

Anticancer effect of Piper betle leaf extract on KB cell lines – an in vitro study.

Sadiya R Veettil¹, E Anuradha Sunil², Archana Mukunda², Arun Mohan², Shanly John³, Meera K Pynadath²

ABSTRACT

Background: Various researchers have stated a causal association of betle quid chewing with oral cancer and other potentially malignant disorders of oral cavity. On the contrary, *Piper betle* leaf when used alone has potential medicinal benefits including anticancer, anti-helminthic, hepato-protective and antioxidant activities. In this study we examined the anti-cancer activity of *Piper betle* extract (aqueous) on KB- cancer cell lines

Aims: To observe the anti- cancer activity of *Piper betle* leaf extract on KB cancer cell lines.

Setting and Design: The study was conducted in Biogenix Research Centre, Thiruvananthapuram. The KB cancer cell lines were procured from NCCS, Pune.

Methods and Material: The cancer cell lines were treated with increasing concentration of *Piper betle* leaf extract 6.25, 12.25, 50 & 100µg/ml. The cytotoxic effect of the extract on the cells was studied by physical indicators of cytotoxic changes by observing the cells under an inverted phase contrast microscope, for any detectable changes in the cell morphology and by MTT assay method to assess the percentage of viability of cells.

Results: The cancer cells showed considerable changes in the cell morphology suggestive of cell cytotoxicity and apoptosis after the treatment with the extract. The results of the MTT assay showed that the percentage viability of the cancer cells decreased with increasing concentrations of the extract, The percentage of viability of cells was noted to be 43.42% with the highest concentration of 100µg/ml of *Piper betle* leaf extract which proves that *Piper betle* leaf extract has anticancer activity.

Conclusion: The cytotoxic potential of *Piper betle* leaf may be used to develop chemotherapeutic agent, but further focused studies of anticancer properties and isolation of compounds from *Piper betle* leaf are necessary to prove its worth in the cancer therapy.

Key words: Anticancer activity, KB cell lines, MTT assay

Oral and Maxillofacial Pathology Journal (2022): <https://www.ompj.org/archives>.

INTRODUCTION

Plant based ayurvedic components are greatly in use today because of excellent beneficial effects without side effects. Hence they have been used for simple illness like cold and cough to life threatening diseases like infections and cancer. Oral cancer is the sixth most common malignancy in the world. It is of major concern in Southeast Asia primarily because of the prevalent oral habits of betle quid chewing, smoking, and alcohol consumption. A working group of the International Agency for Research on Cancer (IARC) concluded that there was adequate evidence of a causal association between tobacco and betle quid (BQ) chewing habits and oral mucosal diseases such as leukoplakia, oral submucous fibrosis and oral cancer.¹ Despite the advancements in therapies like chemotherapy, radiation therapy, hormones and surgery, the five year survival rate has not changed for the past few decades. Studies are still on-going to search for natural-based antiproliferative and chemopreventive agents which can act as alternatives to the chemically-synthesized drugs and which are potentially less toxic with fewer side effects. Studies have reported that various plants and their bioactive compounds have been shown to have anticarcinogenic and anti-proliferative

¹ Oral Pathologist and Private Consultant, Guruvayoor, Kerala;

² Dept of Oral and Maxillofacial Pathology, Royal Dental College, Chalissery, Kerala; ³ Assistant Dental Surgeon, Taluk Hospital, Peravoor, Kannur, Kerala

Corresponding author: Dr Archana Mukunda Professor Dept of Oral and Maxillofacial Pathology, Royal Dental College, Chalissery, Kerala. E-mail: archanamukunda@yahoo.co.in

How to cite this article: Veettil SR, Sunil EA, MukundaA, Mohan A, Shanly John, Pynadath MK. Anticancer effect of Piper betle leaf extract on KB cell lines – an in vitro study. Oral Maxillofac Pathol J 2022; 13(1): page no. 28-31

