.

4 -Ethics Committee, Faculty of Dentistry, Cairo University.

5 -Higher Education and Research Committee.

6- 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.

Background and Scientific Rationale:

Oral squamous cell carcinoma (OSCC) is considered the most common human body malignant tumor. It has poor prognosis regarding its distant lymph node metastasis and local destruction which lead to low survival rate **(Pereira et al., 2007).**

Paclitaxel is a chemotherapeutic drug of the taxols group that is used in the treatment of lung, ovarian and prostate cancer. It is also used to treat breast, head and neck cancer as well as sarcoma and leukemia. Recent studies and researches explored that Paclitaxel has an important role in mitotic arrest activation and induction, which subsequently lead to cell death **(Weaver, 2014).**

Regarding the effect of Paclitaxel on head and neck cancer cell lines, **Maushagen et al. (2016)** noticed a time-dependent decrease in the number of viable cancer cells (24-48 hours) upon a fixed dose of Paclitaxel (10μM).

Inhibition of microtubule union and assembly by Paclitaxel induces apoptosis and cell cycle arrest in the G2/M-phase and this leads to inhibition of cell replication and cancer growth **(Rowinsky and Donehower, 1995; Zhang et al., 2014).**

In another study on gastric cancer, Paclitaxel induced a higher inhibitory effect on tumor cell proliferation than other types of chemotherapy (90.7% for Paclitaxel, 72.6%for Oxaliplatin and 67.7% for Epirubicin **(Zhang et al., 2013)**.

Regarding the apoptotic effect, a study of multiple concentrations of Paclitaxel on breast cancer cells has explored high levels of apoptotic cells (80 % – 87%) at doses of 500nM and 700nM, respectively for 48 hours **(Armat et al., 2016).**

The conventional treatment strategies such as surgery, radiotherapy and chemotherapy come with low levels of expectations regarding prognosis and survival rate **(Jemal et al., 2006)**. On the other hand, serious side effects of chemotherapy such as renal toxicity, gastrointestinal upset and bone marrow suppression remain hazards which cannot be avoided **(Carr et al., 2008)**.Thus, there is a great need to discover new agents and

strategies for treating OSCC or even enhancing the effect of chemotherapy in order to reduce the dose given.

Complementary alternative medicine (CAM) is an additional modality for treating various types of cancers **(Brauer et al., 2010; Lewith et al., 2010)**. Traditional medicinal plants and their derivatives have been widely used as effective therapeutic agents for OSCC.

Licorice extract, Licochalcone A, is one of many natural extracts of Glycyrrhiza inflata plant. It has been used in treatment of inflammation **(Shibata et al.,1991; Kwon et al.,2008)**, infections **(Chen et al., 2001; Ziegler et al., 2004)**and cancer **(Rafi et al., 2002)**.

Regarding its antitumor effect, Licochalcone A reveals interesting results in cell cycle arrest and apoptosis in cancer cells such as prostate **(Yo et al., 2009),** bladder **(Yuan et al., 2013)**, colon **(Lee et al., 2008)** and gastric cancer **(Xiao et al., 2011)**.

Aim and objectives of the study

1- To study the effect of Licochalcone A on cell viability, cell proliferation and apoptosis compared with the effect of Paclitaxel chemotherapy in cultured OSCC cell line (SCC-15).

2- To determine the role of Licochalcone A in enhancing the ant-proliferative and apoptosis inducing effects of Paclitaxel in an attempt to reduce the chemotherapeutic dose in the clinical practice.

PICO:

Pobulation: Cultured human oral squamous cell carcinoma cell line (SCC-15).

Intervention1: Application of Licochalcone A alone on the cultured (SCC-15) cell line.

Intervention2: Application of Paclitaxel chemotherapy alone on the cultured (SCC-15) cell line.

Intervention3: Application of Licochalcone A and Paclitaxel chemotherapy together on the cultured (SCC-15) cell line.

