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Cytotoxicity of various extracts of *Indigofera barberi* Gamble

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Abstract
Indigofera barberi Gamble (Fabaceae) plant traditionally used to treat various skin diseases, renal disease and liver disease. Reported Phytochemicals are glycosides, steroids, tannins and Phenolic compounds and flavonoids. However, no studies have been investigated for cytotoxic effect. The present study carried out to investigate the cytotoxic effect of petroleum ether, chloroform and ethanolic extracts of this plant on the growth of the human liver hepatoma cell lines were examined by MTT assay. The IC_{50} value of petroleum ether, chloroform and ethanolic extracts of *Indigofera barberi* in HepG2 cell line found to be 150mg/mL, 100mg/mL and 75mg/mL respectively. Among the tested cell lines, ethanolic extract of was more selective cytotoxic against HepG2 cell line. The Compound IB-4, isolated by further subjected from ethanolic extracts and its IC_{50} value found to be 40mg/mL.

Keywords: *Indigofera barberi* gamble, MTT assay, HepG2 cell line, cytotoxicity, IC_{50} value

1. Introduction
In worldwide, cancer is one of the leading causes of death. Nearly 0.5% of population in US is diagnosed with cancer. Development of imaging and molecular diagnostic techniques, cancer continues to strike millions of people globally. Cytotoxicity testing is one of the method is use to detect whether a compound or extracts contains notable quantities of biologically active or harmful compounds. Cytotoxicity test methods are useful for screening because they serve to separate toxic from nontoxic materials, providing predictive evidence of compound safety [1]. Cytotoxicity studies involved the analysis of morphological damage or inhibition of zone of outgrowth induced by the chemicals tested. There are different methods used for the evaluation of cytotoxicity, MTT (3-(4, 5-dimethylthiazol-2yl)-2, 5- diphenyl tetrazolium bromide) method is one among them, which measures the metabolic activity of the viable cells. The reaction between MTT and 'mitochondrial dehydrogenase' produces water-insoluble formazan salt which is solubilized and quantified with an ELISA plate reader. The absorbance directly correlates with the cell number [2].
Nature is a reliable source of new drugs including anti-cancer agents. *Indigofera barberi* Gamble (Fabaceae) is a threatened medicinal plant, which plays an important role in folklore medicine. The medicinal plant traditionally used to treat various skin diseases, renal disease and liver disease [3-6]. Preliminary phytochemical analysis of the aerial plant extracts showed the presence of glycosides, steroids, tannins and Phenolic compounds, flavonoids and carbohydrates [7]. However there are no studies have been able to demonstrate the cytotoxic effect of *Indigofera barberi*. The main aim of this study was to investigate the cytotoxic effect of *Indigofera barberi* against HepG2 cell lines.

2. Material and Methods
2.1 Collection and Identification of Plant materials
The aerial parts of *Indigofera barberi* Gamble were collected from Thalakona (Nelakona regions) of Chitoor District of Andhra Pradesh, India in the month of November 2010. The plant material was taxonomically identified by Prof. P. Jayaraman, Plant Anatomy Research Centre, Chennai, Tamil Nadu and India. The voucher herbarium specimen (PARC/2012/1246) has been preserved in our laboratory for further reference. The aerial parts of *Indigofera barberi* were subjected to shade drying for about two and half months, then segregated, pulverized by a mechanical grinder and passed through a 22 mesh sieve. The coarse powdered plant materials were kept in an airtight container for further analysis.

2.2 Preparation of various extracts from *Indigofera barberi*

The aerial parts of *Indigofera barberi* were dried and powdered. The plant powdered materials were successfully extracted with petroleum ether (40-60ºC) by hot continuous percolation method in Soxhlet apparatus for 24 hrs[8]. Then this marc was dried and then subjected to chloroform extraction (60ºC) for 24 hrs, then marc was dried and then it was subjected to ethanol (95%) extraction (80ºC) for 24 hrs. The extracts were filtered and concentrated using rotary flash evaporator and residues were dried in desiccators over sodium sulphite below 60°C.

