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Death receptor pathway genes (Caspase and BID) expression analysis in cancerous cells in response to extracts of amla and turmeric

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Abstract: Plant extracts antiproliferative effects were determined by using mammalian cells along the expression profile of Caspases 3, 8, and the BID gene of the death receptor-induced pathway. Two medicinal plants viz., Turmeric (*Curcuma longa*) and Amla (*Emblica officinalis*) extracts were examined for antiproliferative effect through Neutral Red-Dye uptake assay on Vero and MDA-MB 231 cell lines. A reverse transcriptase polymerase chain reaction was used to determine the expression of genes while GAPDH expression was used as an internal control. Expression of BID was up-regulated in methanolic turmeric extract-induced MDA-MB 231 cells while Caspases 3,8 expressions were the same in induced and uninduced MDA-MB 231 cells. Activated BID cleaved into tBID and activated the intrinsic pathway which caused death in methanolic turmeric extract-induced cancerous cells. Ethanolic extracts of turmeric exerted the strongest antiproliferative effects on Vero and methanolic extracts on MDA-MB 231 cells. The morphological studies of cell lines and gene expression analysis of turmeric methanolic extract-treated cells showed activation of apoptosis via converting BID into t-BID (intrinsic pathway) and activating Caspase-3 and Caspase-8 (extrinsic pathway). With the differential cytotoxicity and induction of apoptosis in induced cancer cells in comparison to uninduced cancerous cells, hence turmeric is a natural source of new anti-cancerous compounds.

Keywords: Cell-death, death receptor pathway, BID, caspases 3,8; *Curcuma longa*; *Emblica officinalis.*

INTRODUCTION

Triggering either apoptosis or cell-cycle arrest by natural, plant-based dietary compounds is a promising approach for inhibiting the progression of carcinogenesis. The main purpose of the current research was to screen two traditional medicinal plants, *Emblica officinalis* (Amla) and *Curcuma longa* (Turmeric) in cell-based antiproliferative assays along with subsequent molecular analyses of Caspase-3, Caspase-8, and BID (BH3 interacting domain death agonist) genes. Understanding the role of plant extracts in triggering such genes related to apoptosis and the development of novel anticancer therapeutic drugs beneficial in the destruction of cancerous cells without being toxic to normal host cells, is highly emphasized. There should be the availability of alternative or complementary natural medicines with minimum side effects as compared to conventional chemotherapy drugs (Nobili *et al*., 2009). The chemotherapeutic drugs severely damage the actively proliferating cells, which further halt mitosis by hampering DNA synthesis. The phytochemicals from spices develop carcinogenesis inhibitors by regulating cell proliferation pathways and metastasis (Butt *et al*., 2013). Similarly, the use of herbal medicines as complementary or adjuvant anticancer therapy is increasing as there is evidence from clinical trials (Wilken *et al*., 2011). The antioxidants from spices, for example, eugenol (clove), capsaicin (red pepper), and curcumin (turmeric) have been found to regulate oxidative stress of cells. Such antioxidants can inhibit ROS formation as they interact with different signal transduction pathways (Zheng *et al*., 2016).

The Caspases are important initiators or effectors which play significant roles in apoptosis during cell death (Degterev *et al*., 2003). There are two important pathways for the activation of Caspases which include ligation of death receptors after extracellular activation (i.e., extrinsic apoptosis pathway) and generation of Cytochrome-c from mitochondria by triggering intracellular signals (i.e., apoptosis pathway) (Vermeulen *et al*., 2005). For an extrinsic apoptosis pathway, the Fas - fibroblast-associated antigen (Apo-1/CD95) is fundamentally actuated at any point on the surface of the receptor which effectively up-regulates its initiator Caspases. The effector Caspases directly cleave or actuate by their initiator Caspase-3 is a regular actuated protease, that catalyzes the cleavage of several key proteins of the cell. The Caspase-8 belongs to Cysteine proteases ensnared both in apoptosis and in cytokines' processing (Kruidering and Evan, 2000). The BID belongs to a Bcl-2 group of proteins controlling the permeabilization of the outer mitochondrial membrane. In apoptosis, BID cleavage occurs not just by Caspase-8 during death receptor signaling of apoptosis, but by other different Caspases,

