

PHYTOCHEMICAL STUDIES AND *IN VITRO* CYTOTOXICITY SCREENING OF *PIPER BETLE* LEAF (PBL) EXTRACT

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ABSTRACT

Piper betle leaves (Family: Piperaceae) have been used in Chinese and Indian folk medicine for centuries, and recently been proposed to be used as a chemopreventive agent because of its anti-oxidant activity. In the present paper, *Piper betle* leaves were standardized for stomatal index, vein islet and vein termination numbers, palisade ratio, UV fluorescence and different ash values. The *Piper betle* leaves are earlier reported to possess anticancer potential. Hence, the aqueous extract of the leaves was subjected to cytotoxicity studies on Hep-2 cell line using MTT and SRB assays. The mean CTC₅₀ was found to be 96.25 µg/mL, which proved the potent cytotoxicity and hence, the probable anticancer property of the selected extract.

KEYWORDS: Betel Leaf, Anticancer, Phytochemical characterization

INTRODUCTION

Cancer is not a single disease but a wide range of different diseases of which there well over a hundred types. Cancers can be classified into two broad types: haematological (malignancies of the blood) or solid tumours¹. Cancer is the number one cause of death, but cancer patients are poorly served by current treatment options. While arsenals of methods, including surgery, chemotherapy and radiation therapy are brought to bear on the disease, success is often marginal and serious side effects are common². Current cancer therapy usually involves intrusive processes including application of catheters to allow chemotherapy, initial chemotherapy to shrink any cancer present, surgery to then remove the tumour(s) if possible, followed by more chemotherapy and radiation. The purpose of the chemotherapy and radiation is to kill the tumour cells as these cells more susceptible to the actions that drug and methods because of their growth at a much faster rate than healthy cells, at least in adults³.

The *Piper betle* Linn., family: Piperaceae, is widely grown in the tropical humid climate of South East Asia, and its leaves, with a strong pungent and aromatic flavour, are widely consumed as a mouth freshener⁴. The leaves are credited with wound healing, digestive, and pancreatic lipase stimulant activities in the traditional medicine⁵. During our exploration of non-toxic and affordable herbal medicinal formulations, the PBL extract and its constituent phenolics were found to show impressive anticancer activities⁶⁻⁸. The deep green heart shaped leaves of betel vine are popularly known as *Paan* in India^{9,10}.

MATERIALS AND METHODS

Collection of *Piper betle* Leaves (PBL)

The PBL leaves were purchased from the local market of Varanasi, cleaned and dried under shade (at ambient temperature), and then in oven at 20-40 °C. The dried leaves were weighed (2.5 kg) and stored in dessicator.

Authentication of *Piper betle* Leaf

The plant material was authenticated from National Bureau of Plant Genetic Resources (ICAR), Pusa Campus, New Delhi. The voucher specimens are preserved in the department (NHCP/NBPGR/2009-6/372).

Standardization of Plant Material¹¹

Stomatal Number

A piece of the leaf (middle part) was cleared by boiling with chloral hydrate solution. The upper and lower epidermis portions were peeled off with the help of the forceps. It was mounted on a slide using glycerin water. Camera lucida was fixed and on the black sheet a square of 1 cm² area was drawn with the help of stage micrometer. The slide with mounted epidermis was kept on the stage and the epidermal cells and stomata were traced on to the black sheet into the square of 1 cm² area. This was done in duplicate and average number of stomata per sq. cm. was calculated.

Stomatal Index

Same procedure as described under stomatal number was followed and finally, along with average number of stomata per unit area and average number of epidermal cells per unit area was also calculated. The stomatal index was calculated with the help of formula:

$$I = [S/(E+S)] \times 100$$

Where, I = Stomatal Index, S = No. of stomata per unit area, E = No. of epidermal cells in the same unit area.

Vein-Islet Number

A piece of the leaf was cleared by boiling with chloral hydrate solution for about 30 min. Camera lucida was fixed and on the black sheet a square of 1 cm² area was drawn with the help of stage micrometer. The slide with mounted epidermis was kept on the stage and veins were traced on to the black sheet into the square of 1 cm² area. The number of vein-islets in the square of 1 cm² area was counted. Where the islets are intersected by the sides of the square, included those on two adjacent sides and excluded those islets on the other sides. This was done in duplicate and average number of vein-islet number per sq. cm. was calculated.