2.3 Isolation of Compounds by column chromatography

2.3.1 Preparation of Admixture

The phytochemical screening of three different extracts shows the presence of secondary metabolites but large classes of phytochemicals are shows the presence in the ethanolic extracts. Hence the ethanolic extracts are selected for the further isolation of biologically active compounds from *Indigofera barberi*. 20 grams of ethanolic extract of *Indigofera barberi* (EEIB) is admixed with 20 grams silica gel (60/120 meshes) to get uniform mixing.

2.3.2 Column Packing

The crude extract of EEIB was subjected to column chromatography for the identification of active phytoconstituents by using column chromatography. The silica gel was used as stationary phase and eluted with various solvent systems for the mobile phase, elution starts from low polar to high polar solvents. EEIB (20.0g) was dissolved in ethanol added with 16 gm of silica gel of mesh size 60 – 120, thoroughly mixed and kept for air drying. The material was packed in the column and by using petroleum ether the admixture was loaded to the top of the column. Then the column was eluted with petroleum ether initially and by the order of increasing polarity the solvents like petroleum ether, benzene, ethyl acetate and ethanol were added. Each and every tube is analyzed by TLC, with single and similar spots are pooled together. The solvents were nullified using rotary evaporator under vacuum.

Elution of the column with petroleum ether: benzene (60:40, v/v) gave a solid, designated as IB-1, and was characterized. The compound IB-1 was found to be homogeneous and tested on TLC in the following solvent systems, Petroleum ether: benzene (4:6, v/v) and Benzene: acetone (8.5:1.5, v/v) and showed very negligible quantity and was not able to weigh.

Elution of the column with benzene (100%) has resulted a semi solid, designated as IB-2 and was characterized. The compound IB-2 was found to be homogeneous and tested on TLC in the following solvent systems, Benzene: acetone (7:3, v/v) and Benzene: ethyl acetate (8:2, v/v) and showed very negligible quantity and was not able to weigh.

Elution of the column with benzene: ethyl acetate (2:8, v/v) has resulted a semi solid, designated as IB-3 and was characterized. The compound IB-3 was found to be homogeneous and tested on TLC in the following solvent systems, Benzene: chloroform (5:5, v/v) and Benzene: ethyl acetate (9:1, v/v) and showed very negligible quantity and was not able to weigh.

Elution of the column with benzene: ethyl acetate (4:6, v/v) has result a semi solid, designated as IB-4 (185 mg) and was characterized. The compound IB-4 was found to be homogeneous and tested on TLC in the following solvent systems, Toluene: ethyl acetate: methanol (5:3:2, v/v) and Toluene: ethyl acetate: formic acid: methanol (5:2:1:1, v/v) and compound IB-4 was colorless semi solid, m.p.314°C.

2.4 Cell lines and culture medium

HepG2 (Human liver hepatoma cells) was used in this study. These were obtained from National Centre for Cell Sciences, Pune.

2.5 Methods for MTT assay [9, 10]

MTT [(3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazoliumbromide] measures the metabolic action of the feasible cells. The assay is non-radioactive and can be performed completely in a microtiter plate (MTP). It is suitable for measuring cell multiplication, cell viability or cytotoxicity. The response between MTT and 'mitochondrial dehydrogenase' produces water-insoluble formazan salt. This technique includes culturing the cells in a 96-well microtiter plate, and after that incubating them with MTT solution for give or take 2 hours. Among incubation period, viable cells change over MTT to a water-insoluble formazan dye. The formazan dye in the MTP is solubilized and measured with an ELISA plate reader. The absorbance specifically associates with the cell number. This is applicable for adherent cells cultured in MTP.

Human liver hepatoma cells (HepG2) (obtained from National Centre for Cell Sciences, Pune, India). 0.1ml of the cell suspension (containing 1x105cells) and 0.1ml of the extracts of *Indigofera barberi* (25-400 µg/ml) in DMSO such that the final concentration of DMSO in media was less than 1% were added to the 96 well plates and kept in carbon dioxide incubator with 5% CO_2, at 370° C for 72 hours. Blank contains only cell suspension and control wells contain 1% DMSO and cell suspension.

