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ORIGINAL ARTICLE

Evaluation of AntiCancer Activity of Methanolic Extract of *Hiptage benghalensis* (L.) Kurz on Cancer Cell Lines

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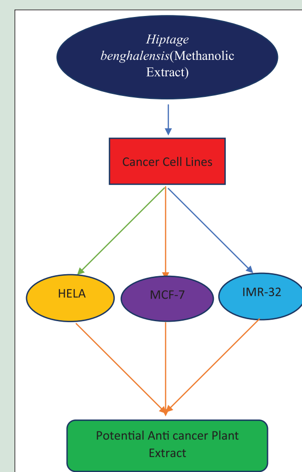
ABSTRACT

Aim and Background: This study aimed to study the anticancer effects of *Hiptage benghalensi*s (L.) kurz using various human cancer cell cultures, *in vitro* using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT assay. **Materials and Methods:** Human cervical carcinoma (HeLa) cells, human breast cancer (MCF-7) cells, and human neuroblastoma (IMR-32) cells were maintained in a 5% CO^2 incubator at 37°C. Different concentrations of an extract of *H. benghalensis* in serum-free culture medium were freshly prepared and used for cytotoxic activity using MTT assay activity. **Results:** Among the plant extract had revealed that, the greater percentage inhibition in all types of cancer cells in a dose-dependent manner using MTT assay. The concentration of the extract causing 50% cell death values of methanolic extract of H. benghalensis were found to be 50.73, 47.90, and 53.76 μg/mL against HeLa, MCF-7, and IMR-32, respectively. **Conclusion:** *H. benghalensis* methanolic extract were showed increased percentage inhibition of MCF 7, HeLa, and IMR 32 cells using MTT assay.

Key words: Caspase-3, cell viability, *Hiptage benghalensis*, reactive oxygen species

SUMMARY

- The anticancer actvity of methanolic extract of *Hiptage benghalensis* were evaluated against three different cancer cell cultures, such as human cervical carcinoma (HeLa), human breast cancer (MCF-7) and human neuroblastoma (IMR-32) cells by using MTT assay, which is based on the reduction of MTT at different concentrations (10, 30, 100, 300 and 500 μg/ml). After 48 h of treatment, methanolic extract of *H. benghalensis* have revealed that greater percentage inhibition in all types of cancer cells in a dose dependent manner. The IC50 values of methanolic extract of *H. benghalensis* were found to be 50.73, 47.90 and 53.76 μg/mL against HeLa, MCF 7, and IMR 32, respectively. We found that exposure of all three cancer cells with these extract dramatically enhanced generation of intracellular ROS at different levels in a dose dependent manner in all cell lines. The results here are expressed as the percentage increase in caspase 3 activity in treated cells compared to cells added with DMSO. Caspase 3 levels were significantly increased with methanolic extract in MCF 7 ($P < 0.05$), HeLa ($P < 0.01$), and IMR 32 ($P < 0.01$) cancer cells. the apoptotic activity revealed that the methanolic extract *Hiptage benghalensis* significantly increased the generation of ROS and caspase-3 activities in all cancer cell lines in a dose dependent fashion.



Abbreviation Used: HBM: Hiptage benghalensis Methanolic extract, MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide, MCF 7: Human breast cancer cells, HeLa: Human cervical carcinoma cells, IMR 32: Human neuroblastoma, H2DCFDA: 2′,7′ dichlorodihydrofluorescein diacetate, PBS: Phosphate-buffered saline, DMSO: Dimethyl sulfoxide, DCF: Dichlorofluorescein, ROS: Reactive oxygen species.

