

Inhibition of cell division by *Piper betle* against B16F10 melanoma in an *in-vivo* experimental model

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ABSTRACT

Medicinal plants occupy an important position for being the paramount sources of drug discovery against cancer. A huge reservoir of bioactive compounds exists in many species of plants of earth. The isolation, identification of these active photochemical compounds and their pharmacological studies may be considered and studied elaborately to treat effectively for various types of cancer. *Piper betle* L. is a well known medicinal plant used in ayurvedic medicines as hepatoprotective, analgesic, antispasmodic, antidiabetic, antiviral and antibacterial agent. The present study was carried out to evaluate the anti melanoma activity of *Piper betle* L. leaves using different extracts viz: Pet. Ether, ethyl acetate, aqueous and ethanol in an *in vivo* model. The different dose concentrations were given per oral as 250mg/kg and 500mg/kg body weight of all the extracts. A significant reduction in size of tumor was observed in a dose dependent manner. The deviation observed in tumor reduction was recorded as $1338 \pm 1.555 \text{ mm}^3$ at 250mg/kgbw and $1307 \pm 1.697 \text{ mm}^3$ at 500mg/kgbw, $1112 \pm 1.343 \text{ mm}^3$ at 250 mg/kgbw and $874 \pm 1.626 \text{ mm}^3$ at 500 mg/kgbw, $964 \pm 1.414 \text{ mm}^3$ at 250 mg/kgbw and $627 \pm 1.838 \text{ mm}^3$ at 500 mg/kgbw, $1108 \pm 1.131 \text{ mm}^3$ at 250 mg/kgbw and $819 \pm 0.919 \text{ mm}^3$ at 500 mg/kgbw for pet ether, aqueous, ethyl acetate and ethanol respectively. Among all the four extracts ethyl acetate 500mg/kgbw with tumor reduction from $1390 \pm 1.838 \text{ mm}^3$ to $627 \pm 1.838 \text{ mm}^3$ showed significant activity. After *in vivo* screening of crude extracts it was concluded that ethyl acetate extract contains some antitumor agents when applied against mice bearing melanoma and depicted significant reduction in tumor size.

Keywords: *Piper betle* L., Melanoma, Anti-cancer properties, Experimental model

I INTRODUCTION

Natural products discovered from medicinal plants have played an important role in treatment of cancer, which is projected to become the major cause of death worldwide. A wide number of plant extracts are used against diseases in various systems of medicine such as ayurveda, unani and siddha. Only a few of them have been scientifically explored. Plant derived natural products such as flavonoids, terpenes and alkaloids [1-3] and soon has received considerable attention in recent years, due to their diverse pharmacological properties including cytotoxic and cancer

chemopreventive effects [4]. Plant based systems continue to play an essential role in healthcare and it has been estimated by the WHO that approximately 80% of the world's inhabitants rely mainly on traditional medicine for their primary health care [5]. The National Cancer Institute collected about 35,000 plant samples from 20 countries and has screened around 114,000 extracts for anticancer activity [6]. Of the 92 anticancer drugs commercially available prior to 1983 in the US and among worldwide approved anticancer drugs between 1983 and 1994, 60% are of natural origin [7]. Scientists all over the world are concentrating on the herbal medicines to boost immune cells of the body against cancer. By understanding the complex synergistic interaction of various constituents of anticancer herbs, the herbal formulations can be designed to attack the cancerous cells without harming normal cells of the body [8,9].

Piper betle Linn.(Family Piperaceae) commonly known as the betel vine is an important medicinal and recreational plant in Southeast Asia. The most probable place of its origin of betel vine is Malaysia but today the plants are cultivated in India, Srilanka, Bangladesh, Burma and Nepal [10,11]. Betel leaves are the most important plant part with medicinal, religious and ceremonial value in the South East Asia. The leaves are credited with wound healing, digestive, and pancreatic lipase stimulant activities in the traditional medicine [12]. Its constituent phenolics were found to show impressive gastroprotective, [13] and anti-inflammatory as well as immunomodulatory properties [14]. In India it is customary to serve betel leaf on various occasions and is also offered to guests as a mark of respect (referred to as tambool) [15]. The therapeutic and medicinal uses of its leaves and roots have been mentioned in literature [16-18]. Based on the color, size, taste and aroma there are many varieties of betel leaves and some of the most popular varieties are the Magadhi, Venmony, Mysore, Salem, Calcutta, Banarasi, Kauri, Ghanagete and Bagerhati [19-20]. Rao (1984) observed that topical application of betel leaf extract inhibited B(a)P-induced oral tumorigenesis in hamsters [21]. Further Anticarcinogenic effects in Swiss male mice was noted from betel leaf extract against tobacco induced carcinogenesis [22]. But no systematic study has been carried out to investigate anti melanoma activity of betel leaves, hence present investigation was undertaken to study anti melanoma efficacy of different extracts of betel leaves in an *in-vivo* experimental model.