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Calpains, Granzyme B, and Cathepsins (Billen *et al*., 2009). The extrinsic pathway of the death receptor principally includes TNFR1 (tumor necrosis factor receptor 1), Fas, DR3, DR4 along with DR5. In these variables, both Fas along with FasL (Fas ligand) are viewed as being imperative effectors for the most apoptosis in a few natural conditions, and what's more, its progressive deregulated expression basically in an assortment of carcinomas (Degterev *et al*., 2003). Moreover, the Fas-dependent cell death pathway likewise incorporates FADD having a death domain along with FADD-related pro-caspase-8 which passes instigating flagging complex (Disk) that results in the apoptotic cell death. In addition to this, pro-caspase-8 ties to the Fas-bound FADD prompting the initiation of Caspase-8 which prompts Caspase-3 activation (Debatin *et al*., 2004a). In the current research, three different genes whose expression profiles were analyzed in the plant extracts, induced mammalian cell lines included Caspase-3, Caspase-8, and BID (Flow Diagram A).

There is clinical evidence that documents that high-level expressions of Bcl-2 proteins communicate with many cancerous cells having chemoresistant phenotypes, including prostate carcinoma, malignant brain tumors, multiple myeloma, acute lymphoblastic lymphoma, etc. (Debatin *et al*., 2004b). Curcumin is rendered as an ingredient of curcuminoids having the capacity of repressing unpredictable multiplication of growth cells, for example, leukemia. The fruit Amla is considered to have therapeutic effects, including anticarcinogenesis, antimutagenic, and most importantly antitumor (Perianayagam *et al*., 2004) by inducing apoptosis (Kim *et al*., 2005). The primary bioactive compound(s) in Amla i.e., Phyllanthus carries anticancer effects and is rich in phenolic acids, flavonoids, and ellagitannins. The University of Malaya has mentioned that Phyllanthus is fit for generating specific toxicity primarily on A549 (lung tumor) and in addition to MCF-7 disease cell lines (Koparal *et al*., 2003).

MATERIALS AND METHODS

Plant extracts and cell lines

Emblica officinalis (Amla) and *Curcuma longa* (Turmeric) plant extracts were prepared in solvents (ethanol, methanol, and water) by crushing 5g of each plant extract till homogenization in 15ml of each solvent with the help of a pestle and mortar. Extracts were centrifuged at 6000 rpm for 15 minutes and the supernatant from each extract was subjected to microfiltration by using a 0.2-micron filter under aseptic conditions. This weight of the pellet was subtracted from the total weight of each plant tissue and hence the weight of total soluble proteins in each solvent was obtained and respective values were expressed as several total proteins per ml of each type of solvent for each plant. Vero cell line (derived from the African green monkey) and MDA-MB 231 cell line (derived from human breast adenocarcinoma cells) was used in the current study.

Medium and propagation of cell lines

The cell lines were maintained as a monolayer in Dulbecco's Modified Eagle Medium (DMEM). One liter medium contained 10% fetal calf serum, streptomycin (100µg/ml), gentamycin (50µg/ml), and Penicillin (100U/ml). sodium bicarbonate (3.7g) and DMEM powder (12g) were all mixed well and kept the pH to 7.4 by 1N NaOH. The medium was used after microfiltration under aseptic conditions. To prepare 50ml cell suspension, media from 3 to 4 cell culture flasks was discarded. The monolayer formed was washed twice with PBS. The 1% Trypsin and 0.5% Versene solution were added and the flask was incubated for 2-5 minutes till the detachment of cells. Extended exposure of cells to trypsin can destroy them. Cells were observed under an inverted microscope to ensure that the cells detached and were not damaged. To the detached cells in flasks, added 50ml DMEM medium and centrifuged at 2500 rpm for 10 minutes. The supernatant was discarded while the pellet was added 50ml DMEM medium with fetal calf serum as mentioned earlier. The final volume of cell suspension was made up to 50ml by adding a prepared DMEM medium as mentioned earlier. The cell suspension was added to flasks of $25cm^2$ for further propagation to maintain cell lines or in 96-cell wells plates for bioassays. The cells were kept at 37^oC in a 5% CO_2 incubator for 48 hours for propagation.