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INTRODUCTION

The current global burden of cancer is quite alarming, and this worrying prospect is even bound to increase significantly in the near future. GLOBOCAN, the database of the International Agency for Research on Cancer, estimates that 14.1 million new cancer cases and 8.2 million cancer-related deaths occurred in 2012, compared to 12.7 million and 7.6 million, respectively, in 2008. Prevalence estimates for 2012 indicate that there were 32.6 million people (above 15 years) alive who had been diagnosed with cancer in the previous 5 years.[1,2] There is also evidence that developing countries are undergoing rapid societal and economic changes, as there are considerable shifts in lifestyles reflecting uptake of lifestyles associated with the industrialized countries, and are thus experiencing a rising burden of cancers.[2-4]

In cancer chemoprevention, natural or synthetic biological or chemical agents are used to reverse, suppress, or prevent carcinogenic progression.[5] Historically, natural products have been employed as anticancer agents for quite a considerable period of human existence, and through the years have been incorporated into both traditional and allopathic medicine. A significant number of chemotherapeutic drugs in current use were either isolated from plant species or derived from a natural prototype.[2,6] The following are some excellent examples of vinca alkaloids, vinblastine, and vincristine, isolated from *Catharanthus roseus* etoposide and teniposide, the semisynthetic

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derivatives of epipodophyllotoxin, isolated from *Podophyllum* species the naturally-derived taxanes isolated from *Taxus* species and the semisynthetic derivatives of camptothecin, irinotecan, and topotecan, isolated from *Camptotheca acuminata*.[7,8] In fact, according to Cragg and Newman, more than 50% of drugs in clinical trials for anticancer activity were isolated from the natural sources or are related to them.[2,9]

Plants materials have a long history of used in the treatment of cancer. Hartwell has reported in his review about the 300 plants species used against cancer.[10,11] Plant-based drug discovery has resulted in the development of many anticancer drugs currently in clinical use. Besides this, it also provides a platform for the design of novel and safe drugs through a proper understanding of the complex synergistic interaction of various constituents of anticancer herbs.[11-13]

Hiptage benghalensis is a herb of the family *Malpighiaceae*. It is distributed in India, Srilanka, and Bangladesh, Andaman Islands and Myanmar to southern China. In Ayurveda, the leaves and bark are considered vulnerary and the leaves are highly regarded for treating skin diseases. The leaf juice possesses insecticidal properties and is used an external application for scabies.[14] Leaves also possess analgesic and anti-inflammatory activity and hepatoprotective activity.[15,16] The leaves and bark are used in treating fungal diseases.[17] The bark, leaves, and flowers are aromatic in nature, and are commonly used as a refrigerant, expectorant, cardiotonic, anti-inflammatory, and insecticidal. They are used in burning sensation, wounds, ulcers, leprosy, cardiac debility, rheumatism, and hyperdipsia.[18,19] The plant is also used in the treatment of chronic rheumatism and asthma.[14] The present study aims to evaluate the anticancer activity of methanolic extract of *Hiptage bengalensis* (L.) kurz leaves in different cancer cell lines.

MATERIALS AND METHODS

Plant material

The leaves of *H. benghalensis* were collected from Thirupathi Hills, Andhra Pradesh, India. It was authenticated by Prof. V. Raju, Department of Botany, Kakatiya University, Warangal, Telangana, India.

Preparation of extracts

H. benghalensis leaf (1.5 kg) is made free from the adherent foreign material and air-dried. Then they were coarsely powdered and macerated with methanol in a round bottom flask for 7 days separately (435.95 mg). The content of the flask was stirred intermittently to ensure the efficiency of the extraction. After a week, they were filtered and concentrated under reduced pressure to yield corresponding extracts, and the extracts were kept in a desiccator to remove moisture and stored properly until used.

Cell culture and treatment

Human cervical carcinoma (HeLa) cells, human breast cancer (MCF-7) cells, and human neuroblastoma (IMR-32) cells were procured from NCCS, Pune. The cells were used between passages 10 and 15. All these cells were grown in suitable culture media supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin antibiotic solution. Cells were seeded at 2,50,000 cells/flask in a total volume of 10 mL. When confluent, all the cells were trypsinized as described above and seeded in 96 well plates at the rate of 1.0×10^4 cells/0.1 mL. All the cell cultures were maintained in a 5% CO_2 incubator at 37°C. Different concentrations of an extract of *H. benghalensis* such as in serum-free culture medium were freshly prepared and used for the cytotoxic activity.

