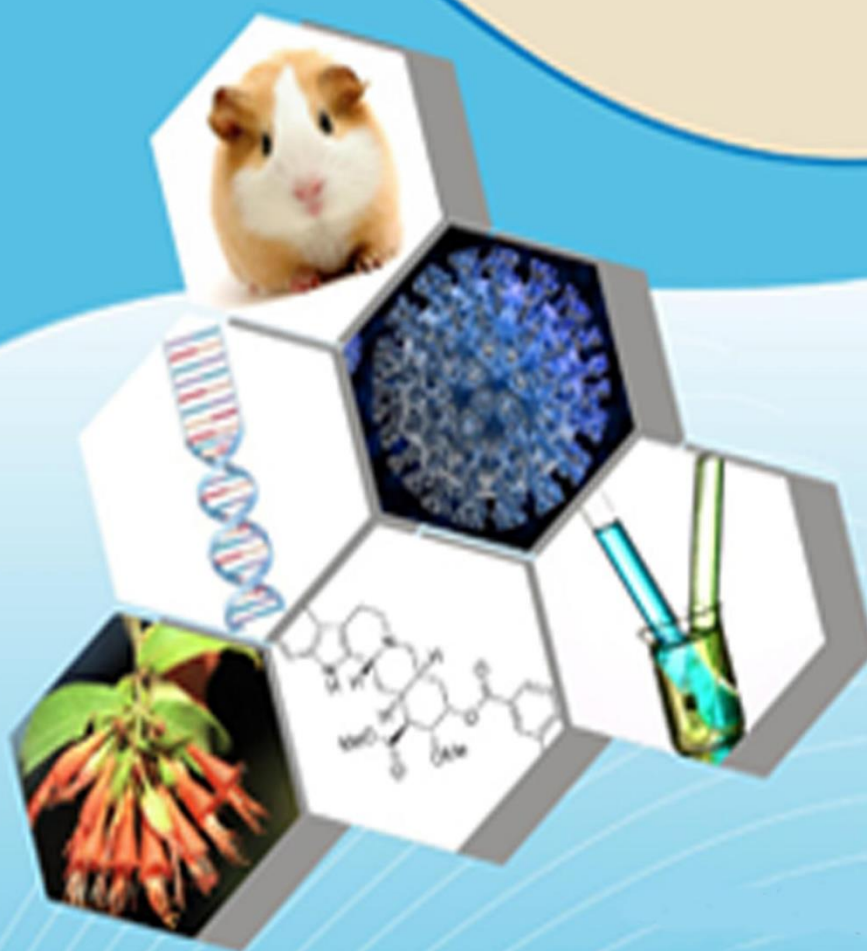




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Anticancer effect of Andrographolide in oral squamous cell carcinoma cell lines - A Systematic Review

Type of research: Systematic review

Running title: Anticancer effect of Andrographolide in oral squamous cell carcinoma cell lines

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STRUCTURED ABSTRACT

Background

Andrographolide, a plant extract of *Andrographis paniculata* has shown promising cytotoxic effects on various cancer in-vitro and animal studies. However, there is scarcity of evidence of its anticancer effect on oral squamous cell carcinoma. Hence, it is important to gather evidence available in literature.

Methods:

Comprehensive databases were used to retrieve the articles. Studies fulfilling the eligibility criteria were included. Due to heterogeneity in methodology, meta-analysis was not attempted. Quality assessment was carried out using ToxRTool, analyzing the toxic effect of agents.

Results:

Six studies have met the inclusion criteria. These studies demonstrated that andrographolide and its combination with silver lipid nanoparticles and cisplatin is important in inhibiting cell proliferation, cell cycle arrest, apoptosis and regulating proteins involved in carcinogenesis pathways. Hence, combination of andrographolide with nanoparticles shows better potential anticancer effect in OSCC cell lines.

CONCLUSION:

Andrographolide has shown anticancer effects in OSCC and lesser IC₅₀ values which ultimately lead to inhibiting the proliferation of tumor cells. Hence, andrographolide with nanoparticles will exhibit better cytotoxicity in lower concentration. Further studies, combination with standard chemotherapeutic drugs should be explored to increase their cytotoxic potency.