Anti-proliferative bioassay of plant extracts

For antiproliferative bioassays, Vero and MDA-MB-231 cell lines were used in 98 wells of flat-bottom cell culture plates. Briefly, 100µl of DMEM was added to all wells in a single row and then 100µl of each plant extract containing 100µg of total soluble plant material was added to the first well of the designated row and serially two folds diluted by transferring 100µl mixture serially from first well to 11^{th} well. The 12^{th} well in each row was the "control well" devoid of any plant extract and containing medium. After serial two-fold dilution of each plant extract, 100µl of the cell suspension (1000 cells per 100µl suspension) was added in all the twelve wells including the control well. The plates were placed in a 5% CO_2 incubator at approximately 37^oC for 48-72 hours and monitored under a microscope. Neutral-Red dye uptake was used to estimate the total number of all viable cells in the cell culture. After 48-72 hours, the cell medium was carefully decanted from all wells, and added 100µl of Neutral red dye from 3.3mg/ml stock solution was mixed in DMEM medium and incubated for two hours at 37^oC under adequate culture conditions. After two hours, Neutral Red-Dye containing DMEM medium was decanted and the plate was washed with PBS thrice. Finally, 150µl of acidified ethanol (stock solution containing 49ml of distilled water, 0.1ml of acetic acid, and 50 ml of absolute ethanol) was added to all the wells.

Data Calculations:

The final optical density (OD) of the Neutral Red-dye extracted from the viable cells was measured at 540 nm by using a microtiter plate reader using the following formula.

$$\text{Percentage of viable cells} = \frac{\text{Absorbance of viable cells in each well containing sample}}{\text{The absorbance of viable cells in control devoid of plant extract}} \times 100$$

The fold dilution that showed a 50% viability, represented IC_{50} for that specific plant extract.

Molecular expression analysis

Based on cell viability analysis, the plant extract which showed the most effective IC_{50} was selected to induce MDA-MB-231 cells for further molecular expression study. The most effective plant extract (methanolic extract of *Curcuma longa*, Turmeric) with IC_{50} achieved at maximum dilution (1024 folds) for MDA-MB-231 cancerous cells, was selected to induce MDA-MB-231 cells for RNA isolation to study expression profiles of different genes belong to death receptor pathway. For gene expression studies Vero cell line was not used as it is an African green monkey-derived cell line and the primers used in reverse transcriptase PCR were for human genes specific. Based on IC_{50} results, the calculated amount (1024 folds diluted) of the most effective plant extract (methanolic extract of *Curcuma longa*, Turmeric) and MDA-MB-231 cell suspension (25 ml; containing 10^5 cells per ml) was taken into five different flasks of 50ml capacity and incubated for 48 hours.

The 1% Trypsin and 0.5% Versene solution were added to each flask and incubated for 2-5 minutes till the detachment of cells. The cell suspension from each flask was centrifuged at 8000 rpm for two minutes and the resulting supernatant was discarded the cells pellet was re-suspended in RNA store buffer and stored at -80^{0}C for subsequent RNA isolation for gene expression studies.

