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Apoptotic induction by *Cassia fistula* leaf extracts against human hepatocarcinoma cell lines

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Abstract

Cassia fistula has therapeutic importance in the field of health care. Several studies reported the use of this plant for the treatment of abdomen, glands, liver and throat cancer indicating its high efficacy and medicinal value. This study was performed to identify the anti-cancer property of traditional medicinal herb, *Cassia fistula* on Hep G2, human hepatic carcinoma cells. The cytotoxicity potential of the plant extract on HepG2 cells was analysed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay followed by determination of its genotoxicity potential by DNA fragmentation assay. Increased level of cysteine-aspartic proteases (CASPASEs) quantified in treated cells validated the DNA fragmentation assay and confirmed the induction of apoptosis. The leaf extracts were prepared using hexane, chloroform and methanol as solvents and subjected to phytochemical investigation. Reverse transcriptase polymerase chain reaction (RT - PCR) suggested the involvement of mitochondrial proteins in the pathway. The database search and text mining approaches performed using liver cancer cells revealed the apoptotic role of B-cell lymphoma (Bcl-2) gene. Results showed that chloroform extract of the plant leaves was most toxic to cancer cells. Further, Bcl-2 gene expression was observed to be low in cells treated with the chloroform extracts of *C. fistula* leaves, suggesting an involvement of mitochondrial proteins in the HepG2 cell apoptosis. Hence, herbs like *Cassia fistula* being a part of traditional medicine in India can also aid as an effective anti-cancer therapy to combat this deadly disease.

Keywords: *Cassia fistula*, Liver cancer, Cytotoxicity, HepG2 cells, Bcl-2 gene, CASPASEs

Introduction

Medicinal properties of plants have been exploited for ages, giving rise to many traditional branches of medicine like Ayurveda. Many plants are considered nutritionally important and bear therapeutic values. These plants include ginger, garlic, Stevia rebaudiana, Macrotyloma uniflorum and Terminalia catappa [1 - 4]. The recent interest in these plants and their healing [5] properties can be attributed to the side effects on intake of their synthetic counterparts [6]. Plant derived medicines with minimal or no industrial processing make up about 25 % of modern pharmacopoeias and they are safer, promote health, prevent diseases and consider

patient's cultural or spiritual inclination in treatment [7]. In addition, many plants such as Catharanthus roseus, Podophyllum emodi, Heliotropium indicum, Ochrosia, Cephalotaxu, and Camptotheca acuminata were reported to possess anticancer property [8]. Cancer has become a global health problem and the second leading cause of deaths in United States with 609,640 cancer deaths estimated to occur along with 1,735,350 estimated new cases in 2018 [9]. Currently, one in four deaths in the United States occurs due to cancer [10]. When ranked within age groups, cancer is one of the five leading causes of death among both males and females and the single largest cause of death worldwide [11]. In a developing country like India with one of the most diverse populations and diets in the world, cancer rates are lower than those seen in western countries, but are on the rise with increasing migration of rural population to the cities, increased life expectancy and changing lifestyles. Liver cancer is the fifth most frequently diagnosed cancer globally and the second leading cause of cancer death in males [12].

They are malignant tumors that grow on the surface or inside the liver. They are formed from either the liver itself or from structures within the liver, including blood vessels or the bile duct [13]. The major primary liver cancer histological subtype is hepatocellular carcinoma (HCC) accounting for 70 to 85 % cases followed by cholangiocarcinoma derived from epithelial lining of the intrahepatic bile ducts [14]. Cancer morbidity may climb to around nine million worldwide. This growing trend indicates a deficiency in the present cancer therapies, which include surgical operation, radiotherapy and chemotherapy. Since the average survival rates have remained essentially unchanged despite such aggressive treatments, there is a critical need for anti-cancer agents with higher efficacy, and less side effects that can be acquired at an affordable cost. Plants are the best alternative as they provide an inexhaustible pool of efficacious agents for treating the disease. Phytochemicals have always been sought after because of their inherent potential to cure disease as demonstrated by ancient medicinal practices [15]. *Cassia fistula*, a semi-wild flowering ornamental plant native to India and adjacent Southeast Asia, also known as Indian Laburnum or Golden Shower tree is a member of the Fabaceae family. It is extensively used worldwide against a wide range of ailments due to the synergistic action of its metabolite production probably responsible for the plant's beneficial effects [16 - 17].

The plant has a high therapeutic value displaying antipyretic, analgesic, anti-inflammatory, and hypoglycaemic effect [18 - 19]. It is useful against skin diseases, liver troubles, tuberculous glands, haematemesis, pruritus, leucoderma and diabetes [20 - 21]. The plant also has anti-bacterial, anti-fungal, anti-parasitic, anti-viral, antioxidant; and anti-fertility properties [22 - 28]. The methanolic extract of *Cassia fistula* seed demonstrated reduction in tumor volume and viable tumor cell count and an improved life span in Ehrlich ascites carcinoma (EAC) tumor bearing mice [29]. The current study is an attempt at elucidating the anti-cancer properties of the ethno-medicinal plant *C. fistula* by analyzing its Phyto-constituents, understanding the cytotoxicity of the extracts on hepatic cancer cells and further analyzing the pathway leading to apoptotic induction in these cells.

Materials and Methods

Sample collection

The leaves of the plant *Cassia fistula* were collected from the suburbs of Chennai, Tamil Nadu, India.

Cell lines

Human hepatocellular carcinoma cell line, Hep G2 was purchased from the National Centre for Cell Sciences, Pune, India. The cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) containing L-Glutamine and 25 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid (HEPES), Penicillin (100 µg/mL), Streptomycin (50 µg/mL) and 10 % Foetal Bovine Serum (FBS). Cells were maintained at 37 °C in a humidified incubator with an atmosphere of 5 % CO_2.

Extract preparation

The leaves were air-dried in shade at room temperature for 2 weeks and ground to a uniform powder. The extracts of the leaf samples were prepared in a sequential procedure by soaking 100 g of dried powder in 900 mL of different solvents (Hexane, Chloroform and Methanol) for 48 h. At the end of each respective extraction, the extracts were filtered using Whatman filter paper. The filtrate was concentrated under reduced pressure in vacuum at 40 °C for 25 min using a rotary evaporator.

Phytochemical analysis

The leaf extracts were screened for the presence of various phytoconstituents like phenols, tannins, flavonoids, coumarins, cardiac glycosides, etc. [30].