Keywords: andrographolide, apoptosis, protein expression level, cell viability, cell survival, *in vivo*, *in vitro*, OSCC

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INTRODUCTION

Oral Squamous cell carcinoma (OSCC) is a major health concern in terms of morbidity and mortality¹. It is the sixth most common cancer worldwide^{1 2}. These tumors primarily develop in individuals with a prolonged usage history of tobacco and alcohol³. Despite the evolving model of multimodality management, which integrates surgical intervention, chemotherapy, and radiation therapy, overall survival remains poor with a five-year relative survival rate below 50%^{4,5}. Currently, there is no effective anticancer drug to improve the survival rate⁶. Those available have various side and adverse effects and have little influence on survivability of oral cancer patients⁷.

Over the last few decades, research has been focusing on developing anti-cancer drugs with better efficacy and potent tolerance.

Various agents from traditional herbal plants have come to focus due to its antioxidant, anti-inflammatory properties⁸. The development of effective, non-toxic, and affordable novel pharmacological agents is an appealing strategy for preventing the early carcinogenesis and development of OSCC⁸. Notably, chemoprevention by phytochemicals is emerging as one such promising strategy to delay or block the carcinogenic processes of OSCC⁹. Recently, an herbal and non-toxic compound from herbal plants, andrographolide, have been identified as a novel and potential candidate for cancer treatment¹⁰.

Andrographolide is extracted from the stem and leaves of *Andrographis paniculata* (Burm.f.) Nees, Andrographolide have antioxidant, anti-inflammatory, immunomodulatory, antiseptic,

antimicrobial, antiviral, cytotoxic, hypolipidemic, cardio-protective, hepato-protective, and neuroprotective effects^{11 12 13}. The previous studies have indicated the therapeutic role of andrographolide in human diseases such as neurodegeneration, Parkinson's disease and rheumatoid arthritis, intestinal inflammation and colitis, acute lung injuries, respiratory tract infection, diarrhea, fever and cold, and hand and foot, and mouth disease (HFMD)¹⁴.

Andrographolide has shown cytotoxic effects against a number of cancer cell lines¹⁵. The andrographolide exerts its anti-cytotoxic effect through different mechanisms. It includes inhibition of JNK-signal transducers and activators of transcription, NF-kB and PI3K signaling pathways, suppression of cyclins and cyclin-dependent kinases (CDKs), metalloproteinase, growth factors and heat shock proteins (hsp-90), and the induction of tumor suppressor proteins p53 and p21^{16 17}. Andrographolide was selectively known to induce apoptosis of human cancer cells via the death-receptor-mediated apoptotic pathway¹⁸. Andrographolide also sensitizes tumor necrosis factor (TNF)-related apoptosis-inducing ligands (TRAIL) to induced apoptosis in TRAIL-resistant human cancer cells¹⁹. In addition, Andrographolide affects the activity of CDK and induces the expression of cell-cycle inhibitor P27 for restraining tumor cells at G0/G1²⁰. Furthermore, Andrographolide inhibits tumor-induced angiogenesis by decreasing the expression of vascular endothelial growth factor (VEGF), nitric oxide (NO), and various inflammatory

cytokines and chemokine while increasing the expression of endogenous anti-angiogenesis factors, such as interleukin (IL)-2 and tissue inhibitors of metalloproteinase²¹. However, its anticancer effect on oral squamous cell carcinoma cells have not been fully explored. This systematic review is intended to gather evidence on the anticancer effect of andrographolide in OSCC cell lines.

STRUCTURED QUESTION

Does andrographolide have an anticancer effect on oral squamous cell carcinoma cell lines?

MATERIALS AND METHODS

The systematic review was reported according to Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA), 2020 guidelines for systematic reviews. (Supplementary S1: PRISMA checklist)

PICO statement

For the present systematic review, following PICO (The population. Intervention, comparison, outcome) was formulated to find answers to questions.

- Population – OSCC Cell lines
- Intervention / Exposure – Andrographolide with or without combination
- Comparator – compared with contro group Outcome – Cell viability rate, survival analysis, apoptosis rate

The inclusion criteria for studies were; 1. Studies on evaluating the anticancer effect of andrographolide on OSCC cell lines either alone or in combination and as compared with other drugs; 2. Studies in English language were included. Studies were excluded based on the following exclusion criteria; 1. Studies that did not treat oral cancer cell lines with andrographolide; 2. Studies done on cancer cell lines other than oral squamous cell carcinoma;

Search and Sources of Information

PubMed Central, Scopus, Web of Science and Google Scholar were searched to retrieve the relevant published papers. Search strategy was prepared using the keywords from MeSH (Medical Subject Headings) and free text (Supplementary file S1). In addition, an internet search was performed using the free words such as “anticancer activity”, “andrographolide” and “Oral Squamous Cell Carcinoma cell lines”. The Search results were exported into the reference manager software to remove duplicates.

