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Methanolic Root Extract of *Calotropis gigantea* Induces Apoptosis in Human Hepatocellular Carcinoma by Altering Bax/Bcl-2 Expression

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Abstract *Calotropis gigantea*, family apocynaceae, is a milkweed traditionally used in ayurveda for its anti-helminthic, anti-pyretic, and anti-malarial activities. It has also been studied widely for its anticancer potential. In the present study, the antioxidant, antibacterial, antifungal and antitumor potential of its three root extracts viz. chloroform extract (CE), ethyl acetate extract (EE) and methanolic extract (ME) were investigate. Antiproliferative activity of the extracts was examined on human hepatocellular carcinoma cells, HepG2 and human breast cancer cells, MCF-7. Maximum cytotoxicity was obtained with ME on HepG2 cells with IC50 value of 85 µg/mL. ME was further investigated at 85 µg/mL, for cell DNA damage using agarose gel electrophoresis and nuclear staining. RT-PCR analysis was done to study expression of bcl-2 family of genes viz., bax, bcl-2, and p53. Bax expression significantly ($p < 0.05$) increased following ME treatment, whereas bcl-2 expression was significantly ($p < 0.05$) lowered. Significant ($p < 0.05$) increase of p53 expression suggested damage to DNA of HepG2 cells. The study showed that methanolic root extract induces apoptosis in HepG2 cells by altering bax/bcl-2 expression. Further studies are required to obtain knowledge about the complete mechanism of its apoptosis inducing activity.

Keywords: *Calotropis gigantea, HepG2, Bax, Bcl-2, p53*

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1. Introduction

Calotropis gigantea R. Br., *Asclepiadacea (Apocynaceae*), known as milkweed, is a plant native to India, Singapore, Malaysia, Thialand, Phillipines, Indonesia, Sri Lanka, and South China (Kumar et al., 2010b). It is distributed widely in tropics and subtropics. In India, it grows on wastelands as a weed. The root, stem, leaves, flower and latex of *C. gigantean are* used in traditional medicine for the treatment of toothache, ear ache, sprain, anxiety, pain, leprosy, eczema, syphilis, elephantiasis, ulceration, cough, epilepsy and mental disorders (Dash, 2002, Pal and Jain, 1998). Its flowers are used for their antiasthamtic and analgesic activity (Pathak and Argal, 2007). The roots of C. gigantea are extensively used for treatment of tuberculosis, leprosy, lupus, eczema and systemic ulceration. Additionally, the roots posses antipytetic activity (Chitme et al., 2005), antimicrobial activity (Kumar et al., 2010a, Kumar et al., 2010c, Alam et al., 2008), CNS activity (Argal and Pathak, 2006), and cytotoxicity (Wang et al., 2008).

Out of the several activities reported, the selective anti-proliferative activity exhibited by the *Calotropis* species is of high importance as an anticancer agent from herbal origin. There is evidence of the pharmacological potential of whole latex of *C. procera*. Some researchers have recently shown that the whole latex of C. procera possesses anticancer and cytotoxic activity against hepatocellular carcinoma (Choedon et al., 2006). Literature survey indicated evidence for anti-proliferative activity of *C. gigantea* roots (Wang et al., 2008, Kiuchi et al., 1998) but very little is known regarding the mechanism of action on tumor cell lines. In the present study, we investigated the role of apoptotic genes that are involved in process of cell death. Although it has been the subject of extensive biological investigations, its cellular mechanisms have not been completely explored yet.

Cancer is one of the major causes of morbidity and mortality throught the world. Despite tremendous progress in oncology therapeutics, the impulse to find and to

develop novel, alternative or synergistic anti-cancer agents still remains. Considering the side effects imposed by chemotherapeutic drugs, herbal medicine is becoming popular among the researchers. However, to pursue these remedies and make them acceptable requires a deep understanding of their action on the cells. In this work, the roots of *C. gigantea* was subjected to successive solvent treatment, the extracts of which were evaluated for the antioxidant, antibacterial, antifungal and antitumor activity.

2. Materials and Methods

2.1. Collection and Authentication of Plant

Calotropis gigantea was collected from Manipal, Udupi district, Karnataka, India in the month of November, 2010. The plant material was identified and authenticated by Dr. K. Gopalkrishna Bhat, Retired Professor, Purnapragya College, Udupi, Karnataka, India.

