

Antiproliferative efficacy of *Tabernaemontana divaricata* against HEP2 cell line and Vero cell line

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ABSTRACT

Background: Laryngeal cancer may also be called cancer of the larynx or laryngeal carcinoma. Conventional plants are a precious source of novel anticancer agents and are still in performance better role in health concern. The study was intended to estimation of the anticancer activity of the chloroformic extract of *Tabernaemontana divaricata* on the human epidermoid larynx carcinoma cell line (Hep 2). **Materials and Methods:** The aerial parts (leaves, stem, and flowers) of *T. divaricata* were tested for its inhibitory effect in 96 microplate formats against Hep 2 cell line. The anticancer activity of samples on Hep 2 and Vero was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and various enzymatic parameters like catalase, reduced glutathione (GSH), GSH peroxidase, and superoxide anion scavenging activity. Viable cells were determined by the absorbance at 540 nm. Measurements were performed, and the concentration required for a 50% inhibition of viability (IC₅₀) was determined graphically. The effect of the samples on the proliferation of Hep 2 and Vero cells was expressed as the % cell viability. **Results:** The extract on Hep 2 cell line up to 7.8 µg/ml and that IC₅₀ value on Hep 2 cell line was 112 µg whereas 94 µg for Vero cell line. Hence, *T. divaricata* has lesser significant action on Vero cell line. **Conclusion:** Medicinal plant drug discovery continues to provide new and important leads against various pharmacological targets including cancer. Our results clearly indicate the anticancer property of the medicinal plant *T. divaricata* against the human laryngeal carcinoma cell lines (Hep 2 cell line).

Key words: Hep2 cell lines, laryngeal carcinoma, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, *Tabernaemontana divaricata*, Vero cell lines

INTRODUCTION

Cancer, known medically as malignant neoplasia, is a broad group of diseases involving unregulated cell growth. The cancer may also spread to more distant parts of the body through the lymphatic system or bloodstream. There are over 200 different known cancers that affect humans. Laryngeal cancer may also be called cancer of the larynx or laryngeal carcinoma.^[1] Most laryngeal cancers are squamous cell carcinomas,^[2] reflecting their origin from the squamous cells which form the majority of the laryngeal epithelium. Cancer can develop in any part of the larynx,^[3] but the cure rate is affected by the location of the tumor. For the purposes of tumor staging, the larynx is divided into three anatomical regions: The glottis (true vocal cords, anterior and posterior commissures); the supraglottis (Epiglottis,

arytenoids and aryepiglottic folds, and false cords); and the subglottis.^[4,5] Smoking is the most important risk factor for laryngeal cancer. Death from laryngeal cancer is 20 times more likely for heaviest smokers than for nonsmokers.^[6] Heavy chronic consumption of alcohol, particularly alcoholic spirits, is also significant.

Tabernaemontana divaricata belongs to the Apocynaceae family. The generic synonym of *T. divaricata* is *Ervatamia coronaria* and widely distributed in tropical countries as a garden plant. *T. divaricata* is a shrub or small tree, usually glabrous, found in the Konkan, North Kanara, Western Ghats in Malabar, throughout North India and Travencore up to 3000 ft.^[6-8] Leaves of *T. divaricata* contain indole alkaloids stapfinine,^[9,10] dimeric indole alkaloids-conophylline, and conophyllidine.^[11] Flowers of *E. coronaria* contains α-amyrin acetate, β-amyrin acetate, lupeol β-sitosterol and stigmasterol, flavone, apigenin, four indole alkaloids harmine, heyneanine, voacristine and apparic-ine, phenolic acids namely salicylic acid, syringic acid, and vanillic acid.^[12] Stems of *E. coronaria* contains

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bisindole alkaloid 19,20-dihydro ervatanine A, other alkaloids coronidine, heyneanine, voacristine, voacamine, descarbomethoxy voacamine and five phenolic acids namely vanillic, gentisic, syrengic, α -hydroxy benzoic, and salicylic acid.^[13]

In traditional medicine, *T. divaricata* (L.) R.Br. is used to treat various diseases such as diarrhea, abdominal tumors, arthralgia, asthma, epilepsy, eye infections, fever, fractures, headache, inflammation, leprosy, mania, edema, paralysis, piles, rabies, rheumatic pain, skin diseases, urinary disorders, strangury, toothache,^[14] ulceration, and vomiting. It is also used as anthelmintic, antihypertensive, aphrodisiac, diuretic, hair growth promoter, purgative, remedy against poisons and tonic to the brain, liver, and spleen.^[15,16]

The present study was thus carried out to investigate the antiproliferative efficacy of the chloroformic extract of *T. divaricata* against Hep 2 cell line and Vero cell line.