3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay method

Measurement of cell viability and proliferation forms the basis for numerous *in vitro* assays of a cell population's response to external factors. The reduction of tetrazolium salts is widely believed as a reliable way to study cell proliferation. The effect of test extract on the cellular proliferation and viability were determined using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay method.[20] The yellow tetrazolium salt was reduced using dehydrogenase enzymes present in metabolically active cells, to produce reducing equivalents such as NADH and NADPH. The formazan product has low aqueous solubility and was present as purple crystals. The resulting formazan was dissolved using dimethyl sulfoxide (DMSO) permitted the convenient quantification of product development. The intensity of the product color was measured at 562 nm, and was directly proportional to the number of living cells in the culture.[21]

The adherent cells were trypsinized according to protocol and were resuspended in fresh medium after centrifugation. The cell suspension was mixed thoroughly by pipetting several times to get a uniform single-cell suspension. Ten to fifteen passages were conducted before performing the experiment to evaluate the cytotoxicity. Different dilutions of extract were made in media with final phosphate buffer solution (PBS) +1% polyethylene glycol (solvent control) concentration in the well to be <1%. About 100 μL (0.1 mL) of cell suspension was transferred aseptically to each well of a 96 well plate, and to it 100 μL of solvent/extract (in triplicate) in media was added. The plate was then incubated at 37°C for 48 h in 5% CO_2 incubator. After 48 h of incubation, 20 μL of MTT was added to each well and the microtiter plate was again incubated for 2 h. A volume of 80 μL of lysis buffer was added to each well, the plate was wrapped in aluminum foil to prevent the oxidation of the dye. The plate was placed on a rotary shaker for 2 h to solubilize the purple formazan crystals. The absorbance was recorded on the ELISA reader at 562 nm wavelength. The absorbance of the test was compared to that of solvent control to get the percentage cytotoxicity.[22,23]

Measurement of reactive oxygen species generation

Generation of reactive oxygen species (ROS) was assessed using a cell-permeable fluorescent signal 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) as an indicator for ROS.[24,25] As described previously, H_2DCFDA is oxidized to a highly green fluorescent 2070-dichlorofluorescein (DCF) by the generation of ROS. Cancer cell lines were pretreated with various concentrations of test extract for 24 h. After 24 h incubation period, the cells were washed with cold PBS and incubated with 100 mmol/L H_2DCFDA for another 30 min at 37°C. DCF fluorescence intensity was measured using the fluorescence plate reader (Varioskan Flash Multimode Reader, Waltham, MA) at excitation/emission of 488/525 nm. The determinations were carried out thrice in triplicate, ensuring each time that the number of cells per treatment group was the same to ensure reproducibility. The values were expressed as % relative fluorescence compared to the control.

Caspase-3 assay

Caspases are members of the aspartate-specific cysteinyl protease family. In cells, caspase-3 exists as an inactive 32 kDa proenzyme, called procaspase-3. Procaspase-3 is cleaved into active 17 and 12 kDa subunits by upstream proteases such as caspase-6, caspase-8, and granzyme B during apoptosis. The over expression of caspase-3 can result in the initiation of apoptosis. Similarly, the inhibition of caspase-3 can

prevent cells from entering into the apoptotic pathway. The activation of caspase-3 is used as a biomarker in the evaluation of apoptosis and understanding mechanisms of apoptosis induction.[26]

The assay uses a colorimetric finding of the chromophore p-nitroaniline (pNA) after cleavage from the DEVD-pNA, a labeled substrate. The pNA light production could be quantified using a microtiter plate reader at 405 nm. Assessment of the absorbance of pNA from an apoptotic sample with a control allows determination of the fold rise in caspase-3 activity.[27]

Statistical analysis

The results of the data were expressed as the mean ± standard error of the mean significance was calculated using one-way ANOVA followed by Dunnet's multiple comparison tests compared to control.

