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Dose and time dependent cytotoxicity of Blepharis maderaspatensis (L) against various human cancer cell lines

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Abstract: *Background:* Traditionally, *Blepharis maderaspatensis* has been assigned antioxidant, anti-inflammatory, antimicrobial, and wound healing properties. *Aim and Objectives:* Present study was aimed to assess dose and time-dependent cytotoxic potential of B. *maderaspatensis*. *Material and Methods:* This was an experimental study with positive and negative control under standard laboratory conditions. *In vitro* cytotoxic activity of ethanolic extract of *B. maderaspatensis* was assessed by MTT assay on MCF7, PA1, HT29, A375, HepG2 cancer cell lines. All the cell lines were treated with increasing concentration of the extract for 24, 48 and 72 hours or standard anticancer drug. IC50 value was calculated for each duration from sigmoid dose-response curve by using GraphPad Prism8 software. *Results:* Ethanolic extract of *B. maderaspatensis* was effective cytotoxic agent against all the tested cancer cell lines in dose and time-dependent manner. Cytotoxicity was maximum against A375 (melanoma) (IC50: 21.46µg/ml), followed by HT29 (colon cancer) cell lines (IC50: 32.66µg/ml). For all other cell lines IC50 values were >100µg/ml. *Conclusions:* Ethanolic extract of *B. maderaspatensis* is effective cytotoxic agent and can be evaluated further to study its mechanism of action. Investigations involving purification, identification of active principles and determination of mechanisms of action can be helpful in drug discovery program.

Keywords: Anticancer, MTT Assay, Melanoma, Colon Cancer.

Introduction

Cancer is a pathological condition which involves "uncontrolled proliferation of abnormal cells that invade the adjacent tissues and cause their destruction and sometimes show metastasis" [1]. According to the International Agency for Research on Cancer (IARC), incidence, mortality and 5 year prevalence of all types of cancer in Asia is 48.4%, 57.3% and 39.7% respectively [2].

Among all types of cancers, breast, lung, mouth, cervix, uterine, and tongue cancers are the most predominant types in India [3]. Current therapeutic strategies like chemotherapy and radiotherapy are effective in various types of cancers but they are associated with high systemic toxicity. Also, many chemotherapeutic agents develop resistance [4]. As a result, successful outcome of the treatment is limited. Medicinal uses of different plants were known to human beings since ancient times. At present, many plant products and their analogues are available in market which possess potent anticancer activity [5]. Still constant search for new plant-based agents for safe and effective drug development continues [6-7]. Medicinal properties of any plant are due to presence of variety of secondary metabolites in them. All plants produce secondary metabolites like phenols, flavonoids, steroids, terpenoids, alkaloids to protect themselves from adverse conditions [8]. Concentration and chemical structure of these compounds depend on habitat, climatic conditions and season of harvest. This in turn, decides efficacy and potency of compound for their medicinal use [8].

Blepharis maderaspatensis (L.) is a prostrate, creeping, wiry plant, belonging to family Acanthaceae [9]. It is known as Dudhiya choti

in Hindi and Kooravaal Chedi or Kozhimookkan in Tamil. It is widely distributed in tropical and southern Africa, southern parts of the Middle East and central Asia, India, and southern China [10]. It grows on loose soil, along the crop fencings and prefers sandy soil with heavy percolation. Traditionally, plant ash is used for treating oedema and gout; dry alcoholic extract of the plant is a potent diuretic. Leaf juice is used in eye infections, pharyngitis, and asthma. Plant is also used in treatment of ulcers, fractures, urinary tract infections and venereal diseases [11-12].

Previous studies have demonstrated that B. maderaspatensis possesses good antioxidant activity [13-15]. A.A. Bhaskar reported that *B. maderaspatensis* exhibited strong DPPH and NO scavenging abilities but was not very effective cytotoxic agent in AGS, A549, MCF-7, and COLO320 DM cancer cell lines [16]. Literature search revealed only one study performed to assess antiproliferative potential of the B. maderaspatensis. Since *B. maderaspatensis* is not well studied for its cytotoxic activities, the present study was undertaken to analyse *In vitro* antiproliferative potential of *B. maderaspatensis*.

Material and Methods

Study protocol was cleared by institutional ethical committee (VMKVMC/ICE/17/70 dated 28/12/2017).

