Toxicology Reports 7 (2020) 443–452

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Phytochemical analysis and *in vitro* cytostatic potential of ethnopharmacological important medicinal plants

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ARTICLE INFO

Keywords:
Annona reticulate
Euphorbia hirta
Hibiscus sabdariffa
Lablab purpureus
Moringa oleifera
Murraya koenigii

ABSTRACT

Annonareticulate (Mart.), *Lablab purpureus* (L.) Sweet, *Murrayakoenigii* (L.) Spreng, *Moringaoleifera* (Lam.), *Hibiscussabdariffa* (L.) and *Euphorbiahirta* (L.) are most commonly used medicinal plants by the traditional healers of Karbi Anglong district of Assam, India against different human ailments including cancer suspected cases. The proposed study includes field survey related to ethnomedicinal aspects of medicinal plants, phytochemical analysis, and evaluation of their cytostatic potential with the possible mode of action against Dalton's lymphoma (DL) cell line. The phytochemical analysis of all the plant's extract was studied using standard protocol. The cytotoxicity of the methanol extracts was determined by MTT reduction assay. The effect of the same extract was also tested for development of apoptosis features in DL cells using a fluorescence microscope and flowcytometry. The underlying mechanism closely associated with apoptotic cell death was also studied by measuring reactive oxygen species (ROS), mitochondrial membrane potential, and expression level of apoptosis inducing proteins. *Murraya koenigii* induced more apoptotic features in DL cells, followed by *Annona reticulate*. The decrease in mitochondrial membrane potential, release of cytochrome- c, increase in ROS level and higher expression of caspases (3 and 9) after plant extracts treatment may cause involvement of mitochondria in the process of apoptosis. From this study, it can be concluded that the plant species mainly *Murraya koenigi* and *Annona reticulate* significantly induced cytotoxicity in DL cells through apoptosis by utilizing mitochondrial pathway.

1. Introduction

Cell cycle regulatory proteins control the entire process of cell proliferation to maintain the correct shape and size in the animals. Sometimes, it has been found that failure in the regulatory mechanisms may lead a cell to divide for an indefinite period and finally lead to develop into cancer [1]. Cancer is a serious health burden and responsible for second leading cause of deathworld wide. In the recent time, its treatment is among the most challenging task due to involvement of several factors during carcinogenesis. As per the recent report released by the World Health Organization (WHO) in 2003, cancer rates could further increase by 50 % to 15 million during the year 2020 [2].

At present time, there are three major methods of cancer treatment namely surgery, radiotherapy and chemotherapy; each treatment modality has a specific role in the treatment or it can be used alone or in combination with others [3]. Chemotherapy is a major treatment option against various type of cancer involving different stages which involves the systemic use of the chemical drug(s) to stop or regulate the cancer cells multiplication.

Reporting of traditional knowledge with full details and their experimental validation against human and domestic animals are an essential part of ethnopharmacology [4,5]. It may help in the management, sustainable utilization and propagation of natural resources for future use. It is reported that more than 80 % of the world's population depends mainly on complementary and alternative medicines, and approximately 60 % of the clinical drugs are prepared from natural resources [6]. It is well documented that cancer natural products derived chemotherapeutic agents or their synthetic analogs occupy crucial position because of limited or no side effects and anti-multidrug resistance [7] in the host. Some potent plant-derived anticancer drugs like vincristine, vinblastine, irinotecan, etoposide, paclitaxel, camptothecin,

Abbreviations: DL, Dalton's ascites lymphoma; ROS, reactive oxygen species; MTT, 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide; ΔΨm, mitochondrial membrane potential; RFC, Relative frequency of citation; FL, fidelity level; DPPH, 2,2-diphenyl-1-picrylhydrazyl; OD, Optical density; IU, International unit; PBMC, peripheral blood mononuclear cell; AO/EB, acridine orange and ethidium bromide

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https://doi.org/10.1016/j.toxrep.2020.02.016
Received 6 August 2019; Received in revised form 11 February 2020; Accepted 24 February 2020
Available online 25 February 2020

and epipodophyllotoxin are being presently used in cancer management [8]. It has been reported that there are 121 drugs currently being used for the cancer treatment at different degree of success of which 90 are derived from the plant sources and 74 % of these drugs were discovered mainly based on traditional practices and beliefs [9].

In terms of the wealth of traditional knowledge, India ranks top in Asia related to plants and animals derived medicine and their applications for the treatment of different ailments (2000 species), Siddha (1121 species), Unani (751 species) and Tibetan (337 species). Studies performed over the past 15 years have demonstrated that virtually all the agents currently utilized to treat cancer can induce apoptosis [10,11]. Morphologically, this process is characterized by plasma membrane blebbing, cell shrinkage, and chromatin condensation followed by disassembly of the cells into multiple membrane-enclosed apoptotic bodies, which are then engulfed by body's phagocytic cells [11]. This biochemical mechanism of cell death is executed by the activation of caspases, a family of cysteine-dependent aspartate-directed proteases. The two major pathways, one starting with the interaction of receptor-ligand (death receptors) and the other is associated with the release of cytochrome c from the damaged mitochondrial membrane result in the transduction of signals which ultimately activates initiator and executioner class of caspases [12].

Thus, the present study was carried out with an objective to document and experimentally validate the most commonly used anticancer medicinal plants by the traditional healers of Karbi Anglong region of Assam against murine malignant DL cell line. In this study, an attempt was made to report phytochemicals compositions of different medicinal plants, their cytostatic potential and anticancer activity with a possible mode of action using apoptosis as a significant parameter.

2. Materials and methods

2.1. Plants extraction and dose preparation

All the plant specimens (Fig. 1) were collected during their flowering or fruiting seasons, and the voucher specimens were deposited for preservation in the herbarium of Cotton University, Assam. Dr. J. Arjun made the precise identification of plants mentioned by the informants and the scientific names of plants were also compared with the available herbarium in Botanical Survey of India, Shillong. The scientific name of plants was further confirmed by National Knowledge and Information System for Biological Species (NKISBS): Scientific names of plants. http://www.nature.go.kr:9001/index.do.

Fresh plants of *Annona reticulate* (leaves), *Lablab purpureus* (apical tendril), *Murraya koenigii* (leaves), *Moringa oleifera* (leaves), *Hibiscus sabdariffa* (fruits) and *Euphorbia hirta* (leaves) were collected from Barlongfer area of Karbi Anglong district of Assam, India. The leaves/fruits/tendrils of plants were washed; shade dried, pulverized in a blender and kept in an airtight container. The dried and pulverized plant materials (leaves/fruits/tendrils) were defatted using petroleum ether for 30 min, followed by Soxhlet extraction with methanol (100 %). After two days of extraction the extract was filtered with cheesecloth, then with Whatman No one filter paper (26 m) and finally centrifuged at 5000 RPM for 30 mints. Then, filtrates were concentrated *in vacuo* using Rotary Evaporator (Model: 8911. DVS.199, Equitron Medica Private Limited, Mumbai, India) at 40 °C for 30 – 45 min. The condensed form of crude extract was collected, and the yield of crude extract was calculated. For dose preparation, methanol extract of different plants was dissolved in 1 ml of Dimethyl sulphoxide (DMSO) to prepare the stock solution (10 mg/ml). Further, six different dosages (100, 200, 300, 400 and 500 µg/mL) of each plant were prepared in PBS (P^H: 7.4) for *in vitro* anticancer study.