MTT Assay

The cytotoxicity of the plant extracts against Hep G2 cells was analyzed using the MTT assay [31]. Approximately 5 X 10^3 cells/well (cell line) were seeded into 96 well plates, 100 µL of Rose well Park Memorial Institute 1640 (RPMI 1640, Sigma) medium was added and incubated at 37 ºC in a 5 % CO_2 in-cubator for the cells to be cultured as monolayer. After 24 h, the medium was discarded and fresh medium was added with different concentrations of plant extract (100, 200 and 300 µg/mL). The plates were incubated for 48 h 37 ºC in a CO_2 incubator. After the incubation period, the medium was discarded and 100 µL fresh medium was added with 10 µL of 3 - (4, 5-Dimethylthiazol-2-yl) - 2,5-diphenyltetrazolium bromide (MTT, 5 mg / bmL). After incubation for 4 h, the medium was discarded and 200 µL of Dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. Next, the absorbance in terms of optical density (OD) was read in a micro plate reader at 570 nm and cell survival was calculated using the formulae:

Viability %= (Test OD/Control OD) X 100 and Cytotoxicity %= 100 –Viability %

Cyclo-90 (Cyclophosphamide) at concentration of 90 µg/mL was used as a positive control.

Apoptosis Assay

HepG2 cell lines were cultured in a 6-well plate at the density of (15 X 10^3 cell/well) at 37 °C. After 24 h, the medium was discarded and fresh medium was added with different concentrations of plant extract (100, 200 and 300 µg/mL).

The plates were incubated for 48 h at 37 ºC in a CO_2 incubator. The cells were then washed twice with 1X PBS and incubated with 2 µl of Annexin V-FITC (Bio Legend, UK) at room temperature for 20 min in the dark. Cells were thoroughly washed and then fixed with 4 % PFA followed by washing steps in PBS. Finally, the samples were read using BD FACS Aria and analyzed by FlowJo 8.8.6.

DNA Fragmentation assay

Formation of an oligo ladder upon DNA fragmentation is a characteristic of apoptosis. To analyze the DNA laddering, Hep G2 cells were seeded in a 24 well plate and kept in a CO_2 incubator. Cells were treated with the extract in three different concentrations (50, 100 and 200 µg / mL) for 48 h. At the end of incubation period, the total DNA was isolated from the cells. DNA samples were electrophoretic ally separated on 2 % agarose gel containing ethidium bromide (0.4 µg/mL). DNA was visualized by a UV (302 nm) transilluminator [32]. Quercetin treatment was used as positive control while untreated cells were used as control.

CASPASEs Assay

CASPASE 3 and 9 activities were determined by chromogenic assays using CASPASE-3 and 9 activation kits according to the manufacturer's protocol (Calbiochem, Merck). After treatment with plant extracts (100, 200 and 300 µg/mL), the cells were lysed using Lysis buffer (50 mM HEPES, 100 mM NaCl, 0.1 % CHAPS, 1 mM DTT, 100 mM EDTA). Lysates were centrifuged at 10,000 rpm for 1 min. The supernatants (cytosolic extract) were collected and protein concentration was determined by Lowry's method using BSA as a standard [33]. 100 µg of protein (cellular extracts) was diluted in 50 µL cell lysis buffer for each assay. Cellular extracts were then incubated with 5 µL of 1 mM DEVD-pNA (CASPASE-3 activity) and 4 mM p-nitroanilide (pNA) substrates (CASPASE-9 activity) at 37 °C for 2 h in a 96 well plate. CASPASEs activity was measured by cleavage of the above substrates to free pNA at 405 nm in a microtiter plate reader. Relative CASPASE-3 and 9 activities were calculated as the ratio of absorbance of treated cells to untreated cells. Camptothecin (1 µM) was used as positive control.

Reverse Transcriptase PCR

Reverse transcriptase polymerase chain reaction (RT - PCR) was performed to analyze the gene expression during cell death due to treatment of the cancer cells with leaf extract. Cells were harvested after treatment with different concentrations (50, 100 and 200 µg/mL) of the extracts. Total Ribonucleic Acid (RNA) was isolated using ONE STEP-RNA Reagent (Biobasic Inc.) and complementary DNA (cDNA) was synthesized according to the manufacturer's protocol (Sigma Aldrich, USA). Using this cDNA as template, PCR was carried out with Bcl-2 and GAPDH gene specific primers. After RNA isolation, RNA was immediately reverse transcribed with Easy Script Plus™ Reverse Transcriptase. The cDNA obtained was amplified by PCR. Gene specific PCR was used to amplify Bcl-2. A constitutively expressed gene, namely GAPDH, was chosen in order to assess the quality of PCR. The sequences of the primers used in the study were:

Bcl-2 F: 5` ACTTGTGGCCCAGATAGGCACCCAG3`

Bcl-2 R: 5` CGACTTCGCCGAGATGTCCAGCCAG 3`

GAPDH F: 5` TCCCATCACCATCTTCCA 3`

GAPDH R: 5` CATCACGCCACAGTTTCC 3`

The annealing temperature was 63 ˚C and 53 ˚C for Bcl-2 and GAPDH respectively. Expression ratio was derived by analyzing the gel photos in the Image J software.

Database search and text mining

STRING v9.01, a database of known and predicted protein interactions was used to construct a protein-protein network [34]. The obtained functional partners were used to plot each functional partner as a node and their interaction as edges. Cytoscape system biology software was used to explore the network and perform various operations such as coloring the interactions between the PPE family of proteins, changing the node size, label size and zooming in and out of the network etc. Further the Platform for Exploration of Significant Concepts Associated to co-Occurrences Relationships (PESCADOR) text mining tool was used to perform concurrence analysis [35].

Results and Discussion

Cancer development and progression is a complex and multistep process involving the alteration of many factors and pathways in tissue types involved in the disease. Commonly used treatment like chemotherapy, radiation therapy and surgery for cancer control have serious side effects like toxicity, resistance to the next rounds of treatment and are partially effective. In recent years, cancer chemoprevention using naturally occurring chemo preventive agents from plant origin is being advocated as a promising approach to prevent cancer development and progression as it is non-toxic, cost-effective, easily available and highly efficient in targeting multiple signaling pathways activated in cancer and induce least or no resistance met with conventional cancer therapies thereby reducing the initiation, progression and spread of cancer [36]. Naturally occurring cancer chemo preventive agents, include flavonoids, isothiocyanates, indoles, dithiolthiones, coumarins, and organ sulfides [37 - 43]. In the present study, anticancer potential of leaves of *C. fistula* was evaluated in Hep G2 human hepatocarcinoma cell line.

Phytochemical analysis

Phytochemicals target various mechanisms for cancer chemoprevention like apoptosis, growth factor signaling pathways, cell survival and mitogenic signaling, cell cycle regulation, angiogenesis, anti-oxidant and free radical scavenging activity. Further, these phytochemicals are known to downregulate expression of apoptosis suppressor proteins, such as Bcl-2 and Bcl-xL in several cancer cell lines and animal models, and induce activation of caspases and other pro-apoptotic molecules [44]. In this study, *C. fistula* leaf extracts were prepared in Hexane, chloroform and methanol by sequential cold percolation extraction method. The phytochemical analysis revealed the presence of various phytochemicals like carbohydrates, tannins, saponins, quinones, cardiac glycosides, flavonoids, coumarins, triterpenoids, phenols in the leaves of *C. fistula* extracted with different solvents.

