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PHYTOCHEMICAL, ANTIBACTERIAL AND CYTOTOXIC PROPERTIES OF *PIPER BETLE* L.

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Abstract

The phytochemical, antibacterial and cytotoxic characteristics of *Piper betle* L. leaf extract was examined. The phytochemical analysis revealed the presence of glycosides, alkaloids, terpenoids, flavonoids, tannins, and saponins. The presence of different phytochemicals will help in anticipating their pharmaco logical action in searching for bioactive agents. Antibacterial effects were evaluated against six bacterial strains. The tested extract exhibited no antibacterial action against any bacterial strains investigated. Cytotoxicity was assessed on HeLa and Vero cell lines employing the Cell Counting Kit-8 test. The methanolic extract exhibited cytotoxicity against HeLa and Vero cells with an IC50 value of 270 µg/ml and 240 µg/ml, respectively. This preliminary investigation highlights the promising anticancer activities of *P. betle* leaf extract *in vitro*, suggesting its potential for developing novel anticancer agents. However, cytotoxicity towards Vero cells raises concern for fundamental research in drug discovery and regulatory decision-making, thereby shaping the course of subsequent studies and applications.

Introduction

Medicinal food plants have served as the foundation of traditional herbal medicine among various cultures since ancient times. Consequently, therapeutic food plants and healing herbs are widely accepted as valuable resources for exploring their traditional uses (Taukoorah *et al*. 2016).They also possess positive bioactivities and pharmacological qualities, making them useful in the development of pharmaceuticals and herbal medicine (Thakur and Kumari 2022). These phytochemicals or secondary metabolites, like alkaloids and glycosides, flavonoids, tannins, terpenes, phenols, and oligosaccharides, have exhibited antimicrobial properties in *in vitro* studies (Ağagündüz *et al.* 2021). Studies on a variety of herbal plants have also demonstrated the cytotoxic and anti-proliferative properties of diverse phytochemicals against distinct cancer cell types (Newman and Cragg 2016).

Piper betle L. belongs to family Piperaceae, also known as betel leaf in English, are widely cultivated and utilized in Bangladesh, Malaysia, Pakistan, India, Indonesia, Madagascar, Sri Lanka, Thailand, and the West Indies (Karak *et al.* 2016). *Piper betle* can treat a variety of ailments, including hypertension, brain toxins, blisters, diabetes, headaches, leucorrhoea, wounds and bruises, ringworm invasion, gum swelling, voice issues, wound recovery, obesity, conjunctivitis, rheumatism, constipation, and abrasion (Chauhan *et al.* 2016). Eugenol, chavibetol, methyl eugenol, hydroxychavicol, α-pinene, hydroxycatechol, β-caryophyllene, 1,8-cineol, estragole, chavicol, and β-pinene are among the phytochemicals found in betel leaves. Globally, the culinary, cosmetic, and pharmaceutical industries use these leaves extensively (Guha and Nandi 2019). Betel leaf contains approximately 3.1% protein, 2.3% minerals, 85.4% moisture, 0.8% fat, 6.1% carbohydrates and 2.3% fiber per 100 grams (Kaintura *et al.* 2020). Calcium, beta-carotene, riboflavin, thiamine, vitamin C and niacin are among the minerals and vitamins found in

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betel. Except lysine, histidine, and arginine, betel leaves contain all of the amino acids. Additionally, the leaves include an extensive amount of polyphenolic and flavonoid content (Guha and Nandi 2019).

The present study aimed to determine the cytotoxic effects of *P. betle* leaf extract on two cell lines: HeLa, derived from human cervical carcinoma, and Vero, originating from African green monkey's kidney cells. Antimicrobial potential and the phytochemical properties of the extract were also evaluated.