2.2. Phytochemical study

Preliminary phytochemicals analysis of all the selected plants were performed to detect the presence of plants secondary metabolites of biological importance such as alkaloids, flavonoids, terpenoids, steroids, carbohydrates, proteins, fats, and saponins, etc. All the experiments were carried out by using the standard methods mentioned by Yadav and Agarwala, and Harborne [13,14].

2.3. Cell line and treatment plan

The anticancer activities of all five plants were carried out in *in vitro* condition for 24 h using Dalton's Ascites Lymphoma (DL) cell line. The DL cells were cultured in RPMI-1640 supplemented with 10 % FBS, gentamycin (20 mg/mL), streptomycin (100 mg/mL), and penicillin (100 IU) in a CO_2 incubator at 37 °C with 5% CO_2; 80 % confluent of exponentially growing cells was sub-cultured and used in the

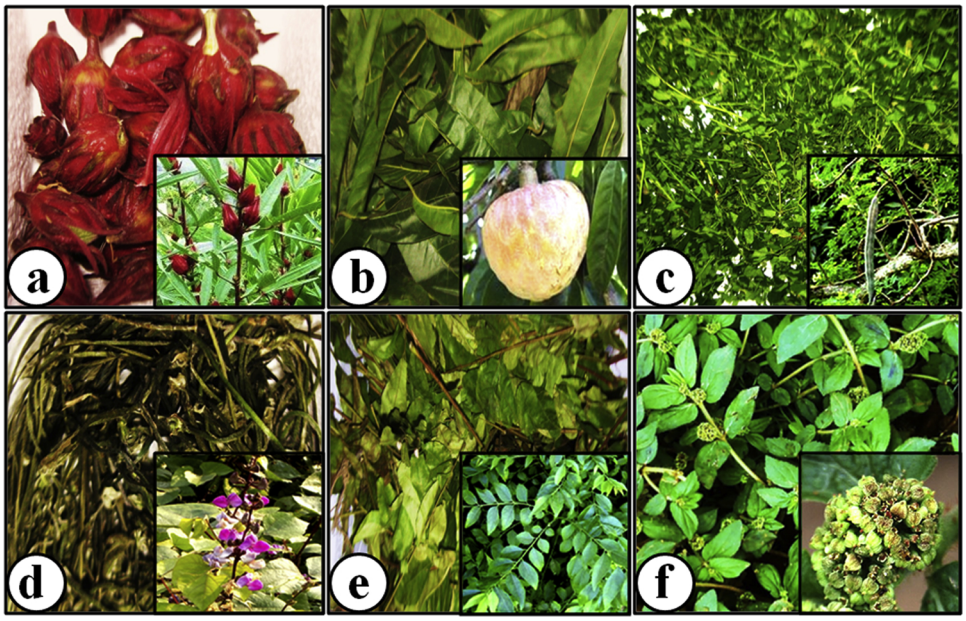

Fig. 1. Photographs are showing the pictures of most commonly used medicinal plants for the treatment of cancer by the traditional healers of Karbi Anglong district of Assam, India. a. *Hibiscus sabdariffa,* b. *Annona reticulate,* c. *Moringa oleifera,* d. *Lablab purpureus,* e. *Murraya koenigii* and f. *Euphorbia hirta.*

experiments. In 1 mL of RPMI-1640 having 10 % FBS, 1×10^6 cells/well were plated in six-well plate overnight in a CO_2 incubator. After 24 h, the condition media were added with six different doses of different plants extract (0, 100, 200, 300, 400 and 500 µg/mL) for *in vitro* anticancer study along with cisplatin as positive reference drug.

2.4. MTT cell proliferation assay

The MTT based cell viability study was performed in DL (cancer cell) and peripheral blood mononuclear cells (PBMC) (normal cell) [15]. 300000 DL cells per 300 µL 1×10^6 cells per mL) in triplicate was taken in a 96-well plate(s), tissue culture grade, flat bottomed, sterile (ThermoFisher Scientific, Waltham, Massachusetts, USA) followed by incubation with different doses (0, 100, 200, 300, 400 and 500 µg/mL) of plants extracts for 24 h, 10 µl of the MTT labeling reagent (0.5 mg/mL) was added into each 96-well plate. The plate was then incubated for four hours in a humidified atmosphere, at 37 °C and 5% CO_2. Following that, 100 µl of the solubilizing solution (DMSO) was added into each well. The plate was checked for complete solubilization of the purple formazan crystals followed by optical density measurement at the wavelength of 550 nm using Elisa Microplate Reader (Manufacturer: Rapid Diagnostics; SKU, LISA-R, India). The percentage of cell viability was calculated with the following formula:

$$\text{Cell viability}(\%) = \frac{\text{OD of Sample} - \text{OD of Blank}}{\text{OD of Control}} \text{X} 100$$

A dose-response curve (% cell viability versus sample concentration) was plotted, and the sample concentration that inhibits 50 % of the cell viability (IC_{50}) was determined. After IC_{50} calculation, range of different IC_{50} i.e. ~500 µg/mL dose was used for further experimental works.

2.5. Apoptosis study

Plants extract mediated apoptotic induction was detected by acridine orange and ethidium bromide (AO/EB) dual staining method [16]. Control and treated DL cells were collected after 24 h treatment, washed with PBS and to the cell suspension; AO/EB (100 µg/mL of each dye) was added, mixed gently and incubated for 5 min. The cells were thoroughly examined under a fluorescence microscope and photographed. About 1000 cells were analyzed, and the percentage of apoptotic nuclei was determined. Bright uniform green nuclei with organized structures were identified as viable cells whereas, apoptotic cells were identified by condensed or fragmented chromatin with red or orange nuclei [17].

2.6. Cell cycle analysis

After treatment with different plant extracts for 24 h, DL cells were centrifuged (1000xg) and washed twice with chilled PBS, and the cells were resuspended in PBS, fixed with 70 % ethanol at 4 °C. To the cell suspension, 10 µl/mL of 10 mg/mL RNase A solution (DNase free, Sigma-Aldrich) was added anf further stained by propidium iodide solution (50 µl/mL, stock: 1 mg/mL) [18] in dark chamber. The cell was incubated at 37 °C for 20 min and the fluorescence from 20,000 events was determined by a BD FACS Calibur machine (Model FACSC420). The populations of cells in the G0/G1, S, and G2/M phases were quantified using BD FACS Diva (v.6.1.2, BD Biosciences, San Jose, CA) software. Apoptotic cells were represented by a sub G0/G1 population seen to the left of the G0/G1 peak [19].