II MATERIAL AND METHODS

Collection and authentication of plant material

Fresh and disease free leaves of *Piper betle* L. were collected from the local market at Bhopal city of M.P. during the month of September to October. The plant was acknowledged by a senior Botanist Dr. Sanjay Sahay, Assist. Professor, Department of Botany, Govt. Science and Commerce College Benazir, Bhopal. After identification of plant a specimen was procured in herbarium record maintained at Govt. Science and Commerce College Benazir, Bhopal, M.P.

Plant Extraction

Maceration Technique

The leaves were washed thoroughly 2 to 3 times in sterile distilled water. The leaf materials were then air dried under shade at room temperature for 8 days and finely powdered. The coarsely powdered dried leaves were cited in a container with 1000 mL pet. Ether solvent for defating and permissible to stand at room temperature for a period of at least 3 days with recurrent agitation until the soluble matter has dissolved [23-24]. Similarly, the defatted leaf extract was further placed in the same container after utterly washing of container with distilled water. The leaf extract was further soaked in the container with different solvents viz, ethyl acetate, ethanol and Aqueous were made in the same manner by using solvents aqueous, ethyl acetate and ethanol respectively.

***In-vivo* Anti-tumor Activity**

Experimental Animals

Healthy C57BL/6 Mice (Both Sex) weighing between 180-250gm was selected for study. Animals were maintained at $25\pm 2^{\circ}\text{C}$ and kept in well ventilated animal house under natural photoperiodic condition in polypropylene cages with paddy husk as bedding with free access to food and water. Animals were fasted prior to dosing (only water was withheld over night). On next day, the fasted body weight of each animal is determined and the dose is calculated according to the body weight.

Method

Hair of dorsal side in a particular area was removed by hair remover (1x 1cm) of each mouse. All mice were kept in a laminar airflow cabinet under pathogen free condition throughout the tumor implantation. The implanted tumor from tumor donors (mouse having melanoma tumor) was removed from the mice. Tumor samples are dissected free of necrotic tissue and blood vessels and were cut into small pieces of about 8mm^3 mechanically and it was munched with the help of bent scissor. Cell suspension was stained with trypan blue stain. All cells which were not stained were counted using haemocytometer. Cell suspension of 5 lack cells/animal was implanted subcutaneous (S.C) for each mouse at the hair removed area. The mice bearing the implanted tumors were randomly divided into 9 groups with 3 mice in each group.

Evaluation of tumor growth

During the treatment, the size of the implanted tumors was measured by vernier caliper to construct the tumor growth curve *in vivo*. The control group was kept on normal diet with DMSO only. The test group was given dose of 250mg/kg and 500mg/kg body weight of all extracts (pet. Ether, aqueous, ethyl acetate, ethanol) throughout the experiment daily orally. Tumor volume was calculated by the formula:

$$\text{Tumor volume} = \text{length} \times \text{width}^2 / 0.5$$

Tumor volume doubling time is defined as the time required for the tumors to grow from 50mm^3 to 100mm^3 for the control group and from 35mm^3 to 70mm^3 for the treatment group, respectively. The tumor growth delay time is

estimated as the time difference for the treated tumors and the controls to reach a volume of 70mm^3 . Data was analyzed by ANOVA.

STATITICAL ANALYSIS:

The experimental results were expressed as mean \pm SEM. Data was assessed by ANOVA followed by the Dunnet's test. Value of $p < 0.05$ was considered as statistically significant.

