



## ***In vitro* evaluation of anticancer property of Pugasaram: a polyherbal gel containing mainly arecanut (*Areca catechu*) and betel (*Piper betle*) leaf on oral squamous cell carcinoma**

**Jeddu Ganapathi Bhat<sup>1\*</sup>, D Shobha<sup>2</sup>, S. Keshava Bhat<sup>3</sup>, Sukesh Bhat<sup>4</sup>, Mythri S<sup>5</sup>**

<sup>1</sup> Jeddu Ayurveda Research Centre, Alike: 574 235, DK, Karnataka, India.

<sup>2</sup> Center for systems Biology and Molecular Medicine, Yenepoya Research Centre, Deralakatte: 575018, DK, Karnataka, India.

<sup>3</sup> Arecanut Research and Development Foundation, Varanashi Towers, Mission Street, Mangaluru: 575 001, Karnataka, India.

<sup>4</sup> Kanachur Institute of Medical Sciences, Deralakatte: 575018, DK, Karnataka, India.

<sup>5</sup> Century International Institute of Dental Science and Research Centre, Poinachi: 671 541, Kasaragod, Kerala, India.

**Corresponding Author: Jeddu Ganapathi Bhat**

**ABSTRACT:** Arecanut, the nut of areca palm (*Areca catechu* L.) is one of the popular chewing substances in the world. It is mostly chewed along with betel leaf (the leaf of *Piper betle* L.) and slaked lime (calcium hydroxide) in the form of betel quid. Both arecanut and betel leaf have immense medicinal values including anticancer activity. A poly herbal gel, called Pugasaram, containing arecanut and betel leaf as major components along with certain other herbs in minor proportions is prepared and tested for its anticancer property against CAL-27 oral squamous cell carcinoma cells. In this *in vitro* study, the Pugasaram exhibited both cytotoxic as well as anticancer property against CAL-27 cells with an IC<sub>50</sub> value of 500 µg/ml. Cells treated with Pugasaram displayed alteration in cell attachment, proliferation and morphology with significant increase in apoptosis, lower migratory potential and fewer colony formation in such cancer cells. With these results in hand, Pugasaram may be exploited further as a chemotherapeutic drug.

**KEYWORDS:** Arecanut, betel leaf, anticancer, Pugasaram, poly herbal gel, oral carcinoma.

### **INTRODUCTION**

Cancer is a dreaded disease wherein the cells undergo uncontrolled multiplication and proliferation throughout the body leading to mortality. Oral squamous cell carcinoma (OSCC) is one of the common cancers on humans in several countries. This cancer is a malignant neoplasia that arises in several parts of oral cavity including the lip, tongue, the skin lining the mouth and gums, beneath the tongue, at the base of the tongue, etc. Even throat cancers are also included in this category of oral cancer. The exact cause for this malady is mostly unknown. Though the use of tobacco is one of the risk factors for oral cancers, other causes are mostly skeptical without conclusive scientific evidences <sup>[1]</sup>.

Though there are several therapeutic options including surgery, radiation therapy and chemotherapy in modern medicine to combat cancer in its early stages, there has been growing demand in recent years to explore the potential of herbal plants as an alternative therapy for such diseases. Umpteen numbers of plants are already

known for their medicinal properties. Areca (*Areca catechu* L.) palm of the family Arecaceae or Palmae is one such plant having lots of medicinal properties [2]. It is a slender (about 15 cm dia) and tall palm (reaching up to 30m height) growing abundantly in several south and Southeast Asian countries [3]. Most of the parts of this palm, including its root, green stem, leaf, inflorescence, fruit and nut show such beneficial effects [4].

The nut of areca palm is known as arecanut. It is misnamed as betel nut in several parts of the world as this nut is commonly chewed along with betel leaf (the leaf of a tropical vine called betel vine (*Piper betle* L.) of Piperaceae family). Except for their ubiquitous presence in the chewing mixture, there is no other similarity between betel vine and areca palm [5]. However, the fact that, both arecanut and betel leaf are reported to exhibit lots of medicinal properties including anticancer activity [2, 6]. Considering these beneficial effects of these two common chewing components an herbal gel called Pugasaram was formulated and evaluated its anticancer property against oral squamous carcinoma cells.

## MATERIALS AND METHODS

### Preparation of Pugasaram

The preparation of Pugasaram is fully described in earlier publication [7]. It mainly contains arecanut, betel leaf, certain other herbs and honey.

