Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

In vitro Anti-Proliferative Effect of *Tephrosia purpurea* on Human Hepatocellular Carcinoma Cells

Ramamoorthy Padmapriya, Loganathan Gayathri[1], Larance Ronsard[2], Mohammad A. Akbarsha[1], Ramasamy Raveendran

Department of Pharmacology, JIPMER, Puducherry, [1]Mahatma Gandhi-Doerenkamp Center, Bharathidasan University, Tiruchirappalli, India, [2]Division of Infectious Diseases, Boston Children's Hospital, Harvard Medical School, Boston, MA, USA

Submitted: 19-05-2016 Revised: 11-07-2016 Published: 07-04-2017

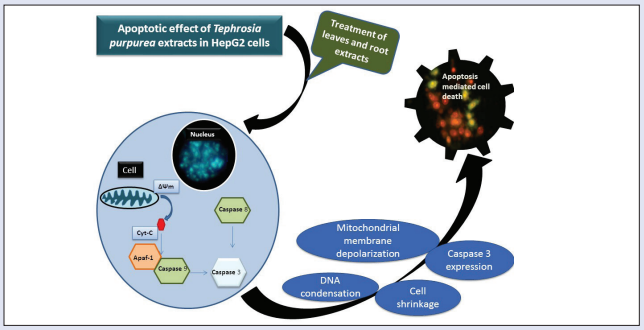
ABSTRACT

Background: *Tephrosia purpurea* is an Indian herb used in traditional medicine to treat various diseases such as jaundice, asthma, liver and urinary disorders. However, the anti-cancer potential of *T. purpurea* on hepatocellular carcinoma (HCC) is poorly understood. Therefore, this study aims to investigate the anti-cancer activity of *T. purpurea* in HepG2 hepatocellular carcinoma cells. **Methods:** The leaves and root of *T. purpurea* were extracted with methanol using soxhlet apparatus. The cytotoxicity of the *T. purpurea* extracts in HepG2 cells was evaluated using MTT assay whereas the mode of cell death was examined by AOEB, Hoechst and JC1 staining under a fluorescence microscope. *T. purpurea* extracts-induced caspase-3 expression was investigated using colorimetric assay. **Results:** The leaves and root extracts inhibited HepG2 cell growth at the IC_{50} of 102.33 ± 10.26 µg/mL and 276.67 ± 20.43 µg/mL respectively at 24 h. Chromatin condensation, nuclear fragmentation, apoptotic bodies formation and mitochondrial membrane depolarization were observed in HepG2 cells treated with both extracts. The caspase-3 expression was significantly ($p < 0.05$) increased in extracts treated cells when compared to control. **Conclusion:** The leaves and root extracts of *T. purpurea* induce apoptosis mediated cell death in HepG2 cells.

Key words: Anticancer, apoptosis, hepatocellular carcinoma, Tephrosia purpurea

SUMMARY

The leaves and root extracts of *T. purpurea* exhibited anticancer activity in HepG2 hepatocellular carcinoma cells. These extracts induced cell shrinkage, DNA condensation and fragmentation, mitochondrial membrane depolarization and upregulated caspase-3 expression indicating *T. purpurea* extracts induce apoptosis in HepG2 cells.



Abbreviation used: AO: acridine orange, DMSO: dimethyl sulfoxide, EB: ethidium bromide, IC_{50}: the concentration at which 50% of cancer cells are dead, JC-1: 5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethyl-imidacarbocyanine iodide, MTT: 3-4, 5-dimethylthiazole-2-yl, 2,5-diphenyl tetrazolium bromide, PBS: phosphate-buffered saline, ΔΨm: mitochondrial trans-membrane potential.

Correspondence:
Dr. R Raveendran, Department of Pharmacology, JIPMER, Puducherry, India.
E-mail: dr.ravee@gmail.com

DOI:10.4103/0973-1296.203981

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Cancer is a global health problem, of which hepatocellular carcinoma (HCC) is the fifth most common malignancy and third most common cause of cancer-related death worldwide. The incidence of HCC is found to be increasing in the recent time.[1] In the early stage, HCC is relatively asymptomatic and remains undetected. At the time of diagnosis, the disease will be too severe to tackle with conventional treatments.[2] The progression of HCC is highly influenced due to multiple factors like alcohol intake, non alcoholic fatty liver, cirrhosis of liver, aflatoxin ingestion, hepatitis B and C virus infection, genetic variations and epigenetic modifications.[3,4] The current major treatments available for the early stage of HCC are surgical resection of the tumor, liver transplantation, radiotherapy and chemotherapy. However, therapeutic options for HCC in advanced stages are limited to palliation.[5] Although substantial advances have been made to treat HCC patients, the one-year survival rate is less than 50%.[6] Moreover, the use of conventional therapies is limited by drug toxicity and resistance indicating the necessity for the development of new complementary and alternative therapy for HCC with the better outcome.[7,8]