III RESULTS

Results of antitumor activity study of pet. Ether, aqueous, ethyl acetate and ethanol extracts of *Piper betle* L. showed that the ethyl acetate extract (500mg/kgbw) exhibited significant antitumor activity. The table and graph depicts that the tumor volume was significantly decreased in comparison to control group when treated with extract. The animals were given drug per oral (p.o.) for test sample and cancer cells obtained from cancer donor mice were injected subcutaneously (s.c.) from cell suspensions. DMSO (Dose-0.2ml) was used as vehicle for control group. The different dose concentrations were given per oral as 250mg/kg and 500mg/kg body weight of all the extracts. A significant reduction in size of tumor was observed in a dose dependent manner. The deviation observed in tumor reduction was recorded as $1338 \pm 1.555 \text{ mm}^3$ at 250mg/kgbw and $1307 \pm 1.697 \text{ mm}^3$ at 500mg/kgbw, $1112 \pm 1.343 \text{ mm}^3$ at 250 mg/kgbw and $874 \pm 1.626 \text{ mm}^3$ at 500 mg/kgbw, $964 \pm 1.414 \text{ mm}^3$ at 250 mg/kgbw and $627 \pm 1.83 \text{ mm}^3$ at 500 mg/kgbw, $1108 \pm 1.131 \text{ mm}^3$ at 250 mg/kgbw and $819 \pm 0.919 \text{ mm}^3$ at 500 mg/kgbw for pet ether, aqueous, ethyl acetate and ethanol respectively. Among all the four extracts ethyl acetate 500mg/kgbw with tumor reduction from $1390 \pm 1.838 \text{ mm}^3$ (control group) to $627 \pm 1.838 \text{ mm}^3$ showed significant activity. The $P < 0.05$ was considered as level of significance. P was found to be less than 0.05 so difference between the groups was considered to be significant. The results are presented as means \pm S.D. Significant differences between control and treatment groups were calculated using One way ANOVA followed by Dunnet's test number of replicates (N) = 6. P values of ≤ 0.05 were considered significant.

IV DISCUSSION

Betle leaf (*Piper betle*) has many medicinal uses and has been recommended in the ancient scriptures of Ayurveda. The betle leaf is known for its acrid, antiseptic, aphrodisiac, aromatic, astringent, bitter, carminative, hot and stimulant properties [25]. The *piper betle* leaves have been earlier reported to possess antioxidant activity [26, 27]. The antioxidant property is correlated with hepatoprotective, antidiabetic, antiarthritis, antistroke and anticancer properties, since free radicals are directly involved in all these diseases [28].

The present investigation was carried out to evaluate the *in vivo* anticancer activity of various extracts (pet. Ether, aqueous, ethyl acetate and ethanol) of *Piper betle* leaves against melanoma induced C57BL/6 mice. Interestingly, we report here that the differential cytotoxic effect of these extracts was related not only to their chemical composition but may be also due to the nature of the tumor cells. In fact, although all the extracts studied showed a comparable tumor reduction efficacy against the melanoma tumor bearing mice, the individual effect of the ethyl acetate 500mg/kgbw extract was higher (tumor reduction $627 \pm 1.838 \text{ mm}^3$) against the melanoma as compared to that of other extracts (pet. ether, aqueous, ethanol: $1307 \pm 1.697 \text{ mm}^3$, $847 \pm 1.626 \text{ mm}^3$, $819 \pm 0.919 \text{ mm}^3$ respectively)

and their individual effects. Our results agree with previous research in which the significant anti-proliferative activity of ethyl acetate extract of *Piper betle* against human lung cancer cell line (A549) was reported [29]. Our observation agrees with the previous results of Fatahilah *et al* 2010 [30], who reported proliferative inhibitor activity against nasopharyngeal epidermoid carcinoma cells. Our findings are also in agreement with the previous results of Widowati *et al* 2011 [31], who showed anticancer activity of aqueous extract of *Piper betle* leaves in an *in-vitro* model against breast cancer cell line (T47D) by using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay and confirmed that *P. betle* is able to inhibit T47D cell proliferation with IC_{50} 55.2 μ g/ml. Similarly, our findings concur with the previous study lead by Patel *et al* 2012 [32], which shows that hydroalcoholic extract of leaves of *Piper betle* take inhibition of transplanted B16F10 melanoma in mice when the extract treated animals were injected with 50mg/kgbw (i.p) daily for ten days and resulted in delaying tumor growth and prolonged mean survival time in mice.