Source of Support: Nil

Conflict of Interest: None

effects towards cancer cells, suggesting the potential use of these extracts in inhibiting cancer cell growth. *Piper betle* leaf, although a main ingredient of the betel quid, has been shown to have anticancer, antioxidant, antiseptic, antifungal, astringent and carminative effects. *Piper betle* leaf is a medicinal plant that is traditionally used in catarrhal and pulmonary affections, as a

digestive and carminative and as a stimulant of pancreatic lipase.² *Piper betle* leaf belongs to the Piperaceae family and is thought to have originated in South East Asia.³ The leaves of the plant are commonly chewed with areca nut, slaked lime and sometimes tobacco. Scientifically, studies have reported the biological benefits of *Piper betle* leaf only to include inhibition of platelet aggregation, anti-diabetic activities, immunomodulatory properties and anti-allergic activities.⁴

KB cell lines are the most commonly used cell lines in research related to oral squamous cell carcinoma as KB cell lines have been presumed to be derived from epidermoid carcinoma of the mouth. They are currently considered as contaminated HeLa cell lines which stain positive for keratin with immunoperoxidase stain and contain Human papilloma virus 18 (HPV 18) sequence.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method is a colorimetric assay based on the activity of mitochondrial succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate to an insoluble purple colored formazan product (Fig. 1).^{5,6} The present study examined the anti-cancer activity of *Piper betle* leaf extract (aqueous extract) on KB-cancer cell lines by MTT assay method and the viability of cells were examined by direct observation of cells in an inverted phase contrast microscope.

MATERIALS AND METHODS

Cell lines and cell culture

We used KB cell lines in our current study as KB cancer cell line was initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecos modified Eagles medium (Gibco, Invitrogen). The cell line was cultured in 25 cm² tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: (Penicillin (100U/ml), Streptomycin (100µg/ml and Amphotericin B (2.5µg/ml)). Cultured cell lines were kept at 37°C in a humidified 5% CO₂ incubator (NBS Eppendorf, Germany).

Preparation of the extract

This current study was conducted using home grown *Piper betle* leaf and processed for extraction as per the Soxhlet Method. We decided to use only *Piper betle* leaf extract in our study as we wanted to test the effect its extract in various concentrations. We preferred *Piper betle* leaf as they are grown here, easily accessible and commonly used in our region. The solid sample is mixed with anhydrous sodium sulfate, placed in an extraction thimble, and extracted using distilled water as solvent in a Soxhlet extractor. The extract is then dried, and as necessary five concentrations of (6.25µg, 12.5µg, 25µg, 50µg, 100µg) solutions were prepared. The sample solution was filtered through 0.22 µm Millipore syringe filter to ensure the sterility.

Cell Treatment Procedure

Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, seeded in 96 well tissue culture plate and incubated at 37°C in a humidified 5% CO₂ incubator. After 24 hours the growth medium was removed, cells were treated with *Piper betle* leaf extract of increasing concentrations (6.25µg, 12.5µg, 25µg, 50µg, 100µg) in respective wells and incubated for 24 hours.

Cytotoxicity Assay by Direct Microscopic observation:

Entire plate was observed at an interval of each 24 hours; up to 72 hours in an inverted phase contrast tissue culture microscope

(Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observations were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.⁷

Cytotoxicity Assay by MTT Assay Method:

15 mg of MTT (Sigma, M-5655) was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization. After 24 hours of incubation period, the sample content in wells were removed and 30µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37°C in a humidified 5% CO₂ incubator for 4 hours. After the incubation period, the supernatant was removed and 100µl of MTT Solubilization Solution (DMSO was added and the wells were mixed gently by pipetting up and down in order to solubilise the formazan crystals. The absorbance values were measured by using microplate reader at a wavelength of 540 nm. Laura B. Talarico et al, 2004.⁸ The percentage of growth inhibition were calculated using the formula.

$$\text{Percentage viability} = \frac{\text{Mean OD Samples}}{\text{Mean OD of control group}} \times 100$$

The LD50 value was calculated using calculated using ED50 PLUS V1.0 Software.

RESULTS

The results indicated that *Piper betle* leaf extract had significant activity against cancer cell lines. Direct microscopic observations under inverted phase contrast microscope, showed detectable changes in the morphology of the cells - such as rounding or shrinking of cells, granulation and cytoplasmic vacuolization which were considered as indicators of cytotoxicity. We treated the KB cell line with increasing concentrations 6.25µg, 12.5µg, 25 µg, 50 µg, 100 µg of *Piper betle* leaf extract. We noted that the cytotoxic changes observed under microscope were directly proportional to the increasing concentration of *Piper betle* leaf extract (Fig.2).