2.7. Mitochondrial membrane potential ($\Delta\Psi m$)

To study changes in mitochondrial membrane potential, DL cell was collected after 24 h treatment, washed twice with PBS and approximately 1×10^7 cells were incubated with cationic lipophilic dye, Rhodamine-123 (1 µM, final concentration) for 10 min [20] and photographed using Confocal microscope. Cells with green fluorescence reflecting high-polarized (bright) and low-polarized (dim) mitochondria were quantified and an average fluorescent intensity was analyzed in all groups with ImageJ 1.x freeware software for Windows (NIH, USA).

2.8. Reactive oxygen species (ROS) measurement

For ROS assessment, nitroblue tetrazolium (NBT) quantification assay was used as described by Choi et al. [21]. Treated and untreated (control) DL cells (1×10^6 cells) were incubated with NBT (1.6 mg/ml) at 37 °C for 45 min. After incubation, the formazan precipitates were dissolved in 560 µl 2 M KOH and 480 µl DMSO. The amount of reduced NBT (O_2^- production) was quantified by determining the absorbance at 630 nm.

2.9. Western blot analysis of apoptosis-related proteins

Total protein samples were isolated from cell lysates using RIPA buffer (Beyotime Biotechnology, Jiangsu, China) as per the manufacturer's instructions. Protein concentrations were determined using the Lowry method. Equal amounts of protein (30 µg) were loaded into each well and were separated using SDS-PAGE, followed by transfer to a polyvinylidene fluoride membrane. The membranes were then blocked with 5% skim milk solution, followed by overnight incubation on a shaker at 4 °C with the primary antibody against mitochondrial and cytosolic cytochrome- c, caspase-3, -8 and - 9. The membranes were probed the following day with secondary antibodies for one hour at room temperature and then washed with Tris buffer saline. The signals were detected using an electrochemiluminescence (ECL) system and scanned. The relative band intensity was quantified using the Image J system.

2.10. Statistical analysis

Data are expressed as mean ± standard deviation (S.D.). To determine the significance of the differences among the groups, one-way ANOVA was performed followed by Post hoc test. $P \leq 0.05$ was considered to be statistically significant.

3. Results

3.1. Field survey, plants extraction, and phytochemicals analysis

During field survey, it was observed that the selected plants' species were also used for other human illness besides cancer such as antihelminthic, skin diseases, diabetes, and blood pressure. The details methods of preparation and treatments methodology are mentioned in Table 1. From the field survey data, it is also evident that *Murraya koenigii* was the most used plant species as shown by Relative frequency of citation (RFC) index (Table 1) followed by *Euphorbia hirta, Annona reticulate, Hibiscus sabdariffa, Moringa oleifera* and *Lablab purpureus.* The fidelity level (FL) of *Murraya koenigii* was higher (98 %) followed by *Euphorbia hirta* (80 %), *Annona reticulate* (78 %), *Lablab purpureus* (76 %), *Hibiscus sabdariffa* (71 %) and *Moringa oleifera* (69 %) (Table 1). For the preparation of crude extract from all mentioned plants, methanol extract was used based on high percentage yield as compared to other solvents. The percent yields of methanol extract of *Annona reticulate* (leaves), *Lablab purpureus* (apical tendril), *Murraya koenigii* (leaves), *Moringa oleifera* (leaves), *Hibiscus sabdariffa* (fruits) and *Euphorbia hirta* (leaves) was found to be 5, 8, 4, 8, 9 and 6 percent respectively as compared to dry weight of each plants. Preliminary phytochemical screening revealed the presence of alkaloid, glycosides, phytosterols, steroids, flavanoids, phlobatanins and terpenoids at different degree in all five selected plants (Table 2).

Table 1
Most commonly used medicinal plants in the ethnomedical practices for the management of cancer and others ailments by Karbi tribe in Karbi Anglong. (Oral- O, Relative frequency of citation- RFC and fidelity level- FL).

Plants	Common name	Family	Voucher no.	Plant part(s) used	Methods of preparation	Some reported use in different human ailments	RFC	FL (%)
1 *Annona reticulate* (Mart.)	Red custard apple	Annona	AKV-11	Roots	Roots boiled and decoction taken twice daily for 3 weeks (O).	Anthelmintic, styptic, suppurant, antidysentric and anticancer.	0.82	78
2 *Lablab purpureus* (L.) Sweet	Country bean	Leguminosae	AKV-12	Tendrils	Tendril measuring 10 cm pounded and juice taken twice daily for one month (O).	Cholera, vomiting, skin cancer, Anti-inflammatory, diarrhea and leucorrhoea.	0.62	76
3 *Murraya koenigii* (L.) Spreng	Curry leaf	Rutaceae	AKV-13	Leaves	Leaves boiled and decoction taken until cure (O).	Diabetes, cancer, stomachache, stimulant, carminative, piles and diarrhea.	0.93	98
4 *Moringa oleifera* (Lam.)	Drumstick tree	Moringaceae	AKV-14	Leaves	Leaves boiled and decoction taken twice daily for seven weeks (O)	Anticancer, antibacterial, antiviral, antihelmintic, diabetes, cardiotonic, ulcer, diarrhea, epilepsy and abortifacient.	0.64	69
5 *Hibiscus sabdariffa* (L.)	Roselle	Malvaceae	AKV-15	Fruits	1 Fruits boiled and decoction taken twice daily for 3 weeks (O). 2 Powder of dry fruits are extracted in boil water and taken for 3 weeks (O).	Cancer, coronary heart disease and atherosclerosis.	0.78	71
6 *Euphorbia hirta* (L.)	Common spurge	Euphorbiaceae	AKV-16	Whole plant	Whole plant boiled and decoction taken twice daily for 6 weeks (O).	Cough, coryza, cancer, bronchitis, asthma, worm infestations, dysentery, gonorrhoea, jaundice and pimples.	0.88	80

3.2. MTT cell viability assay

MTT based cytotoxic activities of crude extract(s) or fraction(s) is most commonly used method for screening of possible cytostatic potential of medicinal plants. In the present study, cytotoxicity effect of different plants extract in DL and PBMC cells were assessed after 24 h of treatment. *Murraya koenigii* induced higher cytotoxicity in DL cells followed by *Annona reticulate, Hibiscus sabdariffa, Moringa oleifera, Lablab purpureus* and *Euphorbia hirta* (Fig. 2). Normal cell (PBMC) showed less cytotoxicity as compared to DL cells at the same dose and time period (Fig. 2). In terms of inducing cytotoxicity in DL cell, *Murraya koenigii* is more effective (IC_{50} = 806 μg/mL) as compared to other plant extracts (Table 3).