Screening and Selection of Studies

The title of the papers were screened to identify relevant articles. In case eligibility cannot be ascertained from the title, papers were further evaluated by reading the abstract. Those papers that have fulfilled preliminary criteria were sought for full text retrieval. Two reviewers read the full text for finalization of these papers for inclusion into systematic review. All the papers relevant to research questions and have

fulfilled eligibility criteria were included. Bibliography of all the identified studies were screened to find additional papers.

Quality assessment of studies

The studies include the in-vivo and in-vitro studies evaluating the cytotoxicity of andrographolide, a compound from plants. Quality assessment was carried out using the TOXR tool²². Studies were scored based on five categories; I: Test substance identification, II: Test system characterization, III: Study design description, IV: Study results documentation, V: Plausibility of study design and data. In total 21 questions for in vivo studies, 18 questions for in vitro studies were assigned. Each question was scored either as “0” or “1”. If a criterion is met, 1 point was given. Total points range was 0-21, 0-18 for *in vivo* and *in vitro* study, respectively. Each study was rated based on total number points into one of reliability categorization. The categories were reliable without restrictions (in vivo study, 18-21; in vitro study, 15-18), reliable with restrictions (in vivo study, 13-17; in vitro study, 11-14), not reliable (in vivo study, <13; in vitro study, <11).

Data Collection

Two authors read the full text and extracted predefined data using the Microsoft excel sheet. The following information was extracted from the full text: author names, year of publication, country, study design, cell lines, type of assays, outcome measures (Cell viability/ survival rate, apoptosis rate).

Data were extracted from text, table, figure/graph (using a digital screen ruler). In addition, authors of articles were contacted to obtain the missing/required data for the experiment.

Synthesis of summary findings

The findings of studies were summarized using descriptive statistics and are presented for each outcome. Quality assessment is presented in tabular format and judged overall as good, moderate and poor based on score. Due to variability and heterogeneity in methodology and material in included studies, meta-analysis was not conducted.

RESULTS

Search findings

The systematic search from the electronic databases of PubMed revealed 222 studies, Google Scholar revealed 3 studies. After removal of duplicates and title scan, 15 studies were identified. After abstract scanning 9 articles were eliminated as they did not meet the inclusion and exclusion criteria. Full text articles for the other 6 studies were obtained for more detailed evaluation (Figure 1: PRISMA Flow chart). The bibliography of these full text articles was scanned manually to include studies apart from the electronic databases. No relevant studies were found from the cross-reference. 6 studies met the inclusion and exclusion criteria of the systematic review.

Study characteristics

The Characteristics of the included studies are presented in Table 1 and 2. Included studies were published between 2011- 2016. 5 studies were from China whereas 1 study was from Taiwan. Three studies evaluated the effect of andrographolide in vitro as well as in vivo. Different cell lines were used in an in-vitro experiment. Only one study extracted the andrographolide from leaves, the rest used the commercially available andrographolide. These included CAL-27, HIOEC, Leuk1, HN6, HN30, SCC-25, HSC-2, HSC-3, HSC-4, Normal human oral cells, HGF, HPLF and HPC, tongue tumor cell (Tb), oral cancer stem cells (ADH+ & CD44+). Cell lines utilized were heterogeneous. Majority studies measured the cell viability and cell apoptosis using different assays (Table 2). Suzuki et al, Wang et al, Huang et al, Suzuki et al compared their efficacy with either saline or other drugs (cisplatin, cannabidiol, 5-FU). Chen et al and Li et al have evaluated andrographolide in combination with cannabidiol and Nano-particles, respectively. The included studies assessed the cell viability or survival using CCK 8, MTT assay, MTS assay, Alamar Blue assay, and Annexin V/7-AAD Binding Assay.

Anti-cancer effect of andrographolide

All studies showed that andrographolide has anticancer efficacy mostly at higher concentration in oral squamous cell carcinoma cell lines. Further, andrographolide was found to increase the cytotoxicity effect in combination with DPP at lower concentration of IC₅₀ values. However, andrographolide alone at low

concentration does not have significant cytotoxic effect compared to the combination with nanoparticles. Li et al reported increased efficacy after addition of loaded solid lipid nanoparticles with andrographolide at low concentration compared to andrographolide alone which shows cell viability at 38.47 µg/ml and 300 µM concentration (IC50).

