Nat. Volatiles & Essent. Oils, 2021; 8(6): 5038-5048

Evaluation Of Antitumor Activity Of Justicia Tranquebariensis L.F.

V. HARI[1,2] , D. JOTHIESWARI[2]* , K. SESHA MAHESWARAMMA[3]

[1]Research Scholar, JNTUA, Anantapur-515-002 Andhra Pradesh, India.

[2] Center for Pharmaceutical Nano Technology**,** Dept. of Pharmaceutical Analysis, Sri Venkateswara College of Pharmacy, RVS Nagar, Tirupati Road, Chittoor- 517127,Andhra Pradesh, India.

[3] Department of Chemistry, Jawaharlal Nehru Technological University Anantapur, College of Engineering, Pulivendula -516390, Andhra Pradesh, India.

ABSTRACT

Objectives: Cancer is the most deadliest disease in the modern world that is affecting all groups of people. Synthetic medicines are suffering with serious side effects and a high demand is created for the natural products based anticancer agents. The current work is aimed to screen the In vitro and In vivo antitumor properties of Justicia tranquebariensis L.f.

Methods: Antitumor activity of Justicia tranquebariensis L.f. will be estimated using MTT assay and Ehrlich Ascites Carcinoma (EAC) in mice. MTT assay was performed for the pet ether, and ethanol extracts of Justicia tranquebariensis L.f. and acute toxicity studies were also conducted according to OECD guidelines-423 using Swiss albino mice. The extracts were screened for cytotoxic potential in the cancer cell lines, namely, A 549 (lung), DU 145 (prostate), HT 29 (colon), MCF-7 (breast) cells. The EAC-bearing mice were treated with various doses of plant extracts, i.e., 200& 400 mg/kg body weight (bw) and standard Doxorubicin. Parameters such as tumor volume, packed cell volume, viable and non-viable cell count, survival time were determined and compared. The hematological parameters (RBC, WBC, Haemoglobin, & differential WBC count) and biochemical parameters (SGPT, SGOT, ASLP, total protein, and Bilirubin levels) were also evaluated.

Results: The results show that the ethanol extracts of Justicia tranquebariensis L.f. are safe at a dose of 2,000mg/kg bw. The ethanol extract has potent cytotoxicity on Breast cancer (MCF-7; 5.01±4.85µM) followed by Lung cancer (A 549; 28.14±2.19), Colon cancer (HT 29; 32.83±5.11µM), and Prostate cancer (DU 145; 39.64±4.18).

Conclusion: The extracts exhibited significant antitumor activity in a dose-dependent manner, and ethanol extract at a dose of 400mg/kg body weight showed better survival, hematological, biochemical, and histological parameters. The treatment also increased the life span of the EAC-bearing mice.

KEYWORDS: Justicia tranquebariensis L.f., MTT assay, Ehrlich Ascites Carcinoma (EAC), cell lines, acute toxicity.

INTRODUCTION

Cancer is a perpetual challenge for the modern world, with substantial mortality (9% of the total deaths worldwide) in people irrespective of age, sex, and geography, cancer [1]. From the literature,

it is traceable that the exploration of antineoplastic drugs is an essential and unremitting effort is being made to find new chemotherapeutics to alleviate cancer disease with the minimum side effect [2].

Herbal medicine is a primary choice for most developing countries, and plant-based medicine has been for centuries in social and community health management. Safety and diverse pharmacological aspects of herbal medicine can give better therapeutic output for chronic diseases [3]. Natural products-based drug discovery is gaining importance in drug discovery, and plant secondary metabolites are becoming the source for the novel lead molecules with good therapeutic efficacy[4,5].

Justicia tranquebariensis L.f. of the Acanthaceae family, called Pindi in Sanskrit, is a small shrub with simple, obovate, and opposite leaves. Plant of this genus has well-known traditional applications in the southern part of India [6]. Traditional healers use it for pain, inflammations, fever, cold, liver diseases, diarrhea, and diabetes [7]. Few phytochemicals such as lignans, including Lariciresinol and Cubebin, were isolated from the aerial parts [8].

The current investigation focuses on screening the In vitro and In vivo antitumor activity of various extracts of Justicia tranquebariensis L.f. and estimating various survival, hematological and biochemical parameters and effects on the life span of the EAC-bearing animals.