Control: Cultured (SCC-15) cell line without treatment.

Outcome: Assessment of cell viability, proliferation and apoptosis.

	Outcome	***Measuring Device/technique***	***Measuring Unit***
Primary	Cell viability	MTT assay and Eliza reader **(Meerloo JV et al., 2011)**	Stained Viable Cell counts
Secondary	Proliferation	IPO-38 immunohistochemical proliferation marker via Image analyzer computer system. **(Prasad K., 2011)**	Area % of immuno-stain.
Tertiary	Apoptosis	Fluorescent microscopy for Annexin-V binding protein **(Rieger AM et al., 2011)**	Stained apoptotic Cell counts

Research Question:

Does Licochalcone A enhance the anti-proliferative and apoptosis inducing effects of Paclitaxel chemotherapy on cultured oral squamous cell carcinoma cell line?

Material and Methods

A- Intervention:

Squamous cell carcinoma cell line (SCC-15) cultured with Licorice extract (Licochalcone A) and chemotherapeutic drug (Paclitaxel).

B- Materials:

1. Squamous cell carcinoma cell line (SCC-15) from Veterinary Serum & Vaccine Research Institute (VACSERA) (51 Wezaret EL Zeraa Street, Agouza, Giza, Egypt).
2. Licorice extract (Licochalcone A) obtained from Sigma-Aldrich company (Eschenstrasse 5,82024, Taufkirchen, Germany).
3. Paclitaxel chemotherapeutic reagent from The Egyptian Pharmaceutical Trading Company (1353 Nile Kornish, Shobra, Cairo, Egypt).
4. Annexin V-FITC Apoptosis Staining / Detection Kit from abcam company, KEMET Medical (14a El Emam Aly St Off Orouba St - Ismailia Square, Heliopolis, 11341).
5. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) solution from Sigma-Aldrich company (Eschenstrasse 5,82024, Taufkirchen, Germany).
6. IPO-38 proliferation marker from US Biological life science company (4 Technology Way Salem, MA 01970, Boston, United States).

C- Methods:

Squamous cell carcinoma cell line will be cultured alone, with Licochalcone A,with Paclitaxel chemotherapeutic agent and with Licochalcone A and Paclitaxel together.

D- Collecting samples:

Squamous cell carcinoma cell lines will be purchased from Veterinary Serum & Vaccine Research Institute(VACSERA).

- *Inclusion criteria:*
  Human squamous cell carcinoma cell line

- *Exclusion criteria:*
  1.Animal cell lines
  2. Cell lines other than squamous cell carcinoma cell lines.

E- Cell culture (Sample) Preparation:

- The stored cell line will be removed from the liquid nitrogen container and thawed in a water bath at 37ºC.
- Then cells will be cultured in modified Eagle's medium(MEM) supplemented with heat inactivated fetal bovine serum (FBS), sodium bicarbonate and 2% streptomycin penicillin in flasks at 37ºC in a 5% CO_2 incubator.
- The culture flasks were examined under the inverted phase contrast microscope to assure viability and sterility.

Sub-culturing:

- Trypsin will be added to the flask and turned over for 15 seconds- 2 minutes.
- (MEM) medium then will be added to neutralize the action of trypsin.
- Cultured cells will be re-suspended in newly fresh medium and centrifuged at 2000 rpm for 10 minutes to obtain a cell pellet.
- Cell pellet will then be dispersed and re-suspended in fresh complete culture media and diluted to the appropriate seeding concentration in the culture flask.

