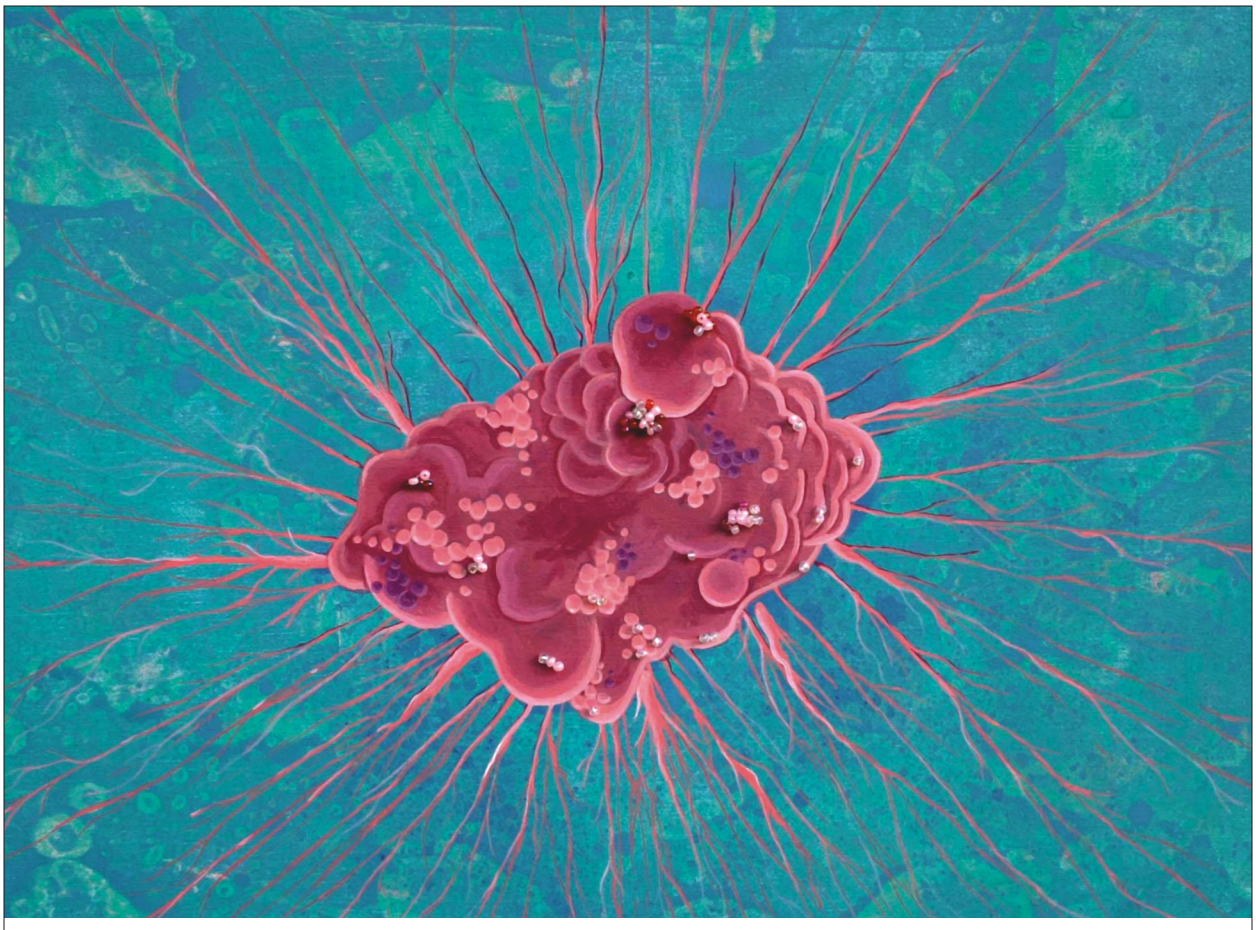

International Journal of Cancer Research

ISSN 1811-9727

Academic Journals Inc.

www.academicjournals.com

OPEN ACCESS International Journal of Cancer Research

ISSN 1811-9727
DOI: 10.3923/ijcr.2018.100.108

Research Article
Evaluation of *in vitro* Antioxidant and Anticancer Activity of *Tabernaemontana divaricata* Leaf Extracts Against T-24 Human Bladder Cancer Cell Lines

[1,2]Sridevi I. Puranik, [2]Murigendra B. Hiremath, [3,4]Rajendra B. Nerli and [5]Shridhar C. Ghagane

[1]Department of Biotechnology and Microbiology, Karnatak University Dharwad, 580003, Karnataka, India
[2]Department of Biotechnology and Microbiology, Karnatak University Dharwad, K.L.E'S R. L. Science Institute (Autonomous), 590001, Karnataka, India
[3]Department of Urology, JN Medical College, KLE Academy of Higher Education and Research (Deemed to be University), JNMC Campus, Nehru Nagar, Belagavi-590010, Karnataka, India
[4]KLES Kidney Foundation, KLES Dr. Prabhakar Kore Hospital and Medical Research Center, Belagavi-590010, Karnataka, India
[5]Department of Urology, KLES Kidney Foundation, KLES Dr. Prabhakar Kore Hospital and Medical Research Center, Belagavi-590010, Karnataka, India