Materials and Methods

Betel leaves were collected from the local market within Dhaka Metropolitan City. The leaves were carefully washed with tap water, dried in a shaded area, and then crushed into a coarse powder with pestles and mortar. They were then kept in sealed containers at room-temperature for further use. 10 gram dried plant samples were fully extracted using 50 ml of methanol for 72 hrs. at 20°C with vigorous shaking. After extraction, the mixture was concentrated at 40°C under decreased pressure with a rotary evaporator after filtering through a Waterman filter paper No. 1. The resultant pellet was then stored at 4°C in airtight screw-cap tubes. To dissolve the pellets of the extract, they were dissolved in a solution containing 2.5% dimethyl sulfoxide (DMSO). Subsequently, the solution was filter-sterilized using a 0.45-µm Millipore filter. Following sterilization, the solution was diluted using distilled water for the antibacterial tests and a culture medium to test for cytotoxicity. Different chemical reagents were prepared for specific phytochemical tests. For alkaloids and tannin tests, extracts were dissolved in 2.5% DMSO, and for glycosides, flavonoids, reducing sugar, terpenoids, steroids, saponins, and volatile oil test plant extracts were dissolved in ethanol (Odebiyi and Sofowora 1978, Njoku and Obi 2009, Talukdar and Chaudhary 2010, Alamzeb *et al*. 2013, Sharma *et al*. 2020).

The leaf extract was also utilized to test its phyto-ingredients. Phytochemical tests for alkaloids, tannins, reducing sugar, glycosides, terpenoids, flavonoids, saponin, steroids, and volatile oils were done following standard procedures (Odebiyi and Sofowora 1978, Njoku and Obi 2009, Talukdar and Chaudhary 2010, Alamzeb *et al*. 2013, Sharma *et al*. 2020).

Gram-negative and Gram-positive bacterial strains *viz.*, *Listeria*, *Escherichia coli*, *Enterobacter cloacae*, *Vibrio cholerae*, *Enterococcus faecalis*, and *Klebsiella pneumoniae* were procured from the Food Analysis and Research Laboratory at the Centre for Advanced Research, in Sciences University of Dhaka. These bacterial cultures were maintained on TSA and stored at 4°C.

The bacterial strains were cultivated in TSB for 24 hrs at 37°C. Subsequently, they were suspended in sterile saline to match the density of the 0.5 McFarland standard. The antimicrobial properties of the extract were assessed using the disc diffusion method (Bauer *et al.* 1966). Each sterile 6 mm dia. paper disc was separately soaked in 30 µl of 200 mg/ml leaf extracts, and each disc was dried to provide 6 mg of dried extract per disc. After being swabbed with the test organisms at a concentration of 10^5 colony-forming units per millilitre (CFU/ml), these discs were then put on Mueller-Hinton agar plates. Control discs containing DMSO without any test compounds were included. The plates were then incubated at 37°C for 24 hrs. Following incubation, the growth inhibition zones around the discs were examined for each microbe, and their dia. were measured and recorded. Each experiment was run in duplicate and repeated three times. The diameter (mm) of the clear inhibitory zones created around the discs was measured, and the results were evaluated using areas devoid of bacterial growth.

The HeLa cell line and Vero cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) provided with streptomycin - 1% penicillin-(1:1), 0.2% gentamicin, and 10% fetal

bovine serum (FBS). Cultures were kept at 37°C in a humidified atmosphere containing 5% CO_2. Subculturing was performed once a monolayer formed in the flask. Cell detachment was achieved using trypsin, followed by the addition of a complete medium to halt the trypsin reaction. Cytotoxicity was measured using Sigma-Aldrich's Cell Counting Kit-8 (CCK-8), a radioactive-free colorimetric test kit. This assay employs WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt], an easily soluble in water tetrazolium salt that generates a water-soluble formazan dye when reduced with an electron mediator. CCK-8 allows for sensitive colorimetric measurements to quantify the total number of living cells in proliferating and cytotoxicity experiments. When cellular dehydrogenases reduce WST-8, an orange-colored formazan end product is produced, and this product is closely correlated with the number of live cells. To conduct the test, 96-well plates were seeded with 2×10^4/100 µl of HeLa cells and 1.5×10^4/100 µl of Vero cells. The cells were then incubated for 24 hours at 37°C with 5% CO_2. Subsequently, the cells were exposed to different doses of *P. betle* methanol extract (375, 700, 1500, and 3000 µg/ml). A control group treated with 2.5% DMSO was also included. After 48 hrs of incubation, cytotoxicity was observed using an inverted light microscope (Trinocular microscope (Optika, Italy). Subsequently, after adding 10 µl of CCK-8 reagents to each well, the wells were incubated for three hours. Following incubation, an ELISA microplate reader (EPOCH, BioTek, USA) was used to assess absorbance at 450 nm. The results were expressed as a percentage of the optical density of cells treated compared to control cells. The test was performed in duplicate to determine the IC50 or LD50 value for the extract using the following equation: Viability (%) = (optical density of sample/optical density of control) × 100.