- Cells will be sub-cultured in a 96-well plate and incubated at 37ºC in the 5% CO2 incubator.
- Then well – plates will be treated with two doses of Licohalcone A (3mg, 6mg) and Paclitaxel chemotherapy (8mg, 16mg) for 24, 48 and 72 hours.
- The control group will be left without treatment.
- ***Study Design Table:***

Intervention / Groups	**Dose (mg)**		**Duration of Treatment (hours)**		
Control group	Untreated		No duration		
LicochalconeA group	3 mg	6 mg	24	48	72
Paclitaxel group	8 mg	16 mg	24	48	72
Licochalcone A and Paclitaxel group	3 mg + 8 mg	6 mg + 16 mg	24	48	72

F- <u>Paraffin embedded cell line pellet preparation:</u>

- Cell suspension will be spanned at 1800 rpm for 10 minutes at room temperature.
- Supernatant decanted
- 5 ml of 70% ethanol is added for 30 minutes and vortexed mildly.
- Suspension is spanned at 1800 rpm for 10 minutes at room temperature.
- Supernatant decanted
- 5 ml of 100% ethanol will be added for 30 minutes and vortexed mildly. Optional: Overnight incubation in 100% ethanol at 4°C makes a very solid pellet
- Suspension will be spanned 1800 rpm for 10 minutes at room temperature.
- Supernatant decanted.
- Using a clean wooden applicator stick, cell pellet will be pulled out carefully of the tube and into a cassette lined with black biopsy filter paper.
- The cassette will be placed in a tissue processor.
- The pellet sample is processed and embedded in paraffin within 24 hours of preparation.

G- <u>Testing the samples:</u>

1- Cell viability will be measured by the MTT assay by adding MTT solution to every treated well plate then incubated with 5 % CO_2 at 37 °C. Records of viable cells will be obtained by the ELISA reader.

2- Area percent of proliferation marker IPO-38 immunohistochemical staining will be measured by image analyzer computer system. IPO-38 will be applied on slides sectioned from paraffin embedded cell line pellet

3- Apoptosis will be measured by Annexin-V binding protein to apoptotic cells in a multi well fluorescence plate reader. Fluorescence of each well will be recorded and images will be taken under the fluorescent microscope.

H- Outcome:

- Primary: Cell viability by MTT assay measured by the ELIZA reader
- Secondary: Cell proliferation by measuring the area percent of immunohistochemical staining of proliferation marker IPO-38.
- Tertiary: percentage of apoptotic cells to the total count of all viable cells via Annexin V binding protein measured by fluorescence microscopy.

I- Sample size:

Based on **(Cumming et al., 2007)** and **(Lazic SE., 2010)**, when cell lines are used, there isn't any biological replication, only technical replication, and it is important to have this replication at the right level in order to have valid inferences. For every single cell line intervention, 6 times applications of drug dose in 6 well plates will be sufficient for every outcome.

J- Randomization:

Randomization of the cell line will not be done as the count of cells in each well plate is standardized and contains the same number of cells **(Amiralaet al., 2013).**

K- 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.

L- Protocol amendments:

Any modifications to the protocol which may affect the progress of the study, including changes of study objectives, study design, sample sizes or study procedures will require a formal amendment to the protocol. Such amendment will be agreed upon by the Council of Oral pathology department.

Search strategy:

1.Title:

Apoptotic and Anti Proliferative Effects of Licorice Extract (Licochalcone A) and Paclitaxel Chemotherapy on Human Oral Squamous Cell Carcinoma Cell Line.

2. Structured Summary:

Objective: To study the effects of Licochalcone A on cell proliferation and apoptosis in cultured OSCC cell lines.

Data Sources: In-vitro study articles in PubMed and Cochran data bases. In addition, using of Egyptian universities libraries web site: www.eulc.edu.eg. Several search items or terms were used as: Licochalcone , licorice extract , OSCC and Paclitaxel.

Study Selection: the selected articles include In-vitro studies only that discuss the uses and activities of licorice extract (Licochalcone) in treating any type of head and neck squamous cell carcinoma cell lines by apoptotic induction or cytotoxic effect of cancer cells and to compare that with similar actions of traditional chemotherapeutics.

Data Extraction: achieved according to the study selection criteria from predefines data bases.

