Research Article (Open access)

Anti-cancer Activity (Oral) of Betel Leaf Extract by *in- vitro*

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Received: 19 April 2016/Revised: 29 May 2016/Accepted: 13 June 2016

ABSTRACT- Cancer is defined as the uncontrollable growth of cells that invade and cause damage to surrounding tissue. Oral cancer appears as a growth or sore in the mouth that does not go away. Nowadays medicinal plants widely used for the treatment of cancer. In our present study we examined the anti- cancer activity of Piper betel leaf extract (aqueous extract) using KB-cell lines obtained from National Centre for Cell Science (NCCS), Pune. The cytotoxic assay was studied by MTT assay method. The LD-50 value was found as 81.157µl/ml extracts necessary for the 50% of cell death. These observations point out that Piper betel extract have considerable activity alongside oral cancer cell lines.

Key-Words: Oral cancer, Cell line, MTT assay, Piper betel

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INTRODUCTION

Oral cancer, which takes account of cancers in lips, tongue, cheeks, floor of the mouth, hard and soft palate, sinuses, and pharynx (throat), can live and life fear-provoking if not diagnosed and care for early. Oral cancer is the eleventh most common cancer globally [1]. There is a wide geographical variation in the incidence of oral cancer, with approximately two-thirds of patients in the developing countries of Southeast Asia, Eastern Europe and Latin America [2]. India has one of the highest incidences of oral cancer (age-standardized rate of 9.8 per 10 000) making it the most common cancer among men (men: women ratio 2:1) and accounts for about 30% of all new cases annually [3].

Access this article online	
Quick Response Code:	Website: www.ijlssr.com
ISSN 2455-1716	DOI: 10.21276/ijlssr.2016.2.4.14

SIGN AND SYMPTOMS

The most common symptoms of oral cancer include swellings/thickenings, lumps or bumps, rough spots/ crusts/or eroded areas on the lips, gums, or other areas inside the mouth and the development of velvety white, red, or speckled (white and red) patches in the mouth. Other symptoms include unexplained bleeding in the mouth, inexplicable numbness, loss of feeling, or pain/ tenderness in any area of the face, mouth, or neck, Persistent sores on the face, neck, or mouth that bleed easily and do not heal within 2 weeks, soreness or feeling that something is caught in the back of the throat, difficulty chewing or swallowing, speaking, or moving the jaw or tongue, hoarseness, chronic sore throat, or change in voice, change in the way your teeth or dentures fit together and dramatic weight loss.

Risk factors for the development of oral cancer include smoking Cigarette, cigar, or pipe smokers, smokeless tobacco users, users of dip, snuff, or chewing tobacco products (50 times more likely to develop cancers of the cheek, gums, and lining of the lips), excessive consumption of alcohol (about six times more common in drinkers than in non drinkers), family history of cancer, excessive sun exposure, and Human papillomavirus (HPV). Certain HPV strains are etiologic risk factors for Oropharyngeal Squamous Cell Carcinoma (OSCC). It is important to note that over 25% of all oral cancers occur in people who do not smoke and who only drink alcohol occasionally.

Aetiology

Tobacco is the single most important risk factor for oral cancer. In comparison to people who never smoked, the relative risk of oral cancer is 5.3 for people smoking <15 cigarettes per day, and 14.3 for people who smoked >25 cigarettes per day. In India the use of smokeless tobacco is rampant in the form of betel quid (*pan*) that contains areca nut and lime with dried tobacco leaves; this form of tobacco has been shown to be highly carcinogenic [4]. Tobacco and alcohol share a synergistic relationship, with alcohol promoting the carcinogenic effects of tobacco leading to a multifold increase in the risk of oral cancer with combined alcohol and tobacco exposure. Heavy drinkers and smokers have 38 times the risk of oral cancer compared with abstainers [5]. Human papillomavirus (HPV) is widely accepted as a causal factor for cancer arising in the lymphoepithelium of the oropharynx; its presence in lesions of oral cavity is less common; and its contribution to oral cancer development is uncertain. The majority of these were oral cavity cancers. However, other studies do not substantiate such a high rate of HPV in oral cancer. Malnutrition, vitamin deficiency, poor dental and oral hygiene are additional predisposing factors for oral cancer [6].

Prevention and control

Routine dental examination is one of the most excellent diagnosed methods; dentist will conduct an oral cancer screening exam. More specifically, dentist will noticed any appetence lumps or irregular tissue changes in neck, head, face, and oral cavity. Dentist may perform an oral brush biopsy if he or she sees tissue in your mouth that looks suspicious. This test is painless and involves taking a small sample of the tissue and analyzing it for abnormal cells. Alternatively, if the tissue looks more suspicious, dentist may recommend a scalpel biopsy. This procedure usually requires local anaesthesia and may be performed with a specialist. These tests are necessary to detect oral cancer early, before it has had a chance to progress and spread.