Most of the phytochemicals were observed in the methanolic extract followed by hexane and chloroform. Flavonoids and carbohydrates were present in all the extracts, while tannins, glycosides, quinones and coumarins were found to be present in hexane and methanolic extracts. Triterpenoids and phenols were detected in the chloroform extracts (Table 1). These plant phytochemicals have a medicinal role in control of various diseases. For example, flavonoids in *C. fistula* may be responsible for the hepatoprotective activity by inhibiting the cytochrome P 450 activity and proliferation of cancer cells and inducing apoptosis [45]. Presence of phenolic compounds in the leaf extract of *C. fistula* acts as an antioxidant agent, by scavenging of the free radicals due to the presence of hydroxyl group in them and they can act as reducing agents, hydrogen donors, metal chelators and singlet oxygen quenchers due to their redox properties [46].

The leaves of *C. fistula* have hepatoprotective activity [47]. Ethyl acetate extract of flowers containing Rhein exhibited dose and time dependent cytotoxicity against human colon adenocarcinoma cell line COLO 320DM by apoptosis [48]. Leaf extracts of *C. fistula* exhibited good hepatoprotective effect against diethylnitrosamine (DENA) induced hepatotoxicity in rats [49]. Thus, the compiled data of the present study was in conformation with the previous findings, wherein the phytochemical analysis of leaf and callus extract of *M. umbellatum* revealed the presence of significant secondary metabolites such as tannins, saponins, quinones, cardiac glycosides, phenols, flavonoids, terpenoids, steroids, and alkaloids [50]. Similarly, various phytochemicals such as phenols, tannins, alkaloids and flavonoids were detected in the active extracts of several medicinal plants [51].

MTT assay

MTT assay is based on the ability to interact with live cells reduced a yellow tetrazolium dye to a purple formazan product [52]. present study was focused to Test the effect of *C. fistula* extract on cell growth and proliferation using MTT assay. Results revealed that 300 µg leaves extracted with chloroform depicted least cell viability (31.98 %) compared to methanol and hexane. Hence, it was most toxic to the cancer cells (Figure 1 and Table 2). The results of the study were compared with the earlier findings wherein anti-proliferative activity of the TCD and ethanol extract of *T. cordifolia* exhibited potent cytotoxic effect against HeLa with an IC_{50} (concentration that inhibits cell growth by 50 %) of 54.23 ± 0.94 µg/mL and 101.26 ± 1.42 µg/mL respectively in MTT assay [53]. Likewise, the leaf extracts of *Kelussia odoratissima* showed selective and dose-dependent cytotoxicity against MDA-MB468, K562, SKOV3, Y79, and A549 cancer cell lines with IC_{50} values of 85, 70, 120, 82 and 145 mg/mL [54].

Apoptosis Assay

Annexin V is a non-quantitative probe used to detect phosphatidylserine expressed on the cell-surface, an indication of apoptosis. HepG2 cells treated with 100, 200 and 300 µg of *C. fistula* leaf extracts dissolved in different solvents (Hexane, Chloroform and Methanol) for were harvested after 48h for quantification of apoptosis by determining changes in cell-surface expression of annexin V. Flow cytometric determination displayed significantly higher levels of Annexin V compared with untreated cells (Figure 2). The observed frequency of cells undergoing apoptosis was found to be much higher in the *Cassia fistula* extract treated cells than in the negative control treated group of HepG2 cells. Similar to MTT assay, apoptosis assay showed that 300 µg leaves extracted with chloroform had the highest apoptotic effect on HepG2 cells. These observations indicate that C. *fistula* leaf extracts might play important regulatory roles in apoptosis.

DNA Fragmentation assay

Apoptosis, the predominant form of programmed cell death has been characterized by the activation of a nuclear endonuclease that cleaves the DNA into multimers of 180 to 200 base pairs and can be visualized as an oligosomal ladder. One of the most prominent biological features of apoptosis is nucleosomal DNA.

Table 1. Phytochemical constituents present in different solvent extracts of *C. fistula*

Phytochemical Tests	Extracts		
	Hexane	Chloroform	Methanol
Molisch's Test (Carbohydrates)	+	+	+
Ferric Chloride Test (Tannins)	++	-	+
Foam Test (Saponins)	-	-	+
Alkaline Test (Flavonoids)	+	+	++
Mayer's Test (Alkaloids)	-	-	-
Quinones	+	-	++
Anthraquinone	-	-	-
Glycosides	-	-	-
Cardiac Glycosides	+	-	+
Terpenoids Test	-	-	-
Triterpenoids	+	+	-
Ferric Chloride Test (Phenols)	-	+	+
Alkaline Test (Coumarins)	+	-	++
Ninhydrin Test (Protein)	-	-	-
Steroids and Phytosteroids	-	-	-
Phlobatannins	-	-	-

+ indicates presence; - indicates absence

Table 2. MTT assay results after treatment of HepG2 cells with plant extracts

Plant extracts		Viability (%)	Cytotoxicity (%)
C		100	0
PC (µg)	90	24.745	75.255
Hexane (µg)	100	68.452	31.548
	200	63.987	36.013
	300	55.321	44.679
Chloroform (µg)	100	42.762	57.238
	200	36.872	63.128
	300	31.986	68.014
Methanol (µg)	100	44.652	55.348
	200	38.873	61.127
	300	32.557	67.443

PC: Positive control (90 µg/mL of Cyclophosphamide)
C: Control cells without treatment containing only DMSO