Plant products are used in the health care system since ancient human civilizations. More than thousands of medicinal plants have been scientifically identified to possess potent anti-cancer activity.[9] Nearly 60% of the currently used anti-cancer drugs are derived from natural agents including plants, micro and marine organisms and many are approved by Food and Drug Administration (FDA). Some natural lead compounds discovered and used as a boon for cancer chemotherapy are vincristine, vinblastine, camptothecin, topotecan, and taxane.[10] Herbal

Cite this article as: Padmapriya R, Gayathri L, Ronsard L, Akbarsha MA, Raveendran R. *In vitro* anti-proliferative effect of Tephrosia purpurea on human hepatocellular carcinoma cells. Phcog Mag 2017;13:16-21.

medicines have received great attention because of their relatively low toxic effect.[11] *Tephrosia purpurea* is an Indian medicinal plant which belongs to the Fabaceae family and popularly known as Wild Indigo. The traditional Ayurvedic medicine uses different parts of this plant to treat various diseases such as jaundice, asthma, rheumatism, ulcer, liver, and urinary disorders.[12] *In vitro* and *in vivo* studies of *T. purpurea* demonstrated the antioxidant, anti-bacterial, anti-inflammatory, anti-plasmodial, anti-diabetic and cytotoxic activities.[13-17]

The current knowledge about the potential mechanisms responsible for the anti-proliferative activity of *T. purpurea* extracts on HCC cells is limited. Therefore, the present study aims to investigate the cytotoxic and the apoptotic properties of methanolic extracts of leaves and root of *T. purpurea* on human hepatocellular carcinoma cell lines.

MATERIALS AND METHODS

Plant material

Fresh *T. purpurea* plant was collected from JIPMER Campus, Puducherry, India and authenticated and deposited in TNAU (Tamil Nadu Agricultural University) Herbarium, Coimbatore, India with voucher No.: BSI/SRC/5/23/2012-13/Tech.368.

Extraction

The leaves and root of *T. purpurea* plant material were washed, shade dried and powdered separately. One hundred gram of each part of the plant materials were extracted with methanol using soxhlet apparatus. Both extracts were concentrated to a paste and stored at 4° C until use.

Chemicals

Plastic wares (Tarson), MTT (Sigma), sterile dimethyl sulfoxide (DMSO) (Merck), RPMI 1640 medium (Sigma), fetal bovine serum (FBS) (Sigma), trypsin (Sigma), penicillin and streptomycin (Himedia), acridine orange and ethidium bromide (Sigma), Hoechst (Sigma), JC-1 (Sigma) and caspase-3 assay kit (Raybiotech) were purchased for this study.

Cell culture condition

HepG2 human hepatocellular carcinoma cells were obtained from the National Center for Cell Science (NCCS), Pune, India and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 µg/mL streptomycin and 100 U/mL penicillin. The HepG2 cells were stored at 37°C and supplemented with 5% CO_2 in a humidified incubator. Once these cells were grown upto 70% confluence, the cells were passaged.

Cell viability assay

Cytotoxic property of *T. purpurea* extracts was evaluated in hepatocellular carcinoma cells (HepG2) using MTT [3-4, 5-dimethylthiazole-2-yl, 2,5-diphenyl tetrazolium bromide] assay.[18] MTT is a standard colorimetric assay to evaluate the percentage of cell death. Approximately 5,000 cells were seeded in 96 well plates and incubated for one day at 37°C in CO_2 incubator. On the following day, media was refreshed and different concentrations of leaves (25-250 µg/mL) and root (50-500 µg/mL) extracts were treated in HepG2 cells and maintained for 24 h. Then, 20µl of MTT (5 mg/mL) was added to each well and incubated for 4 h. After incubation, media was removed, and formazon crystals were dissolved by adding 100 µl of DMSO. Plates were mixed properly for 5 min and absorbance was measured at 570 nm. Data were collected from triplicates of each extract and the mean with the standard deviation were used for analysis.

The percentage of HepG2 cell death was calculated using the following formula:

Percentage of cell death= {(Mean OD of control cells-mean OD of treated cells)/Mean OD of control cells} × 100

The concentration of extracts required to inhibit 50% of the cancer cell growth is termed as IC_{50} value.

Acridine orange and ethidium bromide staining

Acridine orange (AO) and ethidium bromide (EB) dual staining was performed to evaluate the morphological changes of cells due to apoptosis or necrosis. HepG2 cells were seeded in six-well plates, and IC_{50} concentration of methanolic leaves and root extracts were incubated for 24 h and 48 h. After the incubation period, the cells were harvested by trypsinization, washed and resuspended in cold Phosphate Buffered Saline (PBS). The cell suspension was stained with AO/EB (0.1 mg/mL) and a drop of cell suspension was placed on a glass slide and covered with a coverslip. At random 300 cells in triplicate were observed in each sample under a fluorescent microscope (Carl Zeiss, Jena, Germany) using a UV filter (450 - 490 nm). The percentage of live cells and dead cells showing morphological changes such as apoptosis or necrosis were analysed from nuclear and cytoplasmic structural changes.

