

RESEARCH ARTICLE

Editorial Process: Submission:01/04/2023 Acceptance:12/17/2023

Evaluation of the Anti-Carcinogenic Effect of Centella Asiatica on Oral Cancer Cell Line: *In vitro* Study

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Abstract

Objective: To evaluate the anti-carcinogenic effect of Centella Asiatica on to evaluate the Anti-Carcinogenic Effect of Centella Asiatica on Oral Cancer Cell Line oral cancer cell line. **Materials and methods:** Oral Cancer cell line and normal oral keratinocyte cell line were procured. Centella asiatica extract was prepared. The cells were then subjected to the test herbal specimens -Centella asiatica extract in succeeding concentrations of 25 µg/ml, 50 µg/ml, 100 µg/ml at time intervals of 24,48 and 72 hours. Cisplatin (2 µg/ml, 4 µg/ml, 6 µg/ml, 8 µg/ml) was used as a positive control. This experiment was done in triplets. **Results:** The study revealed that the p values were less than 0.05 at concentration 12.5µg/ml, 25µg/ml, 50 µg/ml, 100 µg/ml and time period of 24hrs, 48hrs, 72hrs, thus implying that at these concentrations and time period, the obtained data were statistically significant, thus indicating that there is a statistically significantly decreases in the viable cells as the concentration of the drug as a time period increases The results reveals that centella asiatica possess potential effect of anti-carcinogenic, effect when compared to positive control (Cisplatin). **Conclusion:** The current study reveals that Centella asiatica has an potential anti-carcinogenic effect on oral cancer cell line. So this can be used to treat oral cancer with minimal crippling as compared with allopathic drugs.

Keywords: Centella asiatica- MTT assay- cell viability- oral cancer cell line

Asian Pac J Cancer Prev, 24 (5), 1695-1700

Introduction

Oral cancer is the sixth most common malignancy worldwide. Oral squamous cell carcinoma accounts for about 90% of malignant oral lesions and is widely recognized as the most frequently occurring malignant tumour of oral structures (Ananthi et al., 2018). Oral cancer a major concern Southeast Asia, owing to high prevalence of the disease. Genetic, epigenetic factors are the two basic components that affect most diseases (Kumar et al., 2016) Oral cancer, which affects the mouth, lip, and tongue, is associated with a high rate of morbidity and mortality (Ali et al., 2017). The countries with the highest risk of Oral squamous cell carcinoma (OSCC) in Europe include France, Hungary, Slovakia, and Slovenia. Whereas, on a global scale, India, Sri Lanka, Bangladesh, and Pakistan are the countries with high prevalence of OSCC (Tahmasebi et al., 2020) Oral cancer is one of the most prevalent types of cancer in India, with over 77,000 new cases and 52,000 mortalities diagnosed each year. The increasing rates of oral cancer are the most significant concern for community health because it is one of the most common types of cancer in India (Borse et al., 2020).

Oral cancer effective treatments include surgery, which is the preferred treatment, ionising radiation, which is the most common non-surgical treatment option, or a combination of radiotherapy, chemotherapy, and surgery. Chemotherapy is one of the several modalities in the treatment of OSCC and is known to target rapidly dividing cells in the cell cycle.

The side effects of chemotherapy are Mucositis, osteonecrosis of the jaws, infection, lichenoid reactions, dental anomalies, xerostomia, taste changes, neurologic and nutritional problem (Neville et al., 2002)

Cancer treatment with Ayurveda dates back to the 7th century BC, when Atreya and Dhanwanthari utilised herbal medicines to treat the early stages of cancer. Natural concoctions and herbal medicines have always known to have fewer side effects and is less crippling as compared to allopathic drugs. (Balachandran et al., 2005) Centella Asiatica (CA) is a significant medicine used in India, also termed as mandukparni, Indian pennywort, jalbrahmi, or gotu kola. It is considered one of the “miracle elixirs of life” (also called triterpenoids) (Gohil et al., 2010)

In this study, we intend to analyse the anti-carcinogenic effect of Centella asiatica which, if proven, could be

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useful in preventing frank oral cancer using soil-grown concoctions native to our motherland, as well as reverting carcinomatous changes to an extent that might be a boon in the field of oncology and cancer cure by treating OSCC with minimum possible potential crippling as compared to allopathic chemotherapy.

Materials and Methods

Procurement of cancer and normal oral cell lines:

The oral cancer cell and normal keratinocyte cell line were obtained from The National Centre for Cell Science (NCCS), Pune, India and cultured according to the cell culture instructions provided. The oral cell lines were grown in DMEM containing 10% FBS at 37 °C in an atmosphere containing 5% CO₂.