MATERIALS AND METHODS

Animals

Swiss albino mice (male; weighing around 30-35gm) were used for the study, selected randomly, and acclimatized in cages (polypropylene). Rat pellet diet, water, standard temperature (24±2˚C), humidity (30-70%), and daylight cycle (12:12) were maintained during this period. Prior approval from the institutional animal ethics committee (IAEC) (1447/PO/Re/S/11/CPCSEA-37/A) was taken.

Acute oral toxicity studies:

The oral acute toxicity study of Justicia tranquebariensis L.f. extracts were evaluated according to Organization for Economic Co-operation and Development (OECD) guideline 423 on Swiss albino mice, where the limit test dose of 4000 mg/kg body weight was used. All the animals were kept at overnight fasting before every experiment with free excess to water. The animals were divided into four groups, each comprising five animals. The 1st group served as a negative control, while the 2nd, 3rd, and 4th were considered tested groups that received extracts at a dose of 300 mg/kg, 2000 mg/kg, and 4000 mg/kg body weight orally. Before dose administration, the body weight of each animal was determined, and the dose was calculated according to the body weight. The animals were observed for any toxic effect for the first four hours after the treatment period. Further animals were investigated for three days for parameters such as body weight, urination, food intake, water intake, respiration, convulsion, tremor, temperature, constipation, eye, skin colors, etc [9].

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

The cytotoxic potential of ethanol extracts of Justicia tranquebariensis L.f. was determined by MTT assay using A 549 (lung), DU 145 (prostate), HT 29 (colon), MCF-7 (breast) cells which were obtained from American Type Culture Collection (ATCC), 10801 University Boulevard Manassas, VA 20110, USA. The purple formazan produced by reducing MTT salt with mitochondrial enzymes represents

the viability of cells. The intensity measured spectrophotometrically can be proportionate to the quantity of the living cells and can be expressed as IC_{50} values. The cells are incubated in a 96 well plate with the test samples at standard conditions (37°C, 5% CO_2, 72 hours) with subsequent MTT (20µl, 2mg/ml, phosphate-buffered saline) treatment followed by 3 hours incubation under identical conditions. The colored formazan was extracted with DMSO (100µl), and the intensity was measured using a spectrophotometer (540 nm) in triplicates, and the values were compared with standard (Doxorubicin) and blank [10].

Tumor cells

The transplantable murine tumor cells were obtained from Amala Cancer Research Centre, Thrissur, Kerala, India. The tumor is maintained in the ascitic form by transplanting 1 million tumor cells to the animals (intraperitoneally) every week [11]. Ascitic fluid was collected from the animal, and a concentration of 20 million cells per ml was maintained using sterile ice-cold normal saline solution.

In vivo anticancer evaluation

The animals were divided into seven groups (five animals each). A suspension of the carcinoma cells (20 million cells per ml) was prepared, and 0.1 ml was injected to all the groups, excluding the control [12].

Group 1 : Normal control
Group 2 : EAC control group
Group 3 : EAC + Doxorubicin (0.4mg/kg bw., i.p.)
Group 4 : EAC + pet ether extract (200mg/kg bw., oral) for 9 days
Group 5 : EAC + pet ether extract (400mg/kg bw., oral) for 9 days
Group 6 : EAC + ethanol extract (200mg/kg bw., oral) for 9 days
Group 7 : EAC + ethanol extract (400mg/kg bw., oral) for 9 days

On the next day, all the groups received respective treatments for two weeks. An intragastric catheter was used to administer the extracts orally. After 14 days of treatment followed by one-day fasting, the animals were sacrificed, and the blood & serum parameters, tumor volume, packed cell volume, viable and non-viable cell count mean survival time, the percentage increase in life span were measured [13,14].

Estimation of blood & serum parameters

Blood was collected by cardiac puncture and parameters such as RBC count, WBC count (differential), hemoglobin content. The blood was centrifuged for 20 minutes at 5,000rpm to obtain serum to measure the serum parameters such as SGOT& SGPT levels were also determined to determine the hepatic function [15,16]

Estimation of tumor and packed cell volume

The ascitic fluid was collected from the peritoneal cavity and separated into two equal parts. The first part is centrifuged (1000rpm, 10 minutes) to measure packed cell volume (PCV) using a graduated centrifuge tube.

