Research Article

Cytotoxic Activity and Apoptosis Induction of *Hypericum wightianum* Wall, Ex Wight & Arn

Balaperiasamy B[1]*, Thajuddin N[2], Panneer selvam A[1]
[1]PG & Reasearch department of Botany & Microbiology, AVVM Sri Pushpam College (Autonomous), Poondi, Thanjavur Dist. India.
[2]Depatment of Microbiology, School of Life Sciences, Bharathidasan university, Tiruchirapalli, 620 024, India.
***Corresponding author's E-mail:** drbaloo@rediffmail.com

Accepted on: 10-03-2016; **Finalized on:** 31-05-2016.

ABSTRACT

One of the acquired biological hallmarks of tumor multistep development is the resistance of cancer cells to apoptosis; therefore, induction of apoptosis is an important therapeutic approach. *Hypericum* species are spread throughout the world and have been investigated for their biological properties. Screening of the *Hypericum wightianum* previously proves its cytotoxciy against pathogenic bacteria and the phytochemical and antioxidant properties supports our observation. The objectives of the present study were to determine the most cytotoxic fraction of *Hypericum wightianum* and to assess the apoptosis induction ability of the methanol extract. Selected cell lines i.e. HT29, HEP G2, PNAC 1, A549 and VERO were tested using MTT assay. And the most effective one (A549) tested for its apoptotic induction ability. The methanol extract fraction of *Hypericum wightianum* revealed apoptosis induction ability. Considering the strong historical background about the therapeutic potential of the genus *Hypericum* and the considerable cytotoxic and apoptosis induction ability of *Hypericum wightianum*, suggested for future biological studies.

Keywords: *Hypericum wightianum*, cell lines, MTT assay, apoptosis.

INTRODUCTION

Cancer affects a lot of people all over the world[1] and in many cases cancer cells resist chemotherapy[2]. One of the acquired biological hallmarks during tumour multistep development is the development of cancer cells resistance against programmed cell death, known as apoptosis[3]. The essential roles of apoptosis during tumor progression and chemotherapy resistance have been well defined[4] and restoring apoptosis is an important therapeutic approach[5]. In other words, the apoptosis induction is important as the main mechanism of the cytotoxic effect of the cancer chemotherapeutic agents[6]. There are many common and distinctive natural products known as chemotherapeutic agents against cancer cells[7]. The plants kingdom plays a special role in such performance by introducing the taxanes, vinblastine, and some other drugs[8]. There are still so many hopes to find novel lead compounds from natural sources[9].

In spite of good advancements for diagnosis and treatment, cancer is still a big threat to our society[10]. This is the second most common disease after cardiovascular disorders for maximum deaths in the world[11]. It is believed that in near future the number of cancer patients will increase in the developing and under developed countries, which may rise up to 70%; a serious issue for all of us. The magnitude of cancer problem in the Indian Sub-continent (sheer numbers) is increasing due to poor to moderate living standards[12] and inadequate medical facilities. Most frequently observed cancers in Indian population are of lungs, breast, colon, rectum, stomach and liver[13,14 &15]. The cancer causes in India are almost same as in other parts of the world. The chemical, biological and other environmental identities are responsible for uncontrolled and unorganized proliferation of cells (carcinogens). Basically, under special circumstances carcinogens interact with DNA of the normal cells resulting into a series of complex multistep processes responsible for uncontrolled cell proliferation or tumors[16]. The causes for cancers can be both either internal factors like inherited mutations, hormones, and immune conditions or environmental factors such as tobacco, diet, radiation, and other infectious agents. A significant variation of cancer has been reported due to life styles and food habits[17].

As per nominal Gross Domestic Product (GDP), the economy of India stands on eleventh position in the world, while it is fourth largest by Purchasing Power Parity (PPP) (CIA-The World Factbook, 2009). Indians are at high risk of acquiring cancers due to high rates of smoking, tobacco use, occupational risks, and unhygienic residential living conditions. The prevalence of cancer in India is affecting the economy of the country[18].

