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SHORT COMMUNICATION

In Vitro Antioxidant and Anticancer Potency of *Tabernaemontana divaricata* (L.) Flowers

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Abstract

A study was performed to identify an antioxidant and anticancer property of the flower of *Tabernaemontana divaricata* (L.). *In vitro* antioxidant activity was identified through the execution of various free radical scavenging assays like DPPH (1,1-diphenyl-2-picrylhydrazyl) radical, superoxide anion radical, nitric oxide radicals, hydrogen peroxide radicals in ethanolic flower extract of *T. divaricata* (L.). As a result of these scavenging methods, DPPH radicals were repressed at the percentage of 38.9% in 1000 µg/ml. Likewise in the same concentration of extract, repression of superoxide anion radicals, nitric oxide radicals, and hydrogen peroxide radicals occurred at 49.3%, 56.4% and, 31.2% respectively. *In vitro* antiproliferative activity of the ethanolic flower extract was evaluated using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay to find the percentage of cell inhibition as well cell viability of HepG2 (Hepatocellular carcinoma), HeLa (Cervical adenocarcinoma) and A549 (Lung adenocarcinoma) cancer cell lines. Growth of all these cell lines was inhibited at 89.7%, 91.2% and 90.4% in 500 µg/ml. MTT assay exhibited IC_{50} value at 15.6 µg/ml of extract concentration. The effect of plant extract concentration was identified through decreasing the percent of viable cancer cells when compared to control cells. The results showed that dose dependent cytotoxic activity of *T. divaricata* (L.) against all cancer cell lines. The present work concluded that flower of *T. divaricata* (L.) can act on various cancer cells under *in vitro* condition via neutralizing effect of free radicals.

Keywords: Anticancer Activity, Antioxidant Property, Cancer Cell Lines, Free Radicals, Phenolic Compounds, *Tabernaemontana divaricata* (L.)

1. Introduction

One of the foremost health issues in the public domain is cancer next to cardiovascular disease in developed as well in developing countries. It causes death of many people all over the world. It is expected that in the year 2030, up to 21 million humans will be affected because of cancer[1,2]. Uncontrolled proliferation of abnormal cells has the capacity to assault and detonate contiguous tissues[3]. Hence, the neighbouring normal cells are transformed into malignant cells and it also rushes out to other parts of the body by means of blood stream and lymphatic systems. This migration process is known as metastasis[4,5].

Radiation, smoking, tobacco, contaminants in food, drinking water likewise hormone related problems, and genetic instabilities are referred to as extrinsic and intrinsic elements that are responsible for the origination of cancer[6,7]. Human beings are distressed through 200 forms of cancer[8]. In the northeast province, cervical cancer is a prevalent disease now. Females are distressed through cervical and breast cancer habitually. Commonly, lung cancer mostly affects male[9,10].

One of the free radicals is reactive oxygen species (ROS), contains one or more free electrons in their external orbital. Superoxide anion (O_2^-), ozone (O_3), hydroxyl ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2) radicals have come under the category of ROS [11,12]. The

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unrestrained stage of ROS play a role as an origin for several diseases like cancer, diabetes, neurodegenerative troubles, nephrological problems, and cardiovascular complaints[13,14].

Nowadays cancer growth is regulated using various therapies such as radiation, chemoprevention, immunomodulation, and surgery[15]. Single or combined forms of synthetic or natural agents are used for chemotherapy to treat cancer[16]. Normal cells are afflicted seriously through the usage of radiation as well as medications used for chemotherapy. Some gastrointestinal problems (vomiting, nausea, bone marrow suppression) are arising through the exploitation of cancer medications[17].

So, necessity of less detrimental effect with antioxidant activity obtaining compounds is needed to handle the situation of cancer. Whole part or phytoconstituents from medicinal plants can be used for the treatment of cancer recurrently[18,19]. Secondary metabolites exist in herbs are flavonoids[20], alkaloids[21] and terpenoids[22] which play a vital role in cancer cell eradication by flaunting antioxidant capacities[15,23].