Estimation of viable and non-viable cell count

The second part of ascitic fluid was further subjected to the separation of the cells, and the viability was measured using 0.4% trypan blue solution. The stained cells indicate the non-viability of the cells. It is measured by using the formula

Cell count= (No. of cells × Dilution factor) / (Area × Thickness of liquid film)

Determination of mean survival time and percentage increase in the life span

The mortality will be monitored by the recording percentage increase in life span (% ILS) and mean survival time (MST) as follows:

% ILS = [(Mean survival time of treated group/ Mean survival time of control group)-1] x 100

Histopathological studies

The tumor specimens were separated from the mice and preserved in formalin (adjusted with 10% Phosphate buffer). Before examination of the specimens, they were rinsed with ethyl alcohol followed by xylene and suspended in paraffin wax. 5µm thin specimens were prepared, eosin and hematoxylin were added to stain the specimens. All the specimens were observed under binocular microscope and recorded.

Statistical analysis:

Data will be analyzed using one-way Analysis of Variance (ANOVA) and expressed as mean± S.E.M. Statistical significance was fixed $p< 0.05$.

RESULTS

Acute toxicity studies

The results show that the administration of Justicia tranquebariensis L.f. was safe up to a dose of 2000 mg/kg. No aforementioned toxic symptoms or mortality were observed at this dose.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

From the results, it can be drawn that, after incubating with Justicia tranquebariensis L.f. extracts, for 72hr at 37°C and 5% CO_2, ethanol extract exhibited significant cytotoxicity against the selected cancer cell lines (Table 1). Pet ether extract is showing minimum cytotoxicity against Breast cancer (MCF-7; IC_{50}= 66.65±5.12µM), Lung cancer (A 549; IC_{50}=22.35±1.34 µM) and no significant activity against Colon cancer (HT 29; IC_{50}= >100µM), Prostate cancer (DU 145; IC_{50}= >100µM) cell lines. Whereas, for ethanol extract, it is found that the Breast cancer (MCF-7) cells are more sensitive (5.01±4.85µM) among others when compared to the standard Doxorubicin (1.52±1.76µM) followed by Lung cancer (A 549; 28.14±2.19), Colon cancer (HT 29; 32.83±5.11µM) and Prostate cancer (DU 145; 39.64±4.18).

Table 1. IC_{50} values of extract and Doxorubicin in MTT assay

Treatment	Justicia tranquebariensis L.f.		Doxorubicin (µM)
	Pet ether extract (µM)	Ethanol extract (µM)	

A 549 (lung)	66.65±5.12	28.14±2.19	0.31±1.82
DU 145 (prostate)	>100	39.64±4.18	2.1±1.63
HT 29 (colon)	>100	32.83±5.11	2.34±2.71
MCF-7 (breast)	22.35±1.34	5.01±4.85	1.52±1.76

In vivo anticancer evaluation

Tumor growth

Justicia tranquebariensis L.f. reduced the tumor size and volume in a dose-dependent manner ($p<0.05$). Ethanol extract reduced the tumor volume significantly compared to the pet ether extract. At a dose of 400mg/kg bw, the ethanol extract reduced the tumor volume (1.14±0.07ml) that is comparable with the standard Doxorubicin (0.21±0.02). The packed cell volume was reduced from 3.17±0.14 ml to 0.48±0.07 ml after treating the EAC-bearing mice with ethanol extract with a dose of 400mg/kg bw stood the best treatment among others. Similarly, viable cell count (cells×10^6 /mL) was reduced with Doxorubicin (0.45±0.11) and ethanol extract (0.86±0.17), significant with the values of the EAC-bearing mice group (9.03±0.02). The reduced non-viable cell count was increased as high as 3.97±0.08 cells×10^6/mL in the Doxorubicin followed by ethanol-treated group 2.48±0.13 cells×10^6/mL.

Survival parameters

Median survival time and ILS percentage were significantly improved for ethanol extract treated groups as 200mg/kg bw (31.54±1.06; 68.93%) and at 400mg/kg bw (37.19±0.07; 75.37%). In comparison, the standard Doxorubicin exhibited 43.75±0.04; 101.64%, respectively (Table 2& Figure 1).