Taxonomy of centella asiatica

The family Umbellifere involves the perennial, clonal herbaceous creeper *Centella asiatica* (CA), also known as gotu kola (Apiceae). This particular herb is classified as a member of the Kingdom Plantae, Division Magnoliophyta, Class Mangoliospida, Order Apiales, Family Apiaceae, Genus *Centella*, and Species *Asiatica*

Extract preparation

The Whole plant (*Centella asiatica*) were dried under shade and ground to coarse powder. Afterward, the extract was being sieved through a double layer of sterile fine mesh cloth. To obtain the fine powder, dry spices (weighing 100 g) were crushed and sieved through mesh cloth. Plant that had been grinded were soaked in 300 ml of distilled water for 24 hours at room temperature and then filtered through Whatman's no. 1 filter paper. The filtrate was heated in a water bath at 45°C for 5 days straight to form a thick paste. The thick paste was considered to be 100% extract concentration. These extracts were kept in a refrigerator at 4°C. After herbal preparation, quality control analysis was done to check the purity of extracts both in solid and liquid phase.

Establishment of cell culture:

The cell culture experiments were done at cell culture laboratory. Human oral keratinocyte cell line and oral cancer cell line obtained from the National centre for cell science (NCCS), pune. The cell line was passaged via trypsinization. The cell line flask was filled with trypsin-EDTA solution, and it was then incubated for 1-2 minutes. The detached cells were then resuspended in Dulbecco's medium containing 10 % fetal bovine serum. The medium was then aspirated using a micropipette and inoculated into culture plates. 5000 cells were inoculated in each well along with Dulbecco's medium, 10% fetal bovine serum and antibiotics like streptomycin sulphate and benzyl penicillin. The culture plates were then inoculated in CO₂ incubator. The medium was replaced every 24 hours until 80% confluence was achieved.

Evaluation of cytotoxic effect:

The oral cancer cells with Dulbecco's medium containing 10% fetal bovine serum seeded into six well culture plates and incubated in CO₂ incubator until

the cells reached 80% confluence. The cells were then subjected to 12.5µg/ml of extract for 24 hours. The cells were then subjected to the test herbal specimens -*Centella asiatica* extract in succeeding concentrations of 25 µg/ml, 50 µg/ml, 100 µg/ml at time intervals of 24,48 and 72 hours. Cisplatin (2 µg/ml, 4 µg/ml, 6 µg/ml, 8 µg/ml) was used as a positive control. This experiment was done in triplets. The formula was used to determine the percentage of viable cells.

Cell viability (%) = (Absorbance of sample/Absorbance of control) X 100

MTT assay for evaluating the effects of herbal extracts on normal cell lines

MTT (3,4,5-Dimethyl-2y1-2,5-diphenyltetrazolium bromide) assay is a standard colorimetric assay. (Figure 1) This assay is utilized for measuring cellular metabolism. This assay generally evaluate the cytotoxicity of potential medicinal agents and other toxic agents by measuring the metabolic activity of the cell post the exposure of the drugs, other agents, et

Statistical analysis

The statistical analytical tests are used to determine whether to accept or reject null hypothesis. Mean, Rank values are calculated as descriptive statistics which determines the distribution and the nature of the results obtained by using Spss software version 21.0. Kruskal Wallis test was used as a statistical test for evaluation and comparison of concentration at three time period, the significance of the results obtained.

Results

The following value gives the percentage of cytotoxicity exhibited by the extract of CA and cisplatin (Positive control) on cancer cell lines and normal keratinocytes over a period of 24,48,72 hrs. Each experiment was repeated in triplicates. Since effective results were obtained at the neat concentration (100µg/ml) of both herbal extracts and (8µg/ml) concentration of positive control at a time period of 72hours only these data were utilized for statistical analysis.

Assessment of Cytotoxicity of centella asiatica in oral cancer cell line

24 hours

Lowest viability was observed at 100µg at which 31.20% of the cells were viable, followed by 51.09%,75.81%, 84.03% and 100% at concentrations 50µg, 25µg, 12.5µg and control (untreated cells) respectively.

48 hours

Lowest viability was observed at 100µg at which 25.04% of the cells were viable, followed by 44.56%,70.19%,78.50% and 97.43% at concentrations 50µg,25µg,12.5µg and control (untreated cells) respectively.