### Methodology:

#### a. Cytotoxicity study

The cytotoxicity of Pugasaram on oral squamous carcinoma (CAL-27) cells was conducted *in vitro* by MTT assay and compared with that of the standard anticancer drug Cisplatin.

#### b. Cell viability Assay

The effect of Pugasaram on cell viability was studied at a concentration equivalent to its IC<sub>50</sub> value and compared with that of Cisplatin at its IC<sub>50</sub> concentration on CAL-27 cells. The CAL-27 cells were treated with Cisplatin and Pugasaram at the concentration of their IC<sub>50</sub> values for 48 hours and cell viability was assessed both by capturing the images of cells using crystal violet staining and also by visual counting of cell clusters. ZEISS inverted microscope was used to observe the images.

#### c. Cell Migration Assay

The CAL-27 cells were seeded on a culture plate, a scratch was made in the monolayer using a pipette tip and migrated cells into the gap were noted over a period of 72 hours in both the treatments. Images were captured at the beginning and regular intervals during the cell migration process. Image J software was utilized to measure the distance of cell migration across the gap in response to the drug treatment. The measurements were performed on images taken at different time points during the 72 hours

#### d. Colony Formation Assay

CAL-27 cells were treated with these two compounds and allowed to grow and divide over a period of 3-5 days. During the incubation period, the cells started to organize into visible colonies, which were readily observable to the naked eye. The colonies were then stained with 3% crystal violet stain and incubated in the dark for 1 hour to allow the stain to bind to the cells, and images of the stained colonies were captured. The captured images were then analysed to quantify the number of colonies formed in each well.

#### e. Live-Dead Assay

The live dead assay was performed to differentiate the live and dead cells in both the treatments. Images of the stained cells were captured and Image J software was employed to calculate the ratio of live and dead cells. The obtained images and calculations were used to determine the percentage of cell death in each

treatment group with respect to the control. The percentage of cell death was then converted into fold change values to compare the effect of the drug treatments.

## RESULTS

### a. Cytotoxicity study

The results indicated that both Pugasaram and Cisplatin exhibited dose-dependent cytotoxic effect on CAL-27 cells. As the concentration of these two drugs increased, the percentage of cell viability gradually decreased, indicating their cytotoxic impacts on such cancer cells. The half-maximal inhibitory concentration ( $IC_{50}$ ) of Pugasaram was found to be 500  $\mu\text{g/ml}$ , but for Cisplatin it was much less at 6  $\mu\text{g/ml}$  (Figures 1 and 2).

### b. Cell viability Assay

The crystal violet staining of CAL-27 cells treated with Pugasaram (500  $\mu\text{g/ml}$ ) and Cisplatin (6  $\mu\text{g/ml}$ ) for 48 hours showed a notable effect on cell viability compared to the control. The treated cells exhibited distinct staining patterns compared to the untreated control cells. Images captured using a ZEISS inverted microscope revealed disrupted cellular arrangement with irregular cell shapes and reduced cell density in treated cells compared to control. Specific changes such as changes in cell shape, clustering and detachment from the culture plate were also observed (Figure 3).

Manual counting of cells showed an inhibitory effect of both Cisplatin and Pugasaram on the population of CAL-27 cells (Figure 4). The untreated control group showed a population of 215-245 cells/ $\mu\text{l}$ , whereas it was only 10-20 cells/ $\mu\text{l}$  in Cisplatin treated group and 120-190 cells/ $\mu\text{l}$  in Pugasaram -treated group indicating that both Cisplatin and Pugasaram decreased the population of CAL-27 cells significantly ( $p < 0.001$  and  $p < 0.01$ , respectively).

### c. Cell Migration Assay

Visual inspection of the captured images revealed changes in the migration pattern of CAL-27 cells in response to the drug treatment. The images demonstrated that cells treated with Pugasaram exhibited less migration than the Cisplatin and control group (Figure 5).

### d. Colony Formation Assay

The photographs of CAL-27 cell colonies in different treatments are shown in Figure 6. Stained colonies exhibit varying sizes, shapes, and densities, which are readily observable to the naked eye. A noticeable difference in the number and size of colonies was observed between the control and the drug treated groups.

Statistical analysis of the colony counts revealed a significant decrease ( $p < 0.05$ ) in colony formation in response to the drug treatment compared to the control group (Figure 7).