Abstract

Background and Objective: *Tabernaemontana divaricata* belongs to family *Apocynaceae* is an ornamental, flowering, evergreen shrub that is used in traditional medicine for the treatment of urinary disorders, abdominal tumours and dysentery. The aim of the study was to investigate *in vitro* antitoxicity and anticancer activities from leaf extracts of *Tabernaemontana divaricata*. **Materials and Methods:** The present study involves at screening of preliminary phytochemicals, determination of total phenolic contents, evaluation of methanol, ethanol and aqueous leaf extracts of *T. divaricata* using *in vitro* 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay, ferric ion reducing antioxidant power (FRAP) assay, Phosphomolybdenum (PM) method and evaluation of *in vitro* anticancer activity on T-24 human bladder cancer cell line. **Results:** The preliminary screening of phytochemicals revealed a broad spectrum of secondary metabolites present in all five different solvent extracts. Aqueous extract showed high presence of phenols (62.54±0.18 mg g^{-1} GAE, $r^2 = 0.9882$) among methanol and ethanol extracts in comparison to Gallic acid (80.28±0.14 mg g^{-1} GAE, $r^2 = 0.9954$). Ethanol extracts exhibited potential antioxidant activity at a concentration of 100 mg mL^{-1} (62.41±0.67%). The IC_{50} value was 188.9±07 µg mL^{-1} for T-24 bladder cancer cell line whiles the standard drug Doxorubicin showed an IC_{50} value of 0.23 µM mL^{-1}. Morphological variations was observed in cancerous cells undergoing cell shrinkage and membrane blebbing in human bladder cancer cell line T-24 while the extract showed no cytotoxicity towards normal cells MEF-L929. **Conclusion:** Based on these results it can be concluded that the tested extracts holds significant antioxidant and anticancer properties. However further investigation with lead compounds of *T. divaricata* on *in vivo* trials will enable its therapeutic use.

Key words: Anti-oxidant, anticancer, bladder cancer, phytochemical, *T. divaricata*

Citation: Sridevi I. Puranik, Murigendra B. Hiremath, Rajendra B. Nerli and Shridhar C. Ghagane, 2018. Evaluation of *in vitro* antioxidant and anticancer activity of *Tabernaemontana divaricata* leaf extracts against T-24 human bladder cancer cell lines. Int. J. Cancer Res., 14: 100-108.

Corresponding Author: Murigendra B. Hiremath, Department of Biotechnology and Microbiology, Karnatak University Dharwad, Karnataka, India
Tel: +919886187432

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Bladder cancer (BC), the fourth most common cancer in men and 7th most common in women, the average age at diagnosis is 60 years with male to female ratio of 2.6:1 that represents an important health problem[1]. Bladder carcinogenesis is thought to develop from the interaction of environmental exposures and genetic susceptibility[2]. The early treatment for BC is surgical, radiotherapy and chemotherapy, but post-treatment metastasis occurs in 60% patients, 20% patients are progressed to the middle and advanced stage; moreover, the 5 year survival rate is less[3] than 50%. There is an urgent need for research on herbal treatment using herbal sources[4]. The secondary metabolites may be useful adjuncts in the prevention of cancer recurrence, interrupting carcinogenesis via cell-to-cell interactions or anti-oxidation pathways[5]. The understanding of the role of herbal remedies in the development and course of bladder cancer is expanding rapidly. As experimental design becomes more sophisticated, research will shift its focus from particular synthetic drug to herbal synergistic compounds.

Plants are well known as a major source of modern medicines. From ancient times, humans have utilized plants for the treatment and prevention of diseases, leading to the dawn of traditional medicine[6]. *Tabernaemontana divaricata* belongs to the *Apocynaceae* family, growing evidence suggests that this plant has medicinal benefits and is used to treat various diseases such as diarrhoea, urinary disorders, abdominal tumours, epilepsy, eye infections, inflammation, leprosy, paralysis, rabies, rheumatic pain, skin diseases, ulceration and vomiting[7]. The leaf extracts could possibly be used as pharmacological interventions in many different ailments because of the antioxidants and potential secondary metabolites present in the leaves. The present study was thus carried out to investigate the preliminary phytochemical screening, determination of total phenolic contents, *in vitro* antioxidant properties and *in vitro* anticancer activities of *T. divaricata* leaf extracts against T-24 human bladder cancer cell line and MEF-L929 normal cell lines.

MATERIALS AND METHODS

Materials

Collection of plant material and authentication: The plant *Tabernaemontana divaricata* was collected from different regions of Western Ghats, Karnataka, during the year 2017. It was authenticated by taxonomist Dr. Kotresh. The voucher specimen was deposited in Department of Botany Karnatak Science College, Dharwad. The plant material was washed thoroughly, checked for any microbial contamination. The leaves were separated, shade dried and powdered. The powder was then stored in airtight container at -20°C until used.

Procurement of chemicals and cell line

Chemicals: All chemicals used for the experiments were of analytical grade. Gallic acid and ascorbic acid were procured from Sigma Aldrich (Mumbai, India). Folin-Ciocalteu reagent (FCR), Molybdate reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and other chemicals used during the experiments were procured from Hi media Laboratories, Pvt. Ltd. (Mumbai, India) and Merck Specialties Pvt. Ltd. (Mumbai, India). The sub-culturing, Dulbecco Modified Eagle Medium (DMEM) was obtained from Sigma-Aldrich (St. Louis, MO, USA) which was supplemented with 10% fetal bovine serum, (FBS, from Sigma-Aldrich), 1% penicillin streptomycin and 1% nonessential amino acids from (Abbott Healthcare Pvt. Ltd, Mumbai, India).

Cell lines: The T-24 bladder cancer and MEF-L929 normal mouse embryo fibroblast cell lines were procured by the National Centre for Cell Science (NCCS) Pune, India.

Methods

Extraction: The powdered leaf material was subjected for soxhlet extraction by following the method of Jenson[8] with slight modification in the temperature gradient. The five solvents namely chloroform, ethyl acetate, methanol, ethanol and aqueous were selected for extraction. The extracts were then vacuum dried in rotary vacuum evaporator under the reduced pressure at 40°C.