Oral cancer is treated the same way many other cancers are treated with surgery to remove the cancerous growth, followed by radiation therapy and/or chemotherapy (drug treatments) to destroy any remaining cancer cells. The method of prevention of oral cancers include don't smoke or use any tobacco products, drink alcohol in moderation (and refrain from binge drinking), Eat a well balanced diet, limit your exposure to the sun, (repeated exposure increases the risk of cancer on the lip, especially the lower lip. When in the sun, use UV-A/B-blocking sun protective lotions on your skin, as well as your lips).

MATERIALS AND METHODS

This research work was completed in the period of March to May 2016 in the department of Microbiology Pazhasiraja College, Pulpally Wayanad and *in vitro* studies was carried out by Biogenix research Centre Trivandrum, Kerala.

Collection of Plant material and preparation of crude extract

Piper betel leafs commonly known as betel leafs collected from Wayanad district, Kerala, South India and extracts were prepared by the method of [7] using distilled water as the solvent.

Cell culture

The anti- cancer (oral cancer) activity of Piper betel leaf extracts on KB cells was studied in Biogenix research centre, Trivandrum, Kerala in order to determine the cell viability after plant extract introduction that measures membrane integrity and effect of the plant extract on cell growth.

The cell line (KB- oral cancer) was obtained from National Centre for Cell Science (NCCS), Pune, India and grown in Dulbeccos modified Eagles Medium (EMEM) containing 10% fetal bovine serum (FBS). The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphoteracin B (2.5µg/ml). Cultured cell lines were maintained at 37^0 C, 5% CO2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week [8,9].

Cell Treatment Procedure

Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100µl cell suspension ($5x10^4$ cells/well) was seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator. 1 mg of Piper betel Lyophilized leaf powder was added to 1ml of DMEM and dissolved completely by cyclomixer. After that the extract solution was filtered through 0.22 µm Millipore syringe filter to ensure the sterility.

After 24 hours the growth medium was removed, freshly prepared plant extracts in 5% DMEM were five times serially diluted by two fold dilution (100 µg, 50 µg, 25 µg, 12.5 µg, 6.25 µg in 100 µl of 5% MEM) and each concentration of 100 µl were added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO_2 incubator. The viability of cells were evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method.

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 observation 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.

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 37ºC in a humidified 5% CO_2 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 570 nm (Laura B. Talarico et al., 2004). The percentage of growth inhibition was calculated using the formula:

% Cell viability = Abs (sample)/Abs (control) x 100

RESULTS AND DISCUSSION

The anticancer activity of Piper betel leaf extracts on KB cell lines were studied in Biogenix Research Centre Trivandrum, Kerala. The viability of cells treats with Piper betel leaf extracts reduce in concentration- dependent manner (Table- 1). Higher extract concentrations exhibited stronger anticancer activity. On direct microscopic observations 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. The LD- 50 value was found as 81.157µl/ml extracts needed for the 50% of cell death. Results indicated that Piper betel extract had significant activity against oral cancer cell lines.

Percentage of cell viability determined by MTT Assay method. The % Cell viability was calculated by using the following formula;

% Cell viability = Abs (sample)/Abs (control) x100

Table 1: *in-vitro* anticancer activity of Piper betel leaf extracts

Sample Concentration (µg/ml)	Average OD at 540 nm	Percentage Viability
Control	1.5186	
6.25	1.3822	91.01804
12.5	1.2103	79.69841
25	1.1228	73.93652
50	0.8506	56.01212
100	0.6933	45.65389

LD50 value =81.157 (ED50plus software v1.0)

CONCLUSIONS

Chewing betel quid is addictive and is reportedly done daily by as many as 200- 600 million people globally. Betel leaves are also good for health with lot of medicinal application including anti-diabetic, cardiovascular, anti-inflammatory/ immunomodulatory, anti-ulcer, hepato-protective, anti-infective, etc. Betel leaves has too shown to prevent oral cancer by maintaining the levels of ascorbic acid in the saliva. Ascorbic acid is an excellent source of antioxidant, which helps decrease the free radicals in the body, accordingly preventing cancer. The routine of chew betel leafs in a well manner, do not mix it with any flavourings and nut commonly known as "betel quid" it will cause any adverse effects.

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Source of Financial Support: Nil

Conflict of interest: Nil