Data Synthesis: after searching and date analysis, 4 articles were obtained and they met the selection criteria of data search. 3 studies of them reveal that licorice extract (Licochalcone) has an apoptotic effect on different types of OSCC cell lines by inducing cytotoxicity to the cancer cells and subsequent reduction in its viability. By using different successive doses of Liochalcone A, (25, 50 ,100 ,125 ,200 µg/mL or µM) , the apoptotic effect increase on time dependent manner (12, 24 ,48

hours), with clear decrease in viable cancer cells (78%, 80%, 48% and19%) respectively . the last fourth article explain the apoptotic effect of paclitaxel chemotherapy, and it will be used as a reference in our comparison between Licochalcone A extract and conventional chemotherapy.

Limitations: limited included studies with insufficient data.

Conclusions: on higher concentrations, Licochalcone A appear to be effective apoptotic and cytotoxic natural extract for OSCC cell lines.

Funding: self – funded.

3: Rationale:

Oral squamous cell carcinoma (OSCC) are considered as a one of the most malignant cancers. It has prognosis due to its distant metastasis and obvious destruction which lead to low survival rate.

The conventional treatment modalities such as surgery, radiotherapy and chemotherapy are only the suitable strategies until now. We cannot ignore the serious side effects of chemotherapy such as gastrointestinal upset, bone marrow suppression which cannot be overcome. For all these complications, there is a great need and demand to discover a new agents and strategies for treating OSCC.

Licorice extract, especially Licochalcone A, is one of many natural extracts that have used as a traditional in treatment of inflammation, microbial infections. Regarding antitumor effect, Licochalcone A reveals interesting results in programmed cell death and apoptosis in cancer cells such as prostate**,** bladder, colon and gastric cancer.

4.Objectives:

1- To study the effects of Licochalcone A on cell proliferation and apoptosis in cultured OSCC cell lines trying to support the basis for possible potential application in the clinical practice of OSCC treatment.
2- To determine and distinguish between different effects of natural extract (Licochalcone A) and conventional chemotherapy (taxols).

5: Protocol and Registration:

6: Eligibility criteria:

Type of the study : In-vitro study of the therapeutic effect of natural extract (Licochalcone A) on the oral squamous cell carcinoma cell lines and comparing it with the conventional paclitaxel chemotherapy.

Type of participants: cultured oral squamous cell carcinoma cell line. Any type other than head and neck squamous cell carcinoma and oral squamous cell carcinoma will be excluded.

Type of intervention 1: Application of Licochalcone A.
Type of intervention 2: Application of Paclitaxel chemotherapy.
Type of intervention 3: Application of Licochalcone A with Paclitaxel chemotherapy
Type of control: Cultured cells without intervention.
Type of outcome measures:

- Apoptosis: gene expression of annexin -V
- Proliferation: by cell viability and immuonexpression of IPO38 marker.

Inclusion criteria:

1- All head and neck squamous cell carcinoma cell lines
2- Licochalcone extracts
3- Taxols group of chemotherapy
4- English language

Exclusion criteria:

1- Animal experimental studies
2- Any cancer cell line other than squamous cell carcinoma cell lines.
3- Any chemotherapeutics other than Taxols group.
4- Non-English studies.
5- Systematic review

7: Information sources:

The search done through electronic data bases: PubMed and Cochrane data bases according to the inclusion and exclusion criteria, the date of search was **10/1/2017.** In addition to that, hand search was done by searching in the library of the faculty of oral and dental medicine, Cairo university. Also, searching using the website of the Egyptian Universities Libraries Consortium www.eulc.edu.eg to figure out any related articles to this study.