Veinlet Termination Number

The same procedure as described under vein-islet number was followed and the number of veinlet terminations present within the square was counted and the average veinlet termination number per sq. cm. was calculated.

Palisade Ratio

The palisade ratio is the average number of palisade cells beneath one epidermal cell of a leaf. It is determined by counting the palisade cells beneath four continuous epidermal cells. The following procedures were used for determination of palisade ratio:

A piece of the leaf (middle part) was cleared by boiling with chloral hydrate solution. Camera lucida was fixed and on the black sheet a square of 1 cm² area was drawn with the help of stage micrometer. The outlines of four cells of the epidermis were traced. The microscope was adjusted to focus on the palisade layer and the palisade cells which are covered under the selected epidermal cells were traced. This was done in duplicate and average number of palisade cells beneath a single epidermal cell was calculated.

UV Fluorescence

The fluorescence of dried plant material was observed under UV light of different wavelengths in a UV cabinet to determine the color of dried plant material.

Ash Values

Ash values such as total ash, acid insoluble ash and water-soluble ash were determined by methods described in Indian Pharmacopoeia¹². To determine different ash values, the plant material was powdered, following procedures were adopted for determining ash values.

Total Ash

Accurately weighed dried PB leaves (2 g) were taken in a silica crucible, which was previously ignited and weighed. The powder was spread as a fine, even layer at the bottom of the crucible. The crucible containing drug was incinerated gradually by increasing temperature to make it red hot until free from carbon. The crucible was cooled, placed in dessicator for 30 min and weighed. The procedure was repeated to get constant weight. The percentage of total ash was calculated with reference to air-dried drug.

Acid Insoluble Ash

The ash obtained above was boiled with 25 mL of 2 N hydrochloric acid for 5 min. The insoluble ash was collected on an ashless filter paper and washed with hot water. The insoluble ash was transferred into a silica crucible, ignited, cooled, placed in dessicator for 30 min and weighed. The procedure was repeated to get a constant weight. The percentage of acid insoluble ash was calculated with reference to the air-dried drug.

Water Soluble Ash

The ash obtained as described in the determination of total ash was boiled for 5 min with 25 mL of water. The insoluble matter was collected on ashless filter paper and washed with hot water. The insoluble ash was transferred into silica crucible, ignited for 15 min, and weighed. The procedure was repeated to get a constant weight. The weight of insoluble matter was subtracted from the weight of the total ash. The difference of weight was considered as water-soluble ash. The percentage of water-soluble ash was calculated with reference to the air-dried drug.

Extraction of Plant Material

The extraction was done by simple maceration process¹³. The plant material (725 g) was added to 9250 mL of mixture of distilled water and chloroform (3%), placed for 7 days under room temperature with occasional shaking. The mixture was filtered with muslin cloth, simple filter paper and then finally with whatmann filter paper to obtain clear liquid extracts. The clear liquid extract was lyophilized at -80°C to obtain dark brown color crude extract, which was stored in desiccator and used for further steps of the study.

Organoleptic and Phytochemical Evaluation of PBL Extract

The PBL extract was characterized for organoleptic properties such as color, odor and taste⁴. To determine the different phytochemical constituents in PBL extract, tests for alkaloids, carbohydrates, flavonoids, steroids, amino acids, proteins, vitamins, glycosides, gum, mucilage, enzymes, organic acids, inorganic elements, terpenes, saponins, oils and fats, and tannins and phenols were carried out¹⁰.

Cytotoxicity Screening

Determination of Mitochondrial Synthesis by Microculture Tetrazolium (MTT) Assay

The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays¹⁴. This assay is based on the assumption that dead cells or their products do not reduce Tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The cleavage of MTT (3-(4, 5 dimethylthiazole-2 yl) – 2, 5-diphenyl Tetrazolium bromide) to a blue formazan derivative by living cells in clearly a very effective principle on which the assay is based.

The principle involved is the cleavage of Tetrazolium salt MTT into a blue coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The number of cells is found to be proportional to the extent of formazan production by the cells used.