MATERIALS AND METHODS

Collection of medicinal plant

The medicinal plant used for the experiment was aerial parts (leaves, stem and flowers) of *T. divaricata* collected from the local medicinal garden Chennai, Tamil Nadu. The parts of the medicinal plant were identified and authenticated by the botanist.

Preparation of extracts

Five hundred gram of dried aerial parts of *T. divaricata* was packed in round bottom flask for sample extraction using chloroform. The extraction was conducted by 1000 ml of the solvent mixture for a period of 48 h. At the end of the extraction, the solvent was concentrated under reduced pressure and keep it in the water bath (at 50°C). Now, the extracted experimental solutions were stored in the refrigerator.

Cell culture

Hep2 and Vero cell lines were obtained from National Centre for Cell Sciences Pune. The cells were maintained in Minimal Essential Media supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO₂ at 37°C.

Reagents

Minimum essential medium, FBS, Trypsin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were purchased from Hi-media and Sigma-Aldrich Mumbai.

In vitro assay for cytotoxicity activity (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay)

The anticancer activity of samples on Hep 2 and Vero was determined by the MTT assay.^[17] Cells (1×10^5 /well) were plated in 0.2 ml of medium/well in 96-well plates. Incubate at 5% CO₂ incubator for 72 h. Then, add various concentrations of the samples in 0.1% DMSO for 24 h at 5% CO₂ incubator. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 20 µl/well (5 mg/ml) of 0.5% MTT in phosphate-buffered saline solution was added. After 4 h incubation, 1 ml of DMSO was added. Viable cells were determined by the absorbance at 540 nm. Measurements were performed, and the concentration required for a 50% inhibition of viability (IC₅₀) was determined graphically. The effect of the samples on the proliferation of Hep 2 and Vero cells was expressed as the % cell viability, using the following formula:

Calculation:

$$\% \text{ cell viability} = \frac{\text{A540 of treated cells}}{\text{A540 of control cells} \times 100\%}$$

Enzymatic assays

Catalase activity

The assay mixture contained 1.0 ml of phosphate buffer, 0.4 ml of hydrogen peroxide and 0.1 ml of sample. The reaction mixture was withdrawn and blown into 2 ml of dichromate/acetic acid reagent at 1 min intervals. Then the mixture was heated for 10 min in a boiling water bath. After cooling, the optical density (OD) was measured at 570 nm.

Superoxide anion scavenging activity

Measurement of superoxide radical scavenging activity was done using the standard method.^[18] The superoxide anions generated by phenazinemethosulfate/nicotinamide-adenine-dinucleotide-phosphate, reduced form system, were detected by the reaction with 2,2'-di-p-nitrophenyl-5,5'-diphenyl-(3,3'-dimethoxy-4,4'-diphenylene) ditetrazolium chloride (nitro blue tetrazolium [NBT]). Stock solution of leaf extracts and Quercetin (standard) was prepared to the concentration of 1 mg/ml. The reaction mixture contained 1 ml of NBT solution (312 µM prepared in phosphate buffer, pH-7.4), 1 ml of nicotinamide adenine dinucleotide solution (936 µM prepared in phosphate buffer, pH-7.4) and samples at different concentration (25, 50 and 75 µg) obtained from stock solution were added and finally the reaction was accelerated by adding 100 µl phenazinemethosulfate solution (120 µM prepared in phosphate buffer, pH-7.4). The reaction was incubated at 25°C for 5 min and absorbance was measured at 560 nm against the corresponding blank solutions. Blank consist of

all the reagents, except for the extract, or standard solution is substituted with water. The annihilation activity of free radicals was calculated in % inhibition according to the following relation:

$$\text{Inhibition \%} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample}) \times 100}{\text{Absorbance of control}}$$

Estimation of total reduced glutathione

Total reduced glutathione (GSH) was determined by the method of Sedlak and Lindsay (1968)^[20] modified according to the method of Moron *et al.* (1979), which is based on the reaction with 5, 5'-dithio-bis (2 nitrobenzoic acid) (DTNB or Ellman's reagent) to give a yellow colored compound that absorbs at 412 nm. The absorbance was read at 412 nm against a blank containing trichloroacetic acid (TCA) instead of sample. A series of standards treated in a similar manner was also run to determine the GSH content [Figure 3].

