Original Article

Scientific Validation Of Antihepatocellular Activity Of Terminalia Bellarica Seed Extracts Through In Vitro Cytotoxicity And Anticancer Activity Of HEPG2 Cells

G.Vishnu priya[1],L.Arul Amutha Elizabeth[2*],Tanuja Lella [3],Brigida S .[4]

[1,]Assistant professor, Department of Pharmacology, Sree Balaji Medical College and Hospital, Chromepet, India.
[2,]Professor and HOD, Department of Pharmacology, Sree Balaji Medical College and Hospital, Chromepet, India.
[3,]Assistant professor, Department of Pharmacology, Sree Balaji Medical College and Hospital, Chromepet, India.
[4,]Assistant professor, Department of Pharmacology, Sree Balaji Medical College and Hospital, Chromepet, India.
Corresponding Authors
Dr.Arul Amutha Elizabeth
DOI: 10.47750/pnr.2023.14.04.34

Abstract

Terminalia bellirica (TB) is noted for its medicinal values and associated with many siddha, ayurvedic and unani preparations. It is commonly known as bahera or beleric and seemed to be a common plant on plains and hill areas especially, in Southeast Asia. The present study aimed to compare its cytotoxicity and anti carcinogenic activity of the seed extracts using Vero and HepG-2 (hepatoma) cell lines by MTT method.

T. bellerica seed was extracted with different solvents viz., methanol, ethylacetate, chloroform and aqueous. Among them, aqueous and chloroform extract activity was not confirmed with dose dependency on both of the cell lines, but, methanolic and ethylacetate extracts showed concentration dependent activities. Further, they were analysed on different concentration levels. The ethylacetate and methanolic extract showed IC^{50} value as 20.11± 0.30, and 61.9 ± 0.30µg/ml respectively on HepG2 cells. Their activity was statistically significant and confirmed by evaluation of Hill coefficient method. The treated cells revealed the cell shrinkage and decrease in viable cells than the counterparts in controls. Further the findings of the study revealed that TB extract markedly possessed anticancer and anti-proliferative effects against hepatocellular carcinoma.

Keywords: Terminalia bellirica; anticancer; medicinal plants ; HepG2 cell line.

INTRODUCTION:

Hepatocellular carcinoma (HCC) is sixth most common cause of cancer worldwide and ranks second in cancer related deaths.[1] HCC accounted for approximately 11.75% of all the gastric cancers and liver cancer. In India it is accounting for mortality in 0.7-7.5% of men and 0.2-2.2% of women patients suffering from cancers.[2] Apoptosis inhibition is one of the major causes for its recurrence and chemotherapeutic failure. In tumor microenvironment, due to the irregularity in cancer cells signalling the normal stromal cells highly translated to be cancerous in nature.[3] In HCC commonly used therapeutic options are transplantation of liver, radiofrequency ablation, chemotherapy etc. Though, the drug candidates including sorafenib are efficiently able to block cell divisions in HCC, however, short-term survival, narrow fatality and other immunological consequences (emotional suffering, weakness, ache and anaemia, beside their high cost) make an urgent need for searching the alternatives to those drugs.[5] apoptosis is prospective target for some Chemotherapy. intracellular damage can be caused by drugs, which leads to downstream molecular events.[6] These plant products have different mechanisms of action the growth of the cell inhibition and beginning of apoptosis.[10] The modern era usually try to find out the optimal concentration and useful part of the plants that exhibit an excellent therapeutic value. Hence, phytochemistry and pharmacognosy have become the primary focus of recent research. Naturopathy is considered as an alternative medicine for treatment of cancer.[7] since ancient time plant products had been used traditionally for curing diseases

in various parts of the world.[8] As they possess anticancer property against various types of cancers these have been utilized worldwide for the treatment of various malignant conditions.[9,10]