Determination of Total Cell Protein Content by Sulphorhodamine B (SRB) Assay

SRB is a bright pink aminoxanthene dye with two sulfonic groups. Under mild acidic conditions, SRB binds to protein basic amino acid residues in TCA (trichloro acetic acid) fixed cells to provide a sensitive

index of cellular protein content that is linear over a cell density range of at least two order of magnitude¹⁵.

Colour development in SRB assay is rapid, stable and visible. The developed colour can be measured over a broad range of visible wavelength in either a spectrophotometer or a 96 well plate reader. When TCA-fixed and SRB stained samples are air-dried, they can be stored indefinitely without deterioration.

RESULTS AND DISCUSSION

The results of microscopic characterization of *Piper betle* leaves are shown in Table 1, and Figures 1-3. The sotalmal index, vein-islet number, vein termination number and palisade ratio can be used as identification characteristics for the crude drug. The microscopic observation revealed that the mesophyll of the leaf can be differentiated into palisade and spongy parenchyma. Palisade was single layered and the spongy parenchyma was found to be 3-4 layered, composed of irregular as well as round cells. In the UV fluorescence study, the leaves were found to black to brown color (Table 2). Different ash values of the PBL leaves are shown in Table 3 and can be used for identification and standardization of the crude drug. Simple aqueous maceration of PBL yielded 95.56 g (13.18% w/w with respect to dry leaves) of crude extract. The PBL extract was dark brownish in color, aromatic odor and slightly pungent in taste.

Tests for the presence of different phytochemical constituents in PBL extract showed the presence of carbohydrates, alkaloids, gums, oils, steroids, glycosides, tannins, phenols, vitamins, organic acids and inorganic constituents. Other phytochemical constituents like amino acids, enzymes, flavonoids, mucilages, proteins and non-reducing sugars were absent in PBL extract, shown in Table 4.

In the *in vitro* cytotoxicity study on HEp-2 cell line by MTT and SRB assays, the aqueous extract of PBL exhibited a mean CTC₅₀ value of 96.25 µg/mL (Table 5). This proves the potent cytotoxic property of the PBL extract. This cytotoxicity might be due to the presence of alkaloids, tannins and phenols or organic acids (Table 4). Complete fractionation of the extract and determination of cytotoxicity of each fraction can further give useful information about cytotoxicity of the extract. This study confirms the probable use of PBL aqueous extract as possible anticancer agent. Further detailed anticancer studies and isolation of compounds responsible for the activity are necessary to prove its worth in the cancer therapy.

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Table 1. Microscopic characteristics of *Piper betle* leaves

Parameter	Values
Stomatal number	Upper surface: 45-56 Lower surface: 86-116
Stomatal index	Upper surface: 14.3-16.0 Lower surface: 19.8-23.5
Vein-islet number	76-82
Veinlet termination number	42-110
Palisade ratio	1:4

Table 2. UV fluorescence of dried *Piper betle* leaves

UV Light	Fluorescence
Longer	Black
Shorter	Brown
Visible	Brown

Table 3. Ash values of dried *Piper betle* leaves

Total ash (% w/w)	Acid insoluble ash (% w/w)	Water soluble ash (% w/w)
15.0 ± 1.2	10.5 ± 0.98	9.5 ± 0.64

Table 4. Phytoconstituents present in PBL extract

Phytochemical	Result
Carbohydrates	+
Alkaloids	+
Glycosides	+
Vitamins	+
Gums	+
Mucilages	-
Amino acids	-
Proteins	-
Fats and oils	-
Steroids	+
Flavonoids	-
Tannins and phenols	+
Enzymes	-
Organic acids	+
Inorganic elements	-

+ Present; - Absent

Table 5. *In vitro* cytotoxicity of PBL extract against HEp-2 cell line

Concentration Tested ($\mu\text{g/mL}$)	Mean Absorbance	Growth Inhibition (%)	Mean CTC_{50} ($\mu\text{g/mL}$)
1000	0.129	92.57	96.25
500	0.108	88.16	
250	0.143	84.32	
125	0.320	65.02	
62.5	0.627	31.25	
31.25	0.832	11.96	
15.6	0.865	05.15	
Cell Control	0.912	-	