The toxic effect of *Piper betle* leaf extract on KB cell lines was assessed using MTT method. This assay measures the viability of cells and hence can be done only in metabolically active cells. The viability of cells can be appreciated by change in color from purple to reduction in color graduation. The intensity of the color is directly proportional to the amount of viable cells and is measured using optical density (OD) values. Based on our findings, we observed that the percentage viability of cells decreased with increasing concentrations of *Piper betle* leaf extract (Fig.3). Pearson correlation analysis revealed a significant negative correlation between the concentration of the extracts and the percentage viability of the cancer cells. (R= - 0.96 p= 0.032) (Fig. 4). The percentage of viability calculated using the above mentioned formula using ED50 PLUS V1.0 Software.

DISCUSSION

Piper betle leaf an invaluable medicinal plant where its leaves have been used for many medicinal purposes. Scientific researches on this plant revealed that it possesses many beneficial bioactivities and has a great potential to be used in developing medicinal products. Akira Murakami et al (2014) in their study, tested *Piper betle* leaf extract at a concentration of 8µg/ml where it inhibited EBV activation by 55% without toxicity.^{8,10} Devjani Banerjee et al (2014) observed that the leaf extracts of *Piper betle* leaf have antiproliferative and chemopreventive potential.¹¹ A. R. Fathilah et

al (2010) compared the antiproliferative activity of Psidium guajava and *Piper betle* leaf extracts on the proliferation of cancerous cell lines and found that both plant extracts are equally potent for the treatment of cancerous oral epidermal lesions.¹² Badrul Alam et al (2015) evaluated the methanolic extract of *Piper betle* leaf with regard to antitumor activity against Ehrlich Ascites Carcinoma [EAC]; and the results showed that the *Piper betle* leaf extracts exhibit significant antitumor activity which may be attributed to the augmentation of endogenous antioxidant potential.¹³

Piper betle leaf contains a wide variety of biologically active compounds whose concentration depends on the variety of the plant, season and climate. The aroma of *Piper betle* leaf is due to the presence of essential oils, consisting of phenols and terpenes.¹⁴

Table 1: The percentage of viability of cells with varying concentrations of aqueous extract of Piper betle leaf.

Sample Concentration (µg/mL)	OD Value I	OD Value II	OD Value III	Average OD	Percentage Viability
Control	0.5301	0.5272	0.5226	0.5266	100
6.25	0.3179	0.3090	0.4114	0.3461	65.72
12.5	0.3139	0.3008	0.2969	0.3039	57.70
25	0.3054	0.2985	0.2960	0.3000	56.96
50	0.2996	0.2935	0.2642	0.2858	54.27
100	0.2329	0.2247	0.2284	0.2287	43.42

The various phytochemicals found in the *Piper betle* leaf plants are chavibetol, chavicol, hydroxychavicol, estragole, eugenol, methyl eugenol, hydroxycatechol, caryophyllene, eugenol methyl ether, cadinene, γ-lactone, allyl catechol, p-cymene, cepharadiene A, dotriacontanoic acid, triacontane, p-cymene, terpinene, eucalyptol, carvacrol, sesquiterpenes, cadinene, caryophyllene, dotriacontanoic acid, hentriacontane, pentatriacontane, stearic acid, n-triacontanol, triacontane, piperlonguminine, allylpyrocatecholdiacetate, isoeugenol, 1, 8-cineol, α-pinene, β-pinene, sitosterol, β-sitosterolpalmitate, γ-sitosterol, stigmasterol, ursolic acid, ursolic acid 3β-acetate.¹⁵