Hoechst 33258 staining

Hoechst staining was used to examine the nuclear morphological changes of cancer cells.[19] Like AO/EB staining, after treatment with IC_{50} of methanolic leaves and root extracts for 24 h and 48 h, the cells were harvested and stained with Hoechst 33258 (1 mg/mL in PBS) and incubated for 5 min at room temperature. Nearly 300 cells in triplicate were observed under a fluorescence microscope at 400 × magnification (377-355 nm) and the percentage of cells reflecting nuclear morphological changes were analysed and calculated.

Mitochondrial transmembrane potential

Loss of mitochondrial membrane potential ($\Delta\Psi m$) is an early indicator of apoptosis, and it was detected by lipophilic, cationic dye JC-1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethyl-imidacarbocyanine iodide).[20] The cells were grown on glass coverslips (22 × 22 cm) placed in 6-well plates and the IC_{50} concentration of methanolic leaves and root extracts of *T. purpurea* were treated and incubated for 24 h and 48 h. After the incubation period, the cells were stained for 10 min with the JC-1 dye that selectively enters mitochondria. Here, live cells appear as orange-red and apoptotic cells appear as green based on the extent of loss of mitochondrial membrane potential. The mitochondrial depolarization of cells was observed under fluorescence microscope.

Measurement of caspase-3

T. purpurea extracts induced caspase-3 activity in HepG2 cells was measured by caspase-3 colorimetric assay kits, according to the manufacturer protocol (Ray Biotech Caspase-3 Colorimetric Assay Kit). HepG2 cells were treated with the IC_{50} concentrations of methanolic leaves and root extracts and incubated for 24 h and 48 h. Control and treated cells were pelleted and lysed in 50 µl of chilled lysis buffer and incubated on ice for 10 min. Cells were centrifuged for 1 min at 10,000 x g and the supernatant was transferred to a fresh tube. Protein concentration was estimated by the method of Lowry *et al*. Later, 50 µg of protein in 50 µl of lysis buffer was added to a 96-well plate and mixed with 50 µl of 2X reaction buffer (10 mM DTT). Then 5 µl of 4mM DEVD-pNA substrate for caspase-3 was added and incubated at 37°C for 2 h in the dark and caspase-3 expression was measured at 405 nm.

Statistical analysis

All the results were expressed as mean ± standard deviation (SD) of three independent experiments. The difference between groups was analysed by one-way analysis of variance (ANOVA) followed by Bonferroni

using GraphPad Prism-6.0 (Graphpad Software Inc, USA). $P< 0.05$ was considered statistically significant.

RESULTS

Cytotoxic effects of the *T. purpurea* extracts in HepG2 cells

The leaves and root extracts showed a potent cell growth inhibitory effect in a dose-dependent manner, the IC_{50} (inhibition concentration 50%) values being 102.33 ± 10.26 µg/mL and 276.67 ± 20.43 µg/mL for 24 h respectively [Figure 1A and 1B].

Apoptotic and necrotic effects of the *T. purpurea* extracts in HepG2 cells

Methanolic leaves and root extracts of *T. purpurea* treated HepG2 cells were subjected to AO/EB staining. Usually AO will enter the nucleus and stain live cells as green colour and EB will penetrate the nucleus of dead cells due to loss of membrane integrity and stain as red colour. In this experiment, viable cells appeared as green fluorescence with highly organized nuclei. Early apoptotic cells appeared as green-orange colour nuclei with condensed or fragmented chromatin and late apoptotic cells appeared as orange to red colour with highly condensed or fragmented chromatin and apoptotic bodies. Necrotic cells fluoresced orange to red without chromatin fragmentation. The IC_{50} values of methanolic leaves and root extracts treated cells showed typical apoptotic morphological features such as condensed nuclei, membrane blebbing and formation of apoptotic bodies in a time-dependent manner, which were clearly observed under the fluorescence microscope and quantitated [Figure 2A, 2B and 2C].

Apoptotic nuclear morphological changes in HepG2 cells by the *T. purpurea* extracts

Apoptotic nuclear morphological changes in HepG2 cells by treatment of methanolic leaves and root extracts were evaluated by Hoechst 33258 staining. The IC_{50} values of leaves and root extracts treated HepG2 cells appeared as bright blue with apoptotic nuclear morphological changes at 24 h and 48 h. These changes include chromatin marginalization, DNA condensation and fragmentation and formation of apoptotic bodies. In untreated cells, there were no morphological changes; nuclei fluoresced as faint blue which was homogenous [Figure 3A, 3B and 3C].