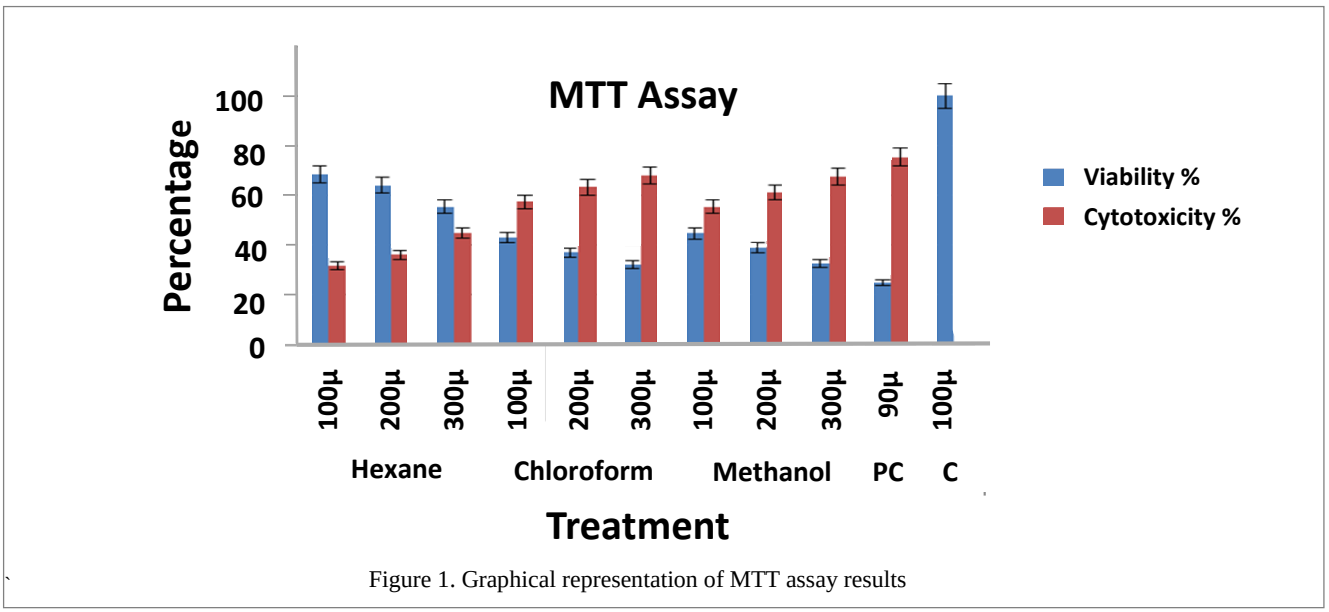


Figure 1. Graphical representation of MTT assay results

DNA fragments are considered to be the hallmark of apoptosis and have been monitored by many researchers by ladder formation on agarose gel electrophoresis [55]. Epigallocatechin gallate (EGCG) is a type of green tea polyphenols with cancer preventive property thereby, induced apoptosis in Hep G2 cells [56].

In the present study, DNA fragmentation assay was performed by seeding HepG2 cells in a 24 well plate and treated with three different concentrations for 48 h. Fragmentation analysis of the DNA was done by calculating cell cycle arrest and its proliferation control. DNA was isolated from these cells and observed using agarose gel electrophoresis. The assay was performed to check genotoxicity potential of the extracts. DNA isolated from the HepG2 cells treated with its chloroform extract demonstrated laddering pattern when visualized by UV transilluminator (Figure 3). This suggested the activation of apoptosis by *C. fistula* leaf extracts in these cells. These outcomes were compared with previous findings wherein clear and similar fragmentation of DNA was observed in all the tested samples of *in vivo* and *in vitro* leaf and callus materials with IC_{50} being used for determining optimum values and typical ladder fragments formation was observed [57]. Similarly, fragmentation of genomic DNA was shown by methanol extract of *C. fistula* treated prostate cancer cell line indicating existence of anticancer agents in the extract and apoptosis regulatory activity due to induction of caspase enzyme [58].

CASPASEs Assay

The apoptotic induction was validated using the CASPASEs quantification assay. The intrinsic and extrinsic apoptotic pathways converge to CASPASE-3, a protease that cleaves the inhibitor of the CASPASE-activated deoxyribonuclease, and the CASPASE activated deoxyribonuclease becomes active leading to nuclear apoptosis.

The upstream caspases that converge to CASPASE-3 are CASPASE-9 and CASPASE-8 in the intrinsic and extrinsic pathways, respectively. Uterus cancer was reported to be effectively managed by unguent topical oil obtained from aerial parts of *A. graveolens*. Modulation of mitochondrial membrane potential showed apoptosis due to activation of caspases 3/7 and 9 [59]. The present study showed upregulation in the CASPASE-3 and 9 activities in HepG2 cells exposed to *C. fistula* leaf extracts. Cells treated with chloroform extracts showed considerable upregulation in CASPASEs level, compared to those treated with hexane and methanolic extracts (Table 3 and Figure 4).

This indicated the activation of apoptosis in HepG2 human hepatocarcinoma cells treated with chloroform extracts of *C. fistula* leaves. This study corroborates with an earlier finding wherein *Terminalia* spp. known for high antioxidant contents and anticancer activity was used. In this, when cells were exposed to *Terminalia* spp., extracts showed considerably elevated Caspase 3 activity in Caco-2 cells indicating apoptosis induction [60].

Hep G2 cells treated with a medicinal polysaccharide, Lentinan (LNT) isolated from shiitake, expressed high levels of caspase-3 and 9 mRNA [61].

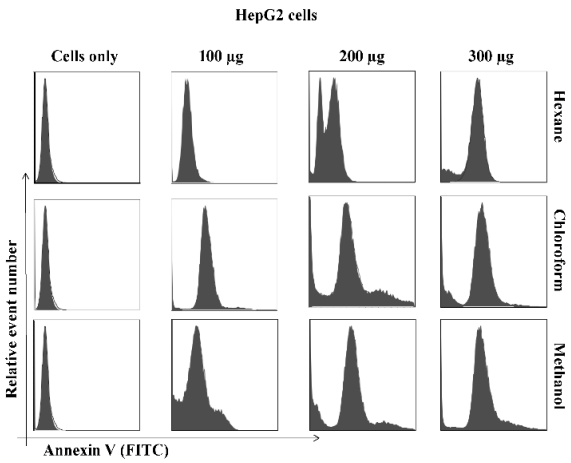
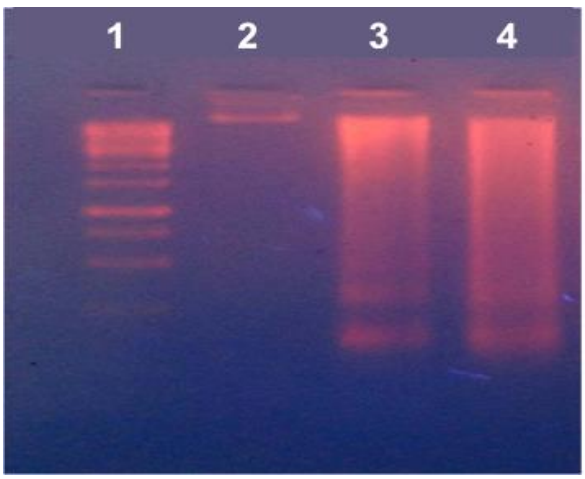


Figure 2. Flow cytometric determination of the effect of *Cassia fistula* extracts on apoptosis of Hep G2 cells



Lane 1 - 1kb DNA ladder,
Lane 2 - DNA from untreated cells,
Lane 3 - DNA from cells treated with Chloroform extract (300µg),
Lane 4 - DNA from cells treated with Chloroform extract (100µg)