Tumor cells have increased production of ROS, causing oxidative stress and disturbing the redox state, leading to DNA damage, mutations and altered gene expression which contributes to carcinogenesis. At the same time, cancer cells have reduced capacity to remove ROS due to altered antioxidant defense systems. However, ROS also play important roles in inducing apoptosis, implying an anti-cancer effect. Hence finding the right balance between ROS and antioxidant defense levels in cancer cells is important to ensure that cancer progression can be inhibited while at the same time maintaining apoptosis. One of the possible action for the antiproliferative effects of this extract occurred through increased activities of antioxidant enzymes which helped in maintaining the balance between ROS production

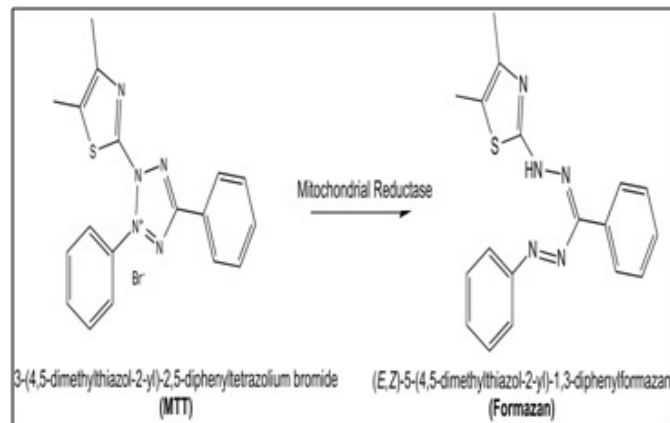


Fig.1: The Principle of MTT assay

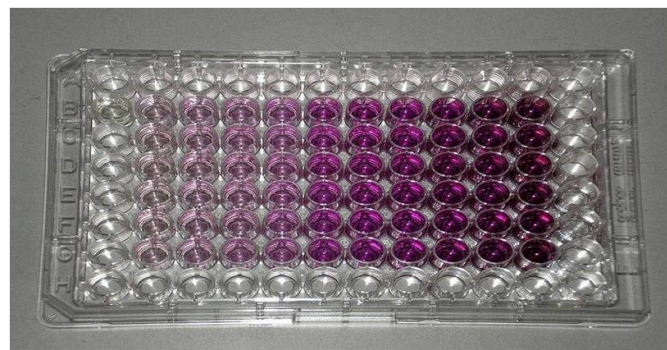


Fig.3: MTT assay showing intensity of the color is directly proportional to the amount of viable cells

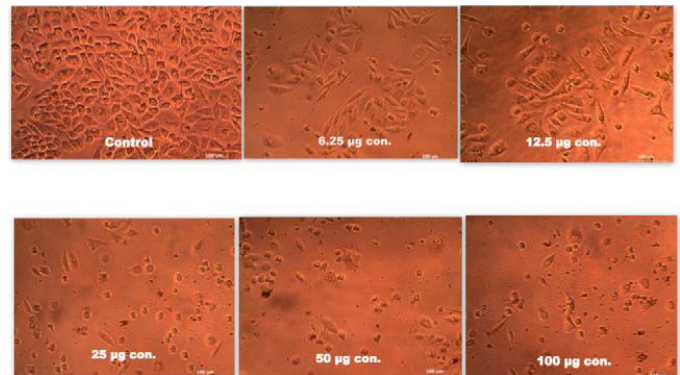


Fig.2: The cytotoxic effect of piper betle leaf extract on control sample and increasing concentration of piper betle leaf extract on KB cell lines

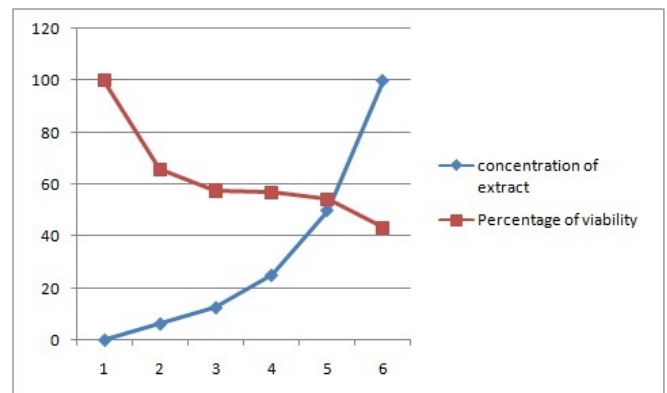


Fig.4: Pearson correlation analysis showing decrease in the percentage of viability of KB cells with increase in concentration of piper betle leaf extract

and removal. Also, previous literatures have cited many reasons for this antiproliferative effect. An active compound of *Piper betle* leaf, hydroxychavicol (HC), inhibited the attachment of KB cells to type I collagen and fibronectin, and subsequently resulted in cell cycle arrest in S and G2/M phases.¹⁶