Preliminary phytochemical analysis: All the five extracts were qualitatively screened for the presence of phytoconstituents namely alkaloids, flavonoids, glycosides, phenols, lignins, saponins, sterols, tannins, anthraquinone and reducing sugar by following the methods of Ghagane *et al.*[9].

Total phenolic content (TPC): The total phenolic content of each of *T. divaricata* leaf extracts were carried by Folin-Ciocalteu method with slight modification[10]. About 1 mg mL^{-1} of extracts were prepared in their respective solvents and were quantified by using spectrophotometer at 760 nm using gallic acid as reference. The results were compared to the standard curves and total phenolic content was expressed as mg g^{-1} gallic acid equivalent (GAE) per gram dry powder for the samples.

In vitro antioxidant assays

2,2-Diphenyl-1-picrylhydrazyl(DPPH) free radical scavenging assay: The free radical scavenging effect of *T. divaricata* (L.) extract was assessed with the stable scavenger DPPH with slight modifications of the method described by Brand-Williams *et al.*[11]. Different concentrations of extracts were prepared in ethanol. Positive control was ascorbic acid. The DPPH solution (0.004%) was prepared in ethanol and 5 mL of this solution was mixed with the same volume of extract and standard solution separately. These solutions were kept in dark for 30 min to read absorbance at 517 nm spectrophotometric by using ethanol as blank. The scavenging activity against DPPH was calculated using the equation:

$$\text{DPPH scavenging activity (\%)} = \frac{\text{A-B}}{\text{A}} \times 100$$

where, A is the absorbance of the control reaction (1 mL of ethanol with 1 mL of DPPH solution) and B is the absorbance of the test sample. The results were analyzed in triplicate. The IC_{50} value is the concentration of sample required to inhibit 50% of the DPPH free radical.

Ferric ion reducing antioxidant power (FRAP) assay: The FRAP assay was performed by following the method of Oyaizu[12] with slight modification. About 0.1% extracts were prepared with different dilutions. About 1 mL of extract was mixed thoroughly with 2.5 mL of 0.2 mM phosphate buffer (pH 7.4) and equal volume of $K_3Fe(CN)_6$ (1% w/v). The mixture was incubated at 50°C for 20 min, followed by the addition of 2.5 mL of TCA (10% w/v). It is then centrifuged at 3000 rpm for 10 min. The upper layer of the solution is collected and mixed with 2.5 mL of distilled water and 0.5 mL of ferrous chloride (0.1% w/v). Finally the absorbance is measured at 700 nm against blank. Ascorbic acid was used as positive reference standard. Increased absorbance of the reaction mixture indicates higher reducing power of the plant extract.

Phosphomolybdenum (PM) assay: The PM assay was determined by the method described by Prieto *et al.*[13]. About 0.1% of *T. divaricata* (L.) (methanol, ethanol and aqueous extracts) leaf extracts was added to each test tube individually containing 3 mL of distilled water and 1 mL of molybdate reagent solution. These tubes were kept incubated at 95°C for 90 min. After incubation the absorbance was measured at 695 nm. Ascorbic acid was used as reference standard.

Cell line assay

Culturing of cell lines: The cells were sub-cultured in Dulbecco Modified Eagle Medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% non-essential amino acids in tissue culture flasks and incubated in a CO_2 incubator in a 5% CO_2 and 95% humidity atmosphere. After trypsinization the cells were counted and the cell viability was tested by trypan blue using haemocytometer. A known number of cells (2×10^3 cell/well in 100 µL of medium) were seeded into 96-well plates, respectively, to execute the MTT assay.

Treatment groups: *Tabernaemontana divaricata* (L.) (5 mg mL^{-1}) of leaf methanol, ethanol and aqueous extract were prepared in 0.1% di-methyl sulfoxide (DMSO) for treating T-24 bladder cancer cell line and the normal cell line (MEF-L929). The reactant mixtures were diluted with media and cells were treated with different concentrations of the extract (3.125-200 µg mL^{-1}) and incubated for 72 h, respectively. The following treatment groups are setup of the study. Negative control: cells alone. Positive control: cells+Doxorubicin (Standard drug). Test groups: cells+methanol extract of *T. divaricata* (L.) leaves cells+ethanol extract of *T. divaricata* (L.) leaves and cells+aqueous extract of *T. divaricata* (L.) leaves. A similar treatment regime was followed for the normal mouse embryo fibroblast (MEF-L929) cell line.

MTT cell viability assays: The MTT cell viability assay was performed by following the methods described[14-16]. After 72 h the media of the treated cells (100 µL) were removed and the cell cultures were incubated with 25 µL of MTT at 37°C for 4 h. After incubation, the formazan produced was solubilized by addition of 100 µL DMSO. The suspension was placed on a micro-vibrator for 5 min and the absorbance was recorded at 540 nm by an ELISA reader and the results were analyzed in triplicate and percentage of inhibition was calculated.

Statistical analysis: All results were expressed as Mean±SD for three replications. Statistical analysis of data was performed by analysis of variance (ANOVA) and level of statistical significance between groups using GraphPad Prism version 7.0 for Windows (GraphPad Software, San Diego, CA, USA). At least three independent analyses were carried out per sample.

RESULTS

Total yield of crude extract: Leaf powder of 100 g of *T. divaricata* gave total yield of 13.88 g in chloroform, 196.84 g in ethyl acetate, 17.92 g in methanol, 14.44 g in ethanol and 11.72 g in aqueous solvents, respectively.