The genus *Hypericum* (Hypericaceae) consists of 484 species[19] of herbs, shrubs and rarely trees distributed in warm temperate, subtropical and tropical montane forests of the world. India harbours 27 species of *Hypericum* in parts of the Himalayas, Aravallis in Central and Western Ghats in peninsular India[20] As per The flora of the Tamilnadu carnatic by K.M.Mathew, Palani hills of Kodaikanal regions four different species of Hypericum was recorded (H.patulum, H. mysorense, H. japonicum and H. wightianum).

© Copyright protected. Unauthorised republication, reproduction, distribution, dissemination and copying of this document in whole or in part is strictly prohibited.

MATERIALS AND METHODS

The plant material was shade dried pulverized (500 g) and extracted with methanol at room temperature for 72 hrs. The extract was filtered and concentrated to dryness under reduced pressure and controlled temperature (40 – 50°C) in a rotary evaporator. The extracts were dark greenish brown solid weighing methanol extract 89.30 gr. (yield, 17.86), and preserved in a vacuum desiccator at 4°C until further use.

Reagents

DMEM (Dulbecco's Modified essential medium) were purchased from In vitrogen Gibco BRL, Dimethyl sulfoxide (DMSO), penicillin, streptomycin, Tryspin-EDTA, MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), 5-Fluorouracil were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from GIBCO BRL (Gaithersburg, MD), Bradford reagent and protease inhibitors (aprotinin, pepstatin A, PMSF, and leupeptin) were obtained from Sigma (St. Louis, MO). Primary antibodies were obtained from Santa Cruz Biotechnology, (Santa Cruz, CA) and Cell Signalling technology (Beverly, MA). HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were of reagent or analytical grade were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture and drug preparations

All cell lines were purchased from National centre for cell sciences (NCCS) Pune. Cell lines were grown as monolayer cultures maintained in Dulbecco's modified Eagle's medium (GIBCO BRL) supplemented with heat inactivated 10% Fetal bovine serum (GIBCO BRL) and 2 mM L-glutamine (Sigma Chemical Co), 100 units/ml penicillin and 100 µg/ml streptomycin and maintained at 37°C in a atmosphere of 5% CO_2 incubator at 95% air humidified. The stock solution was prepared in DMSO and was stored at -20 °C until use. The concentrations used for the study were freshly prepared for each experiment with a final DMSO concentration of 0.1%. All the experiments were performed as three biological replicates with minimum of three independent experiments for compound, and concentration.

Assessment of cell viability

Cell viability was measured by MTT (3-(4, 5-dimethylthiazole-2-yl)-2,5-diphe-nyl tetrazolium bromide) assay described[21]. Approximately (5×10^3 cells/well) HEP G2 cells were plated in each well of 96 well plates and incubated for 24 h for attachment. After incubation, supernatant media was replaced with an equal amount of fresh media containing different concentrations of extracts and 5 Flurouracil (positive control) dissolved in DMSO. After incubation for indicated times, MTT solution was added to the plate at a final concentration of 5 mg/mL. The cells were incubated for 4 hr in dark at 37°C. The resulting MTT-products were dissolved by DMSO. Viability was calculated by measuring optical density at 570 using reference wavelength of 650 nm in ELISA reader (Bio-Rad Instruments Inc., USA).