Results and Discussion

The bioactivity attributes of plants are closely associated with their phytochemical ingredients (Al-Daihan *et al*. 2013). Nine screenings were conducted to analyze the phytochemical compounds present in *P. betle* leaf extract. The present investigation revealed the presence of flavonoids, tannins, saponins, glycosides, and a moderate quantity of alkaloids and terpenoids in the leaf extract of *P. betle*, as summarized in Table 1. Presence of flavonoids, terpenoids, sterols, tannins, alkaloids, and polyphenols were also recorded by Syahidah *et al.* (2017). The extraction of phyto-constituents is greatly impacted by the diluent selection and diverse solvents like petroleum ether, methanol, and ethyl acetate produced discrete findings. Differences in the solubility of active chemicals were also observed in another investigation comparing ethanol, chloroform, petroleum ether, and aqueous extracts; petroleum ether and chloroform extracts could not extract more than two of the evaluated phyto-constituents (Saini *et al.* 2016). These outcomes could indicate the increased solubility of phytochemicals in moderately polar organic solvents like methanol (Cowan 1999). In the investigation, methanol efficiently worked as a solvent for extracting six out of nine bioactive components.

The results of antibacterial properties of the extracts were interpreted following the criteria of Carović-Stanko *et al*. (2010). The methanol extract of *P. betle* displayed inhibition zones less than 10 mm against all the tested bacteria, which means *Listeria, E. cloacae, E. coli, E. faecalis, K. pneumonia* and *V. cholerae* were resistant to this plant extract. Negative controls using DMSO and sterile water also demonstrated no inhibitory effect.

The phytocompounds from betel leaves have been known long to demonstrate antibacterial action against a variety of microorganisms (Chakraborty and Shah 2011, Syahidah *et al.* 2017). In the present findings, the methanol extract of betel leaf failed to inhibit the growth of any of the tested bacteria. These findings stand contrary to the earlier findings. Some possible causes of this include inadequate concentration of active compounds (Li *et al.* 2017), microbial resistance

mechanisms; lack of targeted antimicrobial compounds; environmental conditions and methodological considerations (Cowan 1999, Rios and Recio 2005).

Table 1. Qualitative analysis of phytochemicals in *Piper betle* leaf extract.

Phytochemicals	Test name	Observations	Remark
Alkaloids	Mayer's Test	Pale yellow precipitation	+
Tannins	Ferric Chloride test	Blackish-blue coloration	++
Reducing sugar	Fehling's test	Brick-red precipitation	-
Glycosides	Salkowski's Test	Reddish-brown colour	++
Terpenoids	Salkowski's Test	Reddish-brown colour	++
Flavonoids	Alkaline reagent test	Yellow colour to colourless	++
Saponins	Foam test	Foam's appearance	++
Steroids	Salkowski's test	Reddish colour	+
Volatile oils	General test	White precipitation	-

'+' and '-' denotes the presence and absence of phytochemicals, respectively.

This investigation examined the cytotoxic effects of *P. betle* methanolic extracts. The CCK-8 assay and microscopic examination of the morphological changes in the cell cultures 48 hrs after the extracts were applied to determine the outcomes of cytotoxicity on HeLa cells and Vero cells. The microscopic observations revealed that the number of dead cells grew in proportion to the increasing concentrations of the extract treatment in both cell lines. Cells also showed cellular atrophy, cell shrinkage due to loss of water and cellular content, condensation of the cytoplasm and nucleus, loss of cell-cell contacts, and tissue disintegration denoting cell death (Fig. 1).