Plant material and extraction:

Collection and Extraction of Plant Material: Aerial parts of *B. maderaspatensis* (family Acanthaceae) were collected from Salem district of Tamil Nadu in south India. Plant material was authenticated by the botanists, Dr. N. Karmegam, Salem and voucher specimen was preserved in the department (PHY/001/2018). Collected material was cleaned; air dried in shed and coarsely powdered using electrical grinder. Four hundred gm of powdered material was evenly packed in Soxhlet apparatus and was serially extracted using petroleum ether (60-80^{o}C), chloroform, acetone, and ethanol 95% v/v (75-78^{o}C). For each solvent, continuous hot extraction process was performed for 72 hours. Extract was filtered by using Whatmann filter paper (no.10) and was concentrated by vacuum distillation to 1/10th volume. Remaining solvent from this concentrated extract was removed by evaporation by using hot water bath and stored in desiccater to remove moisture. Dried extract was weighed and then stored in airtight container till further use.

Marc left after petroleum ether extraction was dried completely; weighed and packed in Soxhlet apparatus for extraction with chloroform. Above process was repeated for extraction with acetone and then with ethanol. Marc left after ethanol extraction was dried and soaked in 2L distilled water for 72 hours. Contents were filtered and solvent was removed by using hot water bath. Dried extract was weighed and stored in airtight container. Thus, aqueous extract was obtained by cold maceration.

Phytochemical analysis: Preliminary phytochemical analysis was carried out by chemical methods as described earlier [17]. Alkaloids were detected by Dragendorff's test, Hager's test and Wagner's test. For flavonoids, Shinoda's test and concentrated sulphuric acid test were used. Presence of glycosides and phenols were confirmed by Molisch's test and foam test respectively. Steroids were detected by Liebermann-Burchard's test and Salkowski Reaction.

Phytochemical analysis revealed presence of flavonoids, phenolic compounds, glycosides, saponins and carbohydrates in ethanolic and aqueous extracts. Since these compounds are mainly responsible for antioxidant and cytotoxic activity, ethanolic extract, which has the polarity in between, was selected for further evaluation.

Cell viability studies by MTT assay: Principle: MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) is a water-soluble tetrazolium salt. In viable cells, mitochondrial dehydrogenase converts it to insoluble purple formazan. This water insoluble formazan is solubilized using DMSO and absorbance of resulting purple solution is measured by spectrophotometer.

In the present study, 5 human cancer cell lines viz. breast cancer (MCF7), ovarian cancer (PA1), colon cancer (HT29), skin melanoma (A375) and liver carcinoma (HepG2) were

used. All the cell lines were obtained from National Centre for Cell Sciences (NCCS), Pune. All the chemicals were procured from Hi Media Laboratories. Cells were cultured in minimal essential media supplemented with 10% inactivated Fetal Bovine Serum (FBS), streptomycin (100 µg/ml), penicillin (100 IU/ml) and in humidified atmosphere containing 5% CO_2 at 37^o until confluent. Mycoplasma contamination was ruled out in all the cultures.

TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS) was used to dissociate the cells and their viability was checked. Further, 50,000 cells/well were seeded in a 96 well plate and incubated for 24 hrs at 37^o in 5% CO_2 incubator. After 24 hours, when partial monolayer was formed, supernatant was removed, and monolayer was washed once with medium. To this monolayer, 100 µl of the extract or standard drug was added. Concentrations used were, 3.125, 6.25, 12.5, 25, 50 and 100 µg/ml. Plates were incubated at 37^o in 5% CO_2. Cytotoxic potential of the extract was evaluated after 24, 48 and 72 hours of treatment. Standard anticancer drug was used as positive control. For MCF7, PA1, A375 and HepG2 cell lines, cisplatin was used whereas for HT29 cell line, 5-Fluorouracil (5FU) was used as positive control.

After prescribed duration of treatment with extract, solutions in the wells were discarded and 100 µl of MTT (5 mg/10 ml of MTT in PBS) was added to each well. After incubating the plates for 4 hours at 37^o in 5% CO_2 atmosphere, supernatant was removed and 100 µl DMSO was added. Plates were shaken gently to solubilize formazan and absorbance was read at 570 nm using a microplate reader. Cell viability was calculated by using the formula- Cell viability (%) = [(Absorbance of sample − Absorbance of blank) / (Absorbance of control − Absorbance of blank)] × 100. Direct microscopic observations of cell lines treated with extract was done with inverted microscope.

Statistical analysis: All the tests were performed in triplicates and values were expressed as mean ± SD of the absorbance. Cell viability was calculated from standard curve based on the absorbance values. IC50 (concentration of extract needed to inhibit cell growth by 50%) values were derived from a nonlinear regression analysis (curve-fit) based on sigmoid dose response curve by using GraphPad Prism 8 software.