DNA fragmentation assay

DNA fragmentation was detected by agarose gel electrophoresis. $1X10^6$ HEP G 2 cells were plated in 30 mm culture plate. When the cells reached approximately 70% confluency, increasing concentrations of drug (IC_{50} concentration) were added and the cells were incubated for 48 h. After 48 hours, cells were harvested and pelleted by centrifugation (Eppendorf 5804R, Germany). The harvested cells were washed twice with ice cold PBS. The cell pellet was lysed in a buffer containing 10 mM Tris–HCl, 10 mM EDTA, and 0.2% Triton X-100 (pH 7.5). After 10 min on ice, the lysate was centrifuged (13,000g) for 10 min at 4 °C. Then, the supernatant (containing RNA and fragmented DNA, but not intact chromatin) was extracted first with phenol and then with phenol–chloroform:isoamyl alcohol (24:25:1) as described previously[22]. The aqueous phase was brought to 300 mM NaCl and nucleic acids were precipitated with 2 vol of ethanol. The pellet was washed with 70% ethanol, air-dried, and then dissolved in 20 µL of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5). Following digestion of RNA with RNase A (0.6 mg/ml, at 37 °C for 30 min), The DNA samples obtained were analyzed by 2% agarose gel electrophoresis. After electrophoresis, the gels were stained with ethidium bromide, and visualized as a DNA ladder with UV transillumination.

Western blotting

Western blotting was carried out as described previously[23]. Briefly HEP G2 cells (1.5×10^6) were seeded onto 100-mm culture dishes in the presence or absence of extracts (IC_{50} concentrations) were treated for 24h. The medium was removed and the cells were washed with PBS (0.01M, pH 7.2) for several times and lysed on ice in lysis buffer containing 100 µg/ml phenylmethylsulfonyl fluoride (PMSF), 50 mM Tris–base at pH 8.0, 150 mM NaCl, 0.02% NaNo3, 1% NP-40, 10µM aprotinin,10µM pepstatin A, 10µM leupeptin. The supernatants were collected by centrifugation at 10,000×g for 5min at 4°C, and were used as the cell protein extracts. The harvested protein concentration was measured using a protein assay kit (Bio-Rad). Equal amounts of proteins (50–100 µg) were separated on 7.5%–12.5% SDS-PAGE gel and electro transferred onto PVDF membrane. Proteins were blocked overnight with 5% non-fat dried milk in PBS-T at 2-8°C. After washing in PBS containing 0.1% Tween 20 for 3 times, the membrane was incubated with the specific primary antibodies in 5% (w/v) skim milk in PBST. After overnight incubation at 4°C. The membrane was then washed three times with TBST, incubated further with alkaline phosphatase conjugated goat anti-mouse antibody at room temperature for 2 hours, and then washed three times with TBST. After reaction with horseradish peroxidase-conjugated goat anti-mouse antibody, the immune complexes were

© Copyright protected. Unauthorised republication, reproduction, distribution, dissemination and copying of this document in whole or in part is strictly prohibited.

visualized by using the chemiluminescence ECL PLUS detection reagents following the manufacturer's procedure (Amersham Bioscience). The same nitrocellulose membrane was stripped and incubated with β-actin monoclonal antibody (Sigma) at a 1:2000 dilution for 1 h which acted as a control for loading and blotting.

RESULTS

The inhibitory effect of the methanol extract of *Hypericum wightianum* at different concentration after 24 hrs. on HT29, HEP G2, PNAC 1, A549 and VERO cell lines was investigated adopting MTT assay. The assay was aimed at determining the integrity of mitochondria so as to reflect on the viability or otherwise of the cells. It was recorded that the extract used have significant cytotoxic to all the cell lines used except VERO. The IC50 value obtained (HT29 - 25.34; HEP-G2 - 20.87; PNAC-1 – 70.31; A549 – 11.718 and VERO - >250).

Morphological changes produced the cell lines tested indicating apoptosis, on treatment of the plant extract used. Microscopic evidence for apoptosis was obtained adopting acridine orange (AO) and ethidium bromide (EB) staining, the fluorescence patterns of which depend on viability and membrane integrity of the cells.