8: Search:

	Index Term (synonyms)	PubMed	Cochrane
#1	Oral squamous cell carcinoma	1624	5
#2	Head and neck squamous cell carcinoma	2139	4
#3	Oral cancer	6002	36
#4	HNSCC	654	63
#5	Licochalcone A	17	3
#6	licorice	106	13
#7	paclitaxel	236	4
#8	Taxol	239	2
#9	(oral squamous cell carcinoma) or (head and neck squamous cell carcinoma) or (oral cancer) or (HNSCC)	7445	33
#10	(Licochalcone A) or (licorice)	110	15
#11	(Paclitaxel) or (taxol)	240	4
#12	(oral squamous cell carcinoma) or (head and neck squamous cell carcinoma) or (oral cancer) or (HNSCC) AND (Licochalcone A) or (licorice)	91	39
#13	(oral squamous cell carcinoma) or (head and neck squamous cell carcinoma) or (oral cancer) or (HNSCC) AND (paclitaxel) or (taxol)	150	39
#14	(oral squamous cell carcinoma) or (head and neck squamous cell carcinoma) or (oral cancer) or (HNSCC) AND (Licochalcone A) or (licorice) OR (oral squamous cell carcinoma) or (head and neck squamous cell carcinoma) or (oral cancer) or (HNSCC) AND (paclitaxel) or (taxol)	241	39

(oral cancer) or (HNSCC) AND (paclitaxel) or (taxol)) AND full text[sb] AND ("2016/09/30"[PDat] : "2016/12/27"[PDat]) AND Humans[Mesh])) AND (((oral squamous cell carcinoma) or (head and neck squamous cell carcinoma) or (oral cancer) or (HNSCC) AND (Licochalcone A) or (licorice)) AND full text[sb] AND ("2015/05/20"[PDat] : "2017/01/01"[PDat]) AND Humans[Mesh])) AND (((Paclitaxel) or (taxol)) AND full text[sb] AND ("2016/07/30"[PDat] : "2017/01/01"[PDat]) AND Humans[Mesh])

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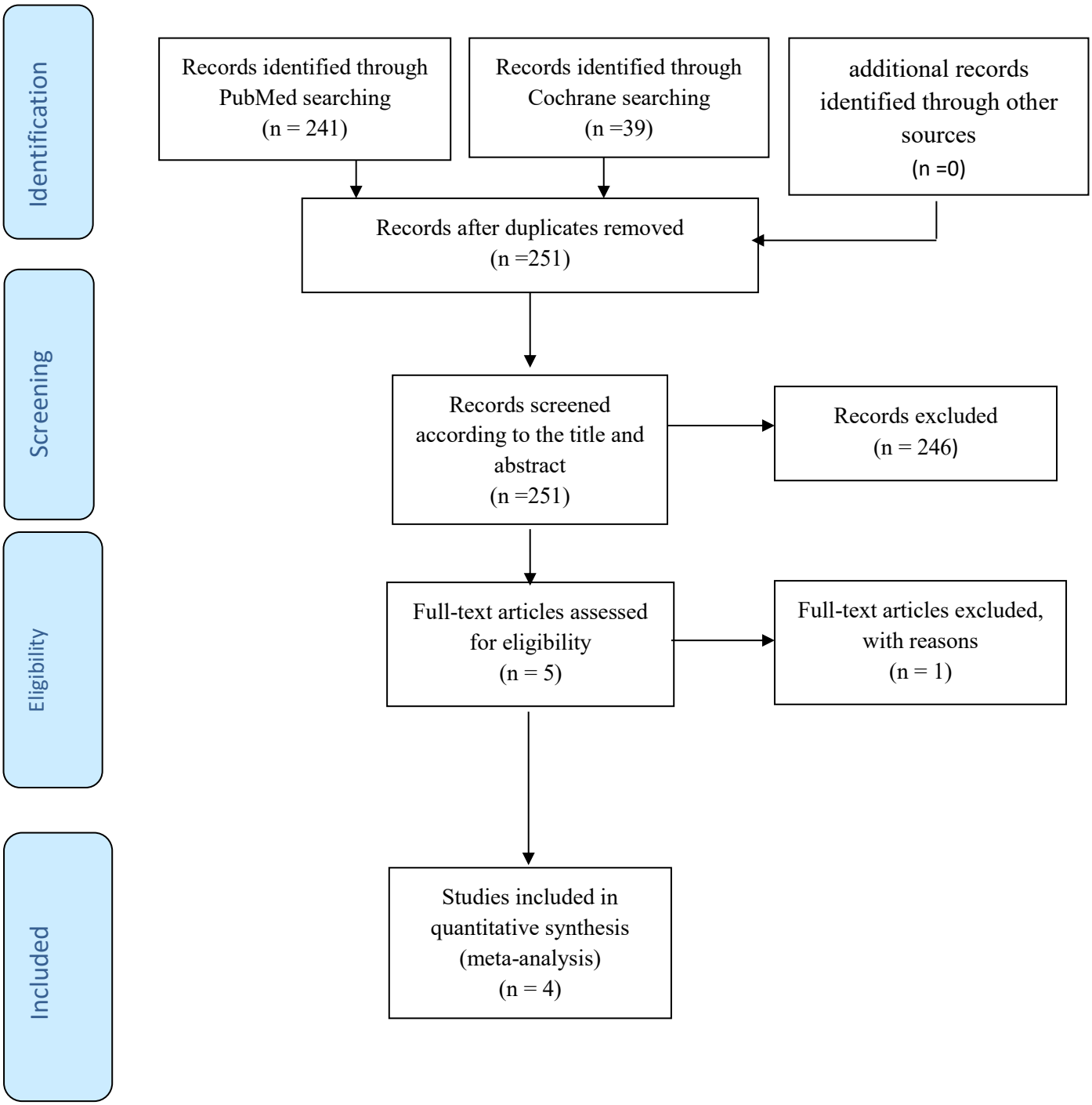
#82	Add	Search oral squamous cell carcinoma) or (head and neck squamous cell carcinoma) or (oral cancer) or (HNSCC) AND (Licochalcone A) or (licorice) OR (oral squamous cell carcinoma) or (head and neck carcinoma) or (oral cancer) or (HNSCC) AND (paclitaxel) or (taxol) Filters: Full text;	242	20:24:20