Results

Phytochemical analysis: Serial extraction of aerial parts of *B. maderaspatensis* gave variable yield. Maximum yield was obtained for ethanol (11%) followed by aqueous extract (8.5%). Phytochemical analysis revealed presence of important phytochemicals like flavonoids, phenolic compounds, glycosides, saponins and carbohydrates in ethanolic extract.

Cytotoxicity analysis: Table 1 shows IC50 values of *B. maderaspatensis* extract against various cell lines, when treated for different durations. It exhibited maximum cytotoxicity against A375 followed by HT29 cell line in time dependent manner. For all other cell lines more than 100 µg/ml concentration of extract was necessary to show effective cytotoxicity for all the duration of treatment.

Table-1: Cytotoxic effect of ethanolic extract of *B. maderaspatensis* on various cell lines

	Duration of treatment (hour)	IC50 values for various cell lines (µg/ml)				
		MCF7	PA1	HT29	A375	HepG2
Extract	24	>100	>100	55.74	31.59	>100
	48	>100	>100	34.86	24.89	>100
	72	>100	>100	32.66	21.46	>100
Cisplatin	24	4.52	3.32	---	2.56	2.54
5 fluorouracil	24	---	---	5.25	---	---

Note: Cytotoxic activity is expressed in terms of IC50. MCF7; Breast cancer cell line, PA1; Ovarian cancer cell line, HT29; Colon cancer cell line, A375; melanoma cell line, HepG2; Liver cancer cell line, effective cytotoxic agent for MCF7 cell line when used for 48 and 72 hours at the concentration of 100 µg/ml (Figure 5). But it becomes more cytotoxic at 100 µg/ml concentration when treated for 72 hours.

Figures 1-5 compare cytotoxic activity of ethanolic extract of *B. maderaspatensis* on various cell lines when treated for 24-, 48- and 72-hours duration, and their comparison with standard anticancer drugs. In case of A375 cell line, extract was effective cytotoxic agent at 50 µg/ml concentration for all the durations (Figure 1). Dose response curve followed similar pattern to that of 5 fluorouracil in case of HT29 cell line (Figure 2). In HepG2 (Figure 3) and PA1 (Figure 4) cell lines, 24, 48- and 72-hour exposure to *B. maderaspatensis* extract exhibited better results at 100 µg/ml concentration.

Fig-1: Dose and time-dependent response of A375 (melanoma) cell line to treatment with B. maderaspatensis extract and standard anticancer drug.

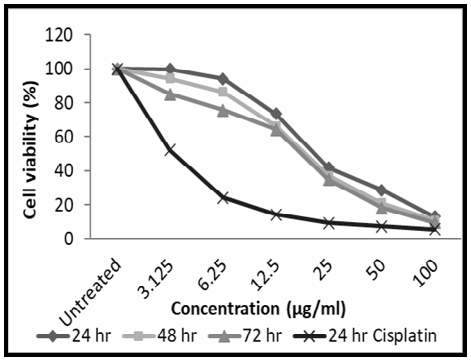


Fig-2: Dose and time-dependent response of HT29 (colon cancer) cell line to treatment with B. maderaspatensis extract and standard anticancer drug.

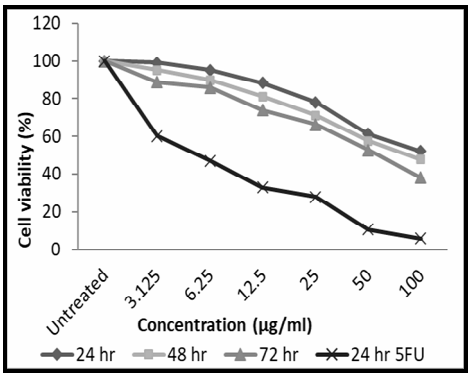


Fig-3: Dose and time-dependent response of HepG2 (liver cancer) cell line to treatment with B. maderaspatensis extract and standard anticancer drug.

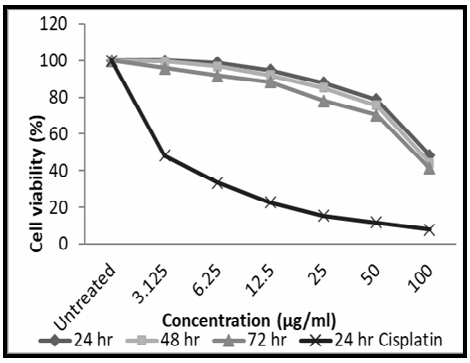


Fig-4: Dose and time-dependent response of PA1 (ovarian cancer) cell line to treatment with B. maderaspatensis extract and standard anticancer drug.