Antioxidants have the capability to act as free radical scavengers, chelating agents as well as controls lipid peroxidation process. Antioxidant defense system precludes the cell damage as a result of terminating sequential reactions of ROS formation by donating an electron to the free radicals[24,25]. Dietary intake of these plant derived phytoconstituents can reduce the risk of disorders instigated by free radicals because of the existence of natural antioxidants in them[26,27].

One of the Apocynaceae family plants is *Tabernaemontana divaricata* (L.). It is known as Crepe Jasmine flore pleno (English) and Adukku nandiyavattai (Tamil) and Chandani (Hindi). Flower juice is used in the treatment of eye infections. Flowers are white, gives cooling, used to treat blistering sense, ophthalmitis, dermatopathy[28], ulcers, and rheumatism[29]. Root has a choking taste. Fondling of oil with its juice on the head cures pain in the eye likewise crushing of root decreases toothache. In sores, it is applied to thwart inflammation with the combination of water[30].

2. Materials and Methods

2.1 Preparation of Extract

Double layered flower variety of *T. divaricata* (L.) had been identified through reference[31]. Flower of this plant is collected, dried in shade for a few days. The dried material is powdered. Then flower powder (5 g) had been blended with 200 ml of 95% ethanol in sterile glass bottles. This mixture is closed tightly and kept for one week with intermittent wavering. Subsequent to the extraction, extracts were filtered through a white muslin cloth in addition to whatmann No.1 filter paper. Filtrates were subjected to evaporation, and further, it was disseminated under vacuum. The dehydrated extract was hoarded in sealed vials for radical scavenging plus anticancer activity assays.

2.2 Free Radical Scavenging Assays

2.2.1 DPPH Radical Scavenging Assay[32]

Different concentrations of ethanolic flower extract were utilized with the range from 200 - 1000 µg/ml. Extract (2.5 ml) was combined with the ethanolic solution of DPPH (0.3 mM, 1 ml) then set aside at room temperature. After half an hour, OD (optical density) was calculated at 518 nm. DPPH with 1 mM morin was exploited as standard. The scavenging capacity in percentage was computed using equation (1) as given below,

DPPH Scavenging capacity (%) = $[(A_C - A_S) / A_C] \times 100$ -------- (1)

where A_C – Control absorbance

A_S – Sample absorbance

2.2.2 SO (Superoxide) Anion Radical Scavenging Assay[33]

PBS (Phosphate Buffered Saline, 0.05 M, pH 7.8), Methionine (13 mM), Riboflavin (2 µM), Ethylene Diamine Tetra Acetic acid (EDTA, 100 µM), NBT (Nitro Blue Tetrazolium, 75 µM) was combined with 1 ml of ethanolic flower extract with various concentrations (200 – 1000 µg/ml). Rutin (1 mM) was used as a standard. Extract and standard tubes were held before 725 lumens, 34 W luminous beams. After 20 minutes,

absorbance values were taken at 560 nm. These tubes were covered in an aluminium foil ruled box.

Superoxide anion radical inhibition was calculated through below equation (2),

Inhibition (%) = $[(A_C - A_S) / A_C] \times 100$ ---------- (2)

2.2.3 NO (Nitric Oxide) Radical Scavenging Assay[34]

PBS with 0.5 ml of sodium nitroprusside (10 mM) was added in 1 ml of different concentrations (200 – 1000 µg/ml) of flower extract tubes. Afterward kept in visible light at 25˚C for 180 min. Nitrite ion was formed after 3 hours, when NO reacts with oxygen. This reaction was enhanced by the addition of an equivalent level of fresh Griess reagent made up of sulphanilamide (1%) in phosphoric acid (2.5%) and naphthyl ethylene diamine dihydrochloride (0.1%). 1 mM gallic acid was utilized as standard. OD was estimated at 546 nm. Scavenging percentage was evaluated using the following expression (3),

Nitric oxide radical scavenging (%) = $[(A_C - A_S) / A_C] \times 100$ ---------- (3)

2.2.4 H_2O_2 (Hydrogen peroxide) Radical Scavenging Assay[35]

Diverse quantities of flower extracts (200 – 1000 µg/ml) were combined with 0.6 ml of phosphate buffer (pH 7.4, 40 mM) containing H_2O_2. After 10 minutes, absorbance values were acquired at 230 nm. Phosphate buffer was employed as blank. 1 mM ascorbic acid was exploited as standard.