Mitochondrial transmembrane depolarization by the *T. purpurea* extracts

The effect of extracts-induced depolarization of mitochondrial transmembrane potential was examined using JC-1 dye. Control cells emitted red fluorescence indicating intact mitochondrial membrane potential. However, both extracts treated HepG2 cells expressed progressive and complete loss of red to green fluorescence at 24 h and 48 h respectively, due to mitochondrial transmembrane depolarization. *T. purpurea* extracts disturbed mitochondrial membrane potential and induced apoptosis in a time-dependent manner [Figure 4].

Effects of leaves and root extract on caspase-3

T. purpurea extracts-triggered apoptosis-mediated death of HepG2 cells for 24 h and 48 h was analysed by caspase-3 assay. As shown in Figure 5, the leaves and root extracts significantly ($p < 0.05$) induced caspase-3 expression when compared to control in a time-dependent manner.

DISCUSSION

In the present study, we have investigated the *T. purpurea* extracts induced apoptosis-mediated cell death in HepG2 cells. Both leaves and root extracts of *T. purpurea* induced cytotoxicity in HepG2 cells. However, leaves extract exhibited more anti-proliferative activity as compared to root extract. Gulecha *et al.* also reported the *in vitro* anticancer activity of fractions of *T. purpurea* leaves on MCF7 cancer cell lines.[17] Despite the cytotoxic activity of *T. purpurea*, the mechanism of cancer cell growth inhibitory effect was not yet explored. Therefore, we have investigated that the *T. purpurea* induced apoptosis-mediated cell death in HepG2 cells.

Apoptosis maintains the balance between cell death and renewal; thereby it eliminates excess, damaged and abnormal cells from the body. The activation of apoptosis results in the formation of apoptotic

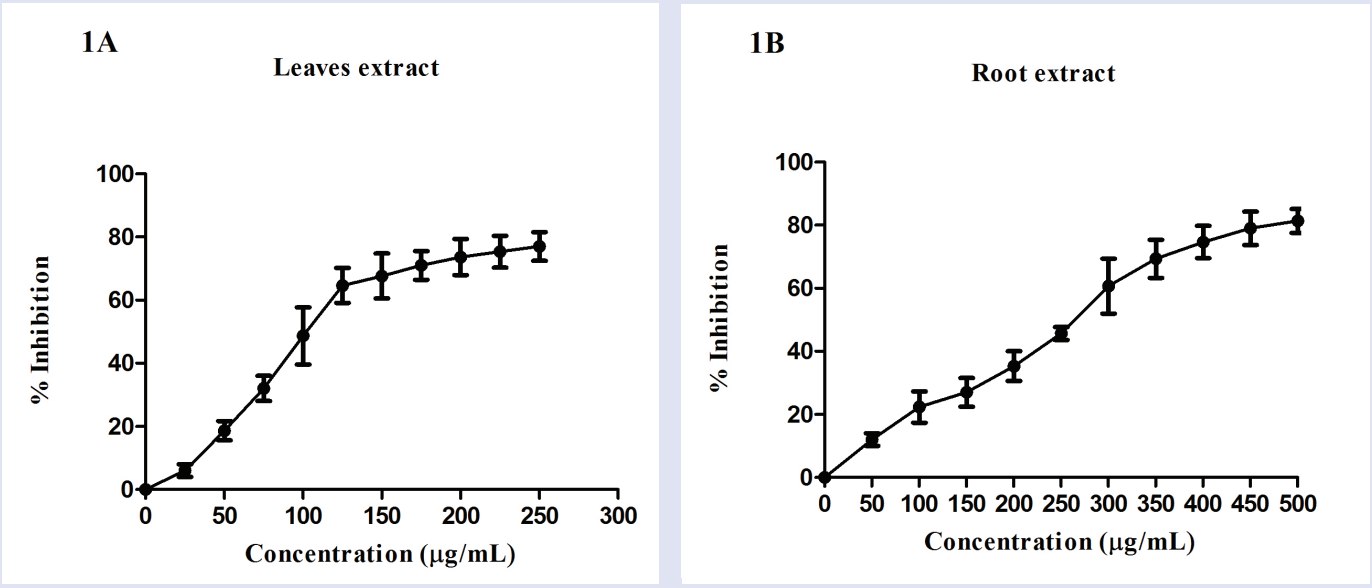


Figure 1: Cytotoxic effect of *T. purpurea* extracts in HepG2 cells after exposure for 24 h. Percentage of inhibition of cell proliferation was determined by MTT assay. Values were expressed as mean ± SD of three independent experiments (n=3). Untreated cells were used as control for comparison.