RESULTS AND DISCUSSION

The spreading of cancer is increasing worldwide, and the percentage of deaths caused by this fatal disease is rising, especially in the developing countries. Scientists and researchers are now giving more of their attention to the herbal medicine to provide treatment for more difficult diseases like cancer due to the fact that, the treatments of cancer patients with chemical therapy have serious side effects. Recently, herbal medicines are coming to play a more vital role in the reduction and prevention of cancer. Plants have been studied in-depth for immunomodulatory and cancer treatment purposes, i.e., *Nigella sativa, Acacia seyal, Allium sativum, Olea europaea,* and *Vitis vinifera*, resulting in isolation of lead compound with promising results in treating cancer. Resveratrol is a leading example isolated from *V. vinifera* applied effectively in treating cancer.[28]

MTT assay was conducted to evaluate the growth inhibitory effects of extract from *H. benghalensis* on the cell viability of three cancer cell lines such as HeLa, MCF-7, and IMR-32 cells, which is based on the reduction of MTT at different concentrations (10, 20, 40, 80, and 160 μg/ml). After 48 h of treatment, methanolic extract of *H. benghalensis* exhibited higher inhibitory effect against all tumor cells, with varying efficiencies and selectivities while others caused marginal cell inhibition [Figure 1]. The evaluation of the anticancer activity of plant extracts is essential for safe treatment. It enables identification of the intrinsic toxicity of the plant and the effects of acute overdose.[29,30] The MTT assay is used in screening the crude extracts as well as in the isolated compounds to assess the toxicity. It could also provide an indication of possible cytotoxic properties of the tested plant extracts. MTT assay is based on the reduction of MTT by mitochondrial dehydrogenase by purple formazan product. It is frequently used as an *in vitro* model system to measure cytotoxic effects of variety of toxic substances and plant extracts against cancer cell lines.[31] The concentration of the extract causing 50% cell death (IC_{50}) values of test extract are given in Table 1. The IC_{50} values

Table 1: The IC_{50} values of HBM using different cell cultures

Extract	IC_{50} values (μg/mL)		
	HeLa	MCF-7	IMR-32
HBM	50.73±2.77**	47.90±3.43*	53.76±3.41**
STD	13.49±1.09***	12.04±1.10***	13.69±0.96***

*P<0.05, **P<0.01, and ***P<0.001 compared to control. The IC_{50} values of HBM using different cell cultures. Data were Mean±SEM. HBM: *Hiptage benghalensis* Methanolic extract; STD: Cisplatin; SEM: Standard error of mean; HeLa: Human cervical carcinoma; MCF: Human breast cancer; IMR-32: Human neuroblastoma; IC_{50}: Concentration of the extract causing 50% cell death