PRISMA flow diagram of study selection:



9: Study Selection:

Data screening was started with 251 articles on the basics of title and abstract and 246 articles were excluded according to the exclusion criteria that restricted the choice only to the head and neck squamous cell carcinoma cell lines and exclude any other cell lines. The remaining five articles were screened by full text and only one article (systematic review) was excludes because it discusses other types of licorice extract and not Licochalcone extract groups.

10: Data collection process:

All data from the selected articles are refined and extracted into data search tables.

11: Data Items:

The extracted data include demographic data including article's title, author name, journal and the year of publication. Methodology and intervention also extruded including the dose, material and duration. In addition, outcome measures are also assessed regarding Apoptosis, cytotoxicity and viability.

Articles data:

Study	name	Author	Type of the study	Year	Journal
(1)	A polysaccharide from Glycyrrhiza inflata Licorice inhibits proliferation of human oral cancer cells by inducing apoptosis via mitochondrial pathway	Huan Shen. et al	In vitro	2015	International Society of Oncology and BioMarkers
(2)	Licochalcone-A Induces Intrinsic and Extrinsic Apoptosis via ERK1/2 and p38 Phosphorylation-mediated TRAIL Expression in Head and Neck Squamous Carcinoma FaDu Cells	Mi-Ra Park. et al	In vitro	2015	Food Chem Toxicol
(3)	Licochalcone B induces apoptosis of human oral squamous cell carcinoma through the extrinsic- and intrinsic-signaling pathways	Hana Oh. et al	In vitro	2015	INTERNATIONAL JOURNAL OF ONCOLOGY
(4)	Effects of paclitaxel on permanent head and neck squamous cell carcinoma cell lines and identification of anti-apoptotic caspase 9b	Maushagen R. et al	In vitro	2016	Journal of Cancer Research and Clinical Oncology