Cell Viability and Survival Assay:

Included studies evaluated the Cell viability after 24 hr, 48 hr and 72 hr of exposure to andrographolide. Cell viability in these studies ranged from 4 % to 115 %. Li et al observed that andrographolide with solid lipid nanoparticles in the concentration 11.74 µg/ml shows better cell viability compared to free andrographolide, which is of 19.98 µg/ml. Wang et al states that 200µm at 72 hr shows 40% of the cell viability rate. Yang et al and Suzuki et al observed that 50-µM concentration of andrographolide show about 40% cell survival rate in OCSC and HNSCC cell lines. Chen et al observed a 5% cell survival rate at 800nM concentration of control.

Apoptosis rate

Apoptosis rates reported in these studies ranged between 4.62% (HN6) and 54.40% (SCC-25). Apoptosis rate varied with the concentration of andrographolide (Table 2). When used with combination there increase apoptosis (10.39 % to 39 %). Chen et al reported the rate of apoptosis for combination of ADG-SLN is about 10.39% and 22.62% for HN6 and HN30 and with DPP (cisplatin) of about 47 % in CAL-27. In

Wang et al study, the apoptosis rate was 39 %, and 49 % for the 150 µm concentration of andrographolide at 24 hr and 48 hr respectively. Yang et al also observed that cancer stem cell markers like ALDH and CD44 show 70% and 50% of apoptosis rate at 12.5 µm concentration of andrographolide.

Risk of bias across studies

The included studies were considered heterogeneous in terms of source of andrographolide, cell lines, assay, dose and concentration. Out of 6, 3 studies performed in vivo experiment, the quality assessment (ToxRtool) score were from 17 to 21. 5 studies performed in vitro experiment. The quality assessment score for the in vitro experiment was from 17 to 19. All studies were categorized as Reliable without Restrictions (Table 3).

DISCUSSION

The chemotherapeutic drugs have undesirable side effects in oral cancer patients and can develop drug resistance over the period. The development of effective, non-toxic, and affordable novel pharmacological agents is crucial for improving long-term survival and better outcomes. Several natural products and plant extracts were shortlisted as potential chemotherapeutic drugs. Andrographolide, a plant extract, was reported to have a potential cytotoxic effect on cancer cells.

The main advantages for using natural extract compounds were so effective in anticancer effect and even in

chemoprevention has been increased in recent years²³. Unfortunately, studies have shown diverse results.

The present systematic review analyzed the studies evaluating the effect of andrographolide in oral cancer cell lines. There are very few studies in literature that have evaluated the andrographolide inhibitory/anticancer activity in OSCC cell lines. The andrographolide has anticancer efficacy with certain limitations. It inhibits the OSCC in both *in vivo* and *in vitro* conditions. It is consistent with observation with studies conducted in other tumor cell lines.²⁰ According to Rajagopal S et al, andrographolide has inhibitory effects on different cancer cell lines at different concentrations/doses²⁰. However, Chen et al study observed that the andrographolide alone did not have any cytotoxic effect on OSCC cells but increased the cytotoxic effect of DPP drug²⁴. Similarly, Li et al observed that there is increased cytotoxic efficacy after it is combined with nano preparation²⁵. This shows that there is potential cytotoxicity in andrographolide; it requires additional treatment with an external agent to be effective²⁵. The minimum inhibitory dose (IC₅₀) of andrographolide for OSCC lines range between 38.47 µg/ml and 300 µM concentration, which can be lowered with help of nano formulation to as low as 0.3371 µg/ml²⁵. Andrographolide is inhibitory to several different tumor cell lines at 5–15 µM (Rajagopal S et al²⁰). Similarly, andrographolide can inhibit several different OSCC cell lines as shown in included studies. This shows that andrographolide is

effective in different oral cell lines and can be effective in OSCC irrespective of tumor site.

Most of the plant extract compounds like andrographolide illustrate minimal solubility and even worse bioavailability and so clinical implication gets reduced²³. Henceforth, Nanotechnology provides surrogate access to conventional therapy that undergoes enhancement in competency and minimal toxicity²⁶. According to Li et al, nano lipids structure will enhance the bioavailability and efficacy of anticancer chemotherapy and prevention of drugs²⁵.