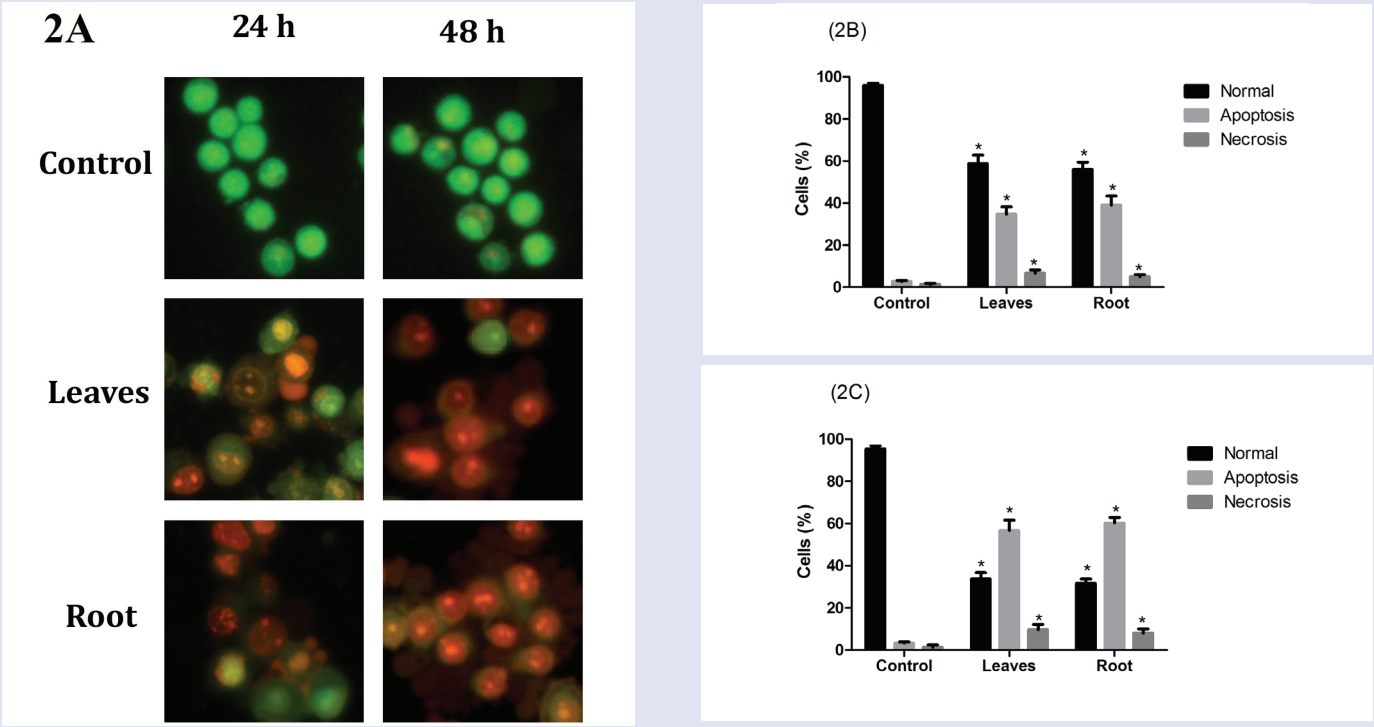


Figure 2: Morphological assessment of apoptosis and necrosis in HepG2 cells stained with acridine orange and ethidium bromide (AO/EB). (2A) The leaves and root and extracts (IC_{50} concentration) induced apoptotic morphological features in HepG2 cells after 24 h and 48 h treatment. [Figure (2B) and (2C)] depict the percentage of normal, apoptotic and necrotic cells after treatment with leaves and root extracts (IC_{50} concentration) at 24 h and 48 h respectively. The values were expressed as mean ± SD of three independent experiments (n = 3). Untreated cells were used as control for comparison. Statistical comparison was made using one-way ANOVA followed by Bonferroni (* denotes $p < 0.05$). Error bar represents standard deviation in triplicates.