Total RNA isolation

The total RNA was extracted by using Thermo-Scientific Gene JET RNA Purification Kit. Briefly, the eppendorf tube containing induced cells suspension from each flask with RNA store buffer was centrifuged at 6000 rpm for five minutes and the resulting supernatant was discarded. To the cells, the pellet was added 600µl lysis buffer and vortexed for 10 seconds. Added 360µL of absolute ethanol and mixed well. All the lysate was loaded on a purification column and centrifuged at 12000 rpm for 1 minute. The flow-through was then discarded and the purification column was positioned back within the collection tube. Added 700µL of the wash buffer 1 (supplemented with ethanol) to the column and centrifuged at 12000 rpm for one minute. The resulting flow-through was again discarded and to the purification, the column added 600µL of wash buffer 2. The column was centrifuged at 12000 rpm for one minute and flow-through was discarded. Finally, 100µL nuclease-free water was added to the column and centrifuged at 12000 rpm for one minute to elute the total RNA contained in the flow-through. The purified RNA quantification was done by Nanodrop. 1 µl of RNA sample absorbance value was taken at two wavelengths, i.e., 260 nm for RNA and 280 nm for protein contamination. The ratio of 260/280 was calculated to check protein contamination in samples. Purified RNA was subjected to DNase treatment before cDNA synthesis. To the 10µg of RNA sample added 2.5µl of 10X DNase buffer, 1µl of DNase enzyme, and double deionized water were added to make a total volume of 25µl. The mixture was incubated at 37^{o}C for 30 minutes.

RT-PCR amplification of genes

In the first step, cDNA synthesis was carried out which involved 5µg of RNA, 2µl of reverse transcriptase, 2.5µl of 5X reaction buffer, 2µl of dNTP (10mM), polyT primer (50 pmol, 1µl) and gene-specific reverse primer (50 pmol, 1µl) (table 1) and the total volume was adjusted to 25µl. The reaction mixture was kept at 45^{o}C in the thermocycler for 60 minutes. Finally, the prepared cDNA was stored at -10^{o}C. The cDNA for each gene was prepared separately by keeping the concentration of purified RNA the same in all cDNA synthesis reactions. The synthesized cDNA of each gene was used in a separate RT-PCR reaction of each gene.

The expression of Caspase-8, Caspase-3, BID, and GAPDH genes was determined. The GAPDH gene expression was used as an internal control. The primers (forward and reverse) used in the current research were mentioned in table 1 and primers parameters were verified by the oligonucleotide properties calculator available at (https://www.biosyn. com). The RT-PCR reaction mixture volume was kept at 50µl for each gene. The reaction mixture contained 10µl of cDNA, 1µl of DNA polymerase (5 units), 5µl of 5X reaction buffer, 2µl of dNTP (10mM), forward and reverse primer (50 pmol, 1µl each), and $MgCl_2$ (1.5mM) (table 1) and the total volume was adjusted to 50µl. The Caspase-3 was amplified at 95^{o}C at an initial denaturation for 3 minutes followed by 30 cycles. These cycles were optimized at the following given conditions: denaturation at 95^{o}C (30 seconds), annealing at 57^{o}C (30 seconds), and extension at 72^{o}C (30 seconds). After 30 cycles, a final extension was done at 72^{o}C (7 minutes) and terminated at 4^{o}C. The Caspase-8 was amplified at the following conditions: initial denaturation at 95^{o}C (3 minutes) followed by 30 cycles. These cycles were optimized at the following given conditions: Denaturation at 95^{o}C (30 seconds), annealing at 56^{o}C (30 seconds), and extension at 72^{o}C (30 minutes). After 30 cycles, a final extension was done at 72^{o}C (7 minutes) and termination at 4^{o}C. The BID gene was amplified at 95^{o}C of an initial denaturation (3 minutes) followed by 30 cycles and each cycle involved denaturation at 95^{o}C (30 seconds), annealing at 60^{o}C (30 seconds), and extension at 72^{o}C (30 seconds) with a final extension at 72^{o}C (30 seconds). The amplified RT-PCR products were observed on 1% agarose gel by using 1X TAE buffer.