72 hours

Lowest viability was observed at 100µg at which

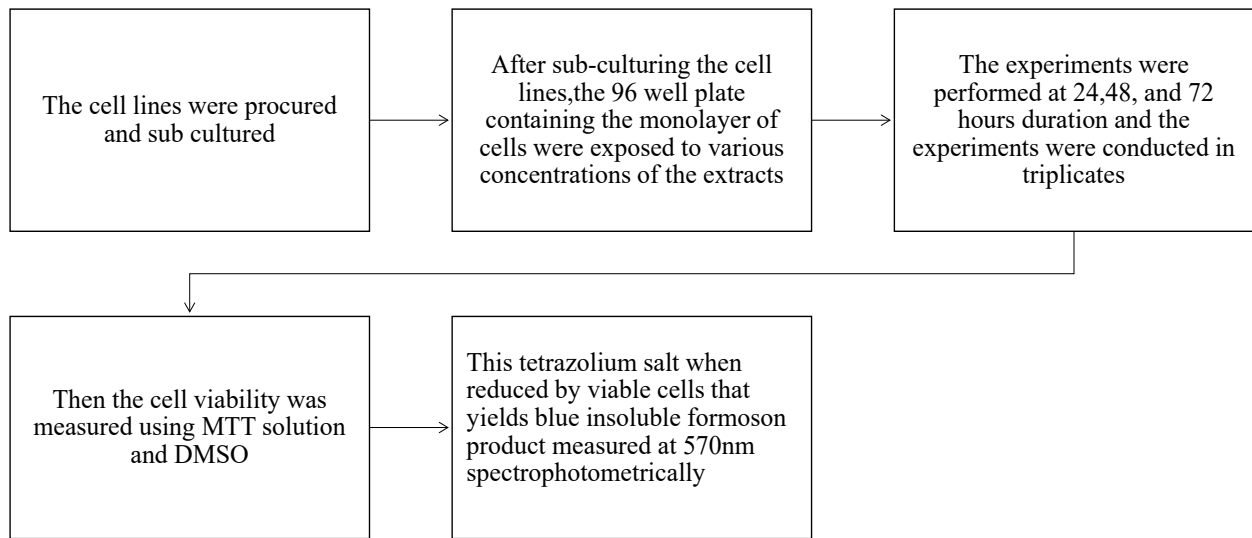


Figure 1. Procedure for MTT Assay

15.40% of the cells were viable, followed by 38.98%, 66.15%, 76.18% and 94.44% at concentration 50 μ g, 25 μ g, 12.5 μ g and control (untreated cells) respectively.

The statistical analysis revealed that the p values were less than 0.05 at concentration 12.5 μ g/ml, 25 μ g/ml, 50 μ g/ml, 100 μ g/ml and time period of 24hrs, 48hrs, 72hrs, thus implying that at these concentrations and time period, the obtained data were statistically significant, thus indicating that there is a statistically significant decrease in the viable cells as the concentration of the drug as a time period increases (Table 1).

Assessment of cytotoxicity of human keratinocyte cell line

The test herbal extracts Centella Asiatica were tested against the epithelial cell lines at dilution of 12.5 μ g, 25 μ g, 50 μ g, 100 μ g at the duration of 24hrs, 48hrs

and 72hrs.

24 hours

Lowest viability was observed at 100 μ g at which 88.91% of the cells were viable, followed by 91.19%, 93.61%, 97.52% and 100 % at concentrations 50 μ g, 25 μ g, 12.5 μ g and control (untreated cells) respectively.

48 hours

Lowest viability was observed at 100 μ g at which 86.93% of the cells were viable, followed by 89.67%, 90.63%, 93.94% and 98.96% at concentrations 50 μ g, 25 μ g, 12.5 μ g and control (untreated cells) respectively.

Table 1. Table Depicting the Statistical Data Obtained from Kruskal Wallis Statistical Test with the level of Significance from at the End of 24 hrs, 48hrs, 72hrs Incubation for CA

	Concentration	Mean	P value	Significance
24 hrs	Control	100	0.022	Significant
	12.5	84.03		
	25	75.81		
	50	51.09		
	100	31.20		
48 hrs	Control	97.43	0.016	Significant
	12.5	78.50		
	25	70.19		
	50	44.56		
	100	25.04		
72 hrs	Control	94.44	0.022	Significant
	12.5	76.18		
	25	66.15		
	50	38.98		
	100	15.40		

Table 2. Table Depicting the Statistical Data Obtained from Kruskal Wallis Statistical Test with the Level of Significance from at the End of 24 hrs, 48hrs, 72hrs Incubation for Cisplatin.