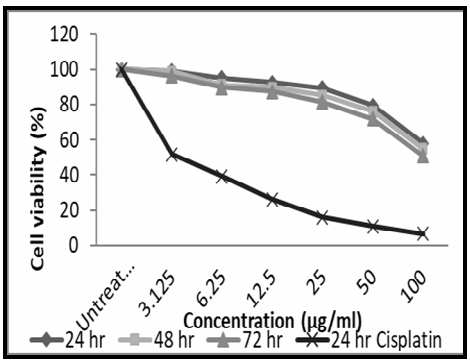


Fig-5: Dose and time-dependent response of MCF7 (breast cancer) cell line to treatment with B. maderaspatensis extract and standard anticancer drug.

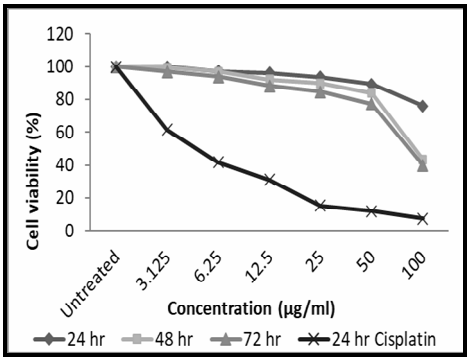
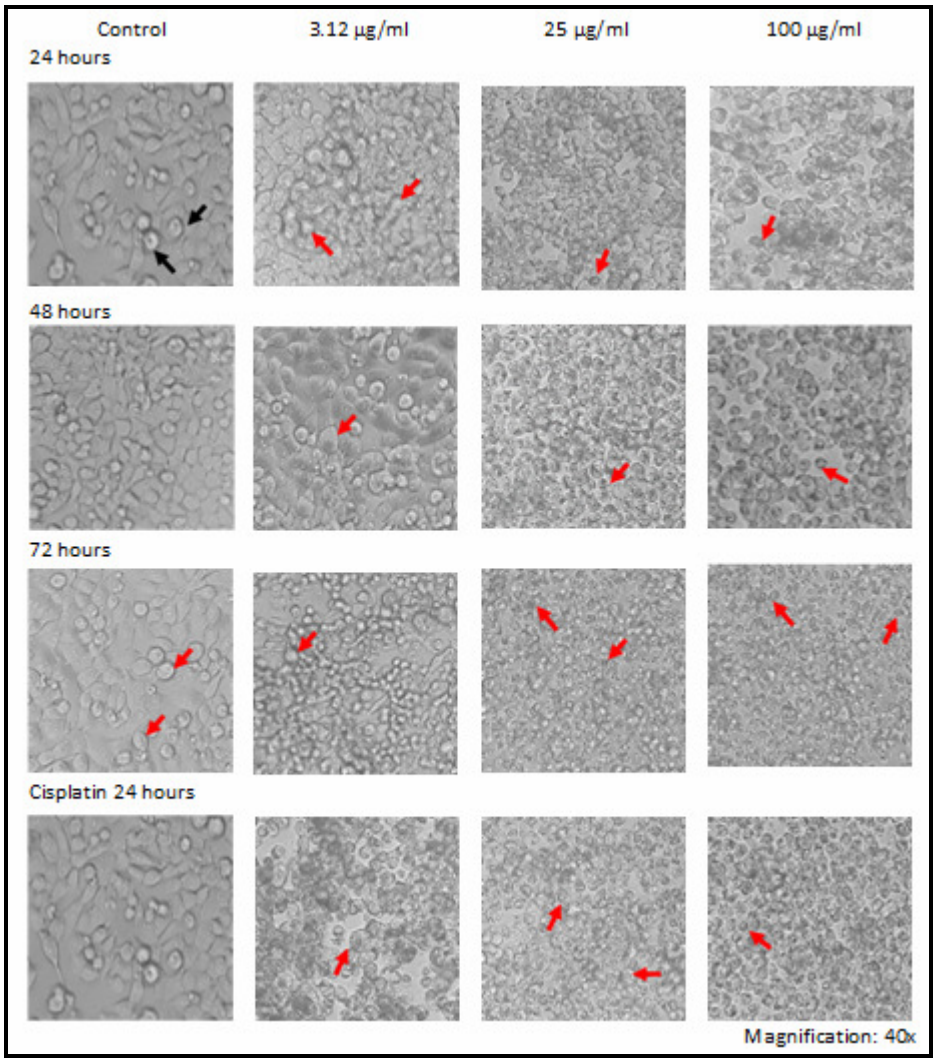


Figure 6 indicates microscopic pictures of melanoma cells demonstrating cytotoxic effect of *B. maderaspatensis* and cisplatin when treated with increasing concentrations of the extract for 3 different durations. Microscopic pictures of melanoma cells

treated with *B. maderaspatensis* indicated apoptotic changes in them in the form of irregularities in nucleal membrane, derangement in chromosomal distribution, formation of membrane blebbing and apoptotic bodies. These changes were comparable to those treated with cisplatin.