Percentage of hydrogen peroxide scavenged was deliberated with the help of given expression (4),

H_2O_2 scavenging (%) = $[(A_C - A_S) / A_C] \times 100$ ------ (4)

2.3 *In Vitro* Anticancer Activity

2.3.1 Types of Cell Lines Used

The liver cancer (HepG2), cervical cancer (HeLa), and lung cancer cell lines (A549) were purchased from the National Centre for Cell Science, Pune, India.

2.3.2 Culture Conditions

HepG2 cell line was cultured in DMEM (Dulbecco's modified Eagle's medium, Himedia, India). These cultured cells were supplemented with 10% FBS (fetal bovine serum) and penicillin-streptomycin (100 U/ml) and then stored at 37˚C, 5% CO_2.

HeLa and A549 cultures were grown in Rosewell Park memorial institute (RPMI1640). The cell lines were added with FBS (10%) after hoarding in a humid environment with 5% CO_2 at 37˚C. The culture medium was changed once in two days.

2.3.3 Cytotoxicity Assay[36]

Aliquots (90 µl) containing 1×10^4 cells of cancer cell line suspensions were seeded into the wells of 96-well titer plates. After 24 h of incubation, 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, and 500 µg/ml of flower extract was added then incubated for further 48 hours. The cytotoxicity test was conducted through the use of MTT assay and detected the formazan dye at 540 nm using a microplate spectrophotometer by taking DMSO (Dimethyl sulphoxide) as a blank[37]. Each treatment was assayed in triplicate. 0.01% of ethanol was utilized as a negative control. Cell growth inhibition percentage was calculated using the formula (5) given below,

Percentage of cells inhibited = 1 – (OD of test wells / OD of control wells) x 100..... (5)

2.3.4 Cell Viability Assay[38]

Tetrazolium salt-MTT (Yellow) converted into formazan crystals (purple) provides the identification of viable cells quantitatively. 96 well plates used at a cell density of 2×10^5/ml (per well) diluted in RPMI 1640 (100 µl). For 24 hours, it was grown in a CO_2 incubator with 37˚C, 5% CO_2. One day later flower extract was disseminated in DMSO. Then this mixture was injected into every well and kept for 48 hours. DMSO alone was given to control groups. Positive control taken for this assay was doxorubicin. 100 µl of MTT solution (0.5 mg/ml) was mixed with all well and set aside for 3 hours. Viable cells were determined by the reduction of MTT to purple formazan. The medium was separated, then DMSO (100 µl) was combined within wells to disperse formazan compound. After that, the plate was agitated for 5 min at 150 rpm. OD was deliberated at 570 nm. Percentage of cell viability was identified using the following equation (6),

Cell viability (%) = (OD of test wells / OD of control wells) x 100..... (6)

2.4 Statistical Analysis

All assays were executed at triplicate. The values are represented as mean ± standard deviation through graphs discussed in the results section.

3. Results and Discussion

3.1 Free Radical Scavenging Activity

Phenolic compounds are one of the secondary metabolites in the plant. It comprises many bioactivities like antibacterial, anti-allergic, anti-inflammatory as well as anticarcinogenic characters. Breaking of free radical chain reactions can occur through quenching of radicals because of the presence of flavonoids[39,40].

Donation of electron or hydrogen to the DPPH radical can lead to deactivation of this radical (DPPH-H)[41], for the reason that antioxidants exist in the flower of *T. divaricata* (L.). Lower percentage inhibition of DPPH radicals was observed when decreased concentration of extracts such as 200 µg/ml (7.3%), 400 µg/ml (15.2%), 600 µg/ml (24.6%) and 800 µg/ml (32.1%). The extract exhibited a higher percentage of DPPH radical inhibition (38.9%) at 1000 µg/ml likewise standard activity is denoted in Figure 1.