	Concentration	Mean	P value	Significance
24 hrs	Control	100	0.009	Significant
	12.5	94.63		
	25	75.31		
	50	32.35		
	100	25.83		
48 hrs	Control	98.39	0.009	Significant
	12.5	84.28		
	25	52.10		
	50	27.94		
	100	21.90		
72 hrs	Control	93.43	0.018	Significant
	12.5	81.39		
	25	48.41		
	50	23.33		
	100	17.89		

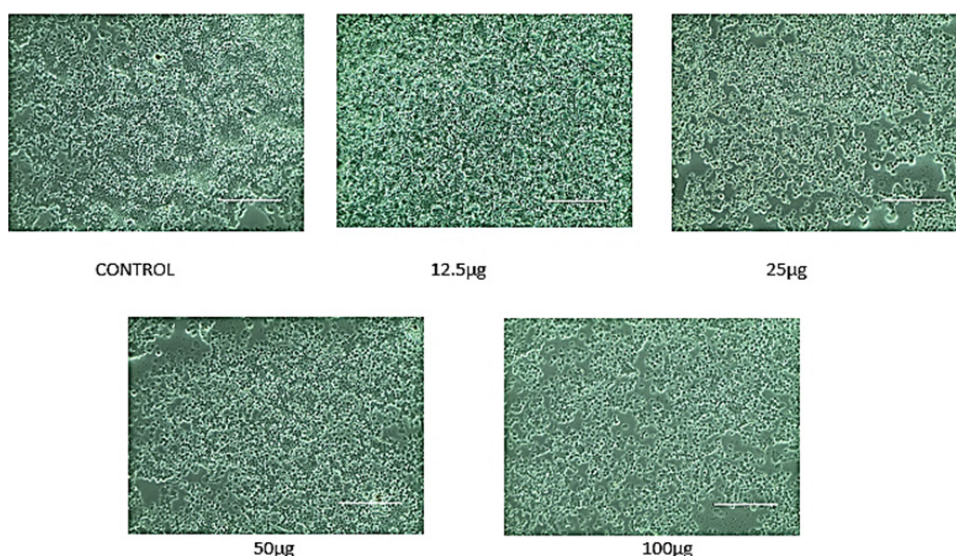


Figure 2. Cytotoxic Effect of Centella Asiatica at 72 hours at Various Concentration

72 hours

Lowest viability was observed at 100µg at which 84.90% of the cells were viable, followed by 88.77% ,89.95%, 91.22% and 97.12% at concentration 50µg, 25µg,12.5µg and control (untreated cells) respectively (Figure 2).

Assessment of cytotoxicity of cisplatin

Cytotoxicity assessment of cisplatin (positive control) on oral cancer stem cells revealed a similar decreasing trend in the percentage of viable cells observed from the end of 24 hours to the end of 72 hours. The study also showed a gradual dose dependent decrease in the concentration of viable cells as the concentration of cisplatin increases (Graph 1).

24 hours

Lowest viability was observed at 8µg at which 17.6% of the cells were viable, followed by 79.90%,66.90%,37.22% and 100 % at concentrations 6µg,4µg,2µg and control (untreated cells) respectively.

48 hours

Lowest viability was observed at 8µg at which 21.28% of the cells were viable, followed by 83.80 %52.13% ,27.66% and 93.38% at concentrations 6µg,4µg,2µg and control (untreated cells) respectively.

72 hours

Lowest viability was observed at 8µg at which 17.94 % of the cells were viable, followed by 81.47%,48.45%,23.72 % and 93.21%, at concentrations 6µg,4µg,2µg and control (untreated cells) respectively.

The statistical analysis revealed that the p values were less than 0.05 at concentration 12.5µg/ml,25µg/ml, 50 µg/ml,100 µg/ml and time period of 24hrs,48hrs,72hrs, thus implying that at these concentrations and time period, the obtained data were statistically significant, thus indicating that there is a statistically significantly decreases in the viable cells as a concentration of the drug as a time period increases (Table 2).