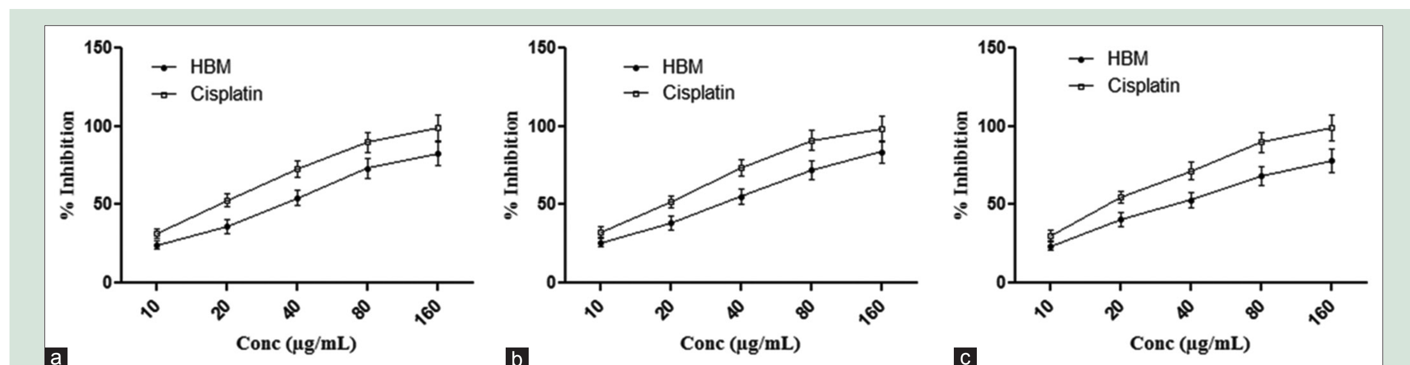

Figure 1: Effect of methanolic extract of Hiptage benghalensison cytotoxicity of (a) human cervical carcinoma, (b) human breast cancer, and (c) human neuroblastoma cancer cell lines; data were mean ± standard error of the mean

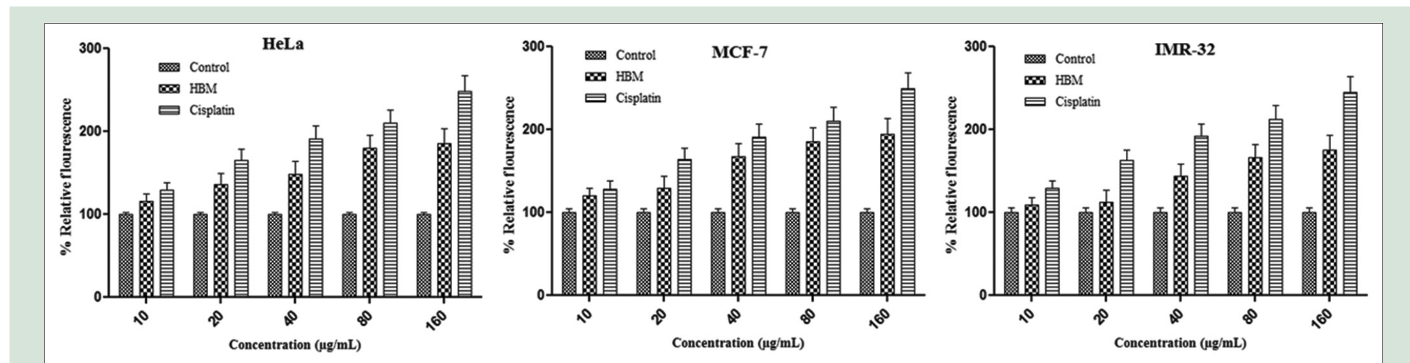

Figure 2: Reactive oxygen species generation effect of methanolic extract of *Hiptage benghalensis* on human cervical carcinoma, human breast cancer, and human neuroblastoma cancer cell lines; data were mean ± standard error of the mean