2.2. Cell Cultures

HepG2, a human hepatocellular carcinoma cell line and MCF-7, human breast cancer cell line were used in the study. Cell lines were purchased from National Center of Cell Sciences, NCCS, Pune, India. Both cell lines were grown in DMEM supplemented with 10% heat-inactivated FBS, 1% antibiotic-antimycotic solution in a humidified incubator (5% CO_2 in air at 37°C) and cultured in T25 flask.

2.3. Preparation of Extract

The dried peeled roots (25 kg) of *Calotropis gigantea* were separated from the plant and washed with tap water, dried under shade and powdered. The powdered material was defatted with petroleum ether (60-80ºC) and successively extracted for 24 h using chloroform, ethyl acetate and methanol. (Murali A. *et al.,* 2010). The extracts were concentrated under vacuum using rotary evaporator and percentage yield was calculated.

2.4. *In Vitro* Anticancer Activity

2.4.1. Antiproliferative Activity

The *in vitro* antiproliferative activities of CE, EE and ME against HepG2 and MCF-7 cells were studied. Different concentrations of the extracts were prepared viz. 1000, 500, 250, 125, 62.5, 31.25, and incubated with cell lines. The results were expressed in IC_{50}, which is the concentration of the extract required to kill 50% of the cells. Cisplatin at 100, 50, 25, 12.5, 6.25, 3.125 µg/mL, was used as standard control and the experiments were repeated 3 times.

a) MTT Assay

The colorimetric assay is based on the capacity of mitochondrial succinate dehydrogenase enzymes in living cells to reduce the yellow, water soluble substrate 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into an insoluble, purple coloured formazan product which can be measured . Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells. Cells were seeded in quadruplicates in 96 well plates at 1×10^4 cells/well. After 24 h, 100 µl of test and control solutions were added over the cells. The plates were incubated for 72 h at 37°C and 5% CO_2 atmosphere (Kumar et al., 2012). After incubation, 50 µl of MTT was added and plates were again incubated for 4 h at 37°C. The supernatant was removed and 50 µl of 1-propanol was added to solubilize the formed formazan. The absorbance was taken at a wavelength of 540 nm after 30 minutes and IC_{50} value was calculated.

b) Sulphorodamine B assay

SRB assay is based on the measurement of total cellular protein. It is a pink aminoxanthene dye which attaches to the basic amino acids of the cells and provides a cellular index to the protein content (Vichai and Kirtikara, 2006). Cells were seeded in quadruplicated in 96 well plates at 1×10^4 cells/well. After 24 h, the compounds were added at different concentrations to the monolayer of cells in microtitre plate. The plates were incubated for 72 hours at 37°C and 5% CO_2 atmosphere. After this incubation period, 25 µl of 50% trichloroacetic acid was added gently to the wells. The plates were again incubated for 1 hour at 4°C and were then flicked and washed with tap water to remove the traces of medium, drug and serum. The air dried plates were stained with SRB dye for 30 minutes and then washed with 1% acetic acid for four times. Finally 100 µl of 10 mM tris base was added. The absorbance was measured at a wavelength of 540 nm in a microtitre plate reader and finally IC_{50} values were calculated.

2.4.2. Fluorescence Cell Staining

Condensation of chromatin is a characteristic event in cells undergoing apoptosis. Nuclear staining was performed as per the standard protocol (Kumar et al., 2012). Briefly, 1×10^5 HepG2 cells /mL were grown in 6-well plates in duplicates and treated with 85µg/mL ME (at IC_{50} concentration). After 24 hours of drug treatment, the cells were fixed with 90% methanol and kept at -20 °C for 20 min. Cells were hydrated using PBS containing 1% bovine serum albumin and 0.1% triton X and stained with acridine orange (0.1 mg/ml) and propidium iodide (10 µg/ml). Immediately cells were washed with PBS and fluorescence was observed under fluorescence microscope.