Table 1: *In vitro* cytotoxicity activity of methanol extract of *Hypericum wightianum*

S. No.	Concentration (µg/mL)	Cytotoxicity activity (%)				
		HT-29	HEP-G2	PNAC-1	A549	VERO
1	250	75.13±0.87	74.70±0.98	74.32±0.63	87.94±0.64	32.43±1.23
2	125	70.68±0.49	67.25±0.95	64.70±1.90	80.03±0.74	20.36±0.39
3	62.5	63.72±0.36	56,26±1.00	48.60±1.21	66.67±0.68	14.98±0.97
4	31.25	54.37±0.49	53.67±1.26	44.00±1.94	26.99±0.41	9.72±0.74
5	15.625	42.30±0.75	46.01±1.51	24.94±0.98	53.63±1.30	7.96±0.88
6	7.8125	35.24±0.95	30.63±0.75	18.59±0.43	47.25±1.01	3.41±0.69
7	3.906	20.5±0.53	22.26±1.16	14.69±0.54	44.41±0.71	1.76±0.91
8	Cell control	100	100	100	100	100

Table 2: Cytotoxic Induction Pathway (Relative intensity (fold change) with ß actin)

Time (Hr.)	P53	Bcl 2	Bax	Cytochrome C	Procaspase 9	Caspase 9	Procaspase 3	Caspase 3	PARP
0	10.36	3.44	0.14	0.89	4	0.66	3.71	0.45	0.96
6	19.16	2.98	0.80	1.26	3.18	1.56	3.81	1.63	1.81
12	36.74	2.32	1.34	1.86	2.78	2.44	2.63	2.81	2.48
24	56.71	1.95	1.99	2.48	1.45	3.48	1.77	3.44	3.70

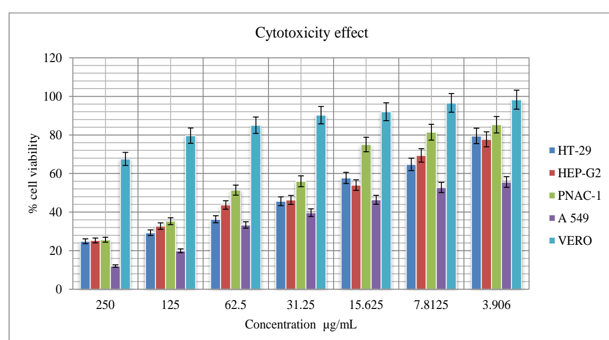


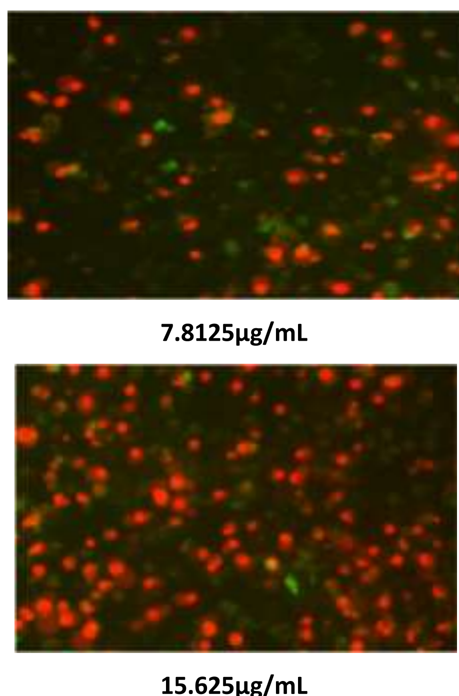
Figure 1: Cytotoxic effect of methanol extract of *Hypericum wightianum* on different cell lines

Uniformly green fluorescing nuclei with a highly organized cellular structure indicated normal and viable cells. Green fluorescing nuclei, with perinuclear chromatin condensation as revealed in bright green patches indicated cells in early phase of apoptosis. Orange to red fluorescing nuclei with highly condensed or fragmented chromatin indicated cells in a late stage of apoptosis. Uniformly orange to red fluorescing nuclei with no indication of chromatin fragmentation but the entire cells as well as nuclei were swollen to large size indicated necrotic cells.