Figure 3. DNA fragmentation assay

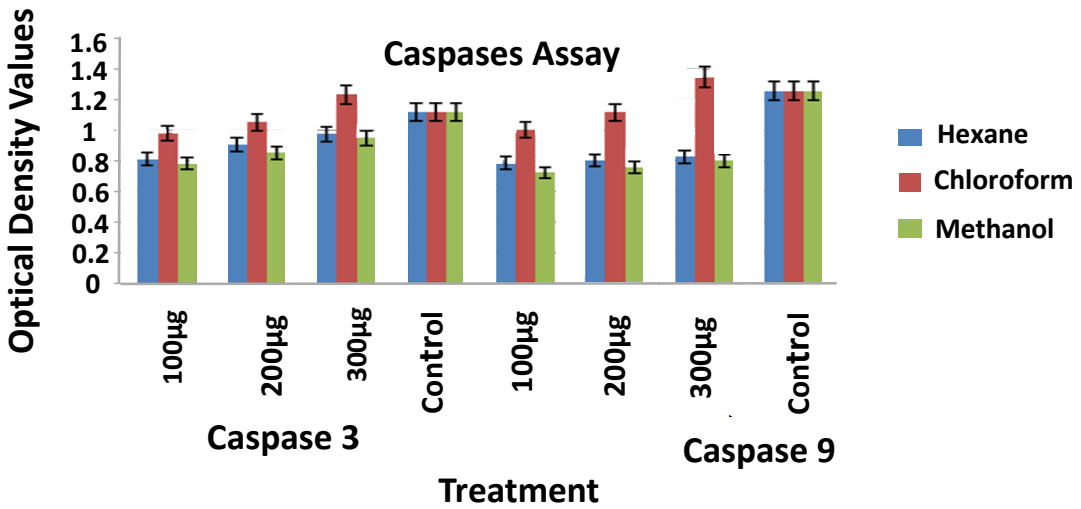


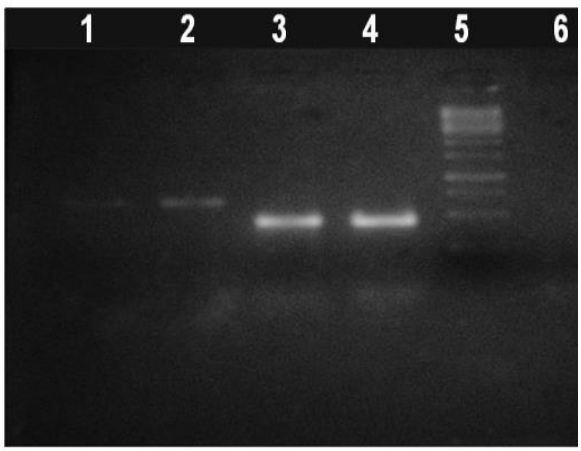
Figure 4. Graphical representation of CASPASEs quantification

Reverse Transcriptase PCR

RT-PCR is a technology by which RNA molecules are converted into their complementary DNA (cDNA) sequences by reverse transcriptase, followed by amplification of the newly synthesized cDNA by standard PCR procedures. This approach of studying gene expression is universally known as RT-PCR, due to the role of reverse transcriptase (RT) in the synthesis of first-strand cDNA [62]. Bcl-2 is a central player in the genetic program of eukaryotic cells favoring survival by inhibiting cell death. Over-expression of Bcl-2 protein has been reported in many types of human cancers, including leukemias, lymphomas, and carcinomas [15]. Bcl-2 is an anti-apoptotic gene involved in an evolutionary conserved pathway crucial to apoptosis and programmed cell death. Bcl-2 blocks the mitochondrial release of cytochrome c and inhibits the activation of CASPASE 9 by the cytoplasmic scaffolding protein Apaf-1. In the current study, cells were harvested after treatment with different concentrations of *C. fistula* leaf extracts. From this RNA was isolated and cDNA was synthesized. Taking cDNA as a template, PCR was carried out with Bcl-2 and GAPDH gene specific primers. Bcl-2 gene expression was observed to be low in cells treated with chloroform extract of *C. fistula* leaves suggesting the involvement of mitochondrial proteins in the apoptosis induced in HepG2 cells by the extracts (Figure 5). GAPDH was taken as internal control for PCR and equal total RNA of each sample (with and without treatment) for PCR was confirmed by GAPDH bands which were equally intense. These results were comparable to an earlier study investigating the anti-proliferative effects of methanolic extract of *F. pseudalliacea* against human colon cancer HCT-116 cell line. When HCT- 116 cells were treated with *F. pseudalliacea*, gene and protein expression of BAX increased whereas Bcl-2 decreased [63]. Similarly, the activity of NF-κB gene expression of Hep G2 cells treated with mulberry leaf extract was suppressed when compared to the control. It was also observed that the levels of AFP, γ-GT and ALP was decreased in Hep G2 cells compared to control in a time dependent manner. The mulberry leaf extracts increased the secretion of ALB [64].

Database search and text mining

Bcl-2 protein interacts functionally with other important proteins in the cell. The primary interactions were depicted in Figure 6. Bcl-2 protein of *Mus musculus* mainly interacted with apoptosis, autophagy, oncogene and nuclear receptor proteins. In addition, it had interactions with other Bcl-2 proteins like agonist, antagonist and interacting killer types. Thus, the protein-protein interaction network of Bcl-2 protein clearly depicted that the Bcl-2 protein interacts with other proteins and plays a vital role in apoptosis. The co-occurring pair AKT-MGMT and CASPASE3 - cell death depicted induction of apoptosis. The occurrences were fetched from two PubMed abstracts with IDs 21405945 and 17201177 respectively. A protein-protein network indicating the predicted functional partners for Bcl-2 proteins in *Mus musculus* was constructed and tabulated (Table 4). By querying the PubMed abstracts tagged with *Cassia fistula*, it was noted that the apoptotic induction property was substantiated for the above plant.