Table 2. Effect of Justicia tranquebariensis L.f. on EAC-bearing mice

Treatment	Body weight (gms)	Tumor volume (ml)	Packed cell volume (ml)	Viable cell count (cells×10^6 /mL)	Non-viable cell count (cells×10^6 /mL)	Median survival time (days)	ILS%
Normal control	31.24±0.12	-	-	-	-	-	-
Disease control	35.37±0.31	4.96±0.31	3.17±0.14	9.03±0.02	0.72±0.03	22.37±0.04	-
Pet ether extract	32.12±0.11	3.17±0.17	3.02±0.05	8.25±0.18	0.97±0.08	23.17±0.03	15.09

(200mg/kg bw)							
Pet ether extract (400mg/kg bw)	31.55±0.33	3.09±0.14	2.97±0.07	7.93±0.08	1.14±0.12	25.11±1.07	19.62
Ethanol extract (200mg/kg bw)	28.54±0.21	2.06±0.12	0.92±0.03	1.27±0.31	2.07±0.17*	31.54±1.06	68.93*
Ethanol extract (400mg/kg bw)	29.34±0.92	1.14±0.07*	0.48±0.07*	0.86±0.17*	2.48±0.13*	37.19±0.07*	75.37*
Positive control (Doxorubicin)	30.08±0.77	0.21±0.02*	0	0.45±0.11*	3.97±0.08*	43.75±0.04*	101.64*

Data represented as mean±SEM, n=5 animals, *p<0.05 is considered as statistically significant

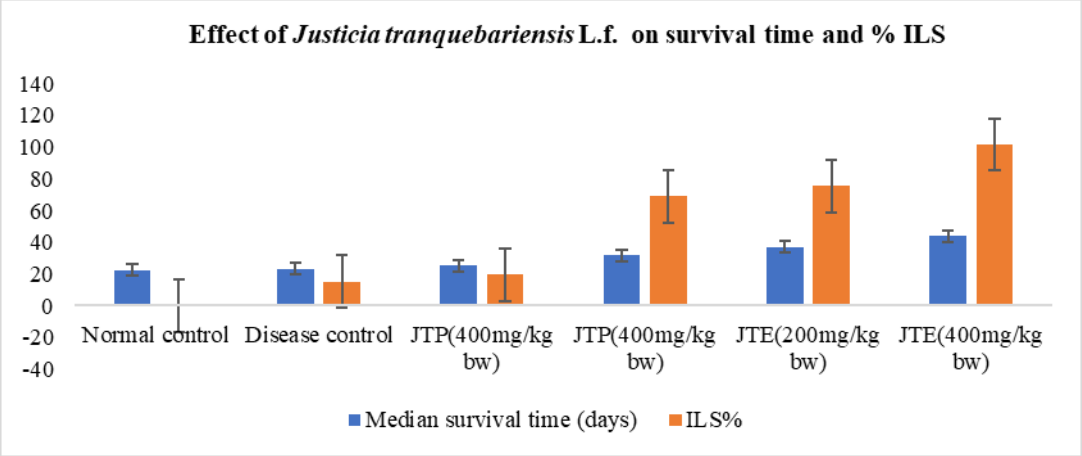


Figure 1. Effect of Justicia tranquebariensis L.f. on survival time and % ILS

Haematological parameters

The RBC count and Haemoglobin content significantly decreased, and the WBC count increased in the EAC-bearing mice. The treatment with the standard Doxorubicin and the extract improved (p<0.05) the haematological parameters. Ethanol extract refurbished the RBC count and Haemoglobin content as 3.05±0.17&8.07±1.05 at 200mg/kg bw and 3.82±0.11&9.18±1.06 at 400mg/kg bw. WBC count was brought to normal by the plant treatment 5.18±0.11&4.92±0.16 at low and high doses with improved differential WBC parameters as mentioned in Table 3& Figure 2.

Table 3. Effect of Justicia tranquebariensis L.f. on haematological parameters of EAC-bearing mice

Treatment	RBC (cells×10^6/ mm^3)	WBC (cells×10^3/ mm^3)	Hb content (%gm)	Differential count		
				Monocytes (%)	Lymphocytes (%)	Neutrophils (%)