3.3. Apoptosis study

To corroborate the mode of cytotoxicity induced by different plants extract, apoptotic study was carried out (Fig. 3 and 4). After treatment with different plants extracts the characteristic cell morphology changes occurs in DL cells. These changes include membrane blebbing, cell shrinkage, nuclear fragmentation and chromatin condensation. *Murraya koenigii* induced higher apoptotic cell death in DL cells followed by *Annona reticulate. Hibiscus sabdariffa* and *Moringa oleifera* have shown almost similar apoptotic effect and the same is also evident by *Euphorbia hirta* and *Lablab purpureus* (Fig. 4).

3.4. Cell cycle analysis

Cell cycle distribution of the DL cell was determined by flowcytometry after treatment with methanol extracts of different plants for 24 h. The results showed that untreated DL cells reflect normal distribution of G_1, S and G_2/M phase (Fig. 5a). Generally, G0/G phase is characterized by 2n (diploid), G2/M phase by 4n (tetraploid), and S phase by somewhere between 2n and 4n. In case of reference drug, cisplatin (Fig. 5b) cell arrest was observed in G_1 phase with doublet peak indicated aneuploid cells population. It is also evident that the DL cells nuclei undergoing apoptosis exhibit decreased PI fluorescence and appear as one subdiploid peak (sub-G0) in a DNA histogram. When apoptotic cells were stained with PI and analyzed with a flow cytometer, they display a broad hypodiploid (sub-G0) peak, which can be easily discriminated from the narrow peak of cells with normal (diploid) DNA content in the red fluorescence channels (Fig. 5. b–f and h). Cells treated with *Lablab purpureus*, *Moringa oleifera* and *Hibiscus sabdariffa* showed cell cycle arrest at G2/M phase (Fig. 5e-g).

3.5. Mitochondrial membrane potential ($\Delta\Psi m$)

Mitochondria are considered to be one of the major sources of signals that initiate apoptotic cell death [20] therefore; the mitochondrial membrane potential was measured in the present study. Several reports have also shown that the decrease in mitochondrial membrane potential is the first step towards apoptosis. In the present study, even after treatment with different plants extract, a significant decline in mitochondrial membrane potential was observed along with reference drug, i.e., cisplatin (Fig. 6). Control group showed high polarized mitochondria showing bright green color. *Murraya koenigii* extract induced large numbers of low polarized (dim) mitochondria followed by *Annona reticulate, Moringa oleifera, Hibiscus sabdariffa, Lablab purpureus,* and *Euphorbia hirta* (Fig. 6).

3.6. ROS measurement

The increase in ROS level (Fig. 7) was also observed after plant extract treatment in DL cell. The ROS generation was high in *Murraya koenigii* followed by *Annona reticulate, Euphorbia hirta* and *Moringa oleifera.* There was no significant ($P \leq 0.05$) increase in ROS level in

Table 2
Results of phytochemical screening of methanol extract of different plants.

Sl nos.	Chemical constituents	Test performed	*Moringa oleifera*	*Murraya koenigii*	*Annona reticulate*	*Euphorbia hirta*	*Lablab purpureus*	*Hibiscus sabdariffa*
1.	Alkaloid	Dragendorff's test	+ + +	+ + +	+ + +	+ + +	+ +	+ +
2.	Carbohydrate	Fehling's test	+ +	+ +	+ +	+	+	+
3.	Glycosides	Borntrager's test	+ +	+ +	+ +	+	+ +	+
4.	Cardiac glycosides	Keller-Killani's Test	-	-	-	+ +	-	-
5.	Cyanogenic glycosides	Sodium picrate test	-	-	-	-	-	-
6.	Terpenoids	Salkowski's test	+ +	+ + +	+ + +	+ +	+	+
7.	Phlobatanins	HCl test	+	+	+ +	+	+	+
8.	Flavanoids	NaOH test	+ +	+ + +	+ +	+ +	+	+ +
9.	Proteins and amino acids	Millon's test	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
10.	Phytosterols	Libermann - Burchard's test	+ + +	+ +	+ +	+ + +	+ +	+ + +
11.	Fixed oils and fats	Spot test	+	+ +	+	+	-	+
12.	Phenolic compoundds and tanins	Ferric chloride test	+	+ +	+ +	+ +	-	-
13.	Gums and mucilages	Ruthenium test	+	-	-	+	-	-
14.	Triterpenoids	Salkowski's test	+ +	+	+	+	+	-
15.	Steroids	Salkowski's test	+ +	+ +	+ +	+ +	+ +	+ +
16.	Anthraquinone	Borntrager's reaction	+ +	+ +	-	-	-	+
17.	Saponins	Foam test	+	+	+	+	-	-

+ + +: highly present, + +: moderately present, +: low and − : absent.

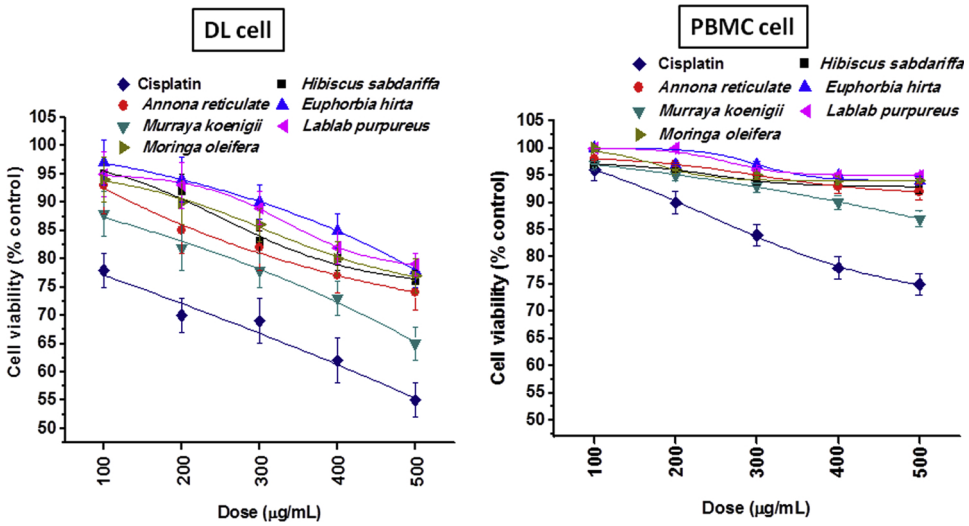

Fig. 2. Cytotoxicity effect of different plants extract in DL (cancer) and PBMC cells (normal) were assessed after 24 h of treatment using MTT assay. Numbers on Y-axis indicates the percentage of viable cells. Cisplatin was used as a positive reference drug.

Table 3
IC_{50} values based on MTT assay in DL cell using different plants extract.