Data on cells indicating apoptosis produced on the treatment with the plant extract in 24hr. Which reveal that the plant extract is highly efficient in bringing apoptosis various amounts in different cell lines. After treatment with IC50 concentrations of the plant extract for 24hrs, the cells were observed for cytological changes. The observation revealed that the treatment brought about chromatin fragmentation, binucleation, cytoplasmic vacuolation, membrane blebbing and late

© Copyright protected. Unauthorised republication, reproduction, distribution, dissemination and copying of this document in whole or in part is strictly prohibited.

apoptosis indication of dot-like chromatin. Data collected from manual counting of cells live and dead.



7.8125µg/mL

15.625µg/mL

Figure 2: AO/ EB staining of A549 cell lines

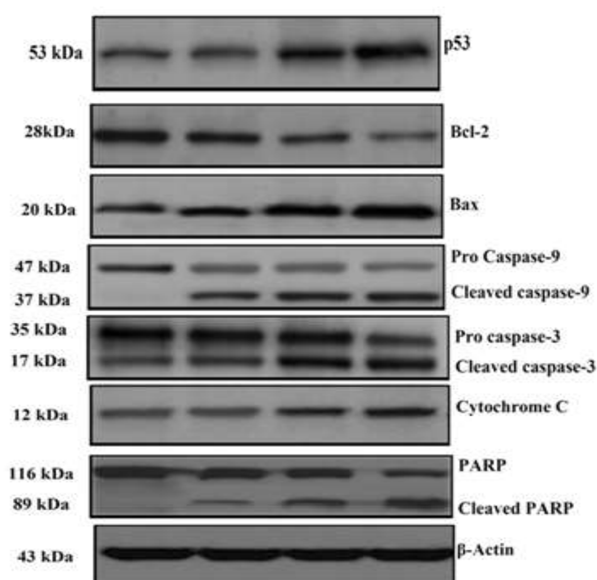


Figure 3: Cytotoxic Induction Pathway

(Relative intensity (fold change) with ß actin)

(Western blotting analysis)

DISCUSSION

The treatment of cancer is still largely based on the use of chemotherapeutic drugs to affect the viability of cancer cells, reduce tumour growth, and alleviate pain. In the 1960s and 1970s, there was a surge of interest in developing anticancer compounds that react chemically with DNA. The strategy of aiming at DNA as a target for anticancer drugs was inspired after the discovery of the alkylating-like platinum agent, cis-diamminedichloroplatinum (II) (cisplatin), which was discovered by an accident during the 1960s. Cisplatin proved to be very effective in treating several solid tumours but its use is limited by toxic side effects in non-target cells as well as the development of drug resistance in the tumour[25].

We tested the growth inhibitory effect of the methanol extract of the plant on the selected cell lines adopting MTT assay. The results evidenced the growth inhibitory effect of the plant extract on different cell lines at different concentration, wherein the normal cells (VERO) was little affected.

Cellular stresses, such as growth factor deprivation, DNA damage and/or oncogen expression, lead to stabilization and activation of the p53 tumour suppressor protein. Depending on the cellular context, the p53 protein would bring about one of the two different outcomes; DNA repair or cell cycle arrest followed by apoptotic cell death. Cell death induced via the p53 pathway is executed by the caspase proteases which, by cleaving their substrates such as PARP, lead to the characteristic apoptotic phenotype[26]. Caspase activation by p53 occurs through the release of apotogenic factors from the mitochondria including cytochrome c and Smac/DIABLO. The release of mitochondrial apoptogenic factors is regulated by the pro- and anti-apoptotic Bcl-2 family proteins, which either induce or prevent the permeabilization of outer mitochondrial membrane[27, 28]. It was shown that some of the pro-apoptotic family members, such as Bax, Noxa and PUMA, are transcriptional targets of p53 [29]. To investigate if the DNA damage brought about by the methanol extract of *Hypericum wightianum* is followed by the induction of apoptosis, we studied the morphological changes, loss of mitochondrial membrane potential and expression of apoptosis-associated proteins in the treated A549 cells.