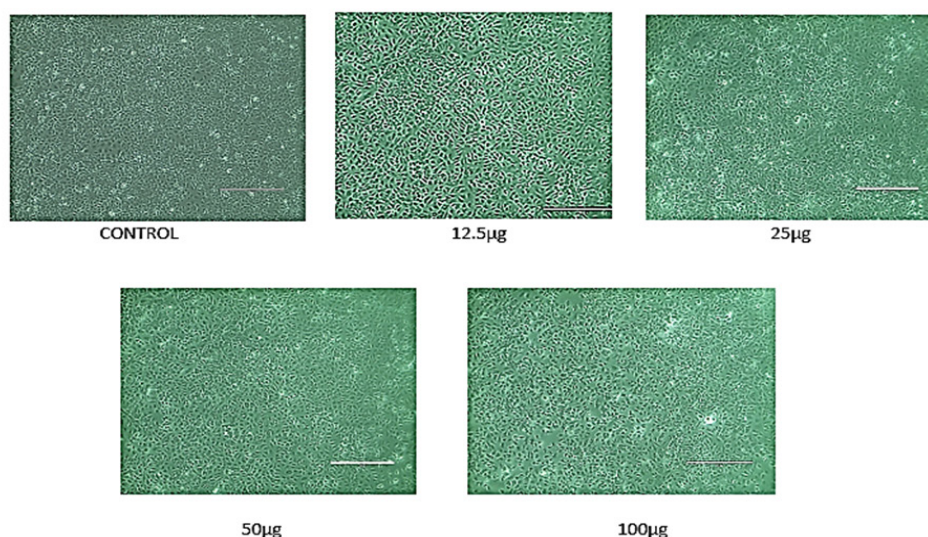
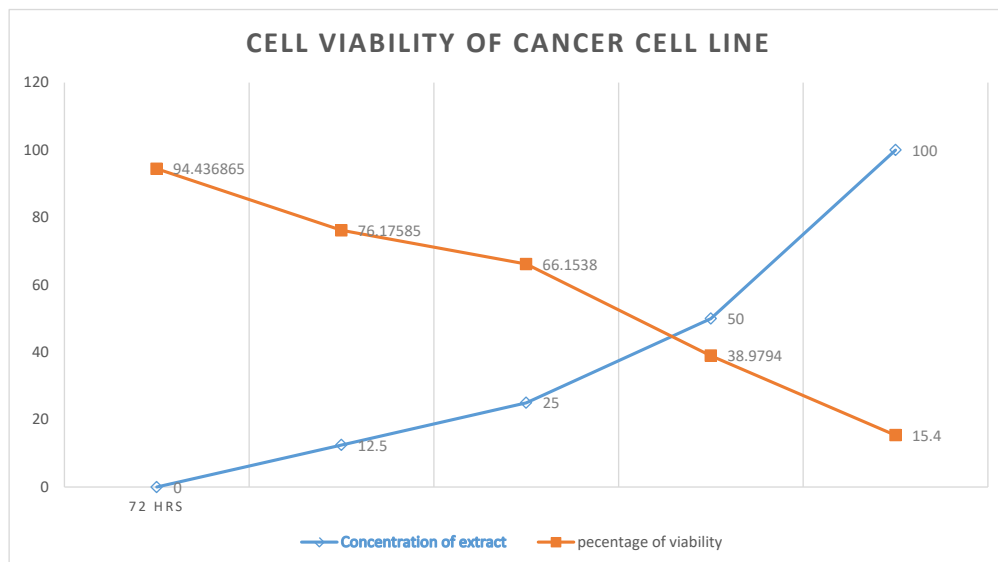


Figure 3. MTT Assay of Centella Asiatica at 72 hours at Various Concentration (Normal Keratinocyte)



Graph 1. Graph Showing Decrease in the Percentage of Viability of Cancer Cell with Increase in Concentration of Centella Asiatica Extract (72hour)

Discussion

Oral and oropharyngeal cancers are the sixth most prevalent malignancy in the world. More than 90% of oral cancers are squamous cell carcinomas (OSCCs). Other tumours of the oral cavity include those of the minor salivary glands, melanomas, and lymphomas. Lymphatic spread into the neck is specifically correlated to the T stage, along with the depth of invasion and tumour thickness. Prolonged tobacco use, particularly the use of smokeless tobacco, chewing betel nut, consuming alcohol, and chronic inflammation, microbial infection was known risk factors for oral cancer (Abati et al., 2020).

Adjuvant therapy for oral cancer is chemotherapy, among that Cisplatin, carboplatin, and oxaliplatin are the three platinum-based medications that are used to treat cancer around the globe. Due to their poor selectivity for cancerous tissue over normal tissue, all cytotoxic chemotherapy drugs, including platinum-based drugs, exhibit a variety of serious side effects. The most common side effects where cisplatin is nephrotoxicity, for carboplatin it is myelosuppression, and for oxaliplatin it is neurotoxicity (Oun et al., 2018).