Superoxide anion radical is a potent ROS species. At the time of energy transduction in mitochondria, electrons drip from this process then combines with oxygen. After this combination, it changes into deleterious types of free radicals such as hydroxyl radical and H_2O_2. These radical species have harmful consequences on macromolecules[42,43]. Figure 2 depicts the maximum superoxide anion radical scavenging percentage (49.3%) at a higher concentration of extract used (1000 µg/ml) as well minimum inhibition (6.9%) recognized at lower concentration (200 µg/ml) with activity of standard.

NO can be exuded by macrophages and neurons; it also regulates our body processes. The excess NO mingles with oxygen and secretes nitrite, peroxynitrite molecules[44,45]. These molecules play a vital role in the cause of several disorders. Greater percentage of NO radical scavenging was (56.4%) identified at 1000 µg/ml which is illustrated in Figure 3. Higher level of NO radical scavenging leads to the reduction of lipid peroxidation as well inflammation caused due to the progression of disorders.

H_2O_2 is a non radical variety of ROS. It has converted into hydroxyl radical ($OH^{\cdot}$) in cells occasionally. Surplus of $OH^{\cdot}$ damage cells in the body. Generation of $OH^{\cdot}$ can be retarded through the reduction of H_2O_2 to H_2O due to the donation of electrons[46]. When using decreased concentrations of extracts such as 200 µg/ml, 400 µg/ml, 600 µg/ml, 800 µg/ml, the percentage of inhibition also reduced as 3.4%, 9.4%, 17.8%, 24.5% respectively. Maximum H_2O_2 radical inhibition (31.2%) was observed at 1000 µg/ml that is represented in Figure 4. In all types of scavenging methods, concentration reliant inhibition was identified.

3.2 In Vitro Anticancer Activity

The current necessity is to improve drugs that can promisingly target cancer cells through their intrinsic

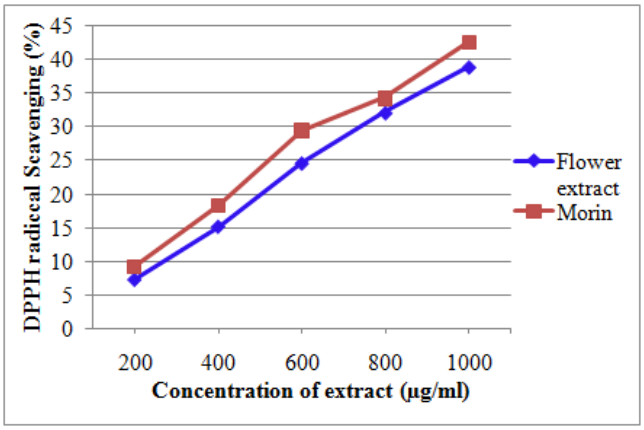

Figure 1. DPPH scavenging ability in the flower of *T. divaricata* (L.).

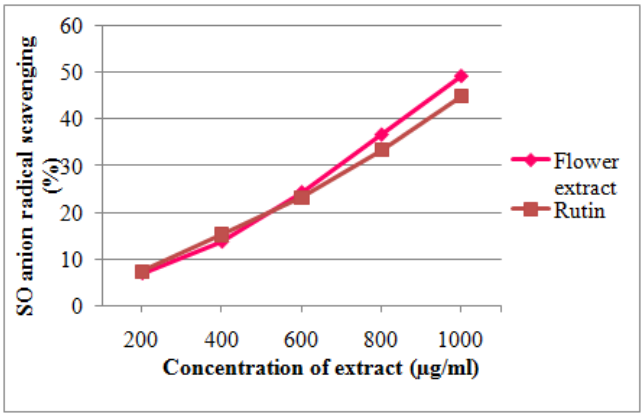

Figure 2. O_2- quenching capability in the flower of *T. divaricata* (L.).