### e. Live-Dead Assay

The visual observations show an increase in apoptosis in response to the drug treatment compared to the control (Figure 8).

There was a significant increase in apoptosis in the Cisplatin and Pugasaram treated groups compared to control ( $p < 0.001$  and  $p < 0.05$ , respectively) indicating the apoptotic effects of the drugs on CAL-27 cells (Figure 9).

## DISCUSSION

In Ayurvedic system of medicine, polyherbal formulation using multiple herbs is advocated to achieve better therapeutic results with reduced side effects. Arecanut is mostly chewed along with the leaf of *P. betle* and slaked lime (calcium hydroxide). This chewing mixture is commonly called betel quid or 'tambula'. There are

reports to show chewing of such traditional form of betel quid is a healthy practice [8, 9]. In a survey conducted on chewers and non-chewers of traditional, uncontaminated form of betel quid not a single instance of cancer was reported among 292 betel quid chewers, whereas there were two cancer patients among 232 non-chewers of betel quid [10]. The present study also confirms that the Pugasaram, which contains areca nut and betel leaf as major components, also exhibits effective anticancer property.

The knowledge on the anticancer property of arecanut is not new. In the year 1974, Kumari et al reported that in mice topical application of benzpyrene (BP), a carcinogenic inducing chemical, at 5 µg per animal three times a week showed tumour growth in all the treated mice (10/10) at the end of 39th week, but when arecanut extract (0.1 ml of 2.0 % solution) was applied along with BP no such tumour (0/10) was observed during that period [11].

Pai *et al* (1981) reported that even saccharin coated arecanut diet reduced the carcinogenic potential of carcinogenic inducing chemical 1,4-dinitrosopiperazine [12]. In the study conducted on mice with more than 20 animals in each group with an experimental period of 40 weeks it was reported that the tumours were more common in the group treated with 1,4-dinitrosopiperazine than in group treated with 1,4-dinitrosopiperazine along with the extract of saccharin coated arecanut.

In a very recent study conducted *in vivo* on Sprague-Dawley rat to find out the chemotherapeutic effect of arecanut extract on tongue cancer it was reported that at the end of 23 weeks of observation in animals treated with 30ppm of cancer inducer (4NQO) in drinking water for 12 weeks the incidence of tongue cancer was 71.43%, whereas it was 0% when arecanut aqueous extract was given in drinking water at doses of 500 and 1000 mg/kg BW for 22 weeks after a gap of one week of treatment of 4NQO for 12 weeks [13].

Arecoline, one of the important phytochemicals present in arecanut, also reported to be anticancerous. *In vitro* treatment of arecoline at concentrations ranging from 10 to 100 µg/ml for 24h inhibited the growth of basal cell carcinoma BCC-1/KMC cells in dose dependent manner, but showed no toxic effect on HaCaT cells [14]. It was also reported that the arecoline hydrobromide (AH) attenuated cancer cell proliferation and tumour growth in xenograft mice and there was complete inhibition of tumour growth at the maximum tolerated dose of 50mg/kg/day of AH treatment [15].

The other most common ingredient of betel quid is the leaf of *P. betle* (betel leaf). Even this leaf exhibits strong anticancer properties against several cancer cells including those causing skin, oral, fore-stomach and breast cancers [16]. The hydroxychavicol, the most abundant phenolic compound in betel leaf was reported to be responsible for such anticancerous activity [17]. This phytochemical of betel leaf was already reported to arrest the growth and multiplication of oral carcinoma KB cells as well [18].

Even studies conducted on laboratory animals substantiated the anticancerous property of betel leaf. A remarkable inhibition of prostate tumour volume by 72% was noticed in xenograft mice when hydroxychavicol was orally fed at a dose of 150 mg/kg bw daily for six weeks [19]. A significant reduction in tumour burden, its volume, weight, viable tumour cell count and increase in the life span was noticed in Ehrlich Ascites Carcinoma bearing mice when they were administered with the methanol extract of betel leaf intraperitoneally at 100 mg/kg bw for nine consecutive days [20].

In the present study the gel Pugasaram showed clear cytotoxicity on oral cancer CAL-27 cells with IC<sub>50</sub> value of 500 µg/ml. Our earlier study reported an IC<sub>50</sub> value of 109.32 µg/ml of Pugasaram against lung cancer A549 cell line [7]. This sort of variation in toxicity level against different cancer cells was reported in other studies as well. The ethanol extract of arecanut reported an IC<sub>50</sub> value of 629.50 µg/ml against the oral cancer HSC - 2 cells but against HSC - 3 cells it was only 164.06 µg/ml [21].