Table 1: Preliminary phytochemical of *T. divaricata* extracts

Constituent	Test	Chloroform	Ethyl acetate	Methanol	Ethanol	Aqueous
Alkaloids	Iodine	+	-	-	-	-
	Wagner's	+	-	-	+	+
	Dragendorff's	+	-	-	+	+
Flavonoids	Pew's	-	-	-	-	-
	Shinoda	-	-	-	-	-
	NaOH	-	+	+	+	-
Glycosides	Keller-Killani	+	++	+	++	+
	Glycosides	+	-	++	++	+
	Conc. H_2SO_4	++	++	_	++	+
	Molisch's	+	-	+	++	++
Phenols	Ellagic acid	-	-	+	++	++
	Phenol	-	-	+	++	++
Lignin's	Labat	++	++	++	++	-
Saponins	Foam test	+	+	-	++	+
Sterols	Salkowski's	++	+	+	-	+
Tannins	Gelatine	-	-	+	+	+
	Lead acetate	+	-	++	++	++
Anthraquinone	Bomtrager's	-	-	-	-	-
Phlobatannins		-	-	-	-	+
Reducing sugar		++	-	-	-	-
Volatile oil		-	-	-	+	-

Table 2: Total phenolic content of *T. divaricata* leaf methanol, ethanol and aqueous extracts

Leaf extracts	TPC	Units equivalent	r^2 values
Methanol extract	51.71±0.90	mg g^{-1} GAE	$R^2 = 0.9806$
Ethanol extract	56.86±0.37	mg g^{-1} GAE	$R^2 = 0.9851$
Aqueous extract	62.54±0.18	mg g^{-1} GAE	$R^2 = 0.9882$
Gallic acid	80.28±0.14	mg g^{-1} GAE	$R^2 = 0.9954$

Table 3: Determination of percentage inhibition of 2, 2-diphenyl-1-picrylhydrazyl radical scavenging activity of *T. divaricata* (%)

Conc. (mg mL^{-1})	Methanol	Ethanol	Aqueous	Ascorbic acid
100	48.58±0.38	62.41±0.67	53.87±0.19	65.25±0.72
200	53.84±0.49	69.55±0.18	59.13±0.25	70.60±0.10
300	62.30±0.20	71.42±0.46	66.23±0.21	79.74±0.67
400	75.89±0.96	82.58±0.18	76.65±0.36	89.46±0.79
500	82.67±0.38	88.74±0.37	85.68±0.42	91.91±0.70

Phytochemical analysis: The different leaf extracts of *T. divaricata* subjected for preliminary phytochemical screening with solvents chloroform, ethyl acetate, methanol, ethanol and aqueous, showed a range of secondary metabolites (Table 1). Alkaloids were detected in chloroform, ethanol and aqueous extract, flavonoids were moderately present in ethyl acetate, methanol and ethanol extracts, glycosides were present in all five solvents, phenols were detected in methanol, ethanol and aqueous extracts, lignin's were present in all extracts except aqueous, saponins were present in chloroform, ethyl acetate, ethanol and aqueous extracts, except ethanol sterols showed its presence in all four extracts except ethanol, tannins were present in methanol, ethanol and aqueous extracts. Anthraquinone were absent, Phlobatannins in aqueous, reducing sugar in chloroform and volatile oil showed its presence in ethanol extract.

Total phenol content: Total phenolic contents vary considerably among extracts of *T. divaricata* methanol, ethanol and aqueous, presented in Table 2. The amount of total phenolic content were calculated using the standard curve of Gallic acid ($R^2 = 0.9954$) value from the equation:

$$y = mx+c$$

where, y is absorbance of unknown, m is the value from the graph, x is concentration, a is value from the graph and the results were expressed as Gallic acid equivalent per gram.

Antioxidant activities

DPPH assay: The antioxidant activities of *T. divaricata* leaf extracts with different concentrations of methanol, ethanol and aqueous were investigate using DPPH free radical scavenging activity. The results were presented in Table 3. The antioxidant capacity of the extract was compared with ascorbic acid as standard antioxidant.

FRAP and PM assay: In the present study, the presence of antioxidants in the ethanol, methanol and aqueous extracts were subjected to FRAP and PM assay along with standard ascorbic acid. The results are presented in triplicates with Mean±SD (Fig. 1a, b).