Assay of glutathione peroxidase

The activity of GSH peroxidase (GPx) was assayed by the method of Rotruck *et al.* (1973).^[21] The reaction mixture consisting 0.2 ml of ethylenediaminetetraacetic acid (EDTA), 0.1 ml of sodium azide, 0.1 ml of H₂O₂, 0.2 ml of GSH, 0.4 ml of phosphate buffer, and 0.2 ml of homogenate was incubated at 37°C for 10 min. The reaction was arrested by the addition of 0.5 ml of TCA, and the tubes were centrifuged at 2000 rpm. To the supernatant, 3 ml of disodium hydrogen phosphate and 1.0 ml of DTNB were added, and the color developed was read at 420 nm immediately. The activity of GPx was expressed as μ moles of GSH oxidized/min/mg of protein.

DNA fragmentation

Isolation of DNA

Two milliliter of cells was taken and centrifuged at 3000 rpm for 5 min. The obtained pellet must be suspended in 200 μL of 1X Tris-EDTA (TE) Buffer and 100 μL of 10% sodium dodecyl sulfate and mixed well. Then the tube is incubated at 50°C for 20 min. 300 μL of phenol: Chloroform: Isoamyl alcohol (25:24:1) were

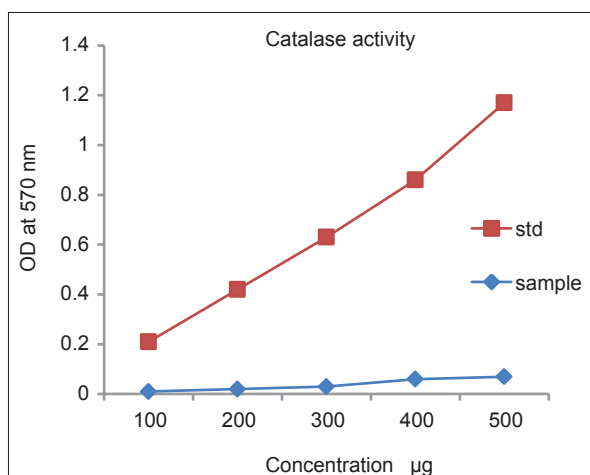


Figure 1: Catalase activity

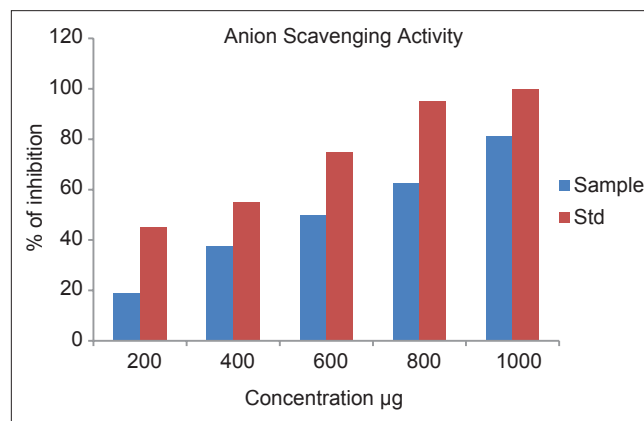


Figure 2: Anion scavenging activity

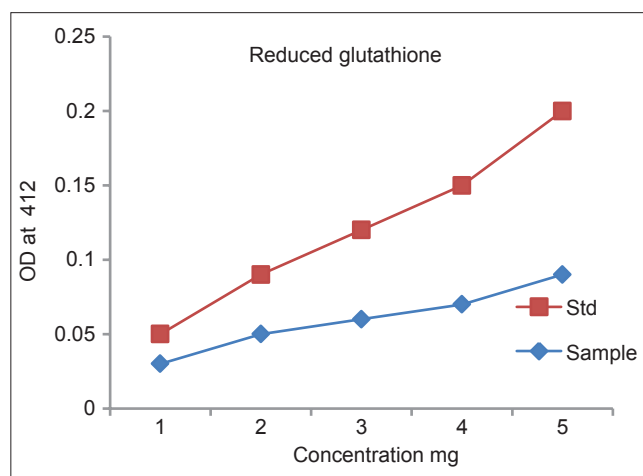


Figure 3: Reduced glutathione activity

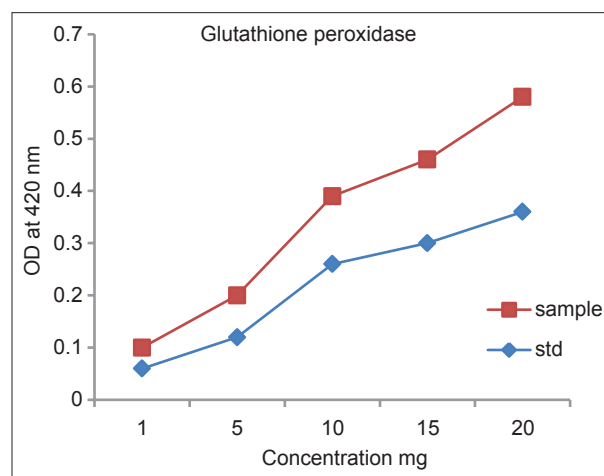


Figure 4: Glutathione peroxidase activity

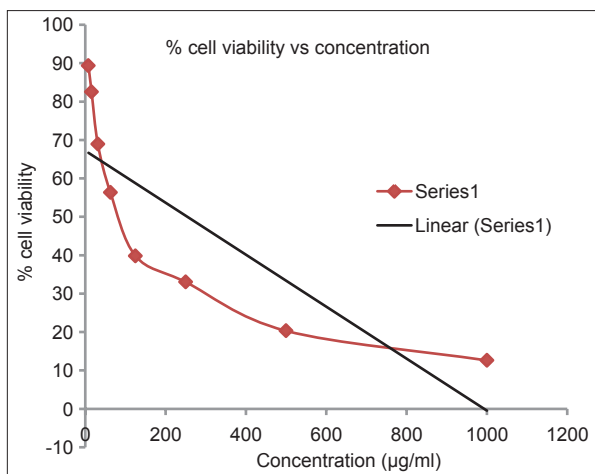


Figure 5: Drug response curve of the chloroformic extract of for *Tabernaemontana divaricata* human laryngeal carcinoma cell lines by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

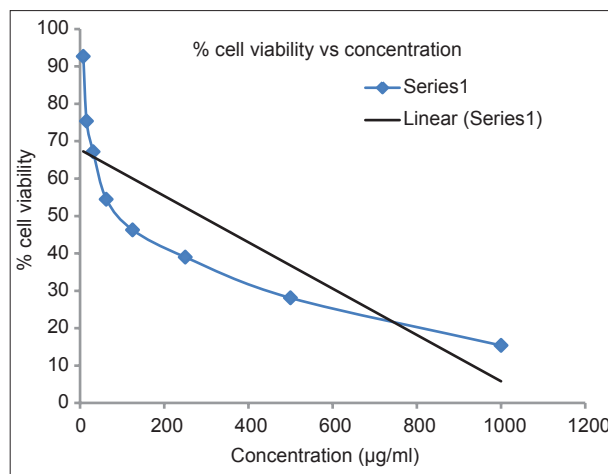


Figure 6: Drug response curve of the chloroformic extract of for *Tabernaemontana divaricata* Vero cell line by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