Sari *et al* (2018) reported apoptotic effect of arecanut ethanolic extract on HSC-2 and HSC-3 oral cancer cells [22]. These authors further postulated that areca nut could be used as a chemotherapeutic agent against human oral squamous cell carcinoma. The results of the present study are also in conformity with this wherein the antiproliferative effect of Pugasaram on CAL-27 oral cancer cells is clearly projected.

Thus, Poogasaram, which contains arecanut and betel leaf, in maximum proportions showed clear cytotoxicity and anticancer property on oral cancer CAL-27 cells. In earlier studies also this gel proved highly cytotoxic on other cancer like A 549 cells [7]. Hence, Pugasaram gel could be effectively exploited further as a chemo preventive ayurvedic drug.

## CONCLUSION

The results of this study demonstrate that Pugasaram gel exhibits dose-dependent cytotoxicity against CAL-27 cells, inducing apoptotic cell death, migration and reducing colony formation. These findings support the potential of Pugasaram as an effective Ayurvedic polyherbal preparation with anticancer properties. Further investigations, including in vivo studies and clinical trials, are warranted to validate its therapeutic potential and to explore its mechanisms of action in more detail.

## ACKNOWLEDGMENTS

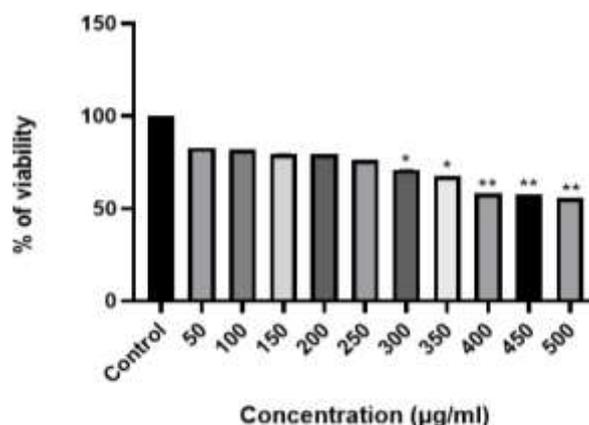
We gratefully acknowledge the financial grant sanctioned by CAMPCO Ltd., Mangaluru, Karnataka, India for this study.

## REFERENCES

1. Keshava Bhat S, Kumar PK, Sukesh Bhat. Arecanut (Areca catechu L) chewing and cancer: conflicting research findings. *Int J Dental Med Sci Res* 2022; 4(4): 21-32.
2. Keshava Bhat S. Arecanut (Areca catechu L): A storehouse of medicines. *Indian J Arecanut Spices Medicinal Plants* 2019; 21(1): 40-55.
3. Ananda KS. Botany. In: Arecanut. Balasimha D, Rajagopal V. (eds). Central Plantation Crops Research Institute, Kasaragod: 671 124, Kerala, India. 2004; pp7-50.
4. Keshava Bhat S, Sukesh Bhat. Bactericidal properties of different morphological parts of areca palm, Areca catechu L. and their potential applications. *Int J Pharmaceut Res Applications* 2024; 9(4): 1419-1426.
5. Keshava Bhat S, Ashwin D, Mythri S. Antidiabetic potential of Arecanut (Areca catechu L.) and certain arecanut formulations available for treating diabetes. *Indian J Arecanut, Spices Medicinal Plants* 2017; 19 (1): 23-31.
6. Toprani K, Patel D. Betel leaf: Revisiting the benefits of an ancient Indian herb. *South Asian J Cancer*. 2013; 2(3): 140–141.
7. Ganapathi Bhat J, Manorama Bhat G, Keshava Bhat S, Sukesh Bhat, Pujari GR. Anticancer property of an ayurvedic polyherbal preparation chiefly containing arecanut and betel leaf. *Int J Research Ayurveda Pharmacy* 2023; 14(1): 13-16.
8. Shukla A, Anupama S, Baghel AS, Vyas M. Ayurvedic Tambula Sevana- A Healthy Traditional Practice. *Int J Herbal Med* 2015; 3(1): 40-44.
9. Keshava Bhat S, Sukesh Bhat, Jagadish, Khandige SN, Kumar P. Chewing farm fresh Betel Quid: a healthy practice in major arecanut (Areca catechu L.) growing region of South India. *IOSR J Pharm Biol Sci* 2020; 15(5) Series 5: 10-21.
10. Jose CT, Keshava Bhat S, Chandran KP, Jayasekhar S, Gowda A. Traditional chewing of arecanut / betel quid and human health- a survey report. *Indian J Arecanut Spices Medicinal Plants* 2020; 22 (1): 3-15.