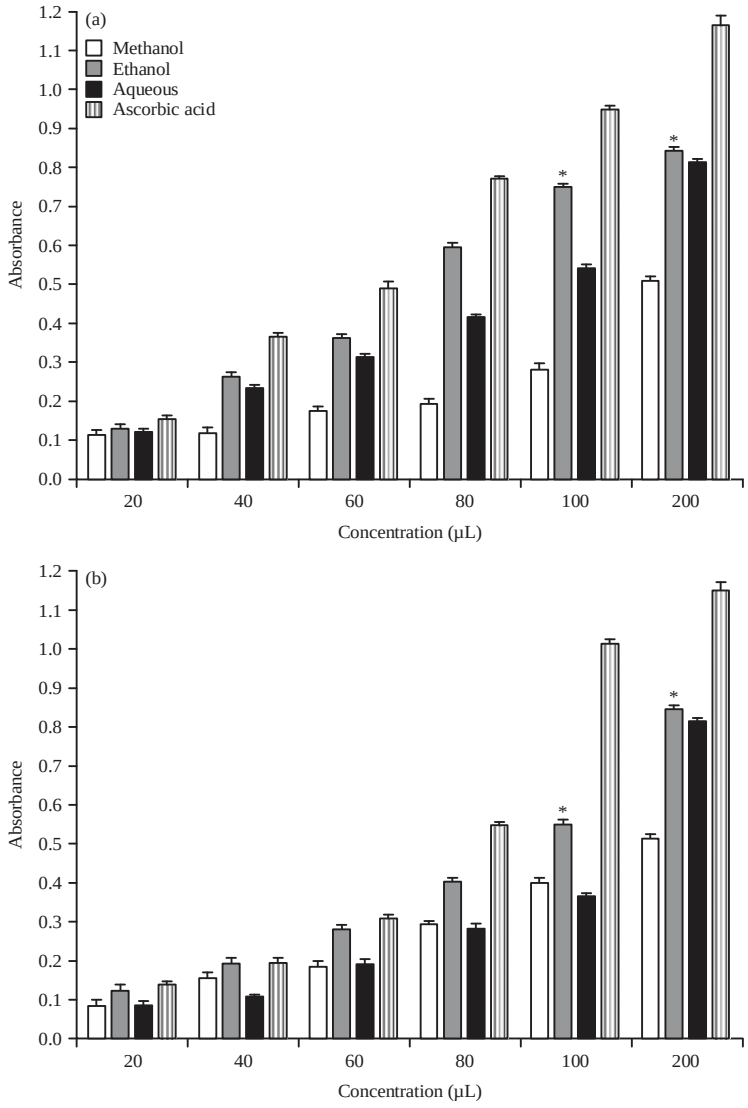

Fig. 1(a-b): (a) FRAP assay for extracts of *T. divaricata* and (b) PM assay for extracts *T. divaricata*
Data is presented as mean SEM (n = 3). *Significant value (p<0.05)

Table 4: IC_{50} values of cell proliferation inhibition of *T. divaricata* leaf extracts (µg mL^{-1})

Cells	Methanol	Ethanol	Aqueous
MEF-L929	97,587.24±17.05	95,475.31±09.08	98,254.24±13.14
T-24	426.6±12.07	188.9±07.05	566.9±15.11
Doxorubicin		0.23 µM mL^{-1}	

Anticancer assay: Anticancer activity of *T. divaricata* leaf methanol, ethanol and aqueous extracts on T-24 bladder cancer cells lines. The effect of methanol, ethanol and aqueous extracts of *T. divaricata* leaves on cell viability were evaluated individually by MTT assay and the IC_{50} values were calculated from dose-dependent response studies assessed 72 h post-treatment. *Tabernaemontana divaricata* leaf extracts inhibited the growth of T-24 bladder cancer cells in a dose-dependent manner. The cytotoxicity for normal mice embryo fibroblast (MEF-L929) cell line was also evaluated and there was no inhibition observed on normal cell lines. The cell viability of the *T. divaricata* leaf extracts was compared to standard chemotherapeutic drug doxorubicin which is commonly used in the treatment of bladder cancer (Fig. 2a, b). The IC_{50} values were calculated in a dose-dependent response and are presented in Table 4. The combination of the extracts along with the standard drug also induced a higher cytotoxicity in cancer cells. These results revealed morphological changes and shrinkage of cells leading to cell apoptosis induced by the extracts in the human bladder cancer cell lines as depicted in Fig. 3a-c.

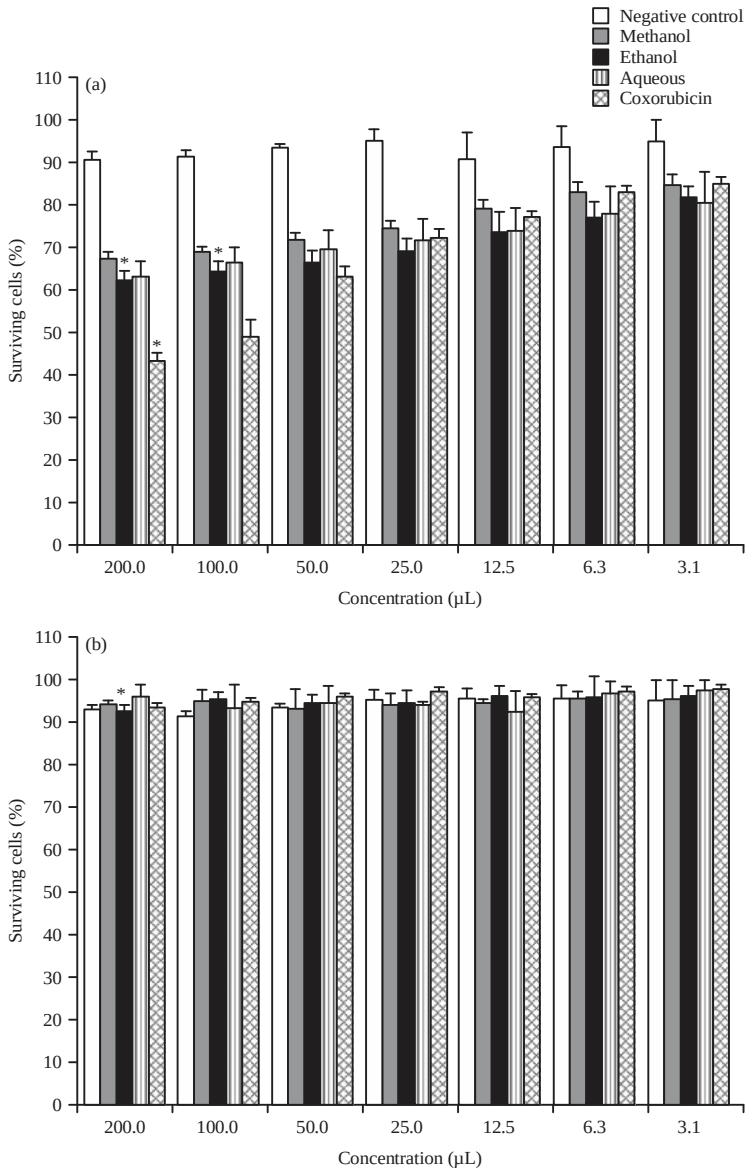

Fig. 2(a-b): (a-b) Viability inhibition (%) of *T. divaricata* methanol, ethanol, aqueous leaf extracts and standard drug (doxorubicin) on T-24 human bladder cancer cell lines