2.4.3. DNA Fragmentation Assay

Important signature of apoptosis is the cleavage of nuclear DNA into small-sized fragments (Basnakian and James, 1994). Cells were grown in T25 flask and subjected to the treatment with the extracts at a concentration below $IC_{50.}$ After 24 h of drug incubation, the genomic DNA was extracted from cells with phenol-chloroform extraction method. In brief, drug treated cells were lysed in a buffer containing Tris HCl (10 mM), NaCl (100 mM), EDTA (1 mM), 1% SDS and proteinase K (50 µg/ml) and incubated for 4 h at 37 °C. RNase A (50 µg/ml) was added and incubated for 2 h at 55 °C. Cell lysate was then subjected to extraction using equilibrated phenol: chloroform: isoamyl alcohol (25:24:1) and centrifuged at 3000 rpm. DNA was precipitated from supernatant with ice-cold ethanol containing 0.3 M

sodium acetate. DNA was dissolved in TE buffer and resolved on 1.5% agarose gel with ethidium bromide. Fragmentation pattern was observed under UV light with the help of Alpha Innotech, Inc gel doc system.

2.4.4. Reverse Transcriptase-PCR Analysis for Bcl-2, Bax, p53

HepG2 cells were cultured and incubated with the extract at a concentration below IC_{50}. After 24 h of incubation, total RNA was isolated from drug treated and untreated cells by the guanidium isothiocyanate/acidic phenol reagent (Trizol, Invitrogen) and quantified using UV spectrometry. cDNA was synthesized from isolated RNA using Invitrogen's SuperScript® III First-Strand Synthesis as per manufacturer's protocol. DNA was synthesized from cDNA for bcl-2, p53, bax, genes with respective primers using Invitrogen's KOD hot start DNA polymerase (Kumar et al., 2012). PCR product was loaded on 1.5% agarose gel and bands were visualized with the help of ethidium bromide. Sequences for primers used are given in Table 2.

3. Results

3.1. Cytotoxicity Studies

Antiproliferative activity of the root extracts was carried out on two cell lines, HepG2 and MCF-7 cell line, using MTT and SRB assay and average of the two assays was calculated. Different concentrations of chloroform, ethyl acetate and methanolic extracts were tested. As observed in Table 1, the methanolic extract showed high cytotoxicity against HepG2 and MCF-7 cell lines with average IC_{50} values 85.05 µg/ml and 92.4 µg/ml respectively. Standard drug cisplatin was significantly cytotoxic over both cell lines.

Table 1. Antiproliferative activity of chloroform, ethyl acetate and methanol extracts on MCF-7 and HepG2 cell lines, represented as IC_{50}

Sample	HepG2			MCF-7		
	MTT	SRB	Average	MTT	SRB	Average
CE	115.3 ± 2.5	130.4 ± 3.1	122.85	125.5 ± 3.2	138.2 ± 3.8	131.85
EE	117.6 ± 1.3	128.9 ± 4.2	123.25	185.1 ± 2.5	201.4 ± 4.1	193.25
ME	82.7 ± 2.2 [a,b]	87.4 ± 1.6 [a,b]	85.05 [a,b]	90.9 ± 6.2 [a,b]	93.9 ± 5.8 [a,b]	92.4 [a,b]
CP	12.2 ± 1.1 [a,b]	18.3 ± 1.3 [a,b]	15.25 [a,b]	11.4 ± 0.4 [a,b]	13.5 ± 0.7 [a,b]	12.45 [a,b]

CE, Chloroform extract; EE, Ethyl acetate extract; ME, Methanolic extract; CP, Cisplatin. All values are mean ± SEM of n=4 in µg/ml. [a] $p < 0.05$ compared to CE, [b] $p < 0.05$ compared to EE.

Table 2. Sequence of forward (F) and reverse (R) primer (5'-3') for PCR analysis

Primer		Sequence (5'-3')	Annealing Temperature (°C)
Bcl-2	F	GGAGCGTCAACAGGGAGATG	56
	R	GATGCCGGTTCAGGTACTCAG	
Bax	F	CCAAGAAGCTGAGCGAGTGTCTC	56
	R	AGTTGCCATCAGCAAACATGTCA	
p53	F	CAGCTTTGAGGTTCGTGTTTGT	51
	R	ATGCTCTTCTTTTTTGCGGAAA	

3.2. Methanolic Extract Causes Nuclear Condensation of HepG2 Cells

Nuclear staining of extracts treated HepG2 cells with acridine orange and propidium iodide indicated nuclear condensation. HepG2 cells on treatment with methanolic extract showed typical apoptotic morphology, like condensed nuclei and formation of apoptotic bodies (Figure 1). In contrast, untreated HepG2 cells showed intact nuclear and cytoplasmic membrane (Figure 1). The nuclear condensation induced by methanolic extract was comparable to standard anticancer drug, cisplatin.