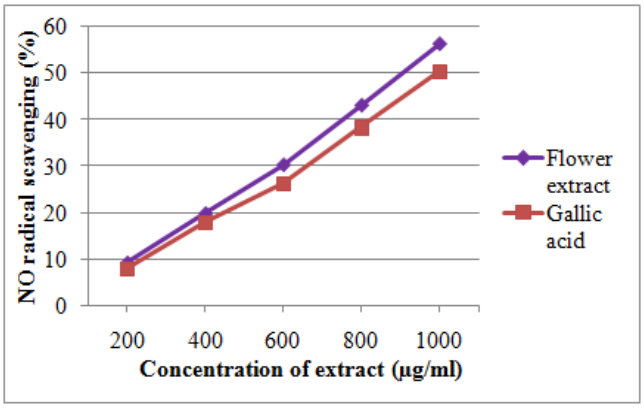

Figure 3. NO radical neutralizing capacity in the flower of *T. divaricata* (L.).

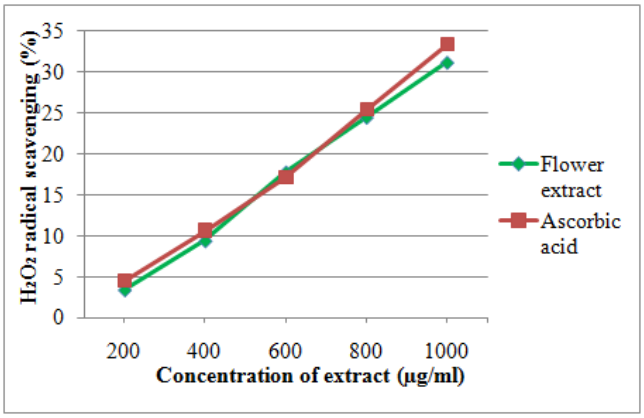

Figure 4. H_2O_2 radical scavenging power in the flower of *T. divaricata* (L.).

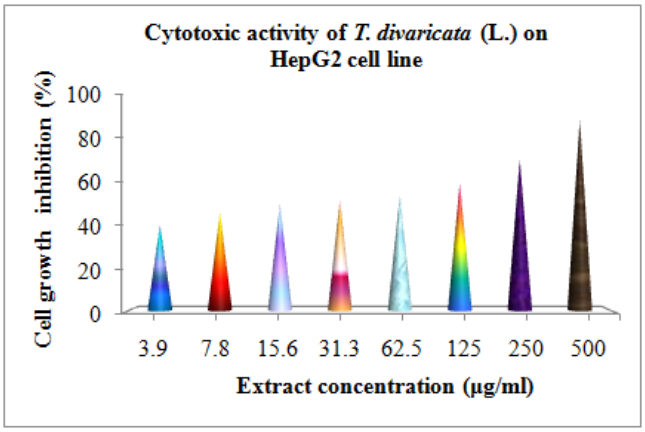

Figure 5. Cytotoxic effect of *T. divaricata* (L.) flower on HepG2 cell growth.

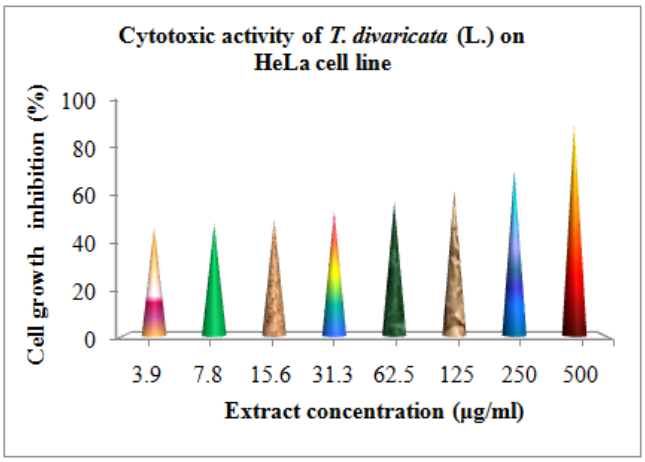

Figure 6. Cytotoxic effect of *T. divaricata* (L.) flower on HeLa cell growth.