Lane 1: Expression of Bcl2 from treated cancer cell
Lane 2: Expression of Bcl2 from undertreatment cancer cell
Lane 3: Expression of GAPDH from cancer cell
Lane 4: Expression of GAPDH from undertreatment cancer cell
Lane 5: 1 Kb DNA ladder
Lane 6: Negative Control

Figure 5. RT-PCR of Bcl-2

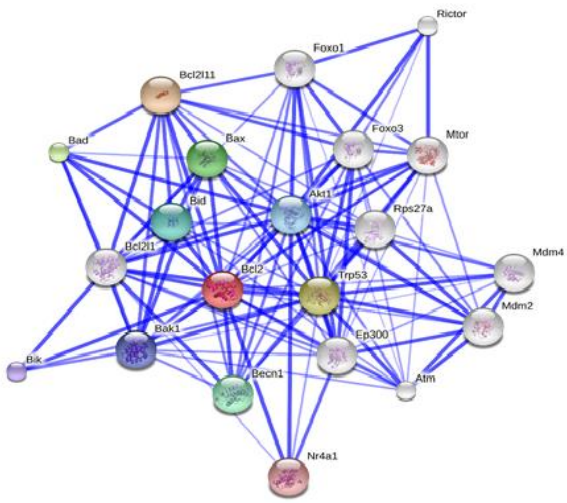


Figure 6. Protein-protein interaction network of Bcl-2 protein in *Mus musculus*

Table 3. CASPASEs quantification after treatment with plant extracts

	CASPASE 3				CASPASE 9			
Extract	Control	100 µg	200 µg	300 µg	Control	100 µg	200 µg	300 µg
Hexane	1.117	0.809	0.902	0.971	1.254	0.785	0.801	0.825
Chloroform	1.117	0.978	1.049	1.232	1.254	1.002	1.114	1.345
Methanol	1.117	0.781	0.851	0.948	1.254	0.721	0.755	0.798

Table 4. Predicted functional partners by STRING database

String ID	Protein Function
Bcl2l11	BCL2-like 11 (apoptosis facilitator); Induces apoptosis and anoikis
Trp53	transformation related protein 53; Acts as a tumor suppressor in many tumor types
Bad	BCL2-associated agonist of cell death; Promotes cell death.
Bax	BCL2-associated X protein; Accelerates programmed cell death by binding
Becn1	Autophagy related; Plays a central role in autophagy. Required for the abcission step
beclin 1	BH3 interacting domain death agonist; Induces caspases and apoptosis
Bid	thymoma viral proto-oncogene 1; AKT1 is one of 3 closely related serine/threonine- protein kinase
Akt1	BCL2-antagonist/killer 1; In the presence of an appropriate stimulus
Bak1	BCL2-interacting killer; Accelerates programmed cell death. Binding to the apoptosis repressors
Bik	BCL2-antagonist/killer 1; In the presence of an appropriate stimulus, accelerates programmed
Nr4a1	nuclear receptor subfamily 4, group A, member 1; Orphan nuclear receptor

Conclusion

The present work is an attempt at exploring the potential of natural resources for the development of drugs for illnesses with a hitherto unknown cure. Secondary metabolites from plants have been known for ages for their potent medicinal and therapeutic values. Herbs like *Cassia fistula* have been a part of the traditional medicine in India. The anti-cancer activity, cytotoxicity and apoptotic potential of leaf extract of this ethno medicinal plant was elucidated against hepatic cancer cell line. DNA laddering, up regulation of CASPASEs and down regulation of Bcl-2 confirmed apoptosis as the mechanism of phytochemical action. Further research on the bioactive principles contributing to its medicinal property along with its structure elucidation, detailed mechanism of action and *in silico* analysis is required. Thus, *Cassia fistula* could be a promising agent for anti-cancer phytotherapy to be used as a combinatorial treatment in conjunction with other cancer-targeted therapies for chemoprevention.

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Conflict of interest

The authors declare that there is no conflict of interest.

References

[1] Grzanna R, Lindmark L, Frondoza CG (2005) Ginger- a herbal medicinal product with broad anti-inflammatory actions. J Med Food. 8 (2): 125 - 132. https://doi.org/10.1089/jmf.2005.8.125

[2] Gupta E, Purwar S, Sundaram S, Rai GK (2013) Nutritional and therapeutic values of *Stevia rebaudiana*: a review. J Med Plants Res. 7 (46): 3343-3353. https://doi.org/10.1089/jmf.2005.8.125

[3] Ranasinghe RLDS, Ediriweera ERHSS (2017) Medicinal and nutritional values of *Macrotyloma uniflorum* (Lam.) Verdc (Kulattha): a conceptual study. Glob J Pharmaceu Sci. 1 (2): 555559. http://dx.doi.org/10.19080/GJPPS.2017.01.555559

[4] Dikshit M, Samudrasok RK (2011) Nutritional evaluation of outer fleshy coat of *Terminalia catappa* fruit in two varieties. Int. J. Food Sci. Nutr. 62 (1): 47-51. http://dx.doi.org/10.19080/GJPPS.2017.01.555559

[5] Gebreyohannes G, Gebreyohannes M (2013) Medicinal values of garlic: a review. Int J Med Med Sci. 5 (9): 401 - 408. https://doi.org/ 10.5897/IJMMS2013.0960

[6] Petrovska BB (2012) Historical review of medicinal plant's usage. Pharmacogn Rev. 11, 1-5. https://doi.org/ 10.5897/IJMMS2013.0960

[7] Jain S, Dwivedi J, Jain PK, Satpathy S, Patra A (2016) Medicinal plants for treatment of cancer: a brief review. Pharmacogn J. 8 (2): 87 - 102. https://doi.org/ 10.5897/IJMMS2013.0960

[8] Kumar S, Shukla YN, Lavania UC, Sharma A, Singh AK (1997) Medicinal and aromatic plants: prospects for India. J Med Arom Pl Sc. 19 (2): 361 - 365.

[9] Siegel RL, Miller KD, Jemal ADVM (2018) Cancer statistics, 2018. CA Cancer J Clin. 68: 7 - 30. https://doi.org/10.3322/caac.21442

[10] Cory S, Adams JM (2002) The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev. 2 (9): 647 - 656. https://doi.org/10.1038/nrc883

[11] Torre LA, Bray F, Siegel RL, Ferlay J, Lortet- Tieulent J, Jemal A (2015) Global cancer statistics, 2012. CA Cancer J Clin. 65 (2): 87 - 108. https://doi.org/10.1038/nrc883

[12] Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D (2011) Global cancer statistics. CA: Cancer J Clin. 61 (2): 69 - 90. https://doi.org/10.3322/caac.20107

[13] Baba AL, Câtoi C (2007) Comparative oncology.The publishing house of the Romanian Academy, Bucharest, pp. 263 - 381.

[14] Srivatanakul P, Sriplung H, Deerasamee S (2004) Epidemiology of liver cancer: an overview. Asian Pac J Cancer Prev. 5 (2):118 - 125. https://dx.doi.org/10.1016%2Fj.jceh.2014.05.013

[15] Sanchez-Beato M, Sanchez-Aguilera A, Piris MA (2003) Cell cycle deregulation in B-cell lymphomas. Blood. 101 (4): 1220 - 1235. https://doi.org/10.1182/blood-2002-07-2009

[16] Bahorun T, Neergheen VS, Aruoma OI (2005) Phytochemical constituents of *Cassia fistula*. Afr. J. Biotechnol. 4 (13): 1530 - 1540. https://doi.org/10.4172/2329-6836.C1.008

[17] Kushawaha M, Agrawal RC (2012) Biological activity of medicinal plant *Cassia fistula*-a review. J Sci Res Pharm. 1 (3): 7 - 11. https://doi.org/ 10.13140/RG.2.2.18492.44167

[18] Kumar JG, Patel N, Sahoo P, Gupta A, Gunjan M (2010) Antipyretic potential of different extracts of *Cassia fistula* linn (Fabaceae) bark. *Int* J Res Ayurveda Pharm. 1 (2): 634 - 636.