Table 1: Set of Forward and Reverse Primers used in RT-PCR

Gene Name	Forward Primer	Reverse Primer	Product Size
Caspase 8 GB|AF102145.1	5' CTG CTG GGG ATG GCCAC 3'	5'CACATAGCACTTTCTAATAGGCT 3'	213
Caspase 3 GB|AF087891.1	5' GAGAACACTGAAAACTCAGTG 3'	5' ACTTCTACAACGATCCCCTC 3'	427
BID GB|BC016926	5' ACC TAC TGG TGT TTG GCT TC 3'	5' CCGGATGATGTCTTCTTGAC 3'	200
GAPDH	5' TGAACGGGAAGCTCACTGG 3'	5' TCCACCACCCTGTTGCTGTA 3'	450

Table 2: Dilutions of amla and turmeric extracts at different concentrations and their cell viability analysis in Vero-Cell Line

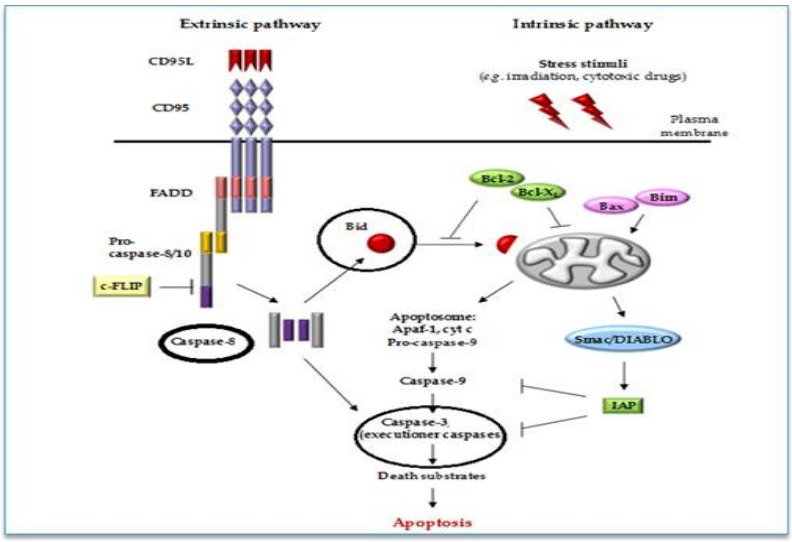
Plant name	OD 540 nm	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	Control	Solvent Control
		0	2	4	8	16	32	64	128	256	512	1024		
Amla	Ethanol	0.043	0.046	0.049	0.052	0.057	0.059	0.062	0.066	0.071	0.075	0.077	0.13	0.12
		33%	35%	37%	40%	43%	45%	47%	50%	54%	57%	59%		
	Methanol	0.044	0.048	0.051	0.054	0.059	0.062	0.066	0.069	0.072	0.075	0.077	0.11	0.11
		40%	43%	46%	49%	53%	56%	60%	62%	65%	69%	70%		
	Water	0.042	0.046	0.049	0.052	0.054	0.059	0.063	0.067	0.07	0.072	0.075	0.11	0.10
		38%	41%	44%	47%	49%	53%	57%	60%	63%	65%	68%		
Turmeric	Ethanol	0.041	0.045	0.049	0.051	0.054	0.056	0.057	0.059	0.062	0.063	0.065	0.13	0.14
		31%	34%	37%	39%	41%	43%	43%	45%	47%	48%	50%		
	Methanol	0.042	0.045	0.047	0.05	0.053	0.056	0.059	0.063	0.067	0.069	0.072	0.12	0.13
		33%	37%	39%	41%	44%	46%	49%	52%	55%	57%	60%		
	Water	0.043	0.046	0.05	0.054	0.057	0.061	0.063	0.067	0.07	0.072	0.075	0.11	0.10
		39%	41%	45%	49%	51%	55%	57%	60%	63%	63%	68%		

