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Short Communication

In vitro Cytotoxic Activity of Ethyl Acetate Fraction of *Hibiscus vitifolius* Flowers Against HeLa Cell Line

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ABSTRACT

Hibiscus vitifolius Linn.(syn. *Fioria vitifolia* (L.) *Mattei),* is a plant native of India and many other tropical regions. It has been used traditionally for the treatment of a number of diseases. It is also well known for its antioxidant activity. The objective of the present study was to perform a preliminary phytochemical analysis and cytotoxic activity of ethyl acetate extract of *Hibiscus vitifolius* (Malavaceae) flowers. The finely powdered flowers were extracted successively with petroleum ether, diethyl ether and ethyl acetate in a soxhlet apparatus for 72 h. The ethyl acetate fraction obtained was tested for the phytochemical constituents by using preliminary phytochemical tests. The cytotoxic activity of the extract was screened by MTT (methylthiazolyl diphenyl- tetrazolium bromide) assay against HeLa (Human cervical cancer) cell lines. The phytochemical analysis revealed the presence of triterpenoids, steroids, flavonoids, tannins and phenolic compounds. The extract was shown to inhibit the cell growth with IC_{50} value of 81.27 µg/mL. These findings suggest that the ethyl acetate fraction obtained from the flower extracts of *Hibiscus vitifolius* has moderate cytotoxic activity.

Keywords: Cytotoxic activity, *Hibiscus vitifolius*, HeLa cell lines, MTT assay.

Introduction

Plants have been used for the treatment of several ailments all over the world even before the advent of modern medicine. Natural phytochemicals are known to possess several substances responsible for their pharmacological activities or acts as the precursor for the synthesis of novel drugs. Nearly 50% of the modern drugs currently in use are of natural origin and as such these natural products play an essential role in drug development. Moreover, naturally obtained compounds are considered safer and easily biodegradable than synthetic compounds and the problem of drug resistance observed in synthetic drugs is also reduced.[1] Plants remain the most common source of several drugs including anticancer agents.

Hibiscus vitifolius Linn (Malvaceae) is one of the largest genera in angiosperms consisting about 200 species, mainly distributed in tropical and subtropical regions of the world. There are around 40 species grown in India and many are valued as ornamental plants and cultivated in gardens.[2,3] Studies have shown that the plants of the *Hibiscus* genus have the potential to provide biologically active compounds that act as anti-oxidants and cardioprotective agents.[4] Plants with good anti-oxidant properties were also found to possess good anti-cancer activity.[5]

These anti-oxidants may prevent and cure cancer and other diseases by protecting the cells from damage caused by free radicals.

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Many antioxidant compounds have been reported in plants such as *H. sabdariffa* (such as cyanidin 3-rutinoside, delphinidin 3-sambubioside, cyanidin 3-sambubioside, cyanidin 3-glucoside, and delphinidin 3-glucoside), *Hibiscus rosa sinensis*, *Hibiscus vitifolius* Linn, etc.[4,6-9] Hence, *Hibiscus* genus may be a great natural source for the development of new drugs and may provide a cost-effective means of treatment for cancer and other diseases in the developing world.[10]

*Hibiscus vitifolius Linn., (*syn. *Fioria vitifolia (L.) Mattei),* is a perennial shrub widely distributed in India. It is used in the treatment of contraceptive, pulsating anterior fontanelle in babies, kidney problems etc.[11-14] Stem barks of *Hibiscus vitifolius* Linn., were shown to possess potential hepatoprotective and antioxidant effect in paracetamol-induced hepatotoxicity in rats. The flower extracts were found to possess significant antiproliferative activity against Hep G2 cell lines. Many other species of *Hibiscus* genera were also found to possess antiproliferative activity against several cancer cell lines. Very limited literature is available on the screening of phytochemicals and anticancer activity of the *Hibiscus vitifolius.* In the present study, we have selected *Hibiscus vitifolius* for the screening of its antiproliferative activity.