Plants have been an add-on source of drugs against diseases since olden days[11.] plants with Polyphenol property have various uses and applications. They have been proven to reduce the risk of cardiovascular diseases, enhance liver regeneration, and increase life expectancy[12]. Hepatic damage is fatal and caused due to genetics, infections, alcohol, chemicals etc. Treatment can be done using plants with antioxidant and hepatoprotective properties. Terminalia from the family Combretaceae has 200 tropical trees and shrubs that are found all over the world, used in management of diarrhea, skin rashes, cancer, inflammation, and different bacterial infections[13,14]. Terminalia bellirica has hepatoprotective[15] and anti-hypercholesterolemia activities[16,17]. T.bellerica seeds consist of phytoconstituents like Glucoside (bellericanin), Gallotannic acid, Coloring matter, resins and a greenish yellow oil. Ellargic acid, gallic acid, lignans (termilignan and thannilignan), 7-hydroxy 3'4' (methylene dioxy) flavone and anolignan B10. Tannins, ellargic acid, ethyl gallate, galloyl glucose [18].
Terminalia belericais an ingredient of 'triphala' used in the treatment of digestion and liver disorders. It has been used in the ancient system of healing and in Ayurveda. Terminalia belericais also known as Vibhitaki in Sanskrit, meaning fearless. It is atoxic and valued for its effectiveness. The plant's fruit has been used in multiple diseases in ancient medicine. Compared to other uses, its significant use is in the management of liver disorders. The seed is also reported to have purgative, cardiac depressant, hypotensive and choleretic effects. It decreases lipid level from the liver and heart, which reduces the risk of disease of those organs.

However, the efficacy of the TB extract against Hepatocellular carcinoma is yet to be analyzed. This study provides a link between the TB extract induced apoptosis and autophagy in relation to reactive oxygen species (ROS).Therefore, the aim of this study was to assess the anticancer effect of T. bellaricia on HepG2 cells, and to outline the elemental mechanism of T. bellaricia-induced apoptosis in HepG2 cells. variation of structural properties and arrangements.[19,20,21]

MATERIALS AND METHODS:

Plant Collection methods:

T. bellirica seed was purchased from the market and certified by botanists at plant Anatomy Research Centre, West Tambaram. 100gms of T. bellirica seed pulverised coarsely using a blender and dried at 40 °C using oven for 24 h

Different Solvent Extracts Preparation:

Extraction of T.bellirica seed extract (10g) was done for 5 hours using soxhlet apparatus with the solvents such as ethanol, acetone, chloroform and water (200ml each). The boiling point was maintained at 20. The extract was collected was filtered using a syringe filter. Buchi rota vapor was used to remove the solvent by evaporation at 45 °C with 5 bars to get a constant mass (1g). The crude extract was then stored at 4 °C for further use [21].

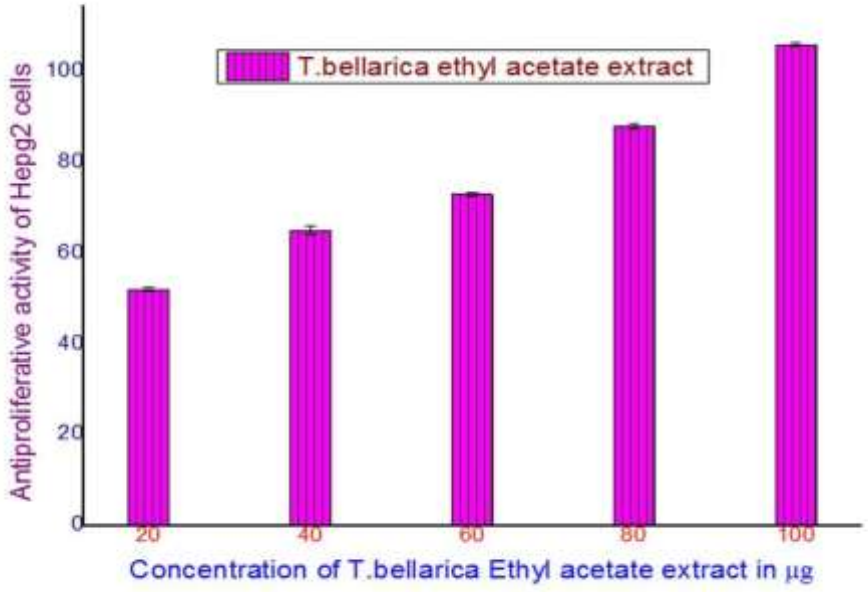
Determination of Phytochemical Constituents:

The phytochemical assays were carried out using standard phytochemical tests [22] to confirm secondary metabolites such as flavonoids, polyphenols, alkaloids, terpenoids, steroids, tannins, saponins, and glycosides are present.