11. Kumari HL, Sirsi M, Bhargava MK. Inhibitory activity of *Areca catechu* on the development of mouse skin tumours induced by the chemical carcinogen 3-4, Benzpyrene. J Plantn Crops 1974; 2 (1): 23-29.
12. Pai SR, Shirke AJ, Gothoskar SV. Long-term feeding study in C17 mice administered saccharin coated betel nut and 1,4-dinitrosopiperazine in combination. Carcinogenesis 1981; 2 (3): 175-177.
13. Sari LM, Novita CF, Andriany P, Sari DK. The chemotherapeutic activity of areca nut extract increased stromal tumor-infiltrating lymphocytes in 4-nutriquinoline-1-oxide-tumor-induced Sprague-Dawley rats. F1000Research 2023; 11: 1571.
14. Huang LW, Hsieh BS, Cheng HL, Hu YC, Chang WT, Chang KL. Arecoline decreases interleukin-6 production and induces apoptosis and cell cycle arrest in human basal cell carcinoma cells. Toxicol Applied Pharmacol 2012; 258 (2): 199-207.
15. Fan J, Lin R, Xia S, Chen D, Elf SE, Liu S, *et al.* Tetrameric acetyl-CoA acetyltransferase 1 is important for tumor growth. Molecular Cell 2016; 64 (5): 859-874.
16. Rai MP, Thilakchand KR, Palatty PL, Rao P, Rao S, Bhat HP, *et al.* *Piper betle* Linn (Betel vine), the maligned Southeast Asian medicinal plant possesses cancer preventive effects: Time to reconsider the wronged opinion. Asian Pacific J Cancer Prev, 2011; 12, 2149-2156.
17. Amonkar AJ, Padma PR, Bhide SV. Protective effect of hydroxychavicol, a phenolic component of betel leaf, against the tobacco-specific carcinogens. Mutation Res 1989; 210 (2): 249–253.
18. Chang MC, Uang BJ, Wu HL, Lee JJ, Hahn LJ, Jeng JH. Inducing the cell cycle arrest and apoptosis of oral KB carcinoma cells by hydroxychavicol: roles of glutathione and reactive oxygen species. British J Pharmacol 2002; 135, 619-630.
19. Gundala SR, Yang C, Rao M, Paranjpe R, Brahmabhatt M, Pannu V, *et al.* Hydroxychavicol, a betel leaf component, inhibits prostate cancer through ROS-driven DNA damage and apoptosis. Toxicol Appl Pharmacol 2014; 280(1): 86–96.
20. Alam B, Majumder R, Akter S, Lee SH. Piper betle extracts exhibit antitumor activity by augmenting antioxidant potential. Oncology Letters 2015; 9: 863-868.
21. Sari LM, Subita GP, Auerkari EI. 2017. Potential antioxidant and cytotoxic activities of arecanut (*Areca catechu* Linn.) extract in human oral squamous cell carcinoma and keratinocyte cells. Asian J Pharmaceutical Clinical Res 2017; 10 (10): 286-291.
22. Sari LM, Subita GP, Auerkari EI. Areca nut extract demonstrated apoptosis-inducing mechanism by increased caspase-3 activities on oral squamous cell carcinoma. F1000 Research 2018; 7: 723.

## Figures



**Figure 1.** The graph illustrates the dose-response relationship between the concentration of Pugasaram and the percentage of cell viability in CAL-27 cells. \* $p < 0.05$ , \*\* $p < 0.01$  compared to control.

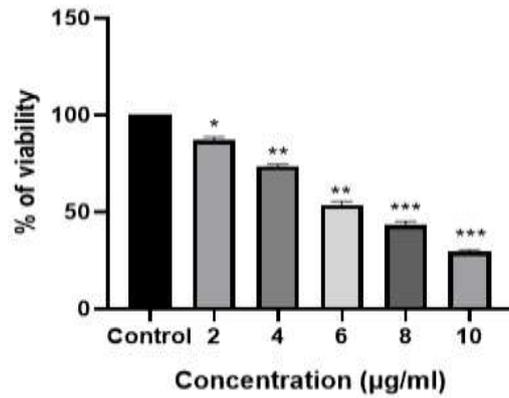


Figure 2. The graph illustrates the dose-dependent relationship between the concentration of Cisplatin and the percentage of cell viability in CAL-27 cells. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared to control.