Materials and Methods

Collection of Flowers

Fresh flowers of *Hibiscus vitifolius* were collected from Bahadurpally village, R.R. District, Telangana, India, during the month of October 2017. The plant material was identified by Dr. Nirmala, Osmania University, Telangana, India. A Voucher specimen (PH- 806) was deposited in the herbarium of the college.

Extraction and fractionation

Fresh flowers (2 kg) of *Hibiscus vitifolius* were separated from the plant, washed under tap water and were shade-dried. Dried flowers were then crushed in a mechanical grinder and the fine powder was collected by passing it through a sieve. The fine powder of the flowers was soaked in 90% ethanol at room temperature (25 - 30°C) for a period of 72 h and

filtered. The resultant extract was further fractionated with petroleum ether, diethyl ether and ethyl acetate. Ethyl acetate fraction on concentration under reduced pressure yielded a dry powder which was dissolved in DMSO to get various concentrations which were used for further study.

Preliminary phytochemical screening
A small portion of the ethyl acetate extract was used for the phytochemical tests for carbohydrates, tannins, alkaloids, flavonoids, steroids, saponins and coumarins.[15-20]

In vitro evaluation of anticancer activity by MTT assay
Cell culture
The human cervical adenocarcinoma cell line (HeLa) was provided by Tata Memorial Research Centre, India and was grown in Eagles Minimum Essential Medium (EMEM) which contained 10% fetal bovine serum (FBS). All cells were maintained at 37°C, 100% relative humidity, 5% CO_2, 95% O_2 and the culture medium was changed twice a week.

Cell treatment
The monolayer cells were detached and single cell suspensions were made using trypsin-ethylenediaminetetra acetic acid (EDTA). A hemocytometer was used to count the viable cells and the cell suspension was diluted with a medium containing 5% FBS in order to obtain a final density of 1 x 10^5 cells/mL. A 96-well plates at plating density of 10,000 cells/well were seeded with 100 µL per well of cell suspension and incubated for cell attachment at 37°C, 5% CO_2, 95% O_2 and 100% relative humidity. The cells were treated with serial concentrations of the test samples after 24 h. Serial dilution method was used for preparing test samples of different concentrations. Cells were initially dissolved in dimethylsulfoxide (DMSO) and further diluted with a serum-free medium to obtain twice the desired final maximum test concentration. The required final sample concentrations of 1.953, 3.906, 7.812, 15.625, 31.25, 62.5, 125 and 250 µg/mL were obtained by adding aliquots of 100 µL of the different sample dilutions to the appropriate wells already containing 100 µL of medium.
After addition of the sample, the plates were incubated for an additional 48 h at 37°C, 5% CO_2, 95% O_2 and 100% relative humidity. The medium without samples served as control and triplicate were maintained for all concentrations. 5-Fluorouracil (150 µg/mL) was used as a positive control. After 48 h of incubation, to each well was added 15 µL of MTT (5 mg/mL) in phosphate buffered saline (PBS) was and incubated at 37°C for 4 h. The medium with MTT was aspirated and the formed formazan crystals were solubilized in 100 µL of DMSO. Using a micro plate reader, the absorbance was measured at 570 nm.[20]
The percentage cell inhibition was determined using the following formula.

$$\% \text{ Cell Viability} = \frac{[(\text{O. D. of control} - \text{O. D. of test compound})]}{\text{O. D. of control}} \times 100$$

The IC_{50} is half the maximal inhibitory concentration of the toxic compound.

Statistical analysis
The absorbance values were denoted as mean ± SEM. IC_{50} was determined using Microsoft excel sheet.