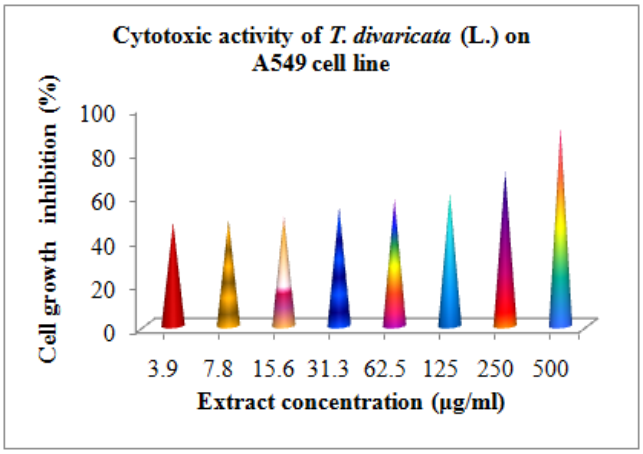

Figure 7. Cytotoxic effect of *T. divaricata* (L.) flower on A549 cell growth.

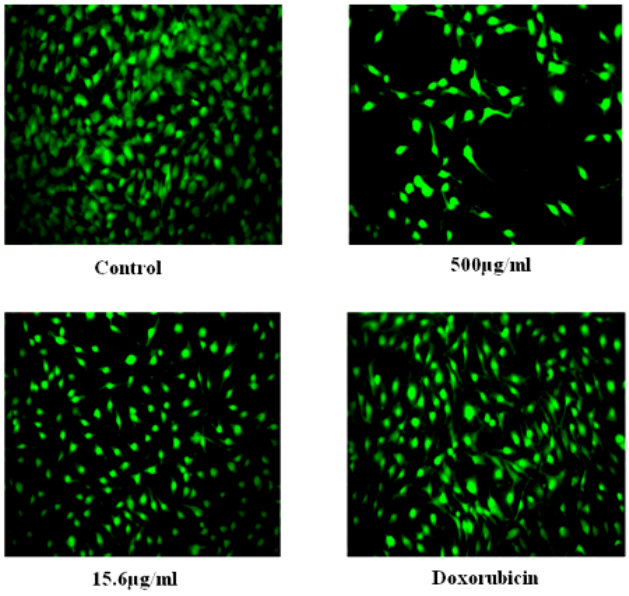

Figure 8. Cytotoxic activity of *T. divaricata* (L.) flower on HepG2 cancer cell line.

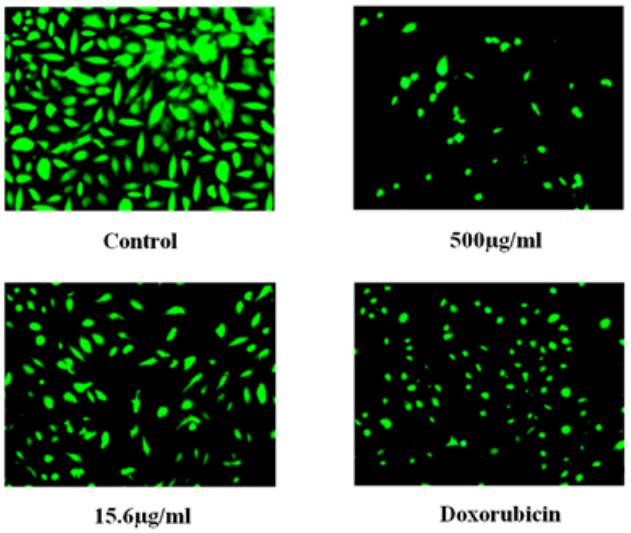

Figure 9. Cytotoxic activity of *T. divaricata* (L.) flower on HeLa cancer cell line.