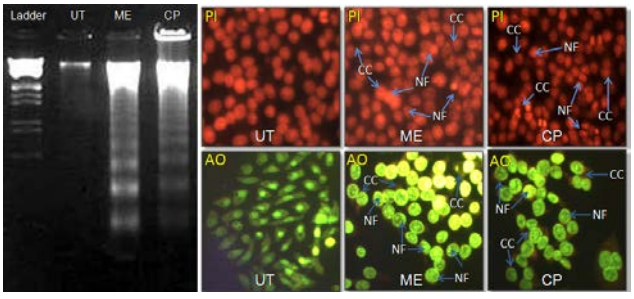

Figure 1. Apoptotic studies. Final image

3.3. Methanolic Extract Induces Degradation of DNA in HepG2 Cells

The effect of the extracts on DNA of HepG2 cells are shown in Figure 1. Agarose gel electrophoresis of the methanolic extract caused damage to DNA, lane 2. Other extracts did not show significant DNA damage.

3.4. Methanolic Extract Downregulates Anti-Apoptotic Gene bcl-2 and Upregulates Pro-Apoptotic Gene bax in HepG2 Cells

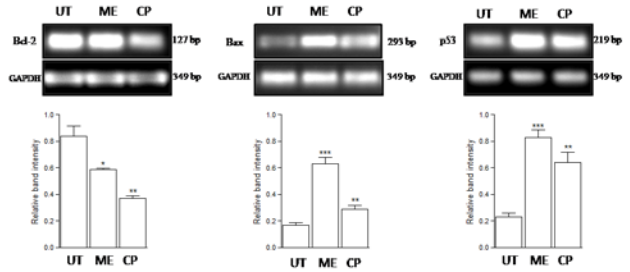

Figure 2. Reverse Transcriptase PCR

The anti-apoptotic protein bcl-2 has been associated with the inhibition of apoptosis and cell survival. Bax is a pro-apoptotic member of bcl-2 family protein and is associated with increased apoptosis. Treatment of HepG2 cells with methanolic extract significantly ($p < 0.05$) decreased the level of bcl-2 mRNA (Figure 2) compared to the untreated cells. At the same time, the levels of bax mRNA were significantly increased ($p < 0.001$). These

results indicated that methanolic extract induced apoptosis by changing the ratio of bax/bcl-2 in HepG2 cells. The level of p53 was significantly increased ($p < 0.001$) in the extract treated cells compared to the untreated cells.

4. Discussion

The quest for the search of novel agents that efficiently interfere with the neoplasic processes or are able to modify specific signaling mechanisms involved in tumor progression are definitely of enormous importance. In this perspective, the plant extracted compounds are a relevant source of molecules that can be exploited for pharmacological purposes. However, there are major limitations in the development of cancer therapy, like the undesired side effects and the low specificity in targeting cancer cells. The continuing hunt for new sources of antineoplastic agents is justified by the lack of specificity and occurrence of unwanted effects by the currently used drugs. Therefore, the first step in a study is to find the selective cytotoxicity by the new compound.

The Apocynaceae family includes around 250 genera and more than 2000 species of tropical trees, vines and shrubs. This species has been used in the traditional medicine for the treatment of gastric problems, fever, pain, malaria and diabetes (Wong et al., 2011). The roots of *Calotropis gigantea* have been used in several diseases like leprosy, eczema, syphilis, elephantiasis, ulceration and cough in the Indian system of traditional medicine (Rajakaruna et al., 2002). In ayurvedic medicine, it is used in the treatment of cancer, and as anti-pyretic, anti-helminthic and antimalarial (Mathen et al., 2011).