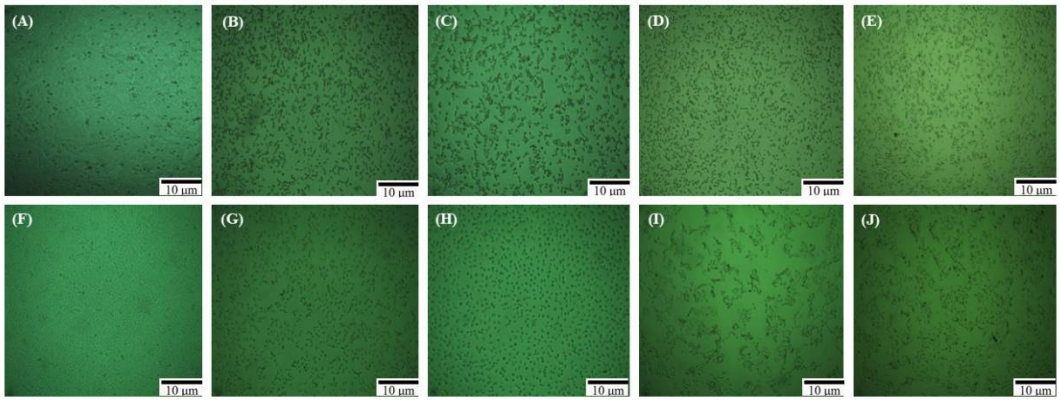

Fig. 1. Morphological changes of the HeLa(A-E) and Vero cells(F-J) with the treatment of *Piper betle* leaf extract at various concentrations. A and F: Control, B and G: 375 µg/ml, C and H: 750 µg/ml, D and I: 1500 µg/ml and E and J: 3000 µg/ml.

In the CCK-8 colorimetric assay, betel leaf extract exhibited an IC50 value of 270 µg/ml and 240 µg/ml on the HeLa and Vero cell line, respectively (Fig. 2). It was found that betel leaf's methanolic extract with the concentration of 375 µg/ml was very effective and 35.36 and 24.78% of HeLa and Vero cells were viable. Usharani and Monisha (2022) reported that only 20.89% of

cells survived when exposed to the highest concentration (200 µg/ml) of betel quid hexane extract. This extract showed a significant cytotoxic effect on HeLa cells, with an IC50 of 96.33 µg/ml.

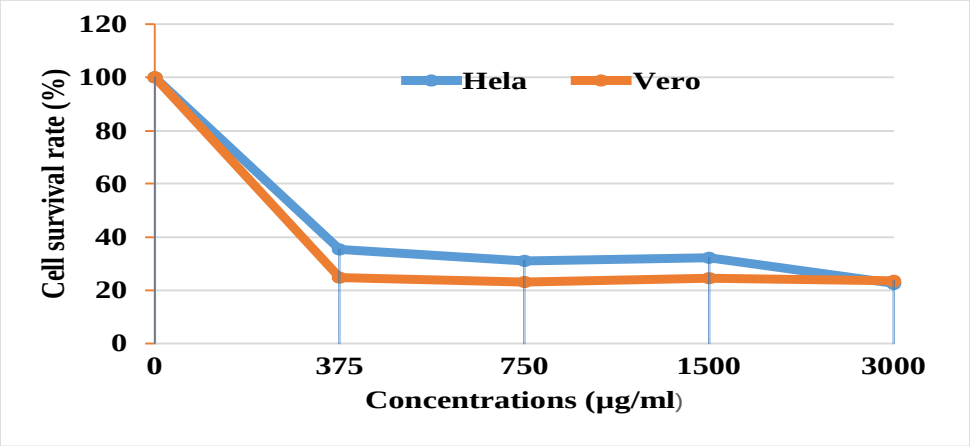

Fig. 2. CCK-8 cell proliferation and cytotoxicity test of methanol extracts of *P. betle* on HeLa and Vero cell lines.

Singh *et al*. (2009) revealed that the n-hexane fraction of crude methanol extract had an IC50 of 184.24 µg/ml, while the n-butanol and chloroform fractions had IC50 values of 233.73 µg/ml and 73.85 µg/ml, respectively, indicating low cytotoxicity against Vero cells. The present result raises concerns regarding the safety of the plant extract in the light of its cytotoxicity against HeLa and Vero cell lines, suggesting that it may have anticancer capabilities overall and against cervical cancer in particular. Furthermore, the extract's dual effects on healthy and malignant cells emphasize the significance of conducting thorough toxicity tests to determine whether or not it is suitable for therapeutic use.

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