Normal control	5.17±0.03	4.27±0.11	12.09±0.11	1.85±0.04	74.19±0.02	19.34±0.02
Disease control	2.83±0.1	6.44±0.13	6.24±0.07	1.12±0.14	30.17±0.02	75.11±0.4
Pet ether extract (200mg/kg bw)	2.54±0.16	6.12±0.13	6.12±0.06	1.19±0.17	35.31±0.16	71.34±0.14
Pet ether extract (400mg/kg bw)	2.36±0.08	5.97±0.09	5.13±0.01	1.23±0.14	41.37±0.17	64.92±0.03
Ethanol extract (200mg/kg bw)	3.05±0.17	5.18±0.11*	8.07±1.05*	1.33±0.17	61.09±0.2	40.37±0.27
Ethanol extract (400mg/kg bw)	3.82±0.11*	4.92±0.16*	9.18±1.06*	1.46±0.19*	69.03±0.21*	28.16±0.37*
Positive control (Doxorubicin)	4.12±0.14*	3.84±0.22*	11.17±1.03*	1.7±0.26*	71.35±0.15*	21.92±0.09*

Data represented as mean±SEM, n=5 animals, p<0.05 is considered as statistically significant

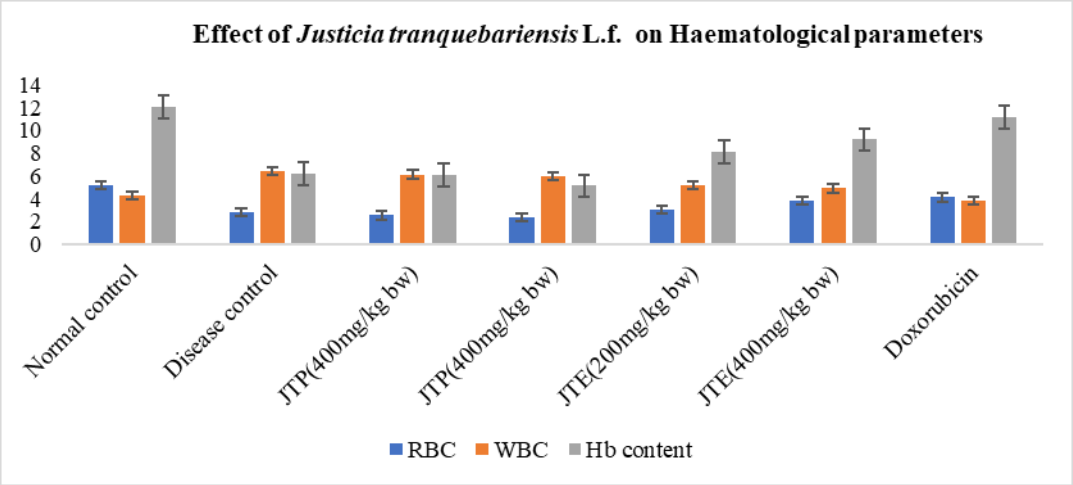


Figure 2. Effect of Justicia tranquebariensis L.f. on Haematological parameters

Biochemical parameters

Similarly, the serum biochemical parameters were significantly (p<0.05) altered in the disease control (Table 4). Treatment with Doxorubicin and Justicia tranquebariensis L.f. for the period of 9 days reestablished the biochemical profile. SGOT, SGPT, SALP, and Bilirubin levels were recovered to normal levels. Treating the EAC-bearing mice with the extracts at a dose of 400mg/kg bw exhibited significant improvement in SGOT levels (43.18±1.09 IU/L), SGPT levels (35.94±1.09 IU/L), SALP levels (86.47±1.24 IU/L), and Bilirubin levels (1.72±1.18 mg/dL) of serum. The total protein content was restored to 8.04±1.34 mg/dL from 5.37±1.09 mg/dL with a dose of 400mg/kg bw.

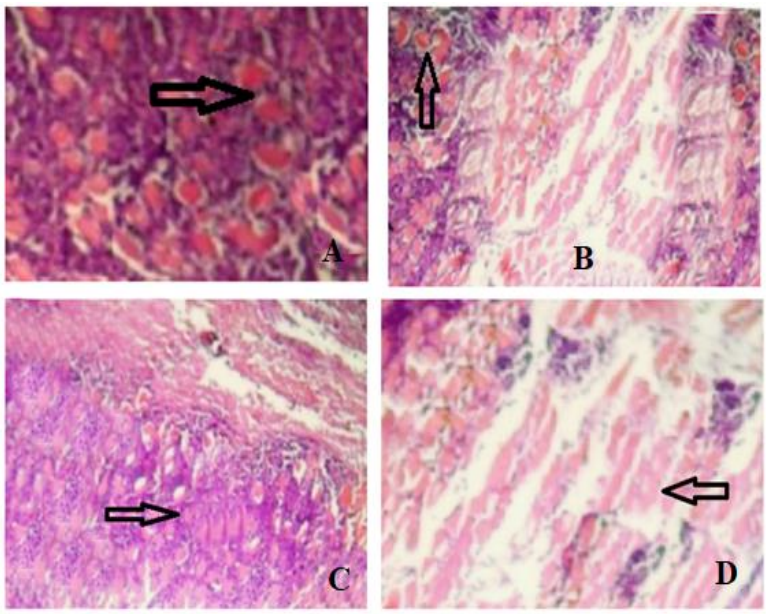
Table 4. Effect of Justicia tranquebariensis L.f. on biochemical parameters of EAC-bearing mice