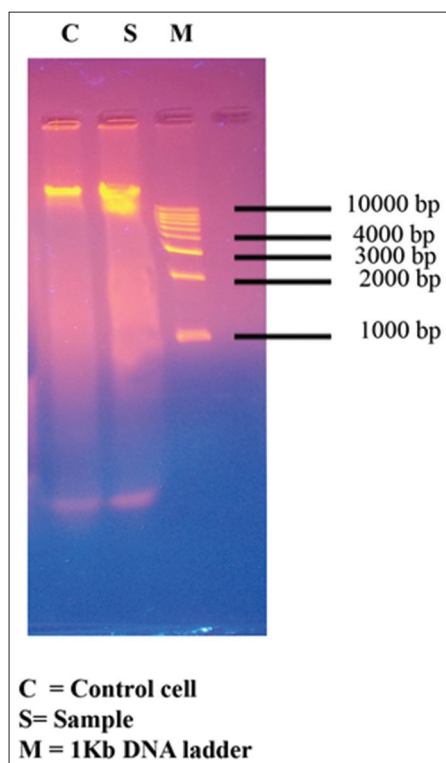


Figure 7: Analysis of DNA fragmentation by agarose gel electrophoresis

added in the incubated tube and centrifuge at 10,000 rpm for 10 min. The supernatant was transferred to new 1.5 mL eppendorf tube, and 1000 µL of isopropanol was added and mixed by inverting the tube (4–5 times), then centrifuged at 1000 rpm for 10 min. The supernatant was discarded, and 500 µL of 70% ethanol was added and centrifuged at 10,000 rpm for 10 min and the supernatant was discarded. Now, air-dry the pellet till there were no traces of ethanol and resuspend the pellet in 20 µL of 1X TE Buffer.

Agarose gel electrophoresis

The extracted DNA was carried out with agarose gel electrophoresis in a horizontal submarine electrophoresis unit Oberhammer *et al.*^[22] The casting was done with the 1.2% agarose gel and 0.72 g of Agarose in 60 mL of diluted 1X Tris-borate-EDTA (TBE) buffer (do not mix), which were dissolved by heating the content to get up to clear solution. The solution was allowed to cool at room temperature, and then 5 µL of ethidium bromide added, mixed and poured the agarose into the casting system with combs. The gel was allowed to solidify, and then carefully disassemble the casting system without disturbing the wells. The gel is transferred to 1X TBE buffer filled electrophoresis tank. 5 µL of gel loading dye added to 20 µL of sample DNA, mixed well, and then the total 25 µL of sample loaded to gel. 10 µL of 1 kb DNA marker added near to the well. The power card terminals were connected at respective positions to run the gel. The unit was switched off after the gel loading dye migrated more than half of the length of gel the unit; the separated DNA bands visualized under ultraviolet transilluminator.

Visualization of propidium iodide-stained cells

To identify those cells undergoing apoptosis, the cells were cytopspined and mounted on the slides. Changes in cell morphology were examined under a differential microscope and a fluorescence microscope.^[23] Cells were seeded in 12-well plates at seeding densities of 5×10^5 cells/well and then treated with the ginger extract at the specified concentration for 24 h. Then, the cells were washed with phosphate-buffered saline (PBS); after washing once with PBS, the cells were stained with 100 µL of a propidium iodide (4 µg/ml). The cells were immediately washed with PBS, cytopspined and mounted on the slides. Changes in