Data is presented as mean SEM (n = 3). *Significant value ($p<0.05$)

DISCUSSION

This study states the importance of herbal medicine in the use of bladder cancer treatment. This study is the ancient practice of herbal medicinal treatment for various diseases. Medicinal plants contain very strong secondary metabolites that bear the antioxidant, radical scavenging agents that help in prevention of cancers and autoimmune disorders[17]. The lack of literature on the use of herbal remedies on the treatment of bladder cancer gave a pavement to evaluate the antioxidant and anticancer activity on bladder cancer cell line[7]. The plant was collected from Western Ghats of Uttar Kannada district and identified according to their taxonomical features as *T. divaricata* and screened for the presences of phytoconstituents with five different solvent extracts. Phytochemical analysis of *T. divaricata* leaf revealed the presences of rich secondary metabolites in the selected leaf extracts. These secondary metabolites are found to have various biological and therapeutic properties[18]. In the present study, alkaloids were noticed in chloroform, ethanol and aqueous extracts, flavonoids were moderately present in ethyl acetate, methanol and ethanol extracts, glycosides were

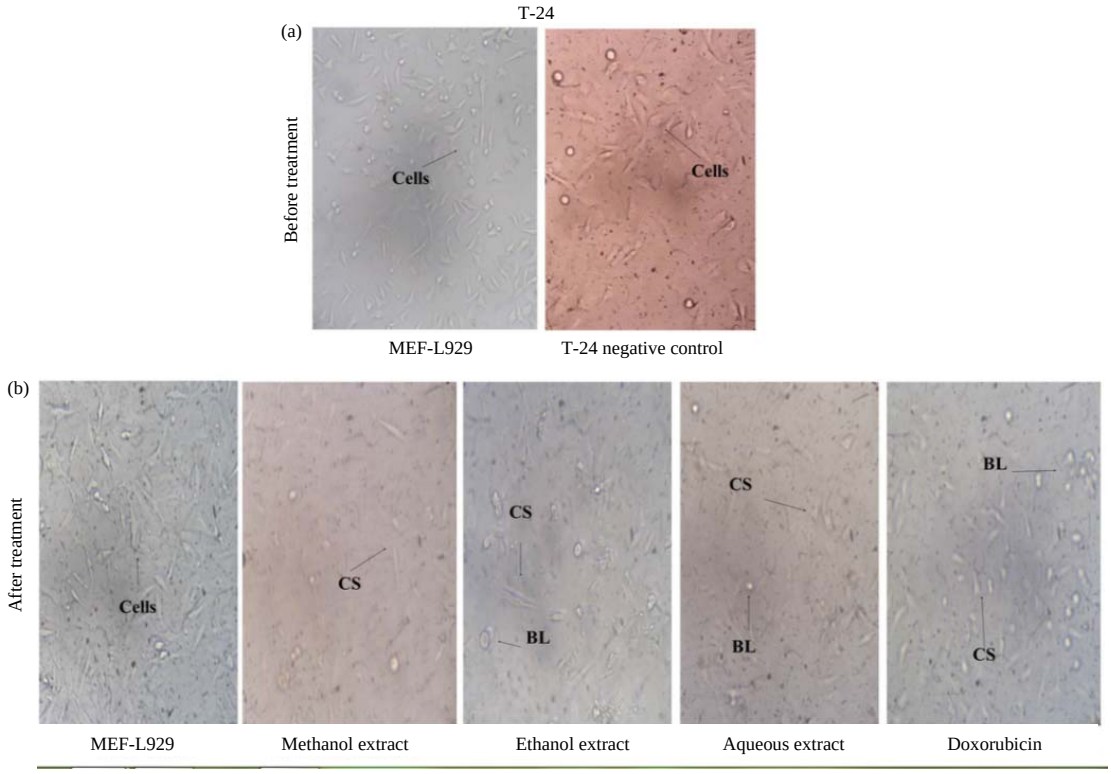

Fig. 3(a-b): (a) Morphological changes of T-24 human bladder cancer cell lines and (b) MEF-L929 normal cell line for 72 h. Morphological changes of standard drug (Doxorubicin). CS: Cellular shrinkage, BL: Membrane blebbing (Magnification for T-24 was 20X and MEF-L929 was 20X

present in all five solvents, phenols were detected in methanol, ethanol and aqueous extracts, lignin's were present in all extracts except aqueous, saponins were present in chloroform, ethyl acetate, ethanol and aqueous extracts, except ethanol sterols showed its presence in all four extracts, tannins were present in methanol, ethanol and aqueous extracts, anthraquinone was absent, phlobatannins in aqueous, reducing sugar in chloroform and volatile oil showed its presence in ethanol extract (Table 1). *Tabernaemontana divaricata*, garden plant in tropical countries is a rich source of alkaloids with various pharmacological properties, the results of phytochemicals revealed the presence of various secondary metabolites. The phenolic compound of methanol, ethanol and aqueous extracts were further quantified using FCR method, the results showed that aqueous extract had higher quantity of phenolic compound present in the leaf sample (62.54±0.18 mg g^{-1} GAE) than methanol (51.71±0.90 mg g^{-1} GAE) and ethanol extracts (56.86±0.37 mg g^{-1} GAE) but slightly lower than the standard Gallic acid (80.28±0.14 mg g^{-1} GAE) (Table 2). The presence of phenolic compound in the leaf extracts of *T. divaricata* is concordance with the properties of the plants that are used in the folk medicine for anti-infection, anti-inflammation, analgesic, anti-tumour, anti-oxidative effect and the effect in neuronal activity[4].