[19] Jerald EE, Joshi SB, Jain DC, Edwin S (2013) Biochemical evaluation of the hypoglycemic effects of extracts and fraction of *Cassia fistula* Linn. Alloxan-induced diabetic rats. Indian J Pharm Sci. 75 (4): 427 - 434. https://doi.org/10.4103/0250-474X.119823

[20] Daisy P, Balasubramanian K, Rajalakshmi M Eliza J, Selvaraj J (2010) Insulin mimetic impact of catechin isolated from *Cassia fistula* on the glucose oxidation and molecular mechanism of glucose uptake on streptozotocin-induced wistar rats. Phytomedicine. 17 (1): 28 - 36. https://doi.org/10.1016/j.phymed.2009.10.018

[21] Einstein JW, Rais MM, Mohd MA (2012) Comparative evaluation of the antidiabetic effects of different parts of *Cassia fistula* Linn, a Southeast Asian Plant. J Chem.1 - 10. https://doi.org/10.1016/j.phymed.2009.10.018

[22] Yadav R, Jain GC; (1995) Antifertility effect of aqueous extract of seeds of *Cassia fistula* in female rats. Adv Contracept. 15: 293 - 301. https://doi.org/ 10.1023/A:1006784224191

[23] Siddhuraju P (2002) Studies on the antioxidant activity of Indian Laburnum (*Cassia fistula* L.): a preliminary assessment of crude extracts from stem bark, leaves, flowers and fruit pulp. Food Chem. 79: 61 - 67. https://doi.org/10.1016/S0308-8146(02)00179-6

[24] Sartorelli P, Carvalho CS, Reimão JQ, Ferreira MJP, Tempone AG (2009) Antiparasitic activity of biochanin A, an isolated isoflavone from fruits of *Cassia fistula* (Leguminosae). Parasitol Res. 104: 311 - 314. https://doi.org/10.1007/s00436-008-1193-z

[25] Sharma A, Laxmi V, Goel A, Sharma V, Bhatia AK (2010) Anti-viral activity of *Cassia fistula* against IBR virus. J Immunol Immunopathol. 12: 114 - 119.

[26] Bhalodia NR, Shukla VJ (2011) Antibacterial and antifungal activities from leaf extracts of *Cassia fistula*: an ethnomedicinal plant. J Adv Pharm Technol Res. 2: 104 - 109. https://doi.org/10.1007/s00436-008-1193-z

[27] Irshad M, Ahmad A, Zafaryab M, Ahmad F, Manzoor N, Singh M (2013) Composition of *Cassia fistula* oil and its antifungal activity by disrupting ergosterol biosynthesis. Nat Prod Commun. 8 (2): 261 - 264.

[28] Irshad M, Zafaryab M, Singh M, Rizvi, MMA (2012) Comparative analysis of the antioxidant activity of *Cassia fistula* extracts. Int J Med Chem. 2012: 1 - 6. http://dx.doi.org/10.1155/2012/157125

[29] Gupta M, Mazumder UK, Rath N, Mukhopadhyay DK (2000) Antitumor activity of methanolic extract of *Cassia fistula* L. seed against Ehrlich Ascites Carcinoma. J Ethnopharmacol. 72 (1): 151 - 156. http://dx.doi.org/10.1155/2012/157125

[30] Harborne JB (1973) Phytochemical methods: a guide to modern techniques of plant analysis. Chapman and Hall, New York, 3: 279 - 302.

[31] Mossman T (1983) Rapid colorimetric assay for cellular growth and survival-application to proliferation and cytotoxicity assays. J Immunol Methods. 65: 55 - 63. http://dx.doi.org/10.1016/0022-1759(83)90303-4

[32] Gavrieli Y, Sherman Y, Ben-Sasson SA (1992) Identification of programmed cell death *in situ* via specific labelling of nuclear DNA fragmentation. J *Cell* Biol.119 (3): 493 - 501. http://dx.doi.org/10.1016/0022-1759(83)90303-4

[33] Lowry OH, Rosenbrough NJ, Farr A. Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem. 193: 265 - 275.

[34] Von Mering C, Huynen M, Jaeggi D, Schmidt S, Bork P (2003) Snel B STRING. A database of predicted functional associations between proteins. Nucleic Acids Res. 31: 258 - 261. http://dx.doi.org/10.1093/nar/gkg034

[35] Barbosa-Silva A, Fontaine JF, Donnard ER, Stussi F, Ortega JM, Andrade-Navarro MA (2011) PESCADOR: A web-based tool to assist text mining of biointeractions extracted from PubMed queries. BMC. Bioinformatics. 12: 435. http://dx.doi.org/10.1093/nar/gkg034

[36] Singh RP, Agarwal R (2006) Mechanisms of action of novel agents for prostate cancer chemoprevention. Endocr Relat Cancer. 13 (3) : 751 - 778. https://doi.org/10.1677/erc.1.01126

[37] Kelloff GJ, Crowell JA, Steele VE, Lubet RA, Boone CW, et al. (1999) Progress in cancer chemoprevention. Ann N Y Acad Sci. 889: 1 - 13. https://doi.org/10.1093/jn/130.2.467S

[38] Agarwal R (2000) Cell signaling and regulators of cell cycle as molecular targets for prostate cancer prevention by dietary agents. Biochem Pharmacol. 60 (8): 1051 - 1059. https://doi.org/10.1016/S0006-2952(00)00385-3

[39] Singh RP, Dhanalakshmi S, Tyagi AK, Chan DC, Agarwal C, et al. (2002) Dietary feeding of silibinin inhibits advance human prostate carcinoma growth in athymic nude mice and increases plasma insulin-like growth factor-binding protein-3 levels. Cancer Res. 62 (11): 3063 - 3069.

[40] Park OJ, Surh YJ (2004) Chemopreventive potential of epigallocatechin gallate and genistein: evidence from epidemiological and laboratory studies. Toxicol Lett. 150 (1): 43 - 56. https://doi.org/10.1016/j.toxlet.2003.06.001

[41] Sarkar FH, Li Y (2004) The role of isoflavones in cancer chemoprevention. Front Biosci. 9: 2714 - 2724.