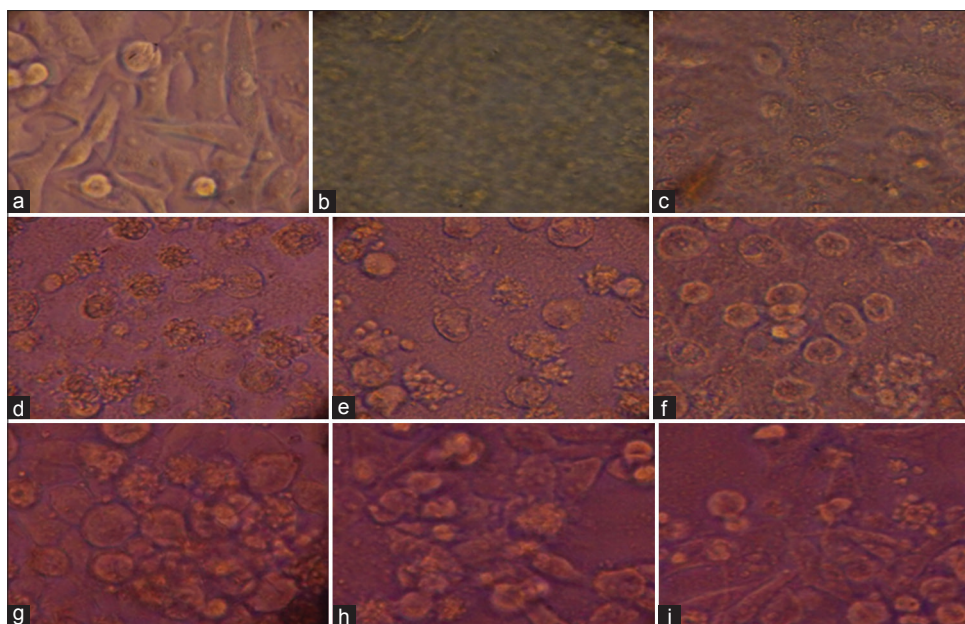


Figure 8: Microscopic examination of morphological changes in Hep2 cells treated with *Tabernaemontana divaricata*. (a) Control cells without treatment; (b-i) cells treated with *Tabernaemontana divaricata* for 24 h

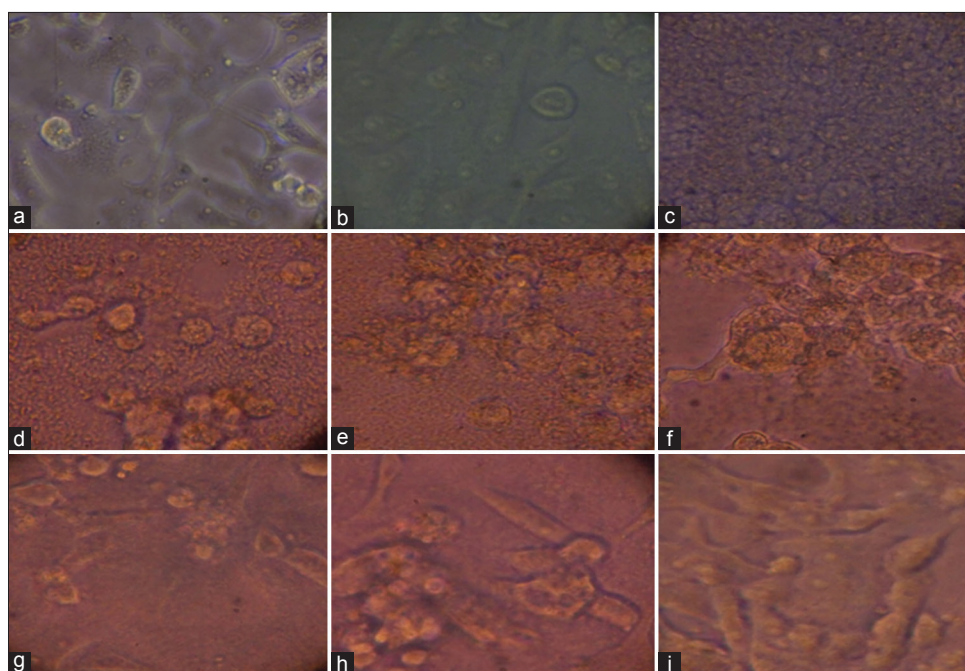


Figure 9: Microscopic examination of morphological changes in Vero cell line treated with *Tabernaemontana divaricata*. (a) Control cells without treatment; (b-i) cells treated with *T. divaricata* for 24 h

cell morphology were examined to identify those cells undergoing apoptosis under Nikon-inverted fluorescent microscope (TE-Eclipse 300, Nikon, Tokyo, Japan) attached with the camera [Figures 8 and 9].

Statistical analysis

Statistical analysis of the data was performed with mean \pm standard deviation between the groups.

RESULTS AND DISCUSSION

The enzymatic activity of the extract of *T. divaricata* was observed by the catalase activity, superoxide anion scavenging activity, and estimation of total reduced GSH. The activity of catalase was expressed as μ mole of H_2O_2 consumed/min/mg protein and OD was measured at 570 nm [Table 1]. The graph is

obtained between concentration (in μg) and measured OD [Figure 1].

For the superoxide anion scavenging activity, the absorbance was measured at 560 nm against the corresponding blank solutions [Table 2]. The annihilation activity of free radicals was calculated in % inhibition [Table 3]. The graph is plotted for the anion scavenging activity between concentration (in μg) and % inhibition [Figure 2].