Results and Discussion

Continuous hot percolation of the powdered flower material with petroleum ether, diethyl ether and ethyl acetate yielded 30.92 g, 19.64 g, 26.46 g and 118.50 g of the respective extracts in a semisolid consistency. In recent years, attention has been focused on the antioxidant and anticancer properties of plant-derived dietary constituents of food.[21] Our preliminary phytochemical screening for *H. vitifolius* flowers (Table 1) revealed the presence of triterpenoids, steroids, flavonoids, tannins and phenolic compounds while carbohydrates, proteins, amino acids, alkaloids, cardiac glycosides, anthraquinone glycosides, saponin glycosides, fixed oils and fats were absent. This was in agreement with previously reported work of Nimila *et al* where the stems of *H. vitifolius* contained proteins, amino acids, fixed oils and fats in the petroleum ether extract. Alkaloids were absent in all the methanol, petroleum ether and ethyl acetate extracts of the plant.[22] Studies have shown that the presence of these phytochemical compounds is known to support the bioactivities of medicinal plants[23,24] and thus may be responsible for the possible anticancer activity.
Recently, many reports are available wherein flowers or their extracts have been shown to exhibit rich antioxidant, anticancer and antimicrobial properties.[25-27] The presence of high level of total phenols, flavonoids, and anthocyanins has been reported in different flowers and their extracts,[28-31] thus supporting the results and observations done in the present study.

In vitro anticancer activity
The results of the MTT assay are presented in Figure 1. The data obtained in the present study revealed that the number of HeLa cells in the negative control wells (without extract) increased from 2 × 10^4 to 6 × 10^4 after 72 h of incubation (Figure 2). In the extract-treated wells, the number of viable cells decreased in a concentration-dependent manner which demonstrated the cytotoxic activity of the plant extract on HeLa cells. It was found that the extract produced 50% of cell death at a concentration of 81.27 µg/mL against cancer cell lines.
In case of HepG2 cell lines, the crude extracts showed moderate to weak cytotoxic activity with IC_{50} of 100 µg/mL.[32] Though very limited literature is available on the cytotoxic activity of the *H. vitifolius* flowers, other species of the genus (*Hibiscus*) have shown promising cytotoxic activity. Extracts from the calyces of *Hibiscus sabdariffa* L. (Malvaceae), were reported to have a variety of pharmacological effects *in vivo* and *in vitro*, including anticancer and antioxidant properties.[33-36] There is very important evidence of the anticancer action of *H. rosa sinensis* extract against the tumor promotion stage of cancer development, in mouse skin irradiated with ultraviolet radiation.[37] The crude extract showed the presence of flavonoids, tannins, triterpenes, phenols and steroids. Phenols and polyphenols, flavonoids, tannins, triterpenes have also been reported to possess anticancer activity.[38] Flavonoids have attracted a great deal of attention in relation to their potential beneficial effects on health.[39] The role of the flavonoids in the treatment of various chronic diseases involving oxidative stress (i.e. in cancer) had also been proved.[40] Flavonoids were found to exhibit anticancer activity via modulation of cell cycle arrest at the G1/S phase, down-regulation of anti-apoptotic gene products.[41] According to Gali *et al*, the anticancer effects of methanol extract of *Argemone mexicana* Linn. leaves may be related to their content of flavonoids.[42] According to Pradhan, flavonoids may exert their chemopreventive role in cancer through their effects on signal transduction in cell proliferation and angiogenesis.[43]
Furthermore, triterpenes also induce apoptotic response on cancer cells by inhibiting nuclear factor kappa B or by causing cell cycle disruption. Nimila *et al*., in their study showed that the hepatoprotective and antioxidant effect of the ethyl acetate extract (stem barks) of *Hibiscus vitifolius* Linn could be possibly due to the presence of flavonoids.[1] The crude extracts exhibited greater and potential hepatoprotective and antioxidant effect in paracetamol-induced hepatotoxicity in rats.
Thus, the cytotoxic effect of the ethyl acetate extract flowers of *Hibiscus vitifolius linn* could be possibly due to the presence of flavonoids, triterpenoids and phenols.