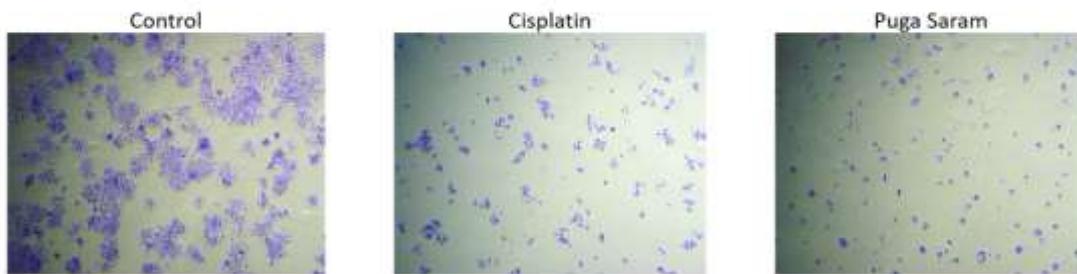


Figure 3. Photographs of CAL-27 cells treated with Cisplatin and Pugasaram compared to the control cells. The treatments with Cisplatin and Pugasaram have induced notable effects on cell morphology and cell attachment in CAL-27 cells.

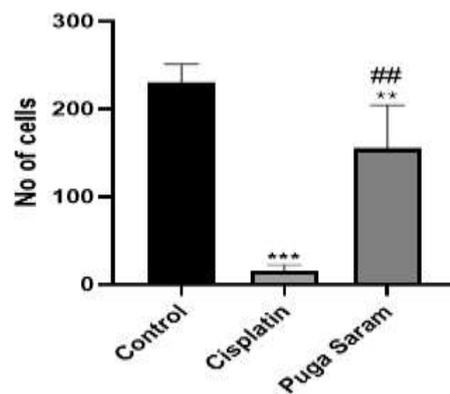


Figure 4. The results of manual counting of CAL-27 cells in all three treatments. \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared to control, ## $p < 0.01$  compared to Cisplatin.

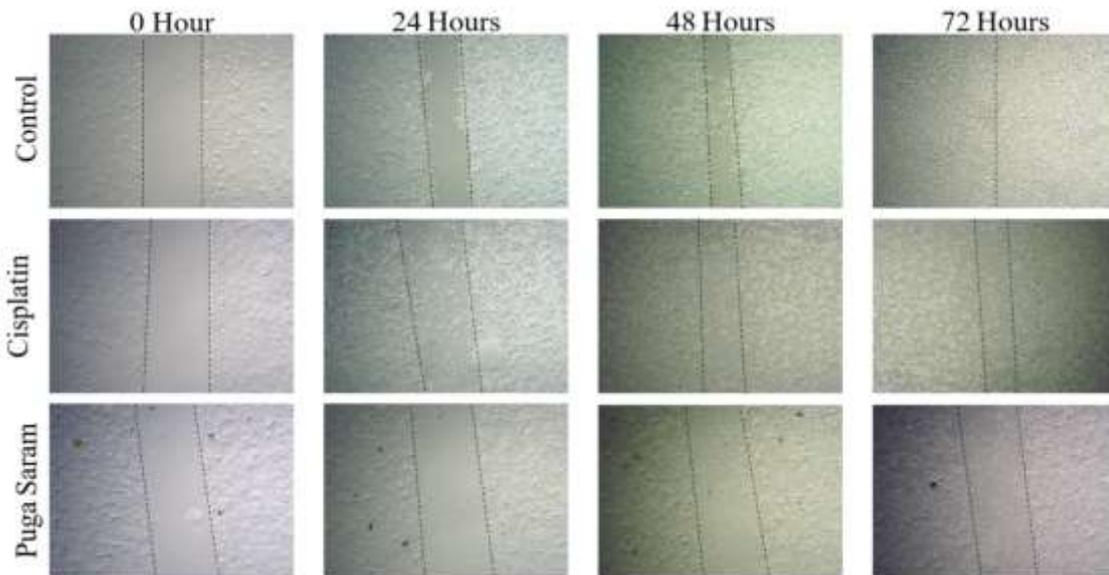


Figure 5. Series of images illustrate the effect of Pugasaram and Cisplatin on the migration of CAL-27 cells in comparison with that of control.