Tabernaemontana divaricata has aroused interest due to the important biologic activity of its extracts, particularly, antioxidant activity, that has been associated with the secondary metabolites present[7]. Radical scavenging activities are very critical due to the lethal properties of free radical ions present in biological systems through reactive oxygen species (ROS) which ultimately impacts many human diseases such as most cancers[17]. Free radical scavenging activity is one of the mechanisms for anti-oxidation[6]. Antioxidant activity of *T. divaricata* leaves extracts were studied by screening its ability to decolorize the stable DPPH radical, FRAP and PM assays.

The results for 100 mg mL^{-1} leaf extracts concentration are presented in Table 3. Ethanol leaf extract proved more potent with (62.41±0.67%) radical scavenging activity for minimum concentration of 100 mg mL^{-1} as compared to methanol (48.58±0.38%) and aqueous extracts (53.87±0.19%). But however the radical scavenging effect of aqueous extract was slightly lower in degree as compared to

the standard ascorbic acid (65.25±0.72%). As like DPPH radical scavenging activity assay, FRAP and PM assay also exhibited maximum potent to ethanol leaf extract and significant as compared to that of methanol and aqueous extracts. The existence of antioxidant, in the various extract have been shown to be an impart antioxidant activity by breaking the free radical chain[11]. This is because of the secondary metabolites like phenols, alkaloids and flavonoids present in the extracts due to which the antioxidants are the compounds capable of donating electron or hydrogen molecule for reduction[19,20]. Higher concentration resolute was unswervingly relative to greater antioxidant potential of the leaf extract. Among the methods tested for antioxidant capacity, DPPH (pink to yellow), FRAP (colourless to green) methods had end point from coloured to colourless whereas, in PM assay it was green to blue (Fig. 1a, b).

The evaluation of anticancer activity of *T. divaricata* leaf methanol, ethanol and aqueous extracts on (T-24) human bladder cancer cell line and (MEF-L929) normal cell line was evaluated for progression and morphology of cells, respectively. The MTT assay an *in vitro* model system to determine the cell viability inhibition was used in screening the extracts that influence basic cellular functions and morphology. As a result significant growth inhibition was observed in bladder cancer cell line (T-24), while there was no adverse effect on the growth of normal cells (MEF-L929). The impact of leaf extracts were showed the concentration (3.125, 6.25, 12.5, 25, 50, 100, 200 μg mL^{-1}) and incubation time period 72 h dependent, where in the inhibition (%) was calculated in triplicates diluted serially. Among the different concentrations gradients 200 μg mL^{-1} of ethanol and aqueous extracts were the most efficient in hampering the progression of cells. The methanol extract showed less effect throughout the range of experimented concentrations and was not statistically significant on (T-24) human bladder cancer cell lines for a single time point of 72 h. The results were compared with the standard Doxorubicin drug[21,22] used in the treatment of bladder cancer showed significant inhibition than the extracts on cancer cell lines (Fig. 2a, b). However, the results showed significant inhibition of ethanol extract was also potent extract with IC_{50} value (188.9±07.05 μg mL^{-1}) for T-24, methanol extract was 426.6±12.07 μg mL^{-1} and aqueous extract showed 566.9±15.11 μg mL^{-1}. Variations in IC_{50} value was observed among methanol and aqueous extracts (Table 4) while the standard drug Doxorubicin exhibited IC_{50} value (0.23 μM mL^{-1}). The results revealed changes at structural and morphological level where the cancerous cells underwent shrinkage and membrane blebbing leading to cell death occurring apoptosis in (T-24) human bladder cancer cell line while the extract showed no cytotoxicity towards normal cell lines (Fig. 3a-c).

CONCLUSION

In the light of these findings, it is evident that *T. divaricata* leaves may be exploited as an important source of antioxidants. This study has gathered experimental evidence that *T. divaricata* leafs are more potent as natural antioxidant for its capacity to protect cells from oxidative DNA damage. It observed that ethanol leaf extracts of *T. divaricata* had significant anticancer activity on T-24 human bladder cancer cell line using *in vitro* model. At *in vitro* model system all the parameters tested *T. divaricata* stood first. Presence of polyphenols in leaves may therefore be responsible for their overall antioxidant potential. Thus *T. divaricata* could serve as an ideal candidate for bio-pharmaceutical therapeutic products and nutraceutical. Further study on various other cancerous cell lines should be carried out to identify the predominant phyto-molecules responsible for the antioxidant and anticancer activity to validate its therapeutic application at large scale.

SIGNIFICANCE STATEMENT

The study provides a platform for implementation of herbal preparations on *in vivo* animal models.

The current conclusions of the study paves the path for development of less toxic molecules in the treatment of cancer. The findings of the research work are unique in the treatment of bladder cancer as there are very less studies around the globe which have used herbal drug.

ACKNOWLEDGMENT

The authors are grateful to Dr. Kotresha K, Department of Botany, Karnataka Science College, Dharwad, Karnataka, for identifying the plant. The authors acknowledge Dr. G. U. Hulepanavarmath, Head, Dept. of English, Rani Chennamma University, Belagavi, for rendering the English Editing of the manuscript.