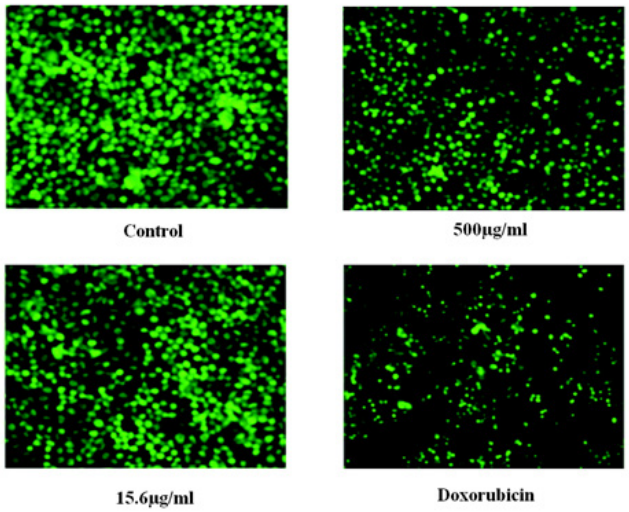

Figure 10. Cytotoxic activity of *T. divaricata* (L.) flower on A549 cancer cell line.

Table 1. Cytotoxic activity of *T. divaricata* (L.) flower against HepG2, HeLa and A549 cancer cell lines

Extract concentration (µg/ml)	HepG2		HeLa		A549	
	Cell inhibition/ cytotoxicity (%)	Cell viability (%)	Cell inhibition/ cytotoxicity (%)	Cell viability (%)	Cell inhibition/ cytotoxicity (%)	Cell viability (%)
500	89.7	10.3	91.2	8.8	90.4	9.6
250	71.32	28.68	71.41	28.59	70.04	29.96
125	60.14	39.86	62.19	37.81	60.10	39.9
62.5	54.53	45.47	57.56	42.44	57.56	42.44
31.3	52.23	47.77	53.83	46.17	53.83	46.17
15.6	50.43	49.57	50.19	49.81	50.19	49.81
7.8	46.01	53.99	48.09	51.91	48.09	51.91
3.9	40.10	59.90	47.06	52.94	47.06	52.94

divergence from normal cells. The enhancement of such drugs with differential action will be very beneficial in cancer chemotherapy without perceived side effects. Plants hold firm secondary metabolites (phytochemicals) which contain the capacity to alter the physiological behaviour of cancer cells and therefore play a role as anticancer drugs to seize the promulgation of cells. Regular ingestion of phytochemicals can diminish the occurrence of numerous cancer forms[47]. So, methodology involves the use of plant extracts to identify its efficiency against cancer cell lines under in vitro conditions.

In order to appraise the cytotoxic activity of ethanolic extract of *T. divaricata* (L.), MTT assay with HepG2, HeLa, and A549 cancer cell lines were executed. Flower extract was used to screen its inhibitory effect at diverse concentrations ranging from 3.9 to 500 µg/ml to ascertain IC_{50} value. In the highest concentration (500 µg/ml) of extract against HepG2, HeLa, and A549, cell inhibition was in the mean value of 89.7%, 91.2%, 90.4% respectively. IC_{50} value for these cell lines was 15.6 µg/ml. Less percentage of cell inhibition was predicted in the lowest concentration of extract taken ie.3.9 µg/ml.

The percentage of HepG2, HeLa, and A549 cell growth inhibition and cell viability were given in Table 1. All the above three cell lines inhibition is graphically denoted in Figures 5–7. Likewise, cytotoxic activity

of *T. divaricata* (L.) against HepG2, HeLa, and A549 cancer cell lines were represented through microscopic images in Figures 8–10 respectively.

Ethanolic flower extract of *T. divaricata* (L.) was used to analyze in vitro anticancer activity against HepG2, HeLa, and A549 cancer cell lines through MTT assay. Apoptosis of these cells can be occurred by destructing the membrane of mitochondria[48]. In case of live cells, succinate dehydrogenase, an enzyme present in mitochondria slashes tetrazolium ring then converts MTT into formazan compound (insoluble, purple colour). The number of viable cells can be identified via formazan production[38].

4. Conclusion

The results acquired from this study reveals that the ethanolic flower extract of *T. divaricata* (L.) has antioxidant as well as anticancer activity against HepG2, HeLa and A549 cancer cell lines. Further examination will be needed to identify antitumour activity through *in vivo* studies and therefore specifically which compound can be responsible for this action on different cell lines. Anticancer effect of *T. divaricata* (L.) flower will afford valuable information to prepare herbal drugs in the potential application to avert and treat cancer.

5. References

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