Treatment	SGOT (IU/L)	SGPT (IU/L)	SALP (IU/L)	Total protein (mg/dL)	Bilirubin (mg/dL)
Normal control	35.36±1.06	25.93±1.06	75.03±1.27	9.06±1.1	0.97±0.34
Disease control	80.69±1.09	69.11±1.17	117.82±1.23	5.37±1.09	3.62±1.06
Pet ether extract (200mg/kg bw)	78.35±1.07	62.13±1.23	112.09±1.21	5.25±1.07	3.28±0.29
Pet ether extract (400mg/kg bw)	74.19±1.15	60.72±1.25	107.33±1.21	5.17±1.07	3.09±1.03
Ethanol extract (200mg/kg bw)	50.32±1.24	48.34±1.16	92.07±1.29	7.83±1.1*	1.93±1.24
Ethanol extract (400mg/kg bw)	43.18±1.09*	35.94±1.09*	86.47±1.24*	8.04±1.34*	1.72±1.18*
Positive control (Doxorubicin)	39.17±1.27*	29.62±1.11*	79.64±1.23*	8.49±1.22*	1.03±1.1*

Data represented as mean±SEM, n=5 animals, $p<0.05$ is considered as statistically significant

Histopathological studies

Histopathological studies of the tissue specimens of the mice were processed and observed under microscope. In the skeletal muscle, it is observed that the subcutaneous tissue of the tumor associated mice was substituted with dead tissue along with angiogenesis. The tumor area was comparatively darker than the normal tissue, with mild inflammation. The regular architecture of the subcutaneous tissue was distorted. The treatment of the plant extracts at the high dose restored the regular anatomical features of the tissue in a dose-dependant manner with improved vascularity (Figure 3). Whereas, Doxorubicin reestablished the homeostasis with minimum effect on the vasculature disarray.



A&B: Disease control C&D: Extract high dose

Figure 3. Histopathological study of solid tumor

DISCUSSIONS

Justicia tranquebariensis L.f. was showing no toxic symptoms and found safe even at 2000 mg/kg concentration. Hence, the present study selected 1/10th and 1/5th of 2000 mg/kg, i.e., 200 mg/kg and 400 mg/kg as working doses.

CONCLUSION

To conclude, Justicia tranquebariensis L.f. exhibited potent cytotoxicity in the selective cancer cell lines. Breast cancer (MCF-7) cells are more sensitive, followed by Lung cancer (A 549), Colon cancer (HT 29), and Prostate cancer (DU 145) when compared with Doxorubicin. In the acute toxicity studies, the extracts are proved to be safe at a dose of 2,000ng/kg bw. In vivo antitumor activity studies revealed that ethanol extract is more potent than pet ether extract. It reduced the tumor size and viability in a dose-dependent manner. The mean survival time and life span also improved with the treatments. The EAC-bearing mice's hematological, biochemical, and histological parameters were also retroceded. This study suggests that the ethanol extract of Justicia tranquebariensis L.f. exhibits significant antitumor activity in the selected models to support the current cancer chemotherapy. Our previous phytochemical analysis identified various secondary metabolites such as alkaloids, carbohydrates, phenols, steroids, terpenoids, glycosides, saponins, especially tannins and flavonoids Justicia tranquebariensis L.f. These secondary metabolites may be responsible for the exhibited antitumor activity either alone or in combination.

Acknowledgements:

The authors thank the management of Sri Venkateswara College of Pharmacy (Autonomous) for their continuous support and encouragement. The authors also thank and acknowledge DST

(F.No.SR/FST/College-280/18.11.2015) under FIST Programe for providing financial support to the institute.