Table 1: Phytochemical screening of *H. vitifolia*

Phytochemical	Inference
Carbohydrates	–
Proteins and amino acids	–
Triterpenoids and steroids	+
Alkaloids	–
Cardiac glycosides	–
Anthraquinone glycosides	–
Saponin glycosides	–
Flavonoids	+
Tannins and phenolic compounds	+
Fixed oil and fats	–

+ indicate presence of phytochemical
- Indicate the absence of phytochmical

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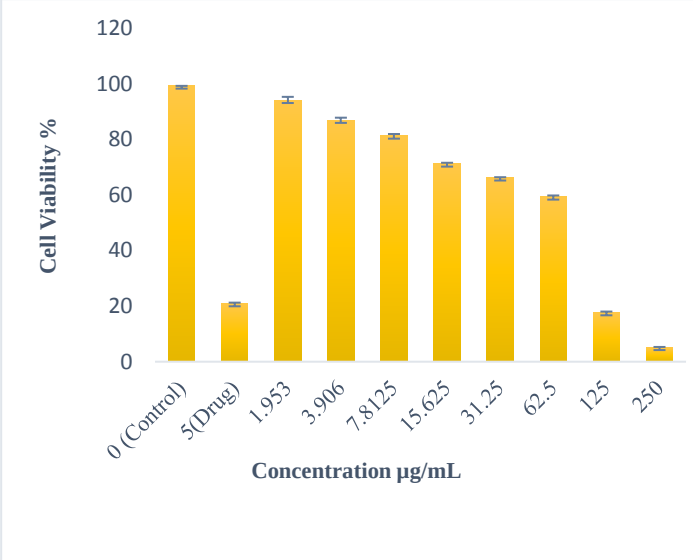

Figure 1: Percentage cell viability of HeLa cell line after treatment with the extract by MTT assay. Values are in mean ± SEM. 5-Fluorouracil was used as a positive control.

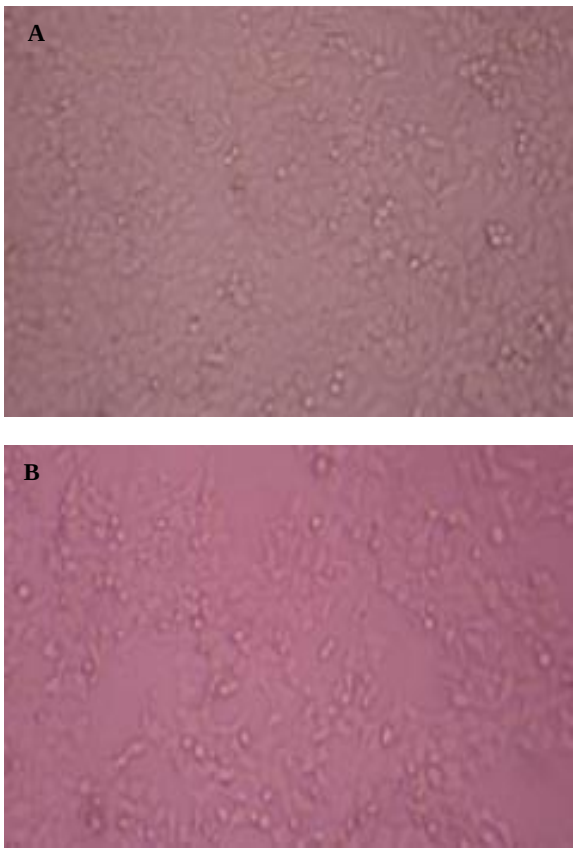

Figure 2: HeLa cells lines after 48 h of incubation **A.** Control (overcrowded cells) **B.** Extract-treated cells at 125 µg/mL extract (decrease in the cells density).

Conclusion

From the present study, it was concluded that the ethyl acetate extract of *Hibiscus vitifolius* flowers were active against Human Cervical Cancer Cell Line (HeLa cell lines) which may be due to synergistic effect of the secondary metabolites present in the extract. Further studies are required for isolation of the active principles responsible for its therapeutic activity and clinical study for evaluation of safety and efficacy of the drug.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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