In-vitro anticancer activity and cytotoxicity

Anticancer activity and cell viability of the T. bellirica seed extract was analysed using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay (MTT) illustrated by Shanthi et al.,(2017). The human liver cancer cell lines HepG2 and normal Vero cell lines were obtained from NCCS (Pune, India) and used in this study. The cells were cultured in MEM, at pH 7.4 supplemented with 10% Fetal calf serum, glutamine (2mM), Penicillin (100 units/ml), and streptomycin (100 mg/mL). Both HepG2 and normal Vero cells were seeded in 96 well plates at a density of $1x10^4$ cells. After attaining the confluence the cells were treated with various drug concentrations

(5-60µg/ml) and incubated at 24 and 48hours. Then the cells were incubated with 100µL (0.5mg/mL) of MTT at 37ºC for 3hours after the treatment period. The formazan crystals were dissolved by adding 100µL of DMSO. The intensity was measured using a multiplate reader at 570nm [21, 22] .



RESULTS:

The anticancer activity of T. bellarica seed extract derived from different solvent systems such as aqueous, methanolic, chloroform and ethyl acetate was evaluated for its cytotoxicity and anti-cancer activity using Vero and HepG2 cell lines by adopting standard methods. The concentration was ranging from 0.05 to 1 mg. Ethyl acetate extract showed highest toxicity in Vero cells in concentration dependent manner. Its cytotoxicity was seemed to be increased with concentration as followed 50%, 90% and 100% at 0.05, 0.1 and 1 mg respectively. On 1mg, there were no cells on the microscopic examination and the monolayer was found to be totally damaged, In addition to, methanolic extract also showed cytotoxicity as followed 42%, 71% and 100% on 0.05mg, 0.1mg and 1mg respectively. Similar to, ethylacetate extract, there was no monolayer on methanolic extract treatment also. Interestingly, chloroform and aqueous extract of the seeds showed relatively lowest cytotoxicity on both cell lines among the type of extracts (Table-1). Though the cytotoxicity was lower, we could not found any dose dependent activity on those two extracts. Hence those two solvent extracts were not selected for further analysis. Only, ethylacetate and methanolic extracts were studied further for their anticancer activity on liver cancer cell line Hep-2.

T. bellarica extracts	**Cytotoxicity of T. bellarica extracts (mg)**		
Concentration of Extracts	**0.05**	**0.1**	**1**
Ethyl acetate	50%	90%	No cells
Chloroform	16%	36%	67%
Methanol	42%	71%	No cells
Aqueous	11%	28%	55%

Table-1: Cytotoxicity of Terminalia bellarica seed extracts on Vero cell line

Figure. 1. Anticancer activity of T. bellaricaseed Ethyl acetate extract in Hepg2 Cell line

The ethylacetate extract of T. bellarica seed extract revealed dose dependent anticancer activity from 10 to 100 µg in liver cancer cell line Hepg2 (Figure-1). On 10 µg, it showed 43±0.83% cytotoxicity followed by 20, 40, 60, 80, and 100µg as 52±0.42%, 65±0.96%, 73±0.48%, 88±0.58% and 96±0.42% respectively with IC_{50} as of 20.11± 0.30µg/ ml. The statistical analysis showed that the results were statistically significant (Hill coefficient of 14.23±3.87, Fig. 2).