Andrographolide exerts its cytotoxic effects through several pathways that are observed in *in vivo* and *in vitro* studies. It includes decrease proliferation of cancer cells, increase IL-2 and IFN- γ , decreasing tumor growth, cell cycle arrest at G2/M phase, decrease in phosphatidylinositol 3-kinase and NF- κ B signaling pathways, suppression of hsp 90, cyclins, and cyclin-dependent kinases, MMPs and growth factors, increase tumor suppressor proteins p53 and p21^{25, 24, 27, 28}.

Andrographolide exposure to OSCC showed variable apoptosis rate, 4.62 % and 54.40 % at low and high concentration, respectively²⁵. There is elevated expression p-AKT and p-p53 levels in CAL-27 cells after the andrographolide and/or DDP treatment resulting in apoptosis²⁴. In Wang et al study, there was inhibition of aberrant NF- κ B activation after andro treats chemically induced oral squamous cell carcinogenesis²⁷. In Li et al study, anti-

proliferative effect was due to cell cycle arrest mainly in the G2/M phase²⁵. Further, andrographolide can cause inhibition by increasing the radiosensitivity of OSCC cell lines through upregulation of miR-218, thereby reducing Bmi1 expression²⁸. Downregulation of Akt and NF-kappaB activity is another mechanism through which andrographolide makes cancer cells susceptible to radiation^{27, 24, 29}.

The review has certain limitations as included studies have heterogeneous methodology. The included studies have variation in different drug dose/concentration, source of andrographolides, cell lines, and assays. In vivo studies have used different animal species such as mice and hamsters with different monitoring systems and maintained in different set-up^{24, 27, 28}. These had reported outcomes using different parameters. Few studies reported cell viability while one study reported cytotoxicity rate²⁹. As a result, it was possible to pool the outcome and do the meta-analysis. Cytotoxicity assays were different in included studies. MTT, CCK-8 assay were used, although both assays give the cell viability rate, these have different sensitivity and specificity which could influence outcome³⁰.

The present review clearly reveals that the field of nanoparticles and its technology has been part of carriers for the andrographolide to enhance its bioavailability and anticancer activity in oral squamous cell carcinoma cell lines. Novel clinical studies and trials will present a done in comprehensively to find the anticancer activity of andrographolide in

patients with different types of cancer. Future studies on clinical trials will absolutely yield some light on the consequences of this compound in the enhancement of new chemotherapeutic and complementary strategies in the Oral Squamous Cell Carcinoma.

CONCLUSION:

The andrographolide extracts obtained from commonly used medicinal plants, *Andrographis paniculata* (Burm.f.) Nees, exhibit anticancer / cytotoxic effect in oral squamous cell carcinoma and can be developed as a potential anticancer agent. However, it is mostly at higher concentration or in combination with drugs or nano-material. Hence, future studies, especially clinical trials, are required to elucidate whether the plant extracts exhibit anticancer activity or cytotoxicity at low concentration.