Key: Yellow: IC_{50} value of strongest extract

Table 3: Dilutions of amla and turmeric extracts at different concentrations and their cell viability analysis in MDA-MB 231 Cell Line

Plant name	OD 540 nm	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	Control	Solvent Control
		0	2	4	8	16	32	64	128	256	512	1024		
Amla	Ethanol	0.085	0.089	0.093	0.096	0.099	0.101	0.105	0.108	0.11	0.113	0.116	0.21	0.19
		40%	42%	44%	45%	47%	48%	50%	51%	52%	53%	55%		
	Methanol	0.091	0.095	0.098	0.104	0.107	0.11	0.114	0.117	0.121	0.123	0.126	0.21	0.19
		43%	45%	46%	49%	51%	52%	54%	55%	57%	58%	60%		
	Water	0.101	0.103	0.105	0.107	0.109	0.112	0.116	0.119	0.121	0.125	0.129	0.22	0.2
		45%	46%	47%	47%	49%	51%	52%	54%	55%	56%	58%		
Turmeric	Ethanol	0.079	0.081	0.084	0.087	0.09	0.094	0.096	0.099	0.102	0.104	0.107	0.21	0.2
		37%	38%	40%	41%	42%	44%	45%	47%	48%	49%	51%		
	Methanol	0.075	0.077	0.08	0.082	0.085	0.088	0.091	0.094	0.097	0.102	0.105	0.21	0.2
		35%	36%	38%	39%	40%	41%	43%	44%	46%	48%	50%		
	Water	0.085	0.088	0.091	0.094	0.097	0.102	0.105	0.108	0.111	0.115	0.119	0.20	0.2
		42%	44%	45%	47%	48%	51%	52%	54%	55%	57%	59%		

(Key: Yellow: IC_{50} Value of strongest extract)



Flow diagram A: Major genes of extrinsic pathways (arrows: genes (Caspase-3, Caspase-8 and BID) studied in the current study

RESULTS

Preparation of plant extracts

Two plants were included in this study, namely *Emblica officinalis* (Amla) and *Curcuma longa* (Turmeric). These were obtained from the Fruit and Vegetable Mundi. --. The plant extracts were prepared using three different solvents, i.e., ethanol, methanol, and water. A 5g of each plant extract was crushed and homogenized in 15 ml of each solvent with the help of a pestle and mortar. The extracts were then centrifuged at 6000 rpm (15 minutes) to separate suspended particles (Selegato *et al*., 2016). After centrifugation, supernatant from each extract was subjected to microfiltration by using a 0.2-micron filter under highly aseptic conditions in a safety cabinet or a laminar flow before a cell-based anticancerous assay.

Anti-proliferative analysis of different plant extracts against vero cell line

After incubation of plates for 48 hours, the cells were washed with PBS. After washing with PBS, each 96-well plate was examined for any morphological changes in response to plant extract treatment. The cell viability was determined by Neutral Red Dye Uptake Assay. Compact growth was observed in control wells that were incubated with a constant number of cells and respective solvents.

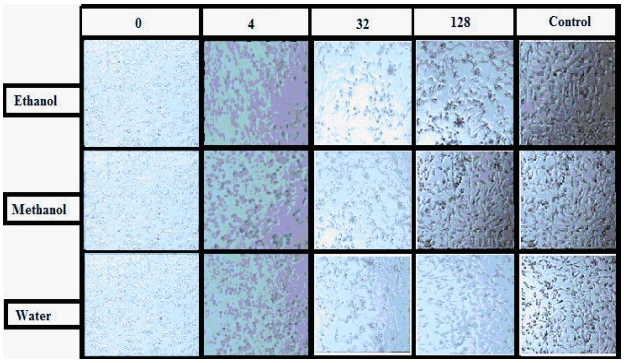


Fig. 1: Microscopic (40X magnification) of Vero-Cells treating with *E. officinalis* (Amla).