Sl. nos.	Plants	Dose (µg/mL)	Duration	R^2	Equation	IC_{50} (µg/mL)
1.	Cisplatin[a]	100, 200, 300, 400 and 500	24 h	0.988	y = -0.054x + 82.8	607[a−f]
2.	*Murraya koenigii*[b]	-do-		0.896	y = -0.053x + 92.7	806 [a−f]
3.	*Hibiscus sabdariffa*[c]	-do-		0.916	y = -0.05x + 100.4	1008 [a−f]
4.	*Annona reticulate*[d]	-do-		0.987	y = -0.048x + 96.8	975 [a−f]
5.	*Moringa oleifera*[e]	-do-		0.895	y = -0.044x + 98.6	1105 [a−d,g]
6.	*Euphorbia hirta*[f]	-do-		0.904	y = -0.049x + 103.3	1088 [a−d,g]
7.	*Lablab purpureus*[g]	-do-		0.987	y = -0.042x + 100	1190 [a−f]

IC_{50} data are mean, n = 3. Letter in superscript indicates significant level at $P \leq 0.05$. For IC_{50} calculation y value was considered as 50 and subsequently x values were determined for respective groups.

case of *Lablab purpureus* and *Hibiscus sabdariffa.* An increase in reactive oxygen species (ROS) production and a decrease in the mitochondrial membrane potential indicated that the mitochondrial-mediated intrinsic pathway might be responsible for apoptotic cell death in DL cells. These results were additionally verified by sub-Go peak (hypodiploid, i.e., apoptotic cells) analysis by flowcytometry.

3.7. Western blot analysis of apoptosis-related proteins

Western blot analyses indicated that different plants extract induced a statistically significant ($P \leq 0.05$) increase in cytochrome- c release into the cytoplasm compared with the untreated cells. We also observed significantly ($P \leq 0.05$) increased in the level of caspase-3 and -9 in DL cells. There was no significant change in the level of caspase-8 as

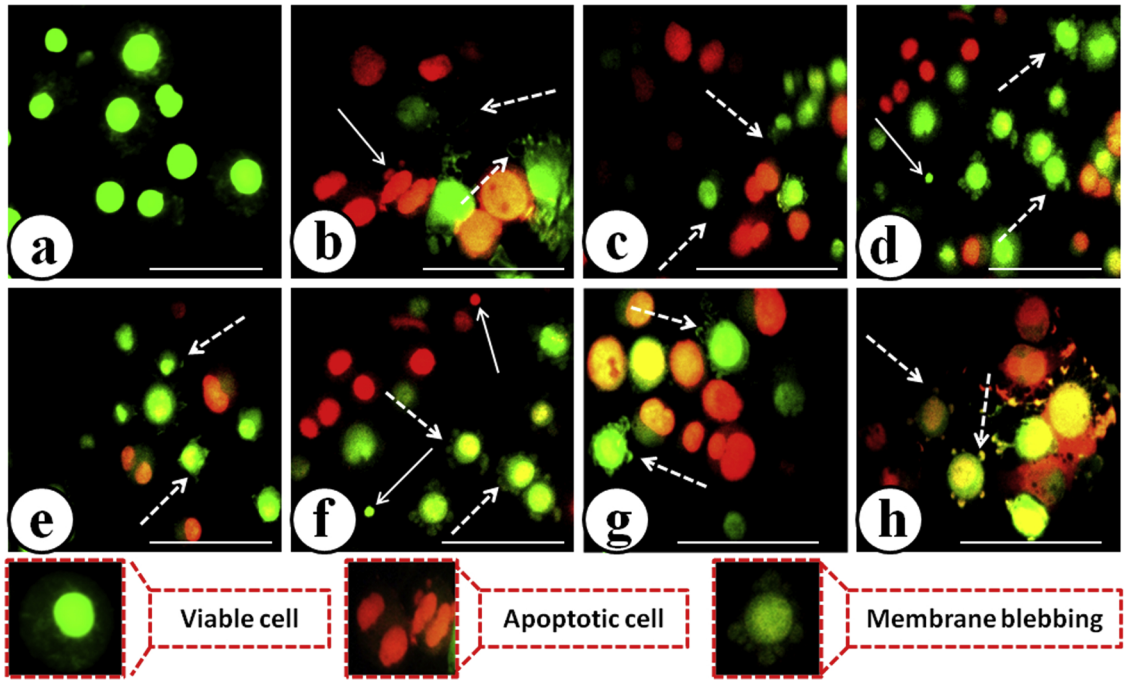

Fig. 3. Morphological features of apoptotic and viable Dalton's lymphoma (DL) cells stained with acridine orange and ethidium bromide. (a) Control DL cells are green and more or less rounded in shape, indicating viable cells. (b) Cisplatin treatment (reference drug) showed severe membrane blebbing and fragmented nuclei. Images (c-h) indicates treatment with *Murraya koenigii, Annona reticulate, Hibiscus sabdariffa, Moringa oleifera, Euphorbia hirta, and Lablab purpureus* extracts respectively (500 µg/mL for 24 h).

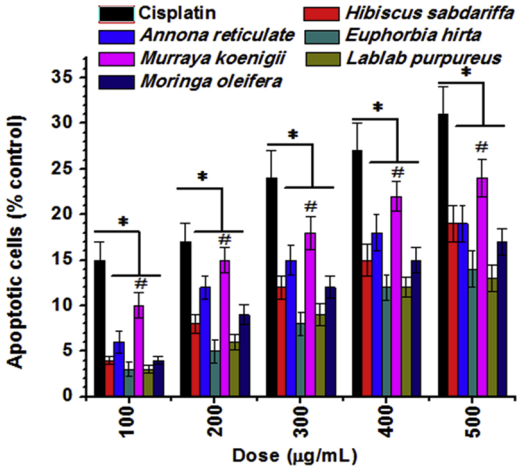

Fig. 4. Graph showing the percentage of apoptotic cell death after plants extract treatment. One thousand cells were counted from each group, and percent of viable and apoptotic cells were counted. Values represent mean ± S.D., n = 3, $^{*}P \leq 0.05$ and $^{\#}P \leq 0.001$.

compared to the untreated group (Fig. 8).

4. Discussion

The present study was aimed to obtained information about traditionally used medicinal plants and to investigate the potential cytostatic activity and apoptotic inducing ability against Dalton's lymphoma cell line (DL) *in vitro*. The high RFC index showed the local importance of a plant species with reference to the almost all informants who cited uses of these plant species [22]. The fidelity level is very useful ethnopharmacological quantitative analysis methods for identifying the resident's most preferred species in use for treating certain diseases [23]. Table 1 showed that *Murraya koenigii* was the most commonly used plant species as demonstrated by Relative frequency of citation (RFC) index whereas, the fidelity level (FL) of *Murraya koenigii* was higher (98 %) followed by *Euphorbia hirta* (80 %), *Annona reticulate* (78 %), *Lablab purpureus* (76 %), *Hibiscus sabdariffa* (71 %) and *Moringa oleifera* (69 %).