We observed that the tumor suppressor protein p53 was upregulated following the DNA damage, after treatment with the methanol extract of *Hypericum wightianum*. It is a regulators of apoptosis and also the transcriptional targets of p53[29]. We found that the pro-apoptotic Bax was up- established fact that Bcl-2 family proteins are central regulated and the antiapoptotic Bcl-2 was down-regulated in the treated cells, thereby disposing the cells to apoptosis through mitochondrial intrinsic pathway. Mitochondrial apoptotic pathway is executed by a family of cysteine proteases. We show that the methanol extract of *Hypericum wightianum* treatment resulted in decrease in the levels of pro-caspases-9 and 3 and concomitant increase in the levels of cleaved caspase-9 and cleaved caspase-3 indicating the activation of caspase cascade culminating in apoptosis which was confirmed by the increase in the levels of cleaved PARP. The morphological observations of the AO/EB -stained cells further substantiated the induction of apoptosis by the methanol extract of *Hypericum wightianum* treatment.

In the recent times, it has been proposed that in PARP-mediated cell death, NAD is rapidly depleted and,

© Copyright protected. Unauthorised republication, reproduction, distribution, dissemination and copying of this document in whole or in part is strictly prohibited.

because this cofactor is necessary for different metabolic processes like glycolysis or the Krebs cycle, leads to ATP loss and a necrotic cell death. In other words, both PARP-mediated cell death and apoptosis are two different processes that occur after DNA damage, in different time frames, wherein p53 itself controls PARP-dependent necrotic death[30,31].

CONCLUSION

Taken together, this study indicates that the methanol extract of *Hypericum wightianum* affects the viability of cell lines tested. Further, the extract is cytotoxic to the normal VERO cells but at concentrations very much higher than for the cancer cells.

Thus, although the IC50 of the extract for VERO cells is very high, there is still an element of concern regarding the safety of the extract to nontarget tissues.

Considering the advantage of the plant extract in terms of dose in therapeutic application, the possibility of targeted delivery of the extract to the tumor site should be worked out. We are currently addressing this issue.