- **Material and methods:**

No. of the study	Type of samples (cell line)	control	Natural extract	Chemotherapy	Western blot analysis						Immunohistochemistry			
					Doses µM			Duration	groups	antibodies	Dose Mg /kg	Duration	Groups	Antibody
1	SCC-25	1	Glycyrrhiza inflata	nil	147	295	591	48 h	nil	Bax Bcl 2 PARP	Not used			
2	FaDu -SCC cell	1	Licochalcone- A	nil	50		100	24 h	nil	TRAIL PARP CASPASE-3	10	3 times per week for 8 weeks	5 µm thick sections	Caspase-3
3	HN22 HSC4	2	Licochalcone-B	nil	10	20	30	NA	nil	Cyclin D P21 P27 Bax Bcl2 PARP	Not used			
4	UT-SCC:24-A,24-B,60-A,60-B	4	nil	paclitaxel	10 µM			NA	4	Caspase 9 Caspace 9b	Not used			

No. of the study	Type of samples (cell line)	control	Natural extract	Chemotherapy	Cell viability assay (MTT assay)							Cell viability assay (Trypan blue assay)							Cell apoptosis assay (Flow cytometric analysis)							
					Doses µM				duration	groups	material	Doses µM			duration		groups	material	Doses µM			Duration		groups	material
1	SCC-25	1	Glycyrrhiza inflata	nil	147		295	591	48 h	96-well plates	20 µg/mL of MTT soloution	Not used							147	295	591	48 h		nil	AnnexinV FITC/PI
2	FaDu - SCC cell	1	Licochalcone - A	nil	25	50	100	125	24 h	96-well plates	20 µg/mL of MTT soloution								100 µM			12 h	24 h	6- well plates	AnnexinV FITC/PI
3	HN22 HSC4	2	Licochalcone -B	nil	Not used							10	20	30	24 h	48 h	12-well plates	Trypan blue solution	10	20	30	48 h		12-well plates	AnnexinV and dead cell kit
4	UT-SCC:24-A,24-B,60-A,60-B	4	nil	paclitaxel	Not used														10 µM			24 h		6 well plates	Annexin V/PI

- **Results:**

No. of study	Drug	Cytotoxicity /Viability												
		Duration (hours)	Dose µM	Effect (cells)	Duration (hours)	Dose µM	Effect (cells)	Duration (hours)	Dose µM	Effect (cells)	Duration (hours)	Dose µM	Effect (cells)	Control (cells)
1	Glycyrrhiza inflata	48	591	4900	48	295	6000	48	147	7000	-	-	-	10000
2	Licochalcone-A	24	25	78000	24	50	80000	24	100	48000	24	125	19000	$1x10^5$
3	Licochalcone-B	24	10	42000	24	20	45000	48	20	34800	48	30	40800	$6x10^4$
4	paclitaxel	24	10	175000	24	10	225000	48	10	157500	48	10	205000	250000

No. of study	Drug	Apoptosis												
		Duration (hours)	Dose µM	Effect (cells)	Duration (hours)	Dose µM	Effect (cells)	Duration (hours)	Dose µM	Effect (cells)	Duration (hours)	Dose µM	Effect (cells)	Control (cells)
1	Glycyrrhiza inflata	48	147	3270	48	295	4140	48	591	5200	-	-	-	10000
2	Licochalcone-A	12	100	32120	24	100	35650	-	-	-	-	-	-	$1x10^5$
3	Licochalcone-B	48	10	7572	48	20	28368	48	30	50016	-	-	-	$6x10^4$
4	paclitaxel	-	10	75000	-	10	117500	-	-	-	-	-	-	250000

List of included studies:

Author,date	Database
Park MR et al (2015)	PubMed
Huan Shen et al (2015)	PubMed
Oh H et al (2016)	PubMed
Maushagen R (2016)	PubMed

Excluded studies:

Title	Author	Reason for exclusion
A Systematic Review of the Anticancer Properties of Compounds Isolated from Licorice (Gancao)	Tang ZH et al (2015)	Systematic review

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