[42] Tsuda H, Ohshima Y, Nomoto H, Fujita K, Matsuda E, et al. (2004) Cancer prevention by natural compounds. Drug Metab Pharmacokinet. 19 (4): 245 - 263.

[43] Bektic J, Guggenberger R, Eder IE, Pelzer AE, Berger AP, et al. (2005) Molecular effects of the isoflavonoid genistein in prostate cancer. Clin Prostate Cancer. 4 (2): 124 - 129. https://doi.org/10.3816/CGC.2005.n.021

[44] Shishodia S, Amin HM, Lai R (2005) Curcumin (diferuloyl methanol) inhibits constitutive NF-kappaB activation, induces G1/S arrest, suppresses proliferation, and induces apoptosis in mantle cell lymphoma. Biochem Pharmacol. 70 (5): 700 - 713. https://doi.org/10.1016/j.bcp.2005.04.043

[45] Wasu JS, Muley PB (2009) Hepatoprotective effect of *Cassia Fistula* Linn. Ethnobota Leaflets. 13 (9):10-16.

[46] Javanraedi J, Stushnoff C, Locke E, Vivanco JM (2003) Antioxidant activity and total phenolic content of Iranian *Ocimum* accessions. Food Chem. 83: 547 - 550. https://doi.org/10.1016/S0308-8146(03)00151-1

[47] Bhakta T, Mukherjee PK, Mukherjee K, Banerjee S, Mandal SC et al. (1999) Evaluation of hepatoprotective activity of *Cassia fistula* leaf extract. J Ethnopharmacol. 66: 277 - 282. https://doi.org/10.1016/S0378-8741(98)00220-7

[48] Duraipandiyan V, Baskar AA, Ignacimuthu S, Muthukumar C, Al-Harbi NA (2012) Anticancer activity of Rhein isolated from *Cassia fistula* L. flower. Asian Pac J Trop Dis. 2 (1): S517 - S523. https://doi.org/10.1016/S2222-1808(12)60213-8

[49] Tawfek NS, Al Azhary DB, Hussien BKA, (2015) Effects of *Cassia fistula* and *Ficus carica* leaf extracts on hepatocarcinogenesis in rats. Middle East J Appl Sci. 5 (2): 462 - 479. https://doi.org/10.1016/j.cbi.2006.12.011

[50] Anbukkarasi M**,** Dhamotharan R, Janarthanam B **(**2017) Studies on phytochemical screening, tannins content and antibacterial activity from leaf and callus extracts of *Memecylon Umbellatum.* Asian J Pharm Clin Res. 10(5): 265 - 269. http://dx.doi.org/10.22159/ajpcr.2017.v10i5.15198

[51] Sakander H, Bhat A, Raveesha KA (2015) Evaluation of antifungal potential of selected medicinal plants against human pathogenic fungi. Int J Green Pharm. 9 (2): 110 - 117.

[52] Mossman T (1983) Rapid colorimetric assay for cellular growth and survival application to proliferation and cytotoxicity assays. J Immunol Methods. 65: 55 - 63. http://dx.doi.org/10.1016/0022-1759(83)90303-4

[53] Polu PR, Nayanbhirama U, Khan S, Maheswari R (2017) Assessment of free radical scavenging and anti-proliferative activities of *Tinospora cordifolia* Miers (Willd). BMC Complement Altern Med. 17 (1): 457. https://doi.org/10.1186/s12906-017-1953-3

[54] Momtazi AA, Askari-Khorasgani O, Abdollahi E, Sadeghi-Aliabadi H, Mortazaeinezhad

[55] F, Sahebkar A (2017) Phytochemical analysis and cytotoxicity evaluation of *Kelussia odoratissima* Mozaff. J Acupunct Meridian Stud. 10 (3): 180 - 186. https://doi.org/10.1016/j.jams.2017.02.002

[56] Mishra T, Khullar M, Bhatia A (2011). Anticancer potential of aqueous ethanol seed extract of *Ziziphus mauritiana* against cancer cell lines and Ehrlich ascites carcinoma. Evid. -Based Complementary Altern. Med. 2011: 1 - 11. http://dx.doi.org/10.1155/2011/765029

[57] Tsang W, Kwok T (2010). Epigallocatechin gallate up-regulation of miR-16 and induction of apoptosis in human cancer cells. J Nutr *Biochem.* 21 (2): 140 - 146. http://dx.doi.org/10.1155/2011/765029

[58] Kamalanathan D, Natarajan D (2018) Anticancer potential of leaf and leaf-derived callus extracts of *Aerva javanica* against MCF-7 breast cancer cell line. J Cancer Res Ther. 14 (2): 321 - 327. https://doi.org/10.4103/0973-1482.171210

[59] Kulkarni A, Govindappa M, Ramachandra YL, Koka P (2015) GC-MS analysis of methanol extract of *Cassia fistula* and it's *in vitro* anticancer activity on human prostate cancer cell line. Indo Am J Pharm Res. 5 (2): 937 - 944.

[60] Mohammed FA, Elkady AI, Syed FQ, Mirza MB, Hakeem KR, Alkarim S (2018) *Anethum graveolens* (dill)-a medicinal herb induces apoptosis and cell cycle arrest in HepG2 cell line. J Ethnopharmacol. 219:15 - 22. https://doi.org/10.1016/j.jep.2018.03.008

[61] Shalom J, Cock IE (2018) *Terminalia ferdinandiana* Exell. fruit and leaf extracts inhibit proliferation and induce apoptosis in selected human cancer cell lines. Nutr cancer. 11: 1 - 15. https://doi.org/10.1080/01635581.2018.1460680

[62] Sun Z, Han Q, Duan L, Yuan Q, Wang H (2018) Oridonin increases anticancer effects of lentinan in HepG2 human hepatoblastoma cells. Oncol Lett. 15 (2): 1999 - 2005. https://doi.org/10.3892/ol.2017.7485

[63] Farrell, RE (2010). RNA methodologies. A laboratory guide for isolation and characterization, 4th ed., Elsevier Inc., USA., pp. 1 - 717.

[64] Bamehr H, Saidijam M, Dastan D, Amini R, Pourjafar M, Najafi R (2018) *Ferula pseudalliacea* induces apoptosis in human colorectal cancer HCT-116 cells via mitochondria-dependent pathway. Arch Physiol Biochem. 1 - 8. https://doi.org/10.1080/13813455.2018.1455710

[65] Fathy SA, Singab ANB, Agwa SA, El Hamid DM A, Zahra FA, et al. (2013) The antiproliferative effect of mulberry (*Morus alba* L.) plant on hepatocarcinoma cell line HepG2. EJMHG. 14 (4): 375 - 382. https://doi. org/10.1016/j.ejmhg.2013.07.001