Figure 6. The photographs of CAL-27 cell colonies in different treatments. A significant decrease in colony formation in response to the drug treatment compared to the control is observed.

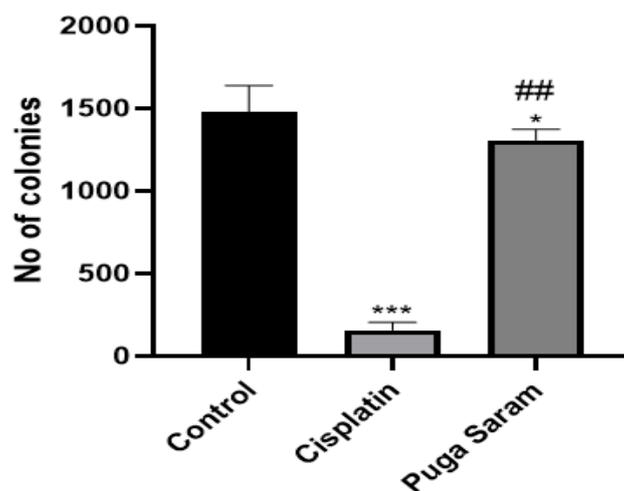


Figure 7. The graph showing the difference in the number of colonies formed between the control and treated groups. \* $p < 0.05$ , \*\*\* $p < 0.001$  compared to control, ## $p < 0.01$  between Pugasaram and Cisplatin.

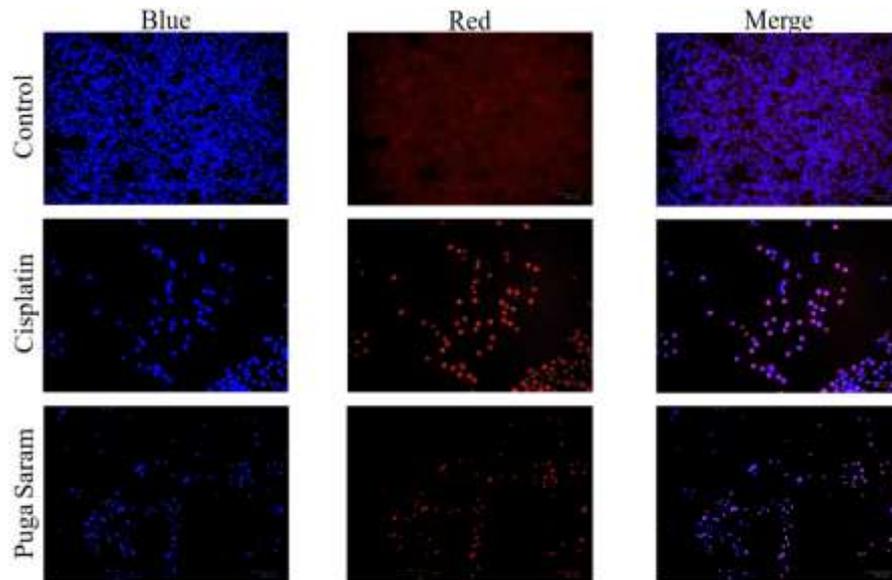


Figure 8. The captured images show stained CAL-27 cells that were treated with Cisplatin and Pugasaram to differentiate between live and dead cells. The images show distinct fluorescence signals: blue fluorescence representing live cells stained with DAPI and red fluorescence indicating apoptotic cells stained with PI.

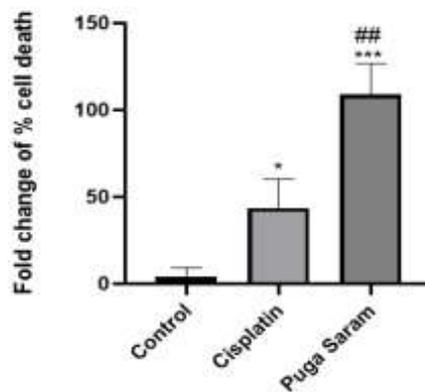


Figure 9. The graph displays the fold change in apoptosis relative to the control group. \* $p < 0.05$ , \*\*\* $p < 0.001$  compared to control, ## $p < 0.01$  compared to Cisplatin.