REFERENCES

1. Antoni, S., J. Ferlay, I. Soerjomataram, A. Znaor, A. Jemal and F. Bray, 2017. Bladder cancer incidence and mortality: A global overview and recent trends. Eur. Urol., 71: 96-108.

2. Breyer, J., S. Shalekenov, A. Aziz, B.W.G. van Rhijn and J. Brundl *et al*., 2017. Increased proliferation as independent predictor of disease recurrence in initial stage pta urothelial bladder cancer. Bladder Cancer, 3: 173-180.
3. Sanchez, A., M.F. Wszolek, A. Niemierko, R.H. Clayman and M. Drumm *et al*., 2018. Incidence, clinicopathological risk factors, management and outcomes of nonmuscle invasive recurrence after complete response to trimodality therapy for muscle invasive bladder cancer. J. Urol., 199: 407-415.
4. Rojas, T.R., G. Bourdy, E. Ruiz, J.P. Cerapio and P. Pineau *et al*., 2018. Herbal medicine practices of patients with liver cancer in Peru: A comprehensive study toward integrative cancer management. Integr. Cancer Ther., 17: 52-64.
5. Raj, P., M. Purushothaman, A. Khusro and S.G. Panicker, 2016. *In-vitro* anticancer and antioxidant activity of gold nanoparticles conjugate with *Tabernaemontana divaricata* flower SMs against MCF-7 breast cancer cells. Korean Chem. Eng. Res., 54: 75-80.
6. Dantu, A.S., P. Shankarguru, D.R. Devi and B.N.V. Hari, 2012. Evaluation of *in vitro* anticancer activity of hydroalcoholic extract of *Tabernaemontana divaricata*. Asian J. Pharm. Clin. Res., 5: 59-61.
7. Bhadane, B.S., M.P. Patil, V.L. Maheshwari and R.H. Patil, 2018. Ethnopharmacology, phytochemistry and biotechnological advances of family Apocynaceae: A review. Phytother. Res., 32: 1181-1210.
8. Jensen, W.B., 2007. The origin of the Soxhlet extractor. J. Chem. Edu., 84: 1913-1914.
9. Ghagane, S.C., S.I. Puranik, R.B. Nerli and M.B. Hiremath, 2017. Evaluation of *in vitro* antioxidant and anticancer activity of *Allophylus cobbe* leaf extracts on DU-145 and PC-3 human prostate cancer cell lines. Cytotechnology, 69: 167-177.
10. Wolfe, K., X. Wu and R.H. Liu, 2003. Antioxidant activity of apple peels. J. Agric. Food Chem., 51: 609-614.
11. Brand-Williams, W., M.E. Cuvelier and C. Berset, 1995. Use of a free radical method to evaluate antioxidant activity. LWT-Food Sci. Technol., 28: 25-30.
12. Oyaizu, M., 1986. Studies on products of browning reaction: Antioxidative activities of products of browning reaction prepared from glucosamine. Jap. J. Nutr. Diet., 44: 307-315.
13. Prieto, P., M. Pineda and M. Aguilar, 1999. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E. Anal. Biochem., 269: 337-341.
14. Igarashi, M. and T. Miyazawa, 2001. The growth inhibitory effect of conjugated linoleic acid on a human hepatoma cell line, HepG2, is induced by a change in fatty acid metabolism, but not the facilitation of lipid peroxidation in the cells. Biochim. Biophys. Acta (BBA)-Mol. Cell Biol. Lipids, 1530: 162-171.
15. Puranik, S.I., S.C. Ghagane, R.B. Nerli, S.S. Jalalpure and M.B. Hiremath, 2017. Evaluation of *in vitro* antioxidant and anticancer activity of *Simarouba glauca* leaf extracts on T-24 bladder cancer cell line. Pharmacogn. J., 9: 906-912.
16. Ghagane, S.C., S.I. Puranik, V.M. Kumbar, R.B. Nerli and S.S. Jalalpure *et al*., 2017. *In vitro* antioxidant and anticancer activity of *Leea indica* leaf extracts on human prostate cancer cell lines. Integrat. Med. Res., 6: 79-87.
17. Jain, S., A. Jain, N. Jain, D.K. Jain and N. Balekar, 2010. Phytochemical investigation and evaluation of *in vitro* free radical scavenging activity of *Tabernaemontana divaricata* Linn. Natl. Prod. Res., 24: 300-304.
18. Eldahshan, O.A. and M.M. Abdel-Daim, 2014. Phytochemical study, cytotoxic, analgesic, antipyretic and anti-inflammatory activities of *Strychnos nux-vomica*. Cytotechnology, 67: 831-844.
19. Ghate, N.B., B. Hazra, R. Sarkar and N. Mandal, 2014. *In vitro* anticancer activity of *Spondias pinnata* bark on human lung and breast carcinoma. Cytotechnology, 66: 209-218.
20. Kumar, A. and S. Selvakumar, 2015. Antiproliferative efficacy of *Tabernaemontana divaricata* against HEP2 cell line and Vero cell line. Pharmacogn. Mag., 11: S46-S52.
21. Liu, Z., Y. Ye, X. Li, S. Guo and L. Jiang *et al*., 2018. The effects of intra-arterial chemotherapy on bladder preservation in patients with T1 stage bladder cancer. World J. Urol., 36: 1191-1200.
22. Jiao, L., L. Bi, Y. Lu, Q. Wang, Y. Gong, J. Shi and L. Xu, 2018. Cancer chemoprevention and therapy using chinese herbal medicine. Biol. Proced. Online, Vol. 20, No. 1. 10.1186/s12575-017-0066-1.