The phytochemicals study of the most commonly used anticancer medicinal plants obtained as per present ethnopharmacological survey revealed the presence of alkaloid, glycosides, phytosterols, steroids, flavanoids, phlobatanins and terpenoids at the different degree in all five selected plants (Table 2). Phytochemicals are diverse groups of naturally occurring secondary metabolites synthesized by plants and show biologically significance by playing an essential role in the plants defense mechanism against various pathogenic microbes. At the same time plants secondary metabolites are considered as useful in discovering the bioactive lead molecules of therapeutic importance [24]. The different phytochemicals such as carotenoids, flavonoids, indoles and glucosinolates, inositol, isoflavones, isothiocyanates, polyphenols, and terpenes have been reported active against various types of cancer both *in vivo* and *in vitro* condition [25–28].

Dalton's lymphomas cell line was used in the present study for the screening of cytostatic potential of plants extract. It is a transplantable T-cell lymphoma first originated in the thymus of murine host and has served as an exciting model for drug testing, because of its usefulness in pre-clinical setting for screening phytochemicals as well as synthetic compounds in the drug discovery process [29]. *In vitro* cytotoxicity study using MTT assay is an essential step in the search of new therapies and it is considered to be one of the most commonly used preliminary screening method for cell proliferation and cytotoxicity [30]. MTT based cell proliferation study showed that *Murraya koenigii* induced higher cytotoxicity in DL cells followed by *Annona reticulate, Hibiscus sabdariffa, Moringa oleifera, Lablab purpureus* and *Euphorbia hirta* (Fig. 3). Normal cell (PBMC) showed negligible cytotoxicity as compared to DL cells at the same dose and time (Fig. 2) this may be due to the well protective capacity of normal cells because of cellular antioxidant or DNA repair mechanism which is lacking in most cancer cells [31].

To understand further about the mechanism of cytotoxicity induced by plants extract, we performed qualitative and quantitative apoptosis

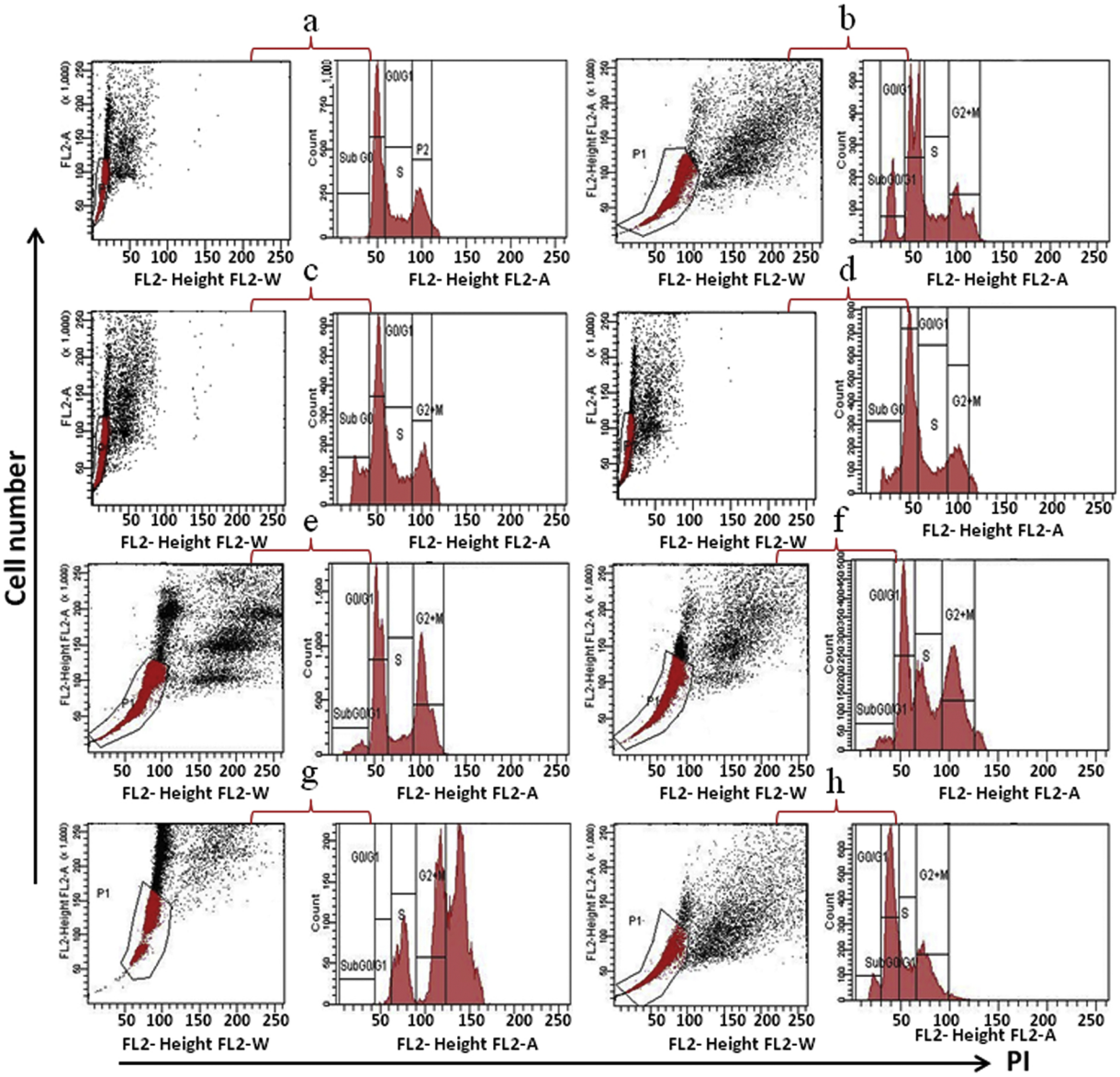

Fig. 5. Cell cycle analysis and DNA fragmentation (apoptosis) study of DL cells exposed to methanol extract of different plants at 500 µg/mL for 24 h and stained with PI and analyzed by flow cytometry. The proportion of sub-G0 phase is an indication of apoptotic cell death. a: control; b: cisplatin; c: *Murraya koenigii*; d: *Annona reticulata*; e: *Lablab purpureus*; f: *Moringa oleifera;* g: *Hibiscus sabdariffa;* h: *Euphorbia hirta.* Scattered plot for each experiments showing gated population for PI-positive cells. The histogram shown here is a representative image of three different experiments.

study using fluorescent-based dual staining method [32]. After treatment with different plants extract the typical apoptotic features were observed, which include membrane blebbing, cell shrinkage, nuclear fragmentation, and chromatin condensation. *Murraya koenigii* induced higher apoptotic cell death in DL cells, followed by *Annona reticulate. Hibiscus sabdariffa* and *Moringa oleifera* have shown almost similar apoptotic effect, and the same is also evident by *Euphorbia hirta* and *Lablab purpureus* (Figs. 3 and 4).