The total reduced GSH was determined, and the absorbance was read at 412 nm against a blank containing

Table 1: The activity of catalase

Serial number	Sample concentration (μg)	Sample OD	OD
1	100	0.01	0.2
2	200	0.02	0.4
3	300	0.03	0.6
4	400	0.06	0.8
5	500	0.07	1.1

Table 2: The superoxide anion scavenging activity

Serial number	Sample concentration (μg)	Sample OD	OD
1	200	0.13	0.11
2	400	0.10	0.09
3	600	0.08	0.05
4	800	0.07	0.01
5	1000	0.03	0.00
Blank		0.16	0.20

Table 3: Annihilation activity of free radicals

Percentage of inhibition	1	2	3	4	5
Sample	18.7	37.5	50	62.5	81.2
Standard	45	55	75	95	100

Table 4: Total reduced glutathione

Serial number	Sample concentration (mg)	Sample OD	OD
1	1	0.03	0.02
2	2	0.05	0.04
3	3	0.06	0.06
4	4	0.07	0.08
5	5	0.09	0.11

Table 5: The activity of glutathione peroxidase

Serial number	Sample concentration (mg)	Sample OD	OD
1	1	0.06	0.04
2	5	0.12	0.08
3	10	0.26	0.13
4	15	0.30	0.16
5	20	0.36	0.22

TCA instead of sample [Table 4]. The graphical representation shows the reduced GSH of sample against standard [Figure 3].

The activity of GPx was assayed, and the color developed was read at 420 nm immediately [Table 5]. The activity of GPx was expressed as μ moles of GSH oxidized/min/mg of protein. The activity of GPx shown by plotted graph of the sample against standard [Figure 4].

In vitro assay for cytotoxicity activity (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay)

The cytotoxicity study was carried out for methanolic extract of *T. divaricata*. The extract was screened for its cytotoxicity against Hep 2 and Vero cell lines at different concentrations to determine the IC₅₀ (50% growth inhibition) by MTT assay. Results are tabulated in Table 6 and 7. Measurements were performed, and IC₅₀ was determined graphically. The percentage growth inhibition was found to be increasing with increasing concentration. The extract on Hep 2 cell line up to 7.8 $\mu\text{g}/\text{ml}$ [Tables 6 and 7, Figures 5 and 6] and that IC₅₀ value on Hep 2 cell line was 112 μg whereas 94 μg for

Table 6: Determination of cytotoxicity by MTT assay on Hep2 cell line

Serial number	Concentration ($\mu\text{g}/\text{ml}$)	Dilution	Absorbance 540 nm	Percentage cell viability
1	1000	Neat	0.13	12.6
2	500	1:1	0.21	20.3
3	250	1:2	0.34	33.0
4	125	1:4	0.41	39.8
5	62.5	1:8	0.58	56.3
6	31.2	1:16	0.71	68.9
7	15.6	1:32	0.85	82.5
8	7.8	1:64	0.92	89.3
9	Control	-	1.03	100

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Table 7: Determination of cytotoxicity by MTT assay on Vero cell line

Concentration ($\mu\text{g}/\text{ml}$)	Dilution	Absorbance 540 nm	Percentage cell viability
1000	Neat	0.17	15.4
500	1:1	0.31	28.1
250	1:2	0.43	39.0
125	1:4	0.51	46.3
62.5	1:8	0.60	54.5
31.2	1:16	0.74	67.2
15.6	1:32	0.83	75.4
7.8	1:64	1.02	92.7
Control	-	1.10	100

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Vero cell line. Hence, *T. divaricata* has lesser significant action on Vero cell line. If the drug has more effect on Vero cell line that denote it cause side effect on normal healthy body cell. While in casing of *T. divaricata*, it gives a superior result on Hep 2 cell but lesser effect on Vero cell. So it gives anticancer activity with no side effect.

The DNA was isolated from the treated cells and subjected to agarose gel electrophoresis and examinations revealed a ladder formation, which is characteristic of apoptosis [Figure 7].