This study confirms the probable use of *Piper betle* leaf extracts as possible anti cancer agents. Further focused study of anticancer properties and isolation of compounds from *Piper betle* are necessary to prove its worth in the cancer therapy.

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Treatment Group	Tumor volume (mm ³)				
	Day 1	DAY 3	DAY 6	DAY 9	DAY 12
Control Group	230 \pm 0.919	357 \pm 0.989	540 \pm 1.414	909 \pm 1.414	1390 \pm 1.838
Pet Ether					
250mg/kg	230 \pm 1.13	352 \pm 1.414	545 \pm 1.697	860 \pm 1.767	1338 \pm 1.555
500mg/kg	230 \pm 1.414	340 \pm 1.131	542 \pm 1.414	823 \pm 1.202	1307 \pm 1.697
Aqueous					
250mg/kg	230 \pm 1.55	281 \pm 1.697	487 \pm 1.414	765 \pm 1.484	1112 \pm 1.343
500mg/kg	230 \pm 1.83	287 \pm 0.848	436 \pm 1.343	617 \pm 1.272	874 \pm 1.626
Ethyl Acetate					
250mg/kg	230 \pm 0.919	268 \pm 1.414	428 \pm 1.343	687 \pm 0.989	964 \pm 1.414
500mg/kg	230 \pm 1.272	274 \pm 1.202	313 \pm 1.484	408 \pm 0.707	627 \pm 1.838
Ethanol					
250mg/kg	230 \pm 1.272	318 \pm 0.919	464 \pm 1.060	660 \pm 1.060	1108 \pm 1.131
500mg/kg	230 \pm 1.202	240 \pm 1.767	375 \pm 1.202	590 \pm 1.202	819 \pm 0.919

Graph 1. Showing difference in control, test groups; 250mg/kg, 500mg/kg respectively.

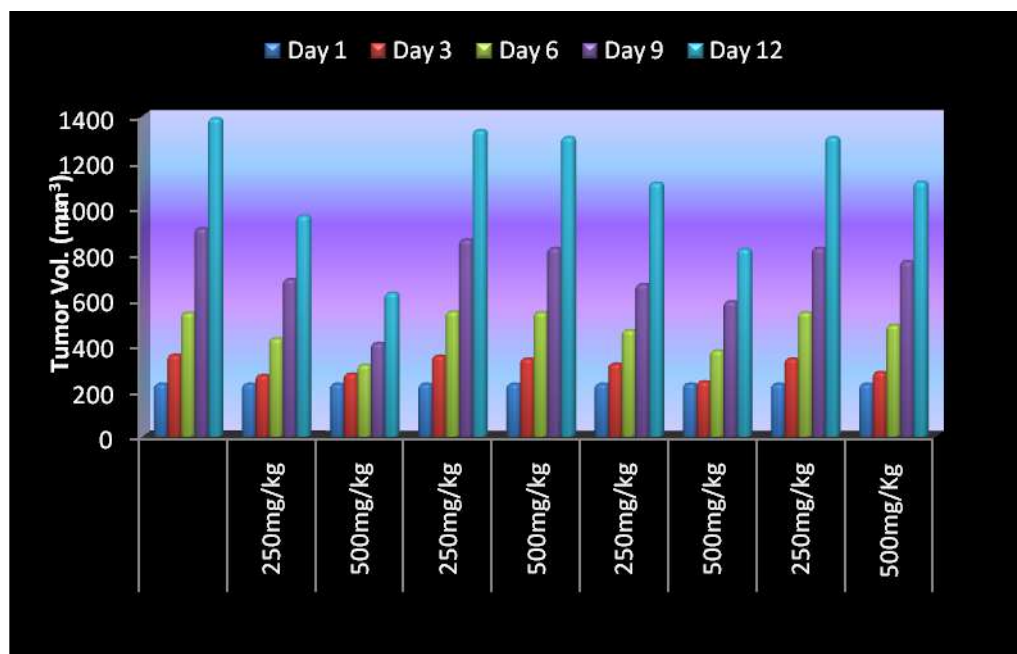


Fig.1: Showing difference in tumor volume in control, test groups; 250mg/kg, 500mg/kg respectively with standard deviation.

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