Fig-6: Microscopic pictures of melanoma (A375) cell line when treated with ethanolic extract of B. maderaspatensis for 24, 48 and 72 hours. First column indicates control where cells were not exposed to the extract. Second, third and fourth column represent cells treated with 3.12, 25 and 100 µg/ml extract, respectively. Last row shows A375 cells treated with standard anticancer drug for 24 hours. Black arrows indicate viable cells with normal nucleus. Red arrows indicate irregularities in nuclear membrane with derangements in chromatin distribution indicating cells undergoing various stages of apoptosis.



Discussion

Traditionally, *B. maderaspatensis* is used as anti-inflammatory agent in treatment of ulcers, oedema, gout and various infections [18-19]. Preliminary phytochemical analysis of ethanolic extract of this plant revealed presence of important phytoconstituents like steroids, phenols, flavonoids and alkaloids.

Our study demonstrated that ethanolic extract of *B. maderaspatensis* shows better cytotoxicity against melanoma and colon cancer cell lines (Table 1, Figure 1-2) in dose and time dependent manner. It also exhibits good cytotoxicity against ovarian, hepatic and breast cancer cell lines (Figure 3-5) at the concentration of 100 µg/ml in time-dependent manner. These findings were comparable to

those of A.A. Baskar et al, with respect to MCF7 cell line (IC50 >100 µg/ml). They also reported that methanolic extract of *B. maderaspatensis* is more cytotoxic to colon cancer cell line (Colo 320DM) as well as VERO (normal renal epithelial cells of monkey) cells. Literature search revealed only one In vitro study that was conducted to evaluate anticancer property of *B. maderaspatensis* [16].

One of the mechanisms of cancer development is oxidative stress to the cells. Free radicals cause DNA damage and mutations in the cells leading to cancerous growth of the cells. Ethanolic extract of *B. maderaspatensis* contains polyphenols and flavonoids which are said to exhibit strong antioxidant activity [20-21]. Flavonoids, alkaloids and phenolic compounds in the extract are said to possess anticancer activities [22]. These phytochemicals which impart antioxidant activity, may explain antiproliferative property of the extract in cancer cells. It is also proposed that antioxidants modulate activity of protein kinases which in turn alter cell signalling pathways and induce cytotoxicity in cancerous cells [20].

Microscopic examination of the cell lines also demonstrated apoptotic changes in the cells in the form of swelling and nuclear disintegration. This indicates that ethanolic extract of *B. maderaspatensis* induces apoptosis in cancerous cells. Apoptosis is regulated by Bcl2 gene family proteins and Caspase gene family proteins [23]. *B. maderaspatensis* may increase the expression of proapoptotic genes like Bax or reduce the expression of antiapoptotic genes like Bcl2 leading to induction of apoptosis in melanoma cells [23]. *B. maderaspatensis* may increase the expression of p53 which in turn, upregulates expression of Bax to induces apoptosis in case of severe DNA damage [24]. Thus, *B. maderaspatensis* may induce DNA damage in melanoma cells and mark them for apoptosis.

According to the guidelines of US National Cancer Institute (NCI) for plant screening program, extract with IC50 value of < 30 µg/ml after 72 hours of treatment is considered as a promising source for purification of a crude extract [25-26]. In the present study, IC50 value of the ethanolic extract of *B.* maderaspatensis after 72 hours of treatment is 21.46 µg/ml for melanoma cell line. Hence it can be the promising candidate for purification and further studies.

Limitation and future scope: This is the preliminary study done to assess efficacy of *B. maderaspatensis* extract against cancer cell growth. As it demonstrated dose and time-dependent inhibition of viability of various cancer cell lines, further studies can be planned to understand its mechanism of action. Extract can be further evaluated for possible identification and characterization of active principles as well as their mechanism of action.

Conclusion

Ethanolic extract of B. maderaspatensis possesses potent cytotoxicity against melanoma cells in dose- and time- dependent manner. It induces apoptosis in melanoma cells and the results are comparable with that of cisplatin. B. maderaspatensis can be the potential source of anticancer agent and further in vivo studies may be undertaken to test its efficacy in animal models. Also active principles can be isolated from the extract and tested in animal models.

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