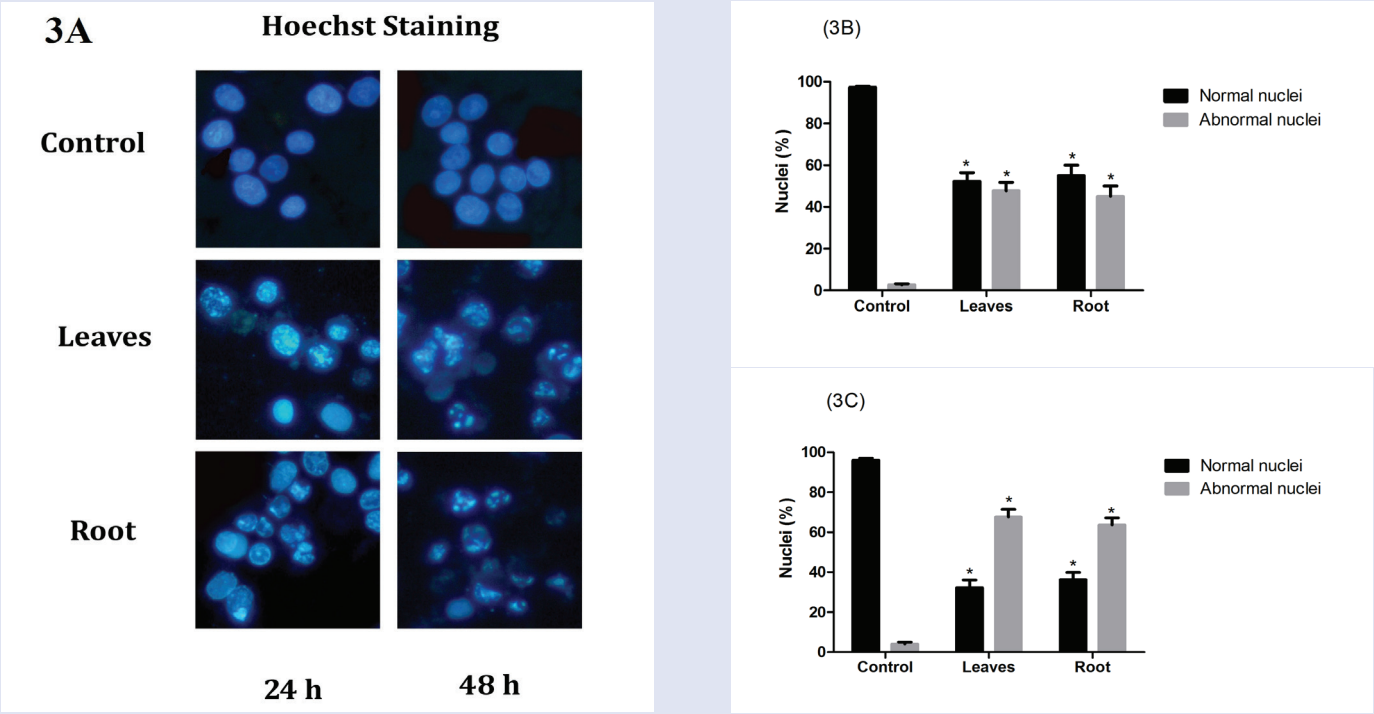


Figure 3: Assessment of nuclear morphological features in HepG2 cells stained with Hoechst. (3A) The leaves and root extracts (IC_{50} concentration) induced nuclear morphological changes in HepG2 cells after 24 h and 48 h treatment. [Figure (3B) and (3C)] depict the percentage of cells with normal and abnormal nuclei after treatment with leaves and root extracts (IC_{50} concentration) at 24 h and 48 h respectively. The values were expressed as mean ± SD of three independent experiments (n = 3). Untreated cells were used as a control for comparison. Statistical comparison was made using one-way ANOVA followed by Bonferroni (* denotes $p < 0.05$). Error bar represents standard deviation in triplicates.

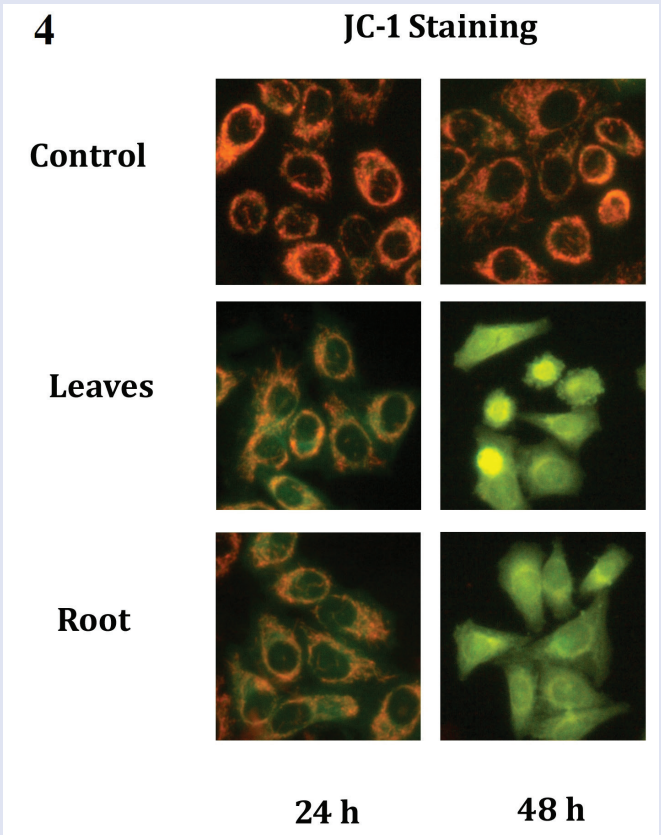


Figure 4: Effect of leaves and root extracts (IC_{50} concentration) induced loss of mitochondrial transmembrane potential in HepG2 cells at 24 h and 48 h as assessed by JC1 staining. Untreated cells were used as control for comparison.