After 72 hours, 20µL of MTT was added and kept in carbon dioxide incubator for 2 hours took after by 80µL of lysis buffer (15% SLS in 1:1 DMF and water). The plate was covered with aluminum foil to protect it from light. At that point the 96 well plates are kept in rotary shaker for 8 hours. Following 8 hours, the 96 well plates are processed on ELISA reader for absorption at 562nm. Blank contains only cell suspension and control wells contain 1% DMSO and cell suspension were also similarly treated. The readings were averaged and viability of the test specimens is compared with DMSO control. The percentage growth inhibition is ascertained utilizing the following formula.

$$\text{\% Growth Inhibition} = \frac{\text{Mean OD of Individual Test Group}}{\text{Mean OD of Control Group}} \times 100$$

3. Results

The effect of petroleum ether, chloroform and ethanolic extracts of *Indigofera barberi* plant on the growth of the human liver hepatoma cell lines were examined by MTT assay. In the experiment the IC_{50} value petroleum ether, chloroform and ethanolic extracts of *Indigofera barberi* in HepG2 cell line found to be 150µg/mL, 100µg/mL and 75µg/mL respectively. The results were provided in the Table 1. Isolation of compounds by column chromatography showed that four compounds were isolated viz. Compound IB-1, IB-2 and IB-3 were not able to weigh; and the compound IB-4 was (185 mg) colourless, semi solid, m.p.314°C is found to be homogeneous. Simple qualitative

chemical analysis confirms that, IB-4 is the flavonoid was isolated from ethanolic extract of *Indigofera barberi*. The IC_{50} value of the isolated compound IB-4 in HepG2 cell line found to be 40µg/mL. The results were provided in the Table 2.

Table 1: Results of cytotoxic effect of various extracts of *Indigofera barberi* on Human liver hepatoma cells (HepG2) by MTT assay

Concentrations (µg/mL)	PEEIB IC_{50} (µg/mL)	CEIB IC_{50} (µg/mL)	EEIB IC_{50} (µg/mL)
400	68	76	83
200	59	61	68
100	38	49	57
50	31	33	40
25	22	19	26
IC_{50} (µg/mL)	150	100	75

Results are expressed as mean±SEM. N=3; IC_{50}, Drug concentration inhibiting 50% cellular growth following 72 h of drug exposure

Table 2: Results of the cytotoxic effect of compound IB-4 on Human liver hepatoma cells (HepG2) by MTT assay

Compound	HepG2 IC_{50} (µg/mL)
IB-4	40

Results are expressed as mean±SEM. N=3; IC_{50}, Drug concentration inhibiting 50% cellular growth following 72 h of drug exposure.

4. Discussion

This study was undertaken to scientifically prove the cytotoxic effect of various extracts of *Indigofera barberi* possessing anticancer activity. *Indigofera barberi* is known and reported for its antioxidant, hepatoprotective and nephroprotective activity. Since there is no studies have been reported for the cytotoxic effect of this plant extacts. An experiment was made to determine the cytotoxicity of different extracts of *Indigofera barberi* by MTT assay method. When the cells were treated for 72 hours with various concentrations of petroleum ether, chloroform and ethanolic extracts (25-400 µg/ml), the relative cell survival progressively decreased in a dose dependant manner. The IC_{50} value of petroleum ether, chloroform and ethanolic extracts of *Indigofera barberi* in HepG2 cell line found to be 150µg/mL, 100µg/mL and 75µg/mL respectively. Among the tested cell lines, ethanolic extract of *Indigofera barberi* was more selective cytotoxic against HepG2 cell line than others in dose dependent manner. Flavonoids are chemo preventive role in cancer through their effects on signal transduction in cell proliferation and angiogenesis [11, 12]. Phytochemical screening of *Indigofera barberi* shows the presence of flavonoids. Moreover flavonoid is the major classes of phytoconstituents presents in all the three different extracts. IB-4, were isolated from ethanolic extracts of *Indigofera barberi*. These results show that cytotoxic properties of the various extracts of *Indigofera barberi* may be due to presence of these compounds. The IC_{50} value of the isolated compound IB-4 in HepG2 cell line found to be 40µg/mL. The results show that *Indigofera barberi* is more cytotoxic against HepG2 cells.

4. Conclusion

In conclusion, the results of cytotoxicity study reveals that various extracts of *Indigofera barberi*. Among all three extracts of *Indigofera barberi*, ethanolic extract possessed the most powerful anticancer activity, with specific selectivity against HepG2 cells and its isolated compounds have significant cytotoxic activity against Human liver hepatoma cell lines. Further studies are essential to characterize the active principles and to elucidate the mechanism of action.

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