CONCLUSION

From our study and the previous literatures, there is great deal of evidence that *Piper betle* leaf has potent anti-carcinogenic activities. Future studies are required to investigate the isolation of lead compounds responsible for the anticancer and anti proliferative activity of this plant.

REFERENCES

1. Nair U, Bartsch H and Nair J. Alert for an epidemic of oral cancer due to use of the betle quid substitutes gutkha and pan masala: a review of agents and causative mechanisms. *Mutagenesis* 2004;19:251-262.
2. Gundala SR and Aneja R. Piper betel leaf: a reservoir of potential xenohormetic nutraceuticals with cancer-fighting properties. *Cancer Prev Res (Phila)* 2014;7(5):477-86.
3. Abraham N.N, Kanthimathi M.S, Aziz A.A. Piper betle shows antioxidant activities, inhibits MCF-7 cell proliferation and increases activities of catalase and superoxide dismutase; *BMC Complementary and Alternative Medicine* 2012;12:220
4. Patra B, Das M T, Mihir & Dey S K. (2016). A review on Piper betle L. *Journal of Medicinal Plants Studies*. 2016;185:185-192.
5. Hossain MD, Anwar M, Akthar S, Numan SM. Uses Impact of Betle Leaf (*Piper betle* L.) *Public Health. Science Journal of Public Health* 2017;5:408-410.
6. Shah GA, Shah TI, Telang S. Anti-proliferative efficacy of piper betle leaf extracts against b16f10 melanoma in an in-vivo experimental model; *World Journal of Pharmacy and Pharmaceutical Sciences* 2016;5:835-843.
7. Roy UB and Vijayalaxmi KK. Evaluation of Cytotoxic Activity of Piper betle Linn. Using Murine and Human Cell Lines In Vitro. *IJSER* 2013;4:221-233.
8. Damonte E.B, Pujol C.A, Coto C.E. Prospects for the therapy and prevention of dengue virus infections. *Adv. Virus Res.* 2004;63:239-285.
9. Dinesh M.D, Athira P.S, Ajma N, Abhisha N.C, Ashitha C. Anti- cancer Activity (Oral) of Betle Leaf Extract by in- vitro. *IJLSSR* 2015;2:412-414.
10. Murakami A, Ali AM, Salleh KM, Koshimizu K & Ohigashi H. Screening for the In Vitro Anti-tumor-promoting activities of Edible Plants from Malaysia. *Bioscience, Biotechnology, and Biochemistry* 2014;64:9-16.
11. Banerjee D & Shah B. Antiproliferative activity of Piper betle leaf extracts on Human Lung Cancer cell line (A549). *IJPPS* 2014;6:432-435.
12. Fathilah AR, Sujata R, Norhanom AW and Adenan MI. Antiproliferative activity of aqueous extract of Piper betle L. and Psidium guajava L. on KB and HeLa cell lines. *JOMPR* 2010;4:987-990.
13. Alam B, Majumder R, Akter S, Lee SH. Piper betle extracts exhibit antitumor activity by augmenting antioxidant potential; *Oncology letters* 2015;9:863-868.
14. Sripradha S. Betle Leaf-The Green Gold. *J. Pharm. Sci. & Res* 2014; 6:36-37.
15. Toprani R, Patel D. Betle leaf: Revisiting the benefits of an ancient Indian herb. *South Asian Journal of Cancer*. 2013;2:140-141.
16. Chang M C, Uang B J, Wu H L, Lee J J, Hahn L J, Jeng J H. Inducing the cell cycle arrest and apoptosis of oral KB carcinoma cells by hydroxychavicol: roles of glutathione and reactive oxygen species. *Br J Pharmacol.* 2002;135: 619-630.