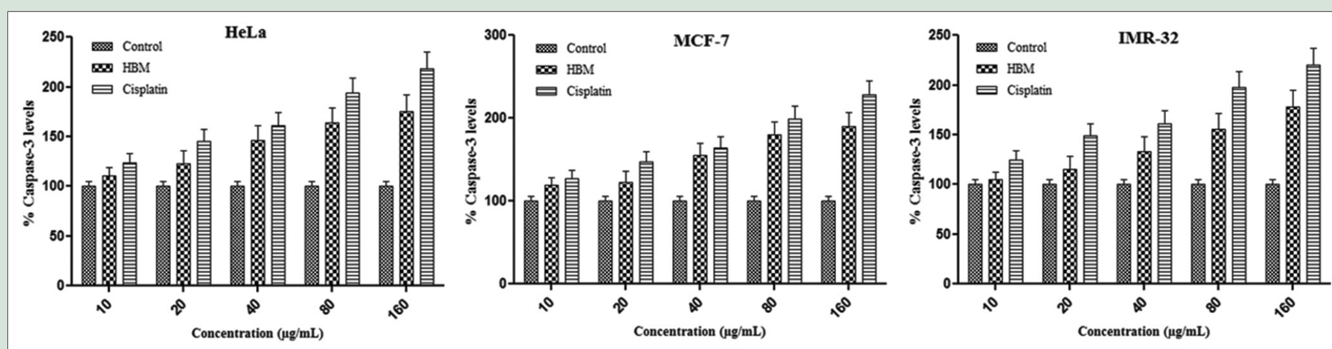

Figure 3: Caspase-3 levels of methanolic extract of *Hiptage benghalensis* on human cervical carcinoma, human breast cancer, and human neuroblastoma cancer cell lines; data were mean ± standard error of the mean

of methanolic extract of *H. benghalensis* were found to be 50.73, 47.90, and 53.76 µg/mL against HeLa, MCF-7, and IMR-32, respectively.

Since the cytotoxicity of methanolic extract of *H. benghalensis* was greater, we further evaluated to reveal the mechanism for its cytotoxicity. For this purpose, we evaluated the apoptotic activity through ROS generation and caspase-3 activities of test extracts against all three cancer cell lines. The results showed that the methanolic extract has significantly increased the ROS production in all the cell cultures [Figure 2]. ROS plays a major role in cellular senescence paving way to cell death; therefore, there is an urgent need for potential therapeutics that may prevent oxidative stress-induced neurodegeneration. It is highly possible that the prooxidant effect is responsible for the apoptotic activity of these extracts, and ROS are key signaling molecules to modulate cell death.[32] Accumulating evidence indicates that cancer cells produce high levels of ROS that lead to a state of increased basal oxidative stress. The increased production of ROS in cancer cells was observed *in vitro* studies.[33] Ahamad *et al*. revealed that naringenin leads to cell death in cancer cells through inducing ROS generation.[34] We, therefore, investigated the effectiveness of methanolic extract in the generation of ROS. We found that exposure of all three cancer cells with these extract dramatically enhanced generation of intracellular ROS at different levels in a dose-dependent manner in all cell lines.

Cancer is characterized by an uncontrolled increase in cell proliferation and/or a reduction in cell apoptosis. Inhibition of growth and induction of apoptosis in cancer cells were considered as the strategies for the cancer treatment.[35,36] As shown in Figure 3 significantly inhibited the growth of cells, which was further confirmed by the decreased cell density. Furthermore, caspases, a family of cysteine proteases, are the key proteins that modulate the apoptotic response. Caspase-3, a key executioner of apoptosis, is considered as a biomarker for cells undergoing apoptosis.[36,37] Different human cancer cells were treated with the plant extract and caspase-3 enzymatic activity within the cells was measured [Figure 3]. Since caspase-3 activity rises while cells die and cell numbers drop, it was essential to normalize caspase-3 activity to the number of cells, to obtain more accurate results. Since caspase-3 activity rises while cells die and cell numbers drop, it was essential to normalize caspase-3 activity to the number of cells, to obtain more accurate results. The results here are expressed as the percentage increase in caspase-3 activity in treated cells compared to cells added with DMSO. Caspase-3 levels were significantly increased with methanolic extract in MCF-7 ($P < 0.05$), HeLa ($P < 0.01$), and IMR-32 ($P < 0.01$) cancer cells.

CONCLUSIONS

In summary, among the *H. benghalensis* methanolic extract were showed increased percentage inhibition of MCF-7, HeLa, and IMR-32 cells using MTT assay. Moreover, the apoptotic activity revealed that the methanolic extract *H. benghalensis* significantly increased the generation of ROS and caspase-3 activities in all cancer cell lines in a dose-dependent fashion. Further studies are warranted to elucidate the molecular mechanisms of isolated compounds of *H. benghalensis***.**

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Nil.

Conflicts of interest

There are no conflicts of interest.

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