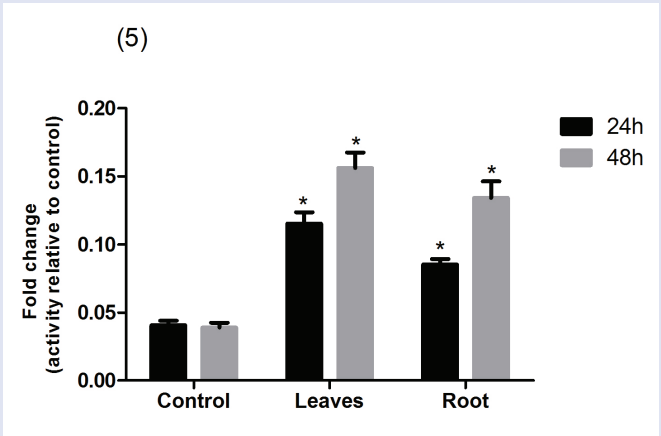


Figure 5: The caspase-3 activity in HepG2 cells treated with the leaves and root extracts (IC_{50} concentration) for 24 h and 48 h (n = 3) as determined by colorimetric assay. Untreated cells were used as control for comparison. Statistical comparison was made using one-way ANOVA followed by Bonferroni (* denotes $p < 0.05$). Error bar represents standard deviation in triplicates.

bodies. These apoptotic bodies do not release their cellular contents into the surrounding tissue, but they are phagocytised by macrophages and surrounding cells. Accordingly, it prevents secondary necrosis and inflammation and the other main purpose of apoptosis is the prevention of cancer.[21] Therefore, activation of apoptosis in cancer cells could be an effective strategy for cancer treatment. Various studies have reported that many plant extracts and plant-derived compounds induce cancer cell death through apoptosis without a significant amount of side effects.[22,23]

The leaves and root extracts of *T. purpurea* induce apoptotic morphological changes in HepG2 cells such as nuclear shrinkage, DNA condensation, fragmentation, membrane blebbing and formation of apoptotic bodies as evidenced by the results of AO/EB and Hoechst staining. The JC1 staining result also suggested that the extracts of *T. purpurea* triggered apoptosis-mediated cell death in HepG2 cells. Mitochondrial swelling is often associated with loss of transmembrane potential and it is considered as an important phenomenon, which occurs as an early event of apoptosis. Loss of mitochondrial membrane potential followed by the release of cytochrome C from mitochondria to the cytosol leads to subsequent activation of caspases resulting in apoptosis.[24] In the present study, the loss of mitochondrial membrane potential was observed in extracts-treated cells and analysed using JC1 dye. These staining results suggest that the extracts of *T. purpurea* triggered apoptosis-mediated cell death in HepG2.

To confirm this apoptosis induction by *T. purpurea* extracts in HepG2 cells, activation of caspase-3 was assessed using the colorimetric method. Caspases are a family of cysteine proteases, and they are strongly considered as important mediators of apoptosis. Caspase-3 activation is positively associated with induction of apoptosis, and it is a downstream enzyme of both intrinsic and extrinsic apoptotic pathways.[25] This colorimetric assay revealed overexpression of caspase-3 in the leaves and root extracts treated samples when compared to control. Previous studies reported that several plant extracts and active compounds such as *Tulbaghia violacea*, *Costus speciosus*, *epigallocatechin-3-gallate (EGCG)* and *silybin* induced caspase-3 mediated apoptotic cell death on HCC.[26-29] The results obtained from the current study also confirmed that the *T. purpurea* extracts have the ability to cause apoptosis-mediated cell death in HepG2 cells through caspase-3.

CONCLUSION

This study concludes that the methanolic leaves and root extracts of *T. purpurea* exert anti-cancer activity in hepatocellular carcinoma cells, and the activity is mediated through apoptosis by induction of caspase-3. In the light of our findings, it may be prudent to isolate the active compounds of the plant and demonstrate their molecular mechanism of anti-cancer activity.

Financial support and sponsorship

The authors thank JIPMER intramural research grant for financial support and MGDC, Bharathidasan University for providing cell culture lab facility.

Conflicts of Interest

There are no conflicts of interest.

REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011;61:69-90.
2. Ryder SD. Guidelines for the diagnosis and treatment of hepatocellular carcinoma (HCC) in adults. Gut 2003;52:1-8.
3. Sanyal AJ, Yoon SK, Lencioni R. The Etiology of Hepatocellular Carcinoma and Consequences for Treatment. Oncologist 2010;15:14-22.
4. El-Serag HB, Rudolph KL. Hepatocellular Carcinoma: Epidemiology and Molecular Carcinogenesis. Gastroenterology 2007;132:2557-76.
5. Osaki Y, Nishikawa H. Treatment for hepatocellular carcinoma in Japan over the last three decades: Our experience and published work review: HCC therapy for three decades. Hepatol Res 2015;45:59-74.