Since ancient times, herbal remedies have been the strongest contenders for treating a wide range of difficult illnesses and always known to have fewer side effects and is less crippling as compared to allopathic drugs. The family Umbellifere involves the perennial, clonal herbaceous creeper Centella asiatica (CA), also known as gotu kola (Apiceae). This particular herb is classified as a member of the Kingdom Plantae, Division Magnoliophyta, Class Mangoliopsida, Order Apiales, Family Apiaceae, Genus Centella, and Species Asiatica. It is used to treat ulcers, varicose veins, chronic venous insufficiency, wound healing, anxiolytic properties, Alzheimer's disease, diabetes, and it has anti-oxidant and anti-inflammatory properties (Shanmugapriya et al., 2022).

Evaluation of anti-carcinogenicity of test herbals on oral cancer cells in comparison with positive control (cisplatin)

Cytotoxicity of CA on cancer cell line was observed to increase with increase in the concentration of extract, which corresponds to the decreasing percentage of viable cells, which was analysed at the end of 24, 48, 72hrs in triplicates. The percentage of viable cells also found to decrease as the time period increases. The anti-carcinogenic effect on oral cancer cells showed lowest activity at neat concentration (100 μ g) with cell viability of 31.2%, 25.14%, 15.41% at 24, 48, 72 hours respectively.

On comparing CA with cisplatin, CA showed better cytotoxic effect on the oral cancer line than Positive control

Despite, there was no relevant studies that discuss the cytotoxic effect of CA on oral cancer cells, there are studies that observed the cell viability by utilizing CA on other cancer cell lines. According to Gonçalves et al., 2016 research, Centella Asiatica could even inhibit tumour growth by preventing cancer cells from proliferating, causing, apoptotic cell death and cell cycle arresting a large number of cancer cells.

The present study was in accordance with a previous study performed by Shima Edalat Fard et al., 2018 evaluated the cytotoxicity effect of CA in MCF-7 breast cancer cell line. They have concluded that highest cytotoxic effect was in 100 μ g as compared to control group. The Cytotoxic effect of extract synthesised by cancer cells to be dependent on the time and dose of the herbal extracts. The present study also showed that highest concentration (100 μ g) produced a highest cytotoxic effect in oral cancer cell line as compared to control group (Fard et al., 2018). Biswas et al studied the anti-proliferating effect of Ocimum sanctum and CA herbal extract on human glioblastoma cells. They evaluated that CA displayed greater cytotoxicity (97% at 2.5 mg/mL). By inhibiting the expression of the survivin gene, both Ocimum sanctum and CA demonstrated promising anti-

proliferative activity and induced apoptosis. Even though our study showed a similar cytotoxic effect on oral cancer cell line (Beg et al.,2022).

Evaluation of anti-carcinogenicity of test herbals on Normal keratinocytes

MTT assessment of Centella Asiatica on oral normal keratinocyte cells revealed a similar decreasing trend in the percentage of viable cells observed from the end of 24 hours to the end of 72 hours. The cell viability of CA in normal oral keratinocytes cells revealed lowest activity at 100µg with cell viability of 88.91%, 86.93 %, 84.90% at 24, 48,72 hours respectively.It is first of its kind to analyze the cytotoxic effect of Centella asiatica on normal oral keratinocytes to observe that the herbal extract only kills the cancer cells, did not affect the normal keratinocytes.

Hussin et al., (2014) evaluated the cytotoxic effects of CA against human normal liver cell line (Chang liver) and hepatocellular carcinoma cell line. Where the extract containing CA did not show any cytotoxic effect and more than 50% of the cells were viable. The less percentage of viable cells can be attributed to the method of extract preparation Even though the author concluded that CA was non-toxic, the results of the current study was far better in the range of 85-99%viable cells.

The limitation is that the current study was conducted in-vitro and this study was only limited to cell viability assay which is often less sensitive and specific as compared to other molecular methods like PCR, ELISA.

In conclusion, this study proves that wide opportunities are available for the therapy of OSCC using phytochemical derivatives. To overcome the side effects of allopathy related anti-oncogenic treatment, natural herbal remedies have gained significance in preventing and treating malignancy (oral cancer). The current study revealed that Centella asiatica was safe and highly effective against oral cancer.

Author Contribution Statement

All authors contributed equally in this study.

Acknowledgements

None.

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