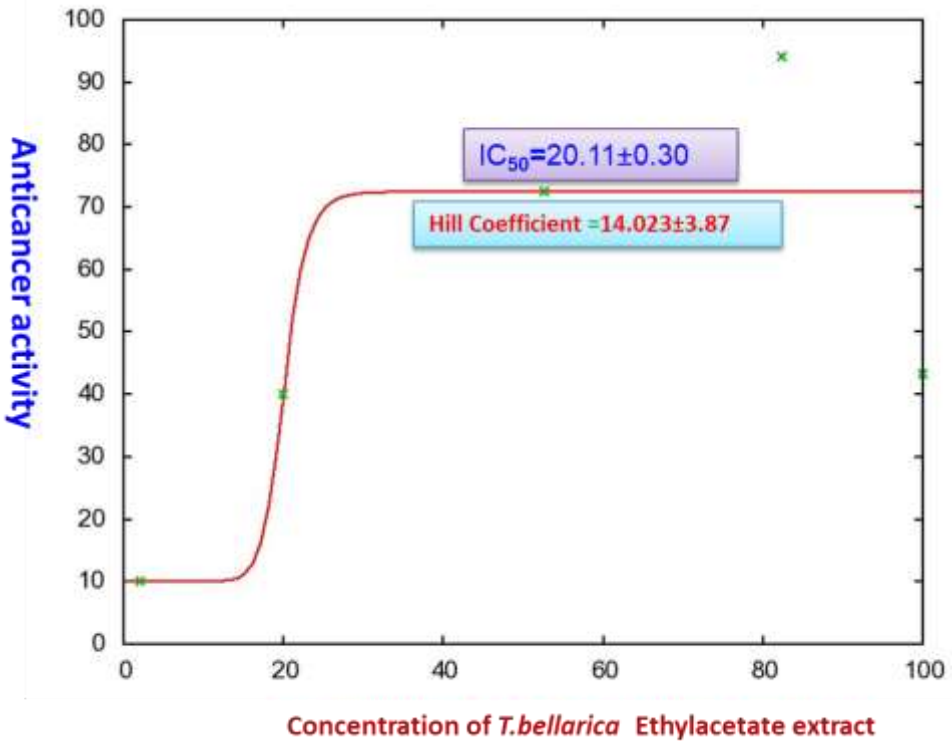


Fig-2. Graphical representation of the Inhibitory Concentration (IC_{50}) T. bellarica ethylacetate seed extract in Hepg2 cell line

The anticancer property of the methanolic extract of T. bellarica was shown in Figure. 3. Different concentration of the extract were used for the study. The concentrations ranged from 10 – 100µg/ ml and its anticancer activity was dose dependent. The activity was found to increase with the concentration as 23±0.63% was at 10µg/ ml followed by 20, 40, 6, 80 and 100µg/ ml as 32±0.12%, 41±0.36%, 53±0.28%, 68±0.18% and 91±0.62% respectively on HepG2 cell lines. It showed IC_{50} as 61.9 ± 0.30µg/ ml against HepG2 cells. The statistical analysis revealed that it was statistically significant with the Hill coefficient of 2.25±2.17 (Figure. 4).

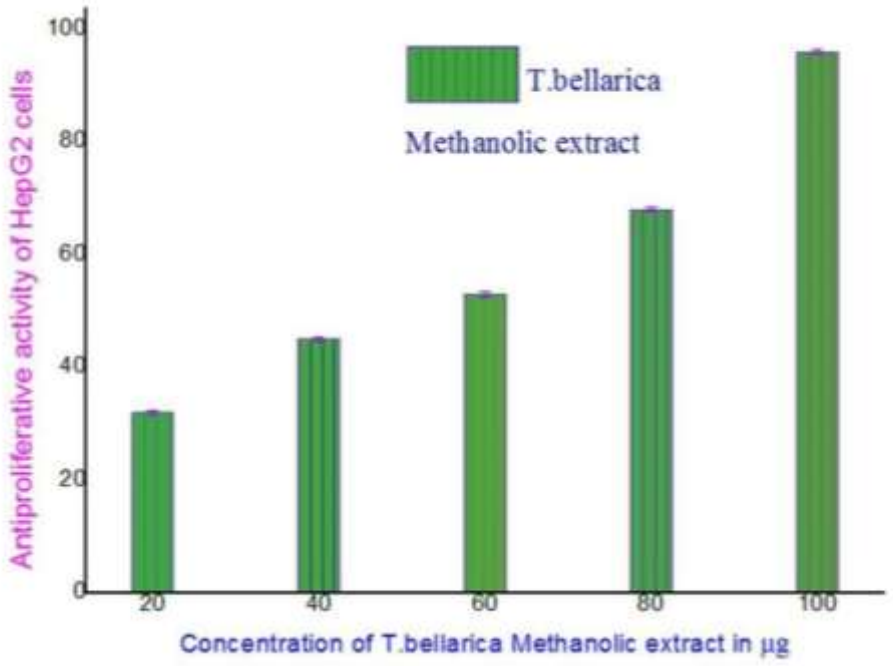


Fig-3:Anticancer activity of Terminalia bellarica seed methanolic extract in Hepg2 Cell line

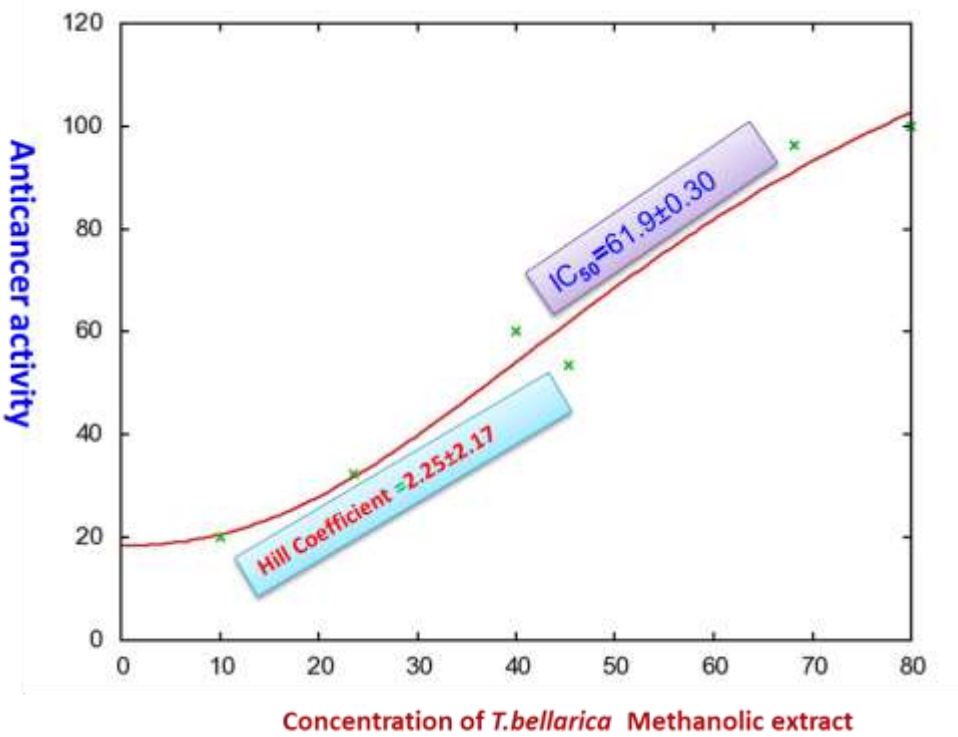


Fig-4:Inhibitory Concentration (IC_{50}) of T. bellaricaseed Methanolic extract in Hepg2 cell line

Further, the cell viability and the cell confluency was analysed in the extract treated cells. The effect of T. bellarica seed ethylacetate and methanolic extract was found to make prominent changes in the cellular morphology

(Figure-5) of HepG2 cells. The reduction of the confluence was evident on the cells treated with ethyacetate (5b) and methanolic extract (5c) compared to the control. In control, the cells were seem to be oblong in nature reflecting their survival fitness; the cells in extract treatment were round in nature indicating their toxic nature of the compounds present in the extract.

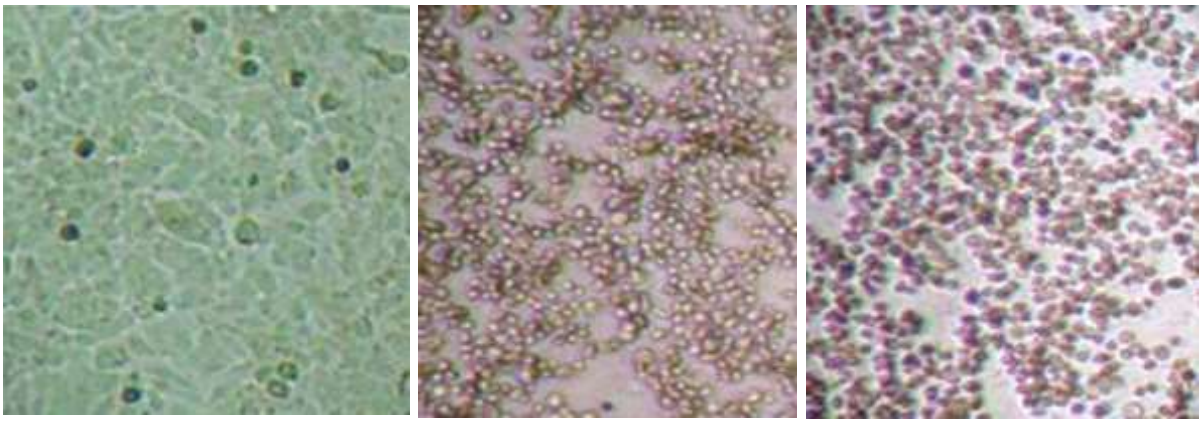


Fig-5: Anticancer activity of T. bellaricaEthyl acetate and Methanolic seed extract in Hepg2 Cell line
a. normal HepG2 cell line, b-Methanolic extract in HepG2 cell line , C- Ethyl acetate extract in HepG2 cell line.

DISCUSSION

Recently, there is an increased demand for safe nutraceutical drugs. Medicinal plants have been an important source to provide raw material to the pharmaceutical industry. plants belonging to the species of Terminalia have produced a number of pharmaceutical efficacious compounds that act on different diseases including many malignant tumours[12,23,16,17].

T. bellirica fruits are used for many medicinal purposes in traditional medicines as the preparations for internal and external applications. Particularly, the seed oil or the paste of its fruit is applied on the swollen and painful parts externally. Those paste preparations are applied as anti-conjunctivitis on the eye-lids also[5]. Those fruit extracts contain many phyto-constituents; such compounds are variably used for treating many chronic diseases as they are working as the multi targeted drug candidates[18,22]. In general, those compounds have the ability to scavenge the free radicals, therefore, they have been used as antibacterial,anti inflammatory and anticancer activities[4,6,10,22,23]. Further, its presence might enhance the anti-cancer activity of the chemotherapeutic agents on a number of haematological malignancies[6,24, 25].

The present study shows both the methanolic and ethyl acetate extract exhibited DPPH radical scavenging potential and anti cancer activity (data not shown). Such results pointed out that the methanol and ethyl acetate extracts of Terminalia bellrica, can be used against cancers, especially hepatocellular origin for their determined anti-oxidative nature[23-25]. Natural products act as a source of novel therapeutics. Various studies highlight that plant sources inhibit or halt the cell division in cancer types since they obstruct the vital hallmarks of cancers[23-25]. Few of them had been shown to have a remarkable inhibitory activity on HCC also. These results suggest that T.Bellrica seeds might be a novel agent for curing HCC.

This is the first study to the best of our knowledge T.bellrica seed extract having anti-apoptotic properties on the hepatomaG2 cell lines and also depicts the cytotoxic effect of T.bellrica. From the findings stated above, suggested that T.bellrica may be suitable for the treatment of human HCC.

REFERENCES:

1. Ferlay, J, Soerjomataram, I, Dikshit, R, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN. Int J Cancer 2015; 136: E359–E386.

2. Spach, DH, Kim, HN. Section 5. Treatment of chronic Hepatitis C infection. Topic 4. Treatment of HCV genotype 4. Hepatitis C Online, 2017.
3. Arıcan, GO, Çakır, O, Arıcan, E, et al. Effects of Geven root extract on proliferation of HeLa cells and BCL-2 gene expressions. Afr J Biotechnol 2014; 11: 4296–4304
4. Best, J, Schotten, C, Theysohn, JM, et al. Novel implications in the treatment of hepatocellular carcinoma. Ann Gastroenterol 2017; 30: 23–32
5. Karaman, B, Battal, B, Sari, S, et al. Hepatocellular carcinoma review: current treatment, and evidence-based medicine. World J Gastroenterol 2014; 20: 18059–18060.
6. Papadopoulos, EI, Yousef, GM, Scorilas, A. Cytotoxic activity of sunitinib and everolimus in Caki-1 renal cancer cells is accompanied by modulations in the expression of apoptosis-related microRNA clusters and BCL2 family genes. Biomed Pharmacother 2015; 70: 33–40.
7. Njamen, D, Zingue, S, Mvondo, MA, et al. The efficacy of some comestible natural products in treatment of cancer. AlternIntegr Med 2014; 3: 158.
8. Soladoye, MO, Amusa, NA, Raji-Esan, SO, et al. Ethnobotanical survey of anti-cancer plants in Ogun State, Nigeria. Ann Biol Res 2010; 1: 261–273
9. Cragg, GM, Newman, DJ. Plants as a source of anti-cancer agents. J Ethnopharmacol 2005; 100: 72–79.
10. Chahar, MK, Sharma, N, Dobhal, MP, et al. Flavonoids: a versatile source of anticancer drugs. Pharmacogn Rev 2011; 5: 1–12.
11. Dias DA, Urban S, Roessner U.A historical overview of natural products in drug discovery. Metabolites. 2012 Apr 16;2(2):303-36.
12. Van Wyk, B. and Wink. Phytomedicines, Herbal Drugs, and Poisons, Medicinal Plants of the World. Timber Press.
13. Pascal Bessongetal. In vitro activity of three selected South African medicinal plants against human immunodeficiency virus type 1 reverse transcriptase: African Journal of Biotechnology Vol. 3 (10), pp. 555-559, October 2004
14. Okatch,H.,Ngwenya,B.,Raletamo,K.M.,etal., :2012.Analytica.Chimica.Acta.730,42-48.
15. Jadon A, Bhadauria M, Shukla S. Protective effect of Terminalia belerica Roxb. and gallic acid against carbon tetrachloride induced damage in albino rats. J Ethnopharmacol. 2007 Jan 19;109(2):214-218.
16. Shaila, H.P., A.L. Udupa and S.L. Udupa, 1995. Preventive actions of terminaliabelerica in experimentally induced atherosclerosis. Int. J. Cardiol, 49: 101-106.
17. Mansour Sobeh et al. Isolation of Myricitrin and 3,5-di-O-Methyl Gossypetin from Syzygiumsamarangense and Evaluation of their Involvement in Protecting Keratinocytes against Oxidative Stress via Activation of the Nrf-2 Pathway. May 2019Molecules 24(9):1839.
18. Shashi B. Mahato, Ashish P.Kundu.13C NMR Spectra of pentacyclic triterpenoids—a compilation and some salient features. December 1994 Phytochemistry 37(6):1517-1575.
19. Hegazy, MG, Emam, MA. Ethanolic extract of TrigonellaFoenumGraecum attenuates cisplatin-induced nephro- and hepatotoxicities in rats. Cell MolBiol 2015; 61: 81–87.
20. El Shemy, HA, Aboul-Enein, AM, Aboul-Enein, KM, et al. Willow leaves' extracts contain anti-tumor agents effective against three cell types. PLoS ONE 2007; 2: e178.
21. MP Shanthi, G Bupesh, S Magesh, K Meenakurmari, K Saravanan, .In Vitro Anticancer Activity of Biophytum Sensitivum Whole Plant Extracts Against Cervical And Liver Cancer Cell Lines,International Journal of Pharmaceutical Sciences and Research 7 (12)2017, 5128.
22. G Lakshmanan, G Bupesh, A Vignesh, A Sathiyaseelan, K Murugesan, Micropropagation and anticancer activity of methanolic extract of Plumbago auriculata Lam, International Journal of Advanced Biotechnology and Research, 2016,7,4,2001-2011
23. L. Arul Amutha Elizabeth, G. Bupesh, In vitro antioxidant efficacy of Terminalia bellirica seed extract against free radicals. International Journal of Pharmaceutical Sciences and Research 8 (11), 4659-4665.
24. Shanthi, M. P., Bupesh, G., Magesh, S., Meenakurmari, K., Saravanan, K., & Muthiah, N. S. (2016). In vitro anticancer activity of Biophytum sensitivum whole plant extracts against cervical and liver cancer cell lines. International Journal of Pharmaceutical Sciences and Research, 7(12), 5128.
25. Kumar, A. G., Bupesh, G., Sankarganesh, P., Vasanth, S., Nandagopal, S., & Joseph, B. (2020). Inhibitory studies of HepG2 and human immune cells using Solanum nigrum. Bioinformation, 16(7), 525.