One of the important criteria for a potential anticancer agent is the ability to induce apoptosis selectively, without harming healthy cells [33]. To corroborate fluorescent based apoptosis study, flowcytometry analysis was also performed. Cell cycle analysis clearly indicates irregularity in the normal distribution in different phases of the cell cycle in all treatment groups. The appearance of a broad hypodiploid (sub-G0) peak, which can be easily discriminated from the narrow peak of cells with normal (diploid) DNA content in the red fluorescence channels (Fig. 5. b–f and h) confirm apoptotic features. Cells treated with *Lablab purpureus*, *Moringa oleifera* and *Hibiscus sabdariffa* showed cell cycle arrest at G2/M phase (Fig. 5e-g). Apoptosis is an essential physiological process related to development and tissues homeostasis in healthy individuals. It is very tightly regulated in the animal's body, but the genetic basis for apoptosis implies that the regulatory pathways of apoptosis can be disrupted anytime by a mutation leading to numbers of human diseases including cancer [34]. Therefore, the research in the field of the natural product is highly encouraged to obtain plants based apoptosis-inducing agents for future drug discovery.

The decrease in mitochondrial membrane potential followed by high levels of reactive oxygen species (ROS) is linked to apoptosis pathways because depolarization of the mitochondrial membrane opens the mitochondrial permeability transition pore, which may lead to the release of apoptosis initiation factors, such as cytochrome c, and trigger the apoptosis process [35]. In the present study also *Murraya koenigii* extract induced large numbers of low polarized (dim) mitochondria followed by *Annona reticulate, Moringa oleifera, Hibiscus sabdariffa, Lablab purpureus* and *Euphorbia hirta* (Fig. 6) whereas, in control group mostly high polarized mitochondria with bright green color was

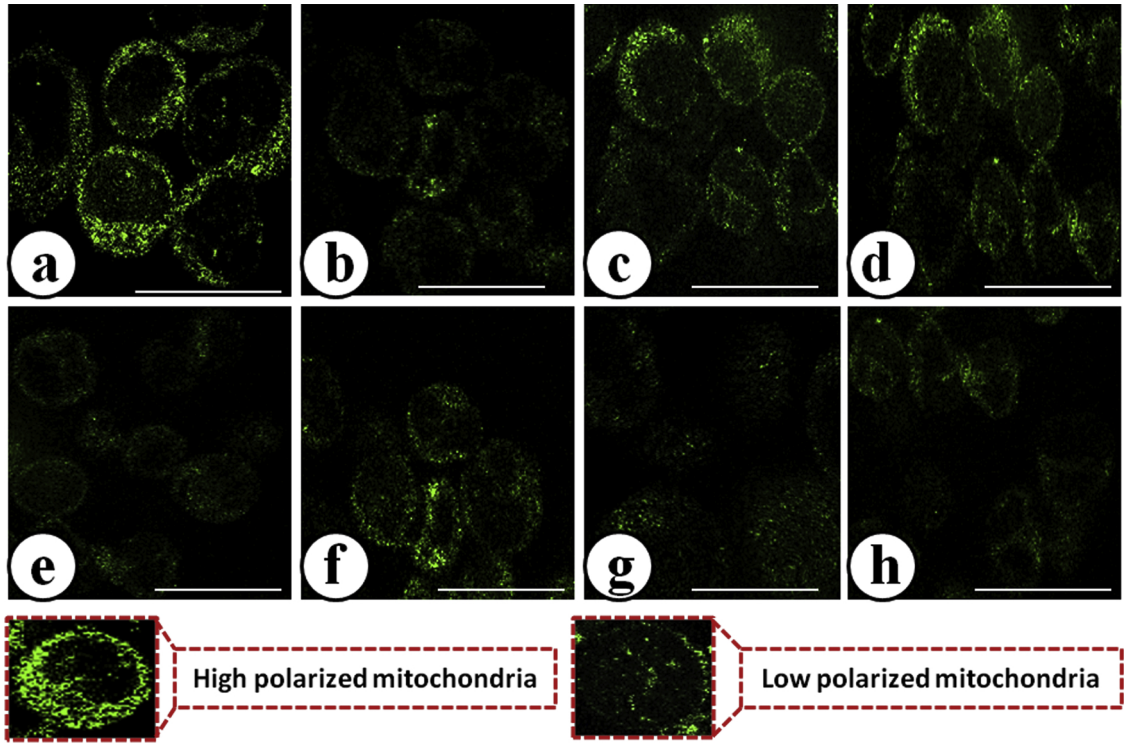

Fig. 6. Alterations on mitochondrial membrane potential induced by methanol extract of different plants in DL cells. A. control, b. reference drug i.e. cisplatin, c. *Euphorbia hirta,* d. *Lablab purpureus,* e. *Hibiscus sabdariffa,* f. *Moringa oleifera,* g. *Annona reticulate* and h. *Murraya koenigii.* Typical imaging was obtained by using confocal microscopy and Rhodamine123 staining. Scale bar: 2 μm.

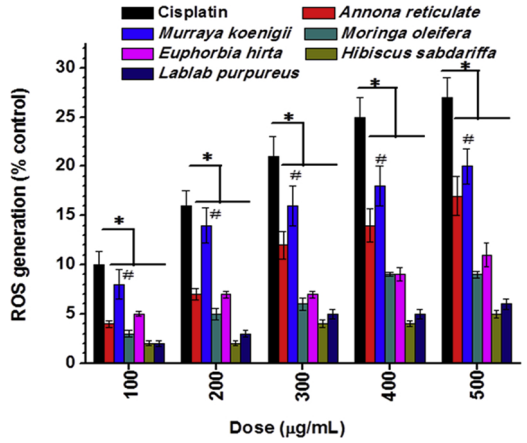

Fig. 7. Effect of methanol extract of different plants on intracellular reactive oxygen species (ROS) generation. Results were expressed in percentage over untreated control. Data are mean ± S.D., n = 3, $*P \leq 0.05$ and $\#P \leq 0.001$.