Conflict of Interest: None

REFERENCES

1. Kakde D, Jain D, Shrivastava V, Kakde R, Patil AT. Cancer therapeutics-opportunities, challenges and advances in drug delivery. J App Pharm Sci. 2011; 1(9): 1-10.
2. Chintalapani S, Swathi MS, Narasu ML. Antiproliferative and apoptosis inducing potential of whole plant extracts of Sesuvium portulacastrum against different cancer cell lines. J App Pharm Sci. 2019; 9(02):038–043.
3. Philomena George. Concerns regarding the safety and toxicity of medicinal plants - An overview. Journal of Applied Pharmaceutical Science. 2011; 01(06): 40-44.
4. Sija SL, Athulya AS, Mahima MR, Vidhya A. Antioxidant and antimicrobial activity of different plant parts of Anacardium occidentale L. and Mangifera indica L.: A Comparative Study. International Journal of Pharmaceutical Sciences and Drug Research. 2019; 11(4):111-5.
5. Padmanabhan P, Jangle SN. Evaluation of DPPH radical scavenging activity and reducing power of four selected medicinal plants and their combinations. International Journal of Pharmaceutical Sciences and Drug Research. 2012; 4(2):143-6.
6. Pavithra T, Hari V, Lavanya P, Aruna Kumari T, Rupesh Kumar M, Bhaskar Reddy K. Pharmacological review on Justicia tranquebariensis Linn. International Journal of Current Advanced Research. 2017; 6(02): 2058-2061.
7. Krishnamoorthi R, Ratha Bai V. Phytochemical Screening and Antioxidant Activity of Justicia tranquebariensis and Bauhinia Racemosa. Open Science Journal of Pharmacy and Pharmacology. 2015; 3(2):10-15.
8. Sandhya B, Thomas S, Isabel W, Shenbagarathai R. Ethnomedicinal plants used by the Valaiyan community of Piranmalai hills (reserved forest), Tamilnadu, India, A Pilot study. Afr. J. Trad. CAM. 2006; 3 (1):101-114.
9. Kifayatullah M, Mustafa MS, Sengupta P, Sarker MM, Das A, Das SK. Evaluation of the acute and sub-acute toxicity of the ethanolic extract of Pericampylus glaucus (Lam.) Merr. in BALB/c mice. Journal of Acute Disease. 2015; 4(4): 309–315.
10. OECD: OECD Guideline for testing of chemicals. Acute oral toxicity-acute toxic class method, guideline no. 423. adopted 2001 Organisation for Economic Co-operation and Development, Rome 2001.
11. Dagli MLZ, Guerra JL, Saldiva PHN. An experimental study on the lymphatic dissemination of the solid Ehrlich tumor in mice. Brazilian Journal of Veterinary Research and Animal Science. 1992; 29: 97-103.
12. Rinku Mathappan, Kesavanarayanan Krishnan Selvarajan, Sabitha Sujeet, Sourav Tribedi. Evaluation of antitumor activity of Urena lobata against Ehrlich ascites carcinoma treated mice. Oriental Pharmacy and Experimental Medicine. 2019; 19: 21–26.
13. Bala A, Kar B, Haldar PK, Mazumder UK, Bera S. Evaluation of anticancer activity of Cleome gynandraon in Ehrlich's Carcinoma treated mice. J Ethnopharm. 2010; 129: 131-4.

14. Brandao EM, Brandão PHDM, Souza IA, Paiva GS, de C. Carvalho M, Lacerda CM. Antineoplasic Effect of Aqueous Extract of Plectranthus Amboinicus in Ehrlich Ascites Carcinoma. J Cancer 2013; 4(7):573-576.
15. Fla´via RF. Ascitic and solid Ehrlich tumor inhibition by Chenopodium ambrosioides L. treatment. F.R.F. Nascimento et al. / Life Sciences. 2006; 78: 2650-2653.
16. Santoshkumar H Dongre, Shrishailappa Badami1, Senthilkumar Natesan, Raghu Chandrashekhar H. Antitumor Activity of the Methanol Extract of Hypericum hookerianum Stem Against Ehrlich Ascites Carcinoma in Swiss Albino Mice. J Pharmacol Sci. 2007; 103: 354-359.