CONCLUSION

Current research in drug discovery from medicinal plants involves a multifaceted approach combining botanical, phytochemical, biological, and molecular techniques. Medicinal plant drug discovery continues to provide new and important leads against various pharmacological targets including cancer. Therefore, it is of interest to investigate the antiproliferative efficacy of aerial parts of *T. divaricata* was carried out. Our results clearly indicate the anticancer property of the medicinal plant *T. divaricata* against the human laryngeal carcinoma cell lines (Hep 2 cell line).

REFERENCES

- Kleinsasser O. Tumors of the larynx and hypopharynx. Stuttgart: Georg Thieme Verlag; 1988.
- Marck PA, Lupin AJ. Cancer of the larynx: The northern Alberta experience. J Otolaryngol 1989;18:344-9.
- Robin PE, Reid A, Powell DJ, McConkey CC. The incidence of cancer of the larynx. Clin Otolaryngol Allied Sci 1991;16:198-201.
- Stephenson WT, Barnes DE, Holmes FF, Norris CW. Gender influences subsite of origin of laryngeal carcinoma. Arch Otolaryngol Head Neck Surg 1991;117:774-8.
- Tuyns AJ. Laryngeal cancer. Cancer Surv 1994;19-20:159-73.
- Ridge JA, Glisson BS, Lango MN, Feigenverg S. Head and Neck tumors. In: Pazdur R, Bagman LD, Camphausen KA, Hoskins WJ (editors). Cancer Management. 12th ed. New York: Multidisciplinary approach Medical, Surgical and Radiation and Oncology, The Oncology Group; 2010.
- Nadkarni KM. Indian Materia Medica. Vol. I. Bombay: Popular Book Depot; 1954. p. 516-8.
- Sharma P, Mehta PM. Dravyaguna Vignyan. Part II and III. Varansi: The Chowkhamba Vidyabhawan; 1969. p. 586.
- Kirtikar KR, Basu BD. Indian Medicinal Plants, Vol.3. 2nd ed. Dehra Dun: International Book Distributors; 1975. p. 1052-3.
- Atta-Ur-Rahman, Anjum M, Nader D. Ervatinine, an indole alkaloid from *E. coronaria*. Phytochemistry 1985;24:2473-4.
- Atta-Ur-Rahman, Anjum M, Nader D. Stapfinine, an indole alkaloid from *E. coronaria*. Phytochemistry 1986;25:1781-2.
- Kam TS, Loh KY, Wei C. Conophylline and conophyllidine: New dimeric alkaloids from *T. divaricata*. J Nat Prod 1993;56:1865-71.
- Joshi AB. Phytochemical and Pharmacological Investigation of the Plant *Ervatamia coronaria*. (Stapf.) (SYN: *Tabernaemontana divaricata* R.Br.), Ph.D. Thesis, Rajiv Gandhi University of Health Sciences, Karnataka; 2004. p. 160.
- Henriques AT, Melo AA, Moreno PR, Ene LL, Henriques JA, Schapoval EE. *Ervatamia coronaria*: Chemical constituents and some pharmacological activities. J Ethnopharmacol 1996;50:19-25.
- Ghani A. Medicinal Plants of Bangladesh: Chemical Constituents and Uses. Vol. 381. Dhaka: Asiatic Society of Bangladesh; 2003. p. 1-16.
- Hoernle AF. The Bower Manuscript. (Archaeological Survey of India, New Imperial Series, Vol. 22). Superintendent of Government Printing, India, Calcutta; 1893-1912. p. 18-20, 22, 91, 104, 107, 128, 133, 159, 173, 188.
- Ali KM. Effect of *Tabernaemontana divaricata* (crepe jasmine) flower methanolic extract on pylorus ligated rats. Malays J Pharm Sci 2010;1:104-5.
- Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55-63.
- Nishikimi M, Appaji N, Yagi K. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochem Biophys Res Commun 1972;46:849-54.
- Sedlak J, Lindsay RH. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal Biochem 1968;25:192-205.
- Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG. Selenium: Biochemical role as a component of glutathione peroxidase. Science 1973;179:588-90.
- Oberhammer F, Wilson JW, Dive C, Morris ID, Hickman JA, Wakeling AE, et al. Apoptotic death in epithelial cells: Cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. EMBO J 1993;12:3679-84.
- Darzynkiewicz Z, Li X, Gong J. Assays of cell viability: Discrimination of cells dying by apoptosis. Methods Cell Biol 1994;41:15-38.

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