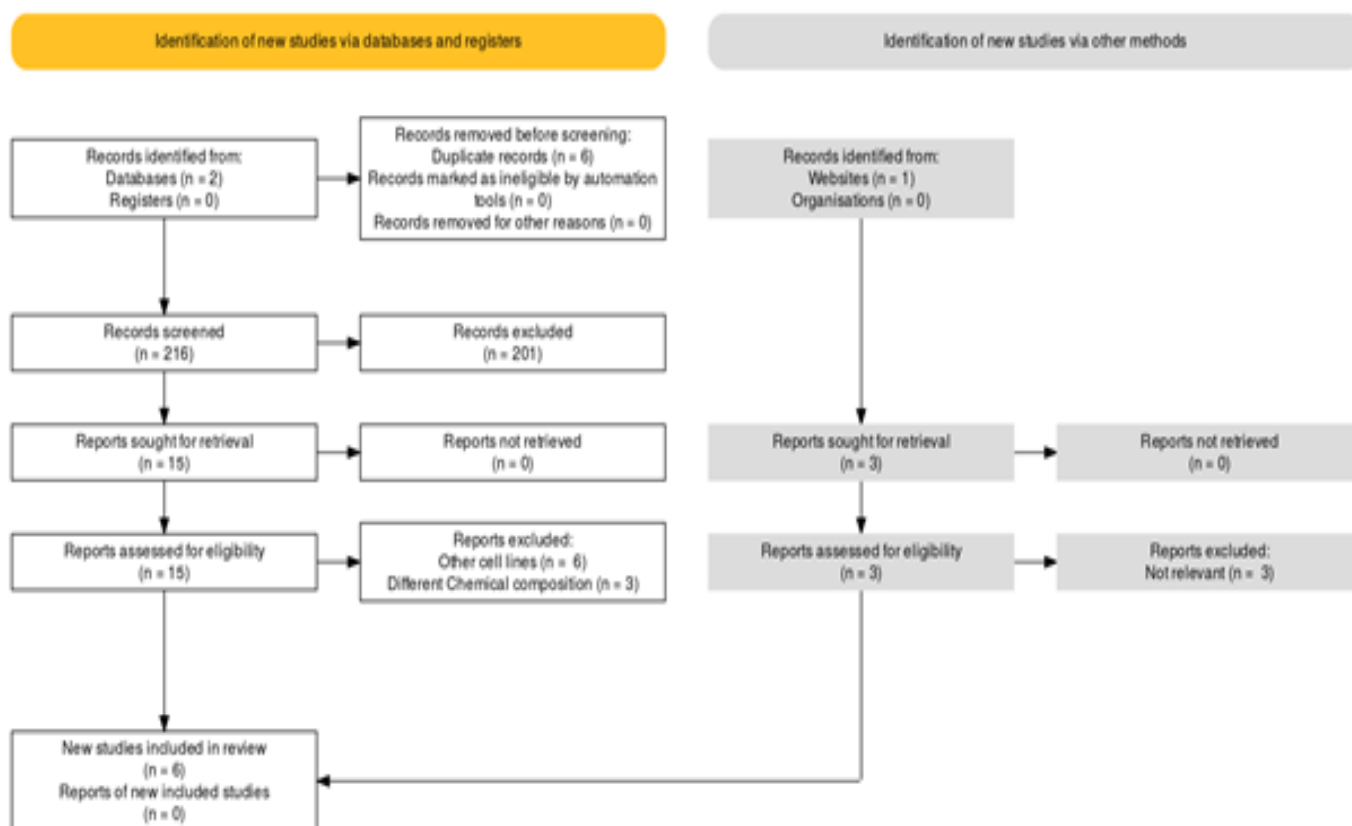
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Figure 1-PRISMA Flow chart



Search strings (Supplementary file S2)

Search (((((((((((oral squamous cell carcinoma cell line) OR (oral squamous cell carcinoma cell lines)) OR (oral cancer cell lines)) OR (oral cancer cell line)) OR (metastatic oral squamous cell carcinoma cell line)) OR (metastatic oral squamous cell carcinoma cell lines)) OR (metastatic oral cancer cell lines)) OR (metastatic oral cancer cell line)) AND ((andrographolide) OR (andrographis paniculata))) AND (((((((((((((((anticancer activity) OR

(anticancer activities)) OR (anticancer effect)) OR (anticancer effects)) OR (anti tumour activity)) OR (antitumor activities)) OR (anti tumour effects)) OR (antitumor effect)) OR (anti tumor activity)) OR (anti tumor activities)) OR (anti tumor effect)) OR (anti tumor effects)) OR (anti proliferative activity)) OR (anti proliferative activities)) OR (anti proliferative effect)) OR (anti proliferative effects)

Table 1: Characteristics of Included Studies

S. No	Author, Country	Study type	Comparator	Control	Study methodology	Parameter(S) studied	Anticancer effect direction

1	Chen et al ,(2017), China	In-vitro study	Andrographolide	Andrographolide + DDP (cisplatin)	1.Cell lines and culture. 2.Cell survival assay (CCK 8 kit) 3.Assessment of cell morphology 4.Apoptosis detection assay 5.Flow cytometric analysis 6.Western blotting	1. Cell proliferation and apoptosis 2.p-AKT and p- p53 levels in CAL -27 cell	No significant cytotoxic effect when used alone
2	Yang et al (2017), China	In-vitro study	Andrographolide	Andrographolide + IR	1.MTT assay 2.Tumorsphere – forming assay 3.ALDH1 activity assay 4.RT-PCR Western blotting 6.Soft agar colony forming assay miR-218 Sponge.	1.Cell proliferation and self-renewal 2. Oncogenicity and radio sensitivity 3. Expression of miR-218 targeting Bmi1	Significant cytotoxic effect
3	Suzuki et al (2016), Japan	In-vitro study	Andrographolide	Doxorubicin (DXR)	1.Cell culture 2.MTT assay 3.Western blotting analysis.	1.Cytotoxicity effect of different compounds of andrographolide 2. Tumor specificity	High cytotoxicity and tumor specificity against oral squamous cell carcinoma.
4	Wang et al (2011), China	In-vitro study	4H- Andrographolide	Saline	1.Cell culture 2.Flow cytometric Analysis. 3.Immunoblotting	1.Cell viability 2.Cell proliferation 3.Cell apoptosis 4. Protein expression (p65, phosphorylated p65 (Ser536), and IκBα (Ser32/36), c- myc, cyclin D1)	Significant cytotoxic effect
5	Huang et al (2020), Taiwan	In-vitro study	CBD and Andrographolide	Cisplatin 5FU	1.Cell culture 2.Impedance Measurement by ECIS. 3.Alar Blue assay. 4.Annexin V/AAD Binding Assay 5.Flow cytometric Analysis	1.Cell viability 2.Cell apoptosis	Significant cytotoxic effect at higher concentration