6. Altekruse SF, McGlynn KA, Reichman ME. Hepatocellular Carcinoma Incidence, Mortality, and Survival Trends in the United States From 1975 to 2005. J Clin Oncol 2009;27: 1485-91.
7. Hollebecque A, Malka D, Ferté C, Ducreux M, Boige V. Systemic treatment of advanced hepatocellular carcinoma: From disillusions to new horizons. Eur J Cancer 2015;51: 327-39.
8. Blanchet B, Billemont B, Barete S, Garrigue H, Cabanes L, Coriat R, *et al*. Toxicity of sorafenib: clinical and molecular aspects. Expert Opin Drug Saf 2010;9:275-87.
9. Mukherjee AK, Basu S, Sarkar N, Ghosh AC. Advances in cancer therapy with plant based natural products. Curr Med Chem 2001;8:1467-86.
10. Cragg GM, Newman DJ. Plants as a source of anti-cancer agents. J Ethnopharmacol 2005;100:72-9.
11. Gokhale SB. Textbook of Pharmacognosy. Nirali Prakashan Pune, 1979.
12. Kirtikar KR, Basu BD. Indian Medicinal Plants. 2nd ed. Allahabad: Lalit Mohan Basu; 1956.
13. Chinniah A, Mohapatra S, Goswami S, Mahapatra A, Kar SK, Mallavadhani UV, *et al*. On the potential of *Tephrosia purpurea* as anti-Helicobacter pylori agent. J Ethnopharmacol 2009;124:642-5.
14. Shenoy S, Shwetha K, Prabhu K, Maradi R, Bairy K, Shanbhag T. Evaluation of antiinflammatory activity of *Tephrosia purpurea* in rats. Asian Pac J Trop Med 2010;3:193-5.
15. Juma WP, Akala HM, Eyase FL, Muiva LM, Heydenreich M, Okalebo FA, *et al*. Terpurinflavone: An antiplasmodial flavone from the stem of *Tephrosia Purpurea*. Phytochem Lett 2011;4: 176-8.
16. Pavana P, Sethupathy S, Santha K, Manoharan S. Effects of *Tephrosia purpurea* aqueous seed extract on blood glucose and antioxidant enzyme activities in *streptozotocin* induced diabetic rats. Afr J Trad CAM 2009;6:78-86.
17. Gulecha V, Sivakuma T. Anticancer activity of *Tephrosia purpurea* and *Ficus religiosa* using MCF 7 cell lines. Asian Pac J Trop Med 2011;4:526-9.
18. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55-63.
19. Latt SA, Stetten G, Juergens LA, Willard HF, Scher CD. Recent developments in the detection of deoxyribonucleic acid synthesis by 33258 Hoechst fluorescence. J Histochem Cytochem 1975;23:493-505.
20. Reers M, Smith TW, Chen LB. J-aggregate formation of a carbocyanine as a quantitative fluorescent indicator of membrane potential. Biochemistry 1991;30:4480-6.
21. Elmore S. Apoptosis: A Review of Programmed Cell Death. Toxicol Pathol 2007;35:495-516.
22. Desai AG, Qazi GN, Ganju RK, El-Tamer M, Singh J, Saxena AK, *et al*. Medicinal Plants and Cancer Chemoprevention. Curr Drug Metab 2008;9:581-91.
23. Li Z-F, Wang Z-D, Ji Y-Y, Zhang S, Huang C, Li J. *et al*. Induction of apoptosis and cell cycle arrest in human HCC MHCC97H cells with Chrysanthemum indicum extract. World J Gastroenterol WJG 2009;15:4538-46.
24. Bras M, Queenan B, Susin SA. Programmed cell death via mitochondria: different modes of dying. Biochemistry (Mosc) 2005;70:231-9.
25. Porter AG, Jänicke RU, Emerging roles of caspase-3 in apoptosis. Cell Death Differ 1999;6:99-104.
26. Saibu GM, Katerere DR, Rees DJG, Meyer M. *In vitro* cytotoxic and pro-apoptotic effects of water extracts of Tulbaghia violacea leaves and bulbs. J Ethnopharmacol 2015;164:203-9.
27. Nair SVG, Hettihewa M, Rupasinghe HPV. Apoptotic and Inhibitory Effects on Cell Proliferation of Hepatocellular Carcinoma HepG2 Cells by Methanol Leaf Extract of Costus speciosus. Bio Med Res Int 2014 2014;637098.
28. Zhang Y, Duan W, Owusu L, Wu D, Xin Y. Epigallocatechin-3-gallate induces the apoptosis of hepatocellular carcinoma LM6 cells but not non-cancerous liver cells. Int J Mol Med 2015;35:117-24.
29. Zhang S, Yang Y, Liang Z, Duan W, Yang J, Yan J, *et al*. Silybin-Mediated Inhibition of Notch Signaling Exerts Antitumor Activity in Human Hepatocellular Carcinoma Cells. PLoS ONE 2013;8:e83699.