The increasing reports on the cytotoxicity of this plant, led us to attempt the evaluation of different extracts of its roots. The roots were cleaned with water, dried under shade and coarsely powdered. The fatty material of the roots was cleared by performing petroleum ether solvent extraction. Thereafter, successive extraction with three different solvents viz. chloroform, ethyl acetate and methanol were carried out. The solvents were removed by rotaevaporator and the extracts were dried under vacuum. The percentage yield of the three extracts was found to be 1.84, 2.14 and 1.95 respectively.

The cytotoxic potential of the organic extracts from *Calotropis gigantea* (Apocyanacea) was firstly evaluated against cancer cell lines, HepG2 and MCF-7 by MTT assay. The cytotoxicity of CE, EE and ME was evaluated by average of MTT and SRB assay. Subsequently, samples considered cytotoxic were tested for apotosis induction potential. Among the three extracts, methanolic extract displayed significantly higher ($p < 0.05$) cytotoxic potential against tumor cells, with IC50 ranging from 85.05 and 92.4 in HepG2 and MCF-7 cell lines respectively. The chloroform extract showed IC_{50} of 122.85 and 131.85 in HepG2 and MCF-7, respectively, while ethyl acetate extract showed IC_{50} of 123.25 and 193.25, respectively. Therefore, further studies were carried out with ME using HepG2 cells, to find out its apoptotic potential and to understand the mechanism of action responsible for this tumor-decreasing potential.

The fragmentation of DNA of HepG2 cells gave evidence of the apoptotic entities present in the methanolic extract (Figure 1). Further, nuclear staining studies also revealed that the extract damages the HepG2 cells (Figure 1). The expression pattern of bax/bcl-2 shifted as the cells received treatment of ME. The bax levels were significantly elevated ($p < 0.001$) in the treatment group compared to the non-treated cells. On the other hand, the bcl-2 expression significantly decreased ($p < 0.05$) in ME treated HepG2 cells. This bax/bcl-2 index is an indicator of the intrinsic pathway of apoptosis and shows that the damage to HepG2 cells is driven by components of ME.

Apoptosis is an elemental process vital for normal tissue homeostasis and development. It is directly related to one of the many signaling pathways that lead to cell suicide. One such pathway is the intrinsic pathway of apoptosis. It involves the activation of tumor suppressor gene p53, upon damage caused to the cell DNA. The damage could be a result of stimuli like free radicals, toxins, or radiation. Due to these stimuli, there is an interruption of inner mitochondrial membrane functioning that involves change of membrane potential. This change leads to the release of apoptosis initiating factors, called as pro-apoptotic genes, e.g., bax, bid, bad, bim. The pro-apotic genes are regulated in conjunction with antiapoptotic gene bcl-2 family members, e.g., bcl-2, bcl-x, etc.

To assess the cell suppressive action of the plant extract, we studied the intrinsic apoptotic signaling pathway. The results of reverse transcriptase PCR showed significant ($p < 0.05$) increase in p53 tumor suppressor gene in the treated HepG2 cells compared to untreated cells. Therefore the damage was caused by the extract to the DNA of cells leading p53 activation. The significant ($p < 0.05$) increase in the bax expression and significant ($p < 0.05$) decrease in bcl-2 expression also confirmed the involvement of intrinsic pathway of cell death.

From the findings of the present study it can be concluded that the methanolic extract of *C. gigantea* has potential anticancer activity against liver carcinoma, indicating the prospect of developing *C. gigantea* as a powerful and inexpensive anticancer agent. Although the study wraps up the role of bcl-2 and bax in the apoptotic mechanism of suppression of HepG2 cells by methanolic root extract, yet more evidence is needed to elucidate the component responsible for this activity. Further investigations are also necessary to establish these extracts as promising lead candidates for anti-neoplastic activity.

5. Conclusion

The present study revealed that the extracts of *Calotropis gigantea* exhibited anticancer activity on different cell lines. The highest potency was observed in the methanolic extract. Investigation of the molecular mechanism gave insight that Bax:Bcl-2 ratio tilts from 4:1 to 1:1. Thus further studies are needed to investigate and isolate its active compounds and also to evaluate its anticancer potential on other cell lines.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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