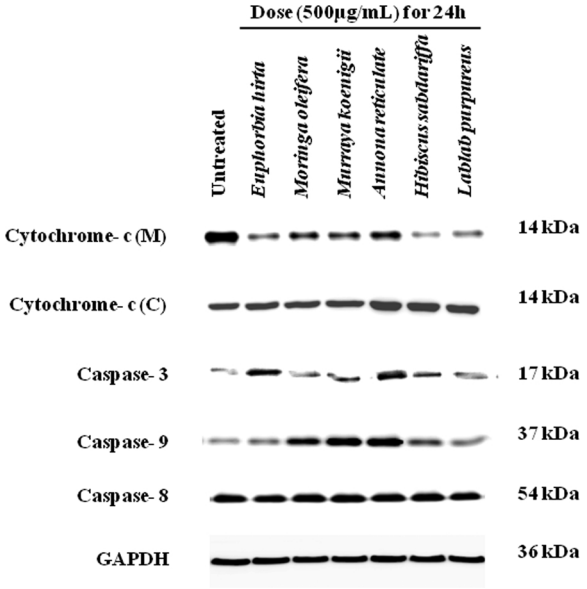

Fig. 8. Effect of different plant extracts treatment (500 μg/mL for 24 h) on the expression of proteins regulating mitochondria-mediated apoptosis.

observed. The involvement of ROS in the execution of apoptosis *via* mitochondria is also reported by several workers [36] in various cancers. The increase in ROS level (Fig. 7) was also observed after plant extract treatment in DL cell. The ROS generation was high in *Murraya koenigii* followed by *Annona reticulate, Euphorbia hirta* and *Moringa oleifera.* An increase in reactive oxygen species (ROS) production and a decrease in the mitochondrial membrane potential indicated that the mitochondrial-mediated intrinsic pathway may be responsible for apoptotic cell death in DL cells. ROS generation is also associated with decreased in mitochondrial membrane potential followed by DNA fragmentation and an increase in expression of pro-apoptotic and inflammatory markers [37].

To further confirm the involvement of mitochondria in apoptosis related cell death, we performed western blot analysis of apoptotic-inducing proteins. Western blot analyses indicated that different plants extract induced a statistically significant ($P \leq 0.05$) increase in cytochrome- c release into the cytoplasm compared with the untreated cells. Plant extracts treatment further lead to higher expression of caspase-3 and -9 ($P \leq 0.05$) in DL cells. No significant change in the level of caspase-8 was observed (Figs. 8 and 9). The increase in cytoplasmic cytochrome- c followed by a subsequent increase in caspase-3 and -9 expressions indicates the involvement of mitochondria in plant extract mediated apoptosis [38,39].

The overall finding of the present study suggest that plant extracts

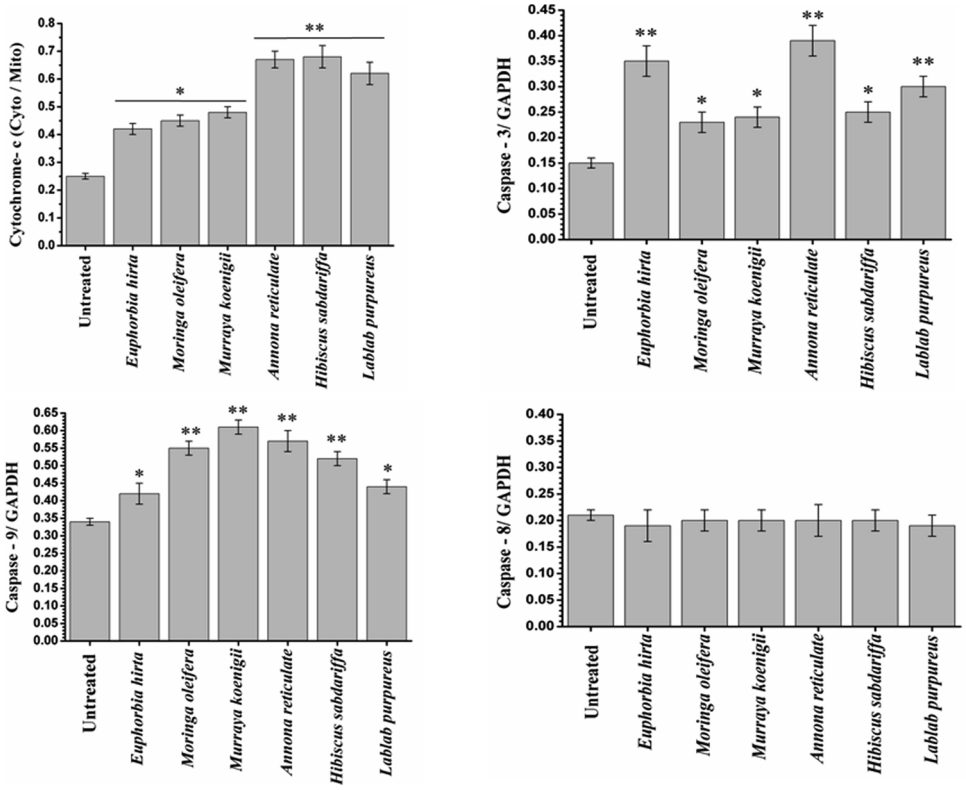

Fig. 9. Effect of different plant extracts treatment (500 µg/mL) for 24 h on the levels of apoptotic related proteins. The level of proteins was quantified using densitometry and normalized against GADPH. Data are presented as mean ± SD., n = 3. *$P \leq 0.05$ and ** $P \leq 0.001$.

(mainly *Murraya koenigii* and *Annona reticulate*) treatment causes increased in ROS level which may alters the mitochondrial structure and triggers changes in permeability. Which may further results in functional impairment of the mitochondria, including decrease in the ΔΨm, release of cytochrome- c into the cytoplasm followed by high expression of caspases- 3 and -9, resulting apoptotic cell death.

5. Conclusion

In conclusion, this study demonstrated that *Murraya koenigii* induced higher apoptotic cell death in DL cells as compared to *Annona reticulate. Hibiscus sabdariffa* and *Moringa oleifera* have shown almost similar apoptotic effect. The plant extract mediated apoptotic cell death is mediated by ROS and mitochondrial pathway in DL cells, but the detail mechanism needs further investigations. Together with the earlier report, the present *in vitro* results support the ethnomedicinal use of these plant extracts against cancer and points out the need of establishing cytostatic efficacy against other human cancer cell lines. Based on the present study, it is also recommended that proper documentation of medicinal plants, gene bank, taxonomist, and conservation organization would eventually need to document this indigenous traditional knowledge (TK).

Funding

The present study was carried out mostly with personal expenditure and supported from colleagues and friends. Some part of work was also carried out by utilizing some fund from DST-SERB project (ECR2016/000079) funded to the author.

Authors' contributions

AKV designed the study, conducted the field work whereas, SS performed data analysis and prepared the first draft with AKV.

Declaration of Competing Interest

None to declare.

Acknowledgements

Administrative staff and HOD, Department of Zoology, Cotton University is highly acknowledged for providing laboratory facility to carry out the present work. DST-SERB (ECR2016/000079), New Delhi, India is also acknowledged for funding support. We are also thankful to all traditional healers of Karbi Anglong district of Assam (India) who helped and shared the required information during field survey and plants collection.

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