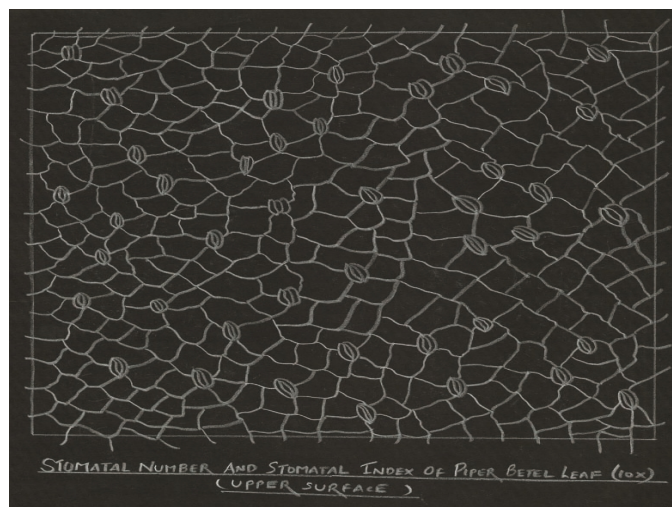


Figure 1. Stomatal number and stomatal index of *Piper betle* leaves (upper surface)

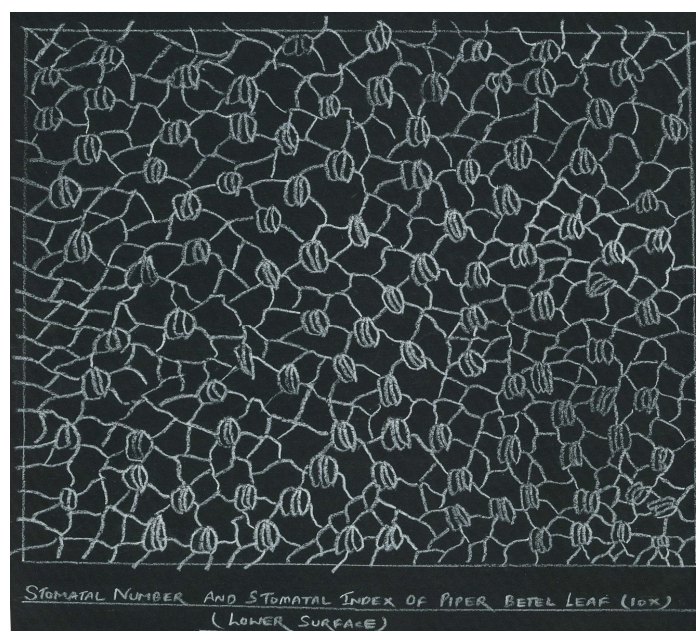


Figure 2. Stomatal number and stomatal index of *Piper betle* leaves (lower surface)

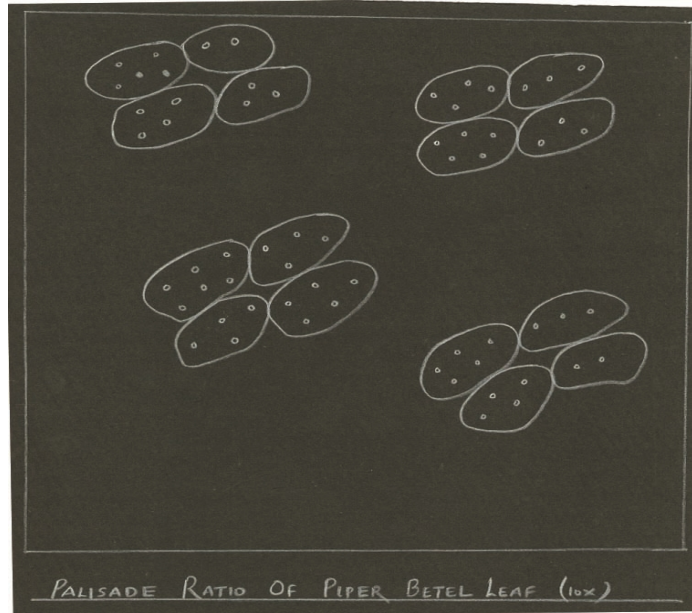


Figure 3. Palisade ratio of *Piper betle* leaves

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