6	Li et al (2020), China	In-vitro study	Andrographolide	ALG-SLN	1.Nano-formulation 2.MTS assay 3.Flow cytometric Analysis 4. HPLC.	1.Cell viability 2.Cell cycle arrest 3.Cell apoptosis 4. Cellular uptake	No significant cytotoxic effect when used alone
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Table 2: Summation table for included studies

→Author, year	Sources Extraction Method	Cell line	Cell viability	Andro (IC50)	Andro combination (IC50) µg/m	Apoptosis rate (%)	Cytotoxicity (CC50)	Tumor growth (gram)	Dose in-vivo (Animal model)
Chen et al 2017	Andrographolide (Sigma-Aldrich) stock solution	CAL-27	48 Hr;	-	-	Andro Only = 18 % +DPP = 39 % Control(DDDP) = 4 %	-	Andro only (1.58 ± 0.18) + DDP (0.62 ± 0.10) Control (1.79 ± 0.20)	Andro (50 mg/kg, once daily); DDP (5 mg/kg, once per week) combinatorial treatment

Li H et al	Free Andrographolide extracted from high performance liquid chromatography (HPLC)	HIOEC Leuk1 HN6 HN30	48 hr	38.47 µg/ml 26.14 µg/ml 13.75 µg/ml 19.98 µg/ml	0.3371 µg/ml 0.717 µg/ml 6.087 µg/ml 11.74 µg/ml	Andro only 9.56% (HIOEC) 15.27% (Leuk1) 4.62% (HN6) 8.01% (HN30) ADG-SLN 12.20% (HIOEC) 23.91% (Leuk1) 10.39% (HN6) 22.62% (HN30)	-	-	-
Huang CC et al 2020	Andrographolide (Sigma-Aldrich) stock solution	SCC-25	24 hr	100 µM	-	Andrographolide (36.40%) Cannabidiol (100%) 5-FU (62.55%) Cisplatin (17.80%)	-	-	-
				300 µM	-	Andrographolide (54.40%) Cannabidiol (100%) 5-FU (70.30%) Cisplatin (64.95%)			
				-	-	Control (DMSO)- (4.40%)			

Suzuki et al 2016	Methanolic extract of leaves of A. paniculata	HSC-2 HSC-3, HSC-4 Normal human oral cells, HGF, HPLF and HPC	48 hr	-	-	-	8±1.0 (HSC-2) 7±1.5 (HSC-3) 10±4.0(HSC-4) 8 (Mean)	-	-
Wang et al (2011)	Andrographolide (Sigma-Aldrich) stock solution	Tb	48 hr	200 µM	-	49 % (Andro 100)* 18 % (Andro 150)*	NA	NA	NA
Yang et al (2016)	Andrographolide (Sigma-Aldrich) stock solution	oral cancer stem cells (ADH ⁺ & CD44 ⁺)	24 hr	50 µM	-	-	-	-	-

* Data extrapolated from graphs/charts

Table: 3 – Quality assessment of selected studies

S.NO	Study	In vivo	In vitro	Reliability Categorization
1	Chen et al (2017)	20	17	Reliable Without Restrictions
2	Yang et al (2016)	17	19	Reliable Without Restrictions
3	Suzuki et al (2016)	-	17	Reliable Without Restrictions

4	Wang et al (2011)	21	17	Reliable Without Restrictions
5	Huang et al (2020)		17	Reliable Without Restrictions
6	Li et al (2020)		17	Reliable Without Restrictions