REFERENCES

1. Aggarwal BB, Danda D, Gupta S and Gehlot P, Models for prevention and treatment of cancer: problems vs promises. *Biochem Pharmacol,* 78(9), 2009, 1083–94.
2. Lanzotti V, Taglialatela-Scafati O, Fattorusso E and Di Pietro A, Plant Compounds and Derivatives as Inhibitors of Cancer Cell Multidrug Resistance. In: Tringali C, editor. *Bioavtive compounds from Natural Sources*. Boca Raton: CRC press, 2011, 409–50.
3. Hanahan D and Weinberg RA, Hallmarks of cancer: the next generation. *Cell,* 144(5), 2011, 646–74.
4. Cotter TG, Apoptosis and cancer: the genesis of a research field. *Nat Rev Cancer,* 9(7), 2009, 501–7.
5. Letai AG, Diagnosing and exploiting cancer's addiction to blocks in apoptosis. *Nat Rev Cancer,* 8(2), 2008, 121–32.
6. Werner JM and Steinfelder HJ, A microscopic technique to study kinetics and concentration-response of drug-induced caspase-3 activation on a single cell level. *J Pharmacol Toxicol Methods.* 57(2), 2008, 131–7.
7. Cragg GM, Kingston DGI and Newman DJ, *Anticancer Agents from Natural Products.* Boca Raton: Taylor & Francis Group 2005.
8. Brunton LL. *Goodman and Gilman, manual of pharmacology and therapeutics.* McGraw Hill Professional; 2013.
9. Cragg GM and Newman DJ, Natural products: a continuing source of novel drug leads. *Biochim Biophys Acta.* 1830(6), 2013, 3670–95.
10. Kotnis A, Sarin R and Mulherkar R, Genotype, phenotype and cancer: Role of low penetrance genes and environment in tumor susceptibility. J Biosci, 30, 2005, 93-102.
11. Jemal A, Siegel R, Ward E, Murray T, Xu J and Thun MJ, Cancer statistics, CA Cancer J Clin, 57, 2007, 43-66.
12. Wynder EL, Covey LS and Mabuchi K, Current smoking habits by selected background variables: Their effect on future disease trends. Am J Epidemiol 100, 1974, 168-177.
13. Nandakumar A. Consolidated report of the population based cancer registries. National Cancer Registry Programme. Indian Council of Medical Research, 1990-96; New Delhi, India.
14. Rao DN and Ganesh B, Estimate of cancer incidence in India in 1991. Indian J Cancer 35, 1998, 10-8.
15. Murthy NS and Mathew A, Cancer epidemiology, prevention and control. Curr Sci, 2004, 4-25.
16. Carmaeia B, Molecular mechanisms in cancer induction and Prevention. Environmental Health perspectives supplements 101, 1993, 237-245.
17. Helbock HJ, Beckman KB, Shigenaga MK, Walter PB, Woodall AA, Yeo HC and Ames BN, DNA oxidation matters: The HPLC- electrochemical detection assay of 8-oxodeoxyguanosine and 8-oxo-guanine. Proc Nat Acad Sci USA, 95, 1998, 288-293.
18. Imran Ali, Waseem A. Wani and Kishwar Saleem, Cancer Scenario in India with Future Perspectives. Cancer Therapy Vol 8, 2011, 56-70.
19. Cossuta D, Vatai T, Bathori M, Hohmann J, Keve T and Simondi B, "Extraction of hyperforin and hypericin from St. John's wort (*Hypericum perforatum L.*) with different solvents", Journal of Food Process Engineering. 2011.
20. Sanchez CC, Prado B and Rabanal RM, Antidepressant effects of the methanol extract of several *Hypericum* species from the Canary Islands. J Ethnopharmacol. 79, 2002, 119-127.
21. Mossman T, Rapid colorimetric assay for cellular growth and survival: an application to proliferation and cytotoxicity assays. J. Immunol. Methods, 65, 1983, 55–63.
22. Sambrook J and Russell DW, Molecular Cloning: A Laboratory Manual. Third edition. Cold Spring Harbor Laboratory Press, Plainview, NY. 2001.
23. Tu L.C., Chou C.K., Chen C.Y., Chang Y.T., Shen Y.C. and Yeh S.F., Characterization of the cytotoxic mechanism of Mana-Hox, an analog of manzamine alkaloids. Biochimica et Biophysica Acta, 1672(3), 2004, 148–156.

© Copyright protected. Unauthorised republication, reproduction, distribution, dissemination and copying of this document in whole or in part is strictly prohibited.

24. Krishan, Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining, J. Cell Biol. 66, 1975, 188–193.

25. Bruijnincx PC and Sadler PJ, New trends for metal complexes with anticancer activity. CurrOpinChemBiol, 12, 2008, 197–206.

26. Schuler M and Green DR, Mechanisms of p53-dependent apoptosis. BiochemSoc Trans, 29, 2001, 684–687.

27. Haupt S, Berger M and Goldberg Z, Apoptosis—the p53 network. J Cell Sci, 116, 2003, 4077–4085.

28. Hofseth LJ, Hussain SP and Harris CC, p53: 25 years after its discovery. Trends Pharmacol Sci, 25, 2004, 177–181.

29. Cory S and Adams JM, The Bcl-2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer, 2, 2002, 647–656.

30. Montero J, Dutta C, Van Bodegom D, Weinstock D and Letai A, p53 regulates a non-apoptotic death induced by ROS. Cell Death Differ 20, 2013, 1465–1474.

31. Elkholi R and Chipuk JE, How do I kill thee? Let me count the ways: p53 regulates PARP-1 dependent necrosis. BioEssays, 36, 2014, 46–51.

Source of Support: Nil, **Conflict of Interest:** None.

© Copyright protected. Unauthorised republication, reproduction, distribution, dissemination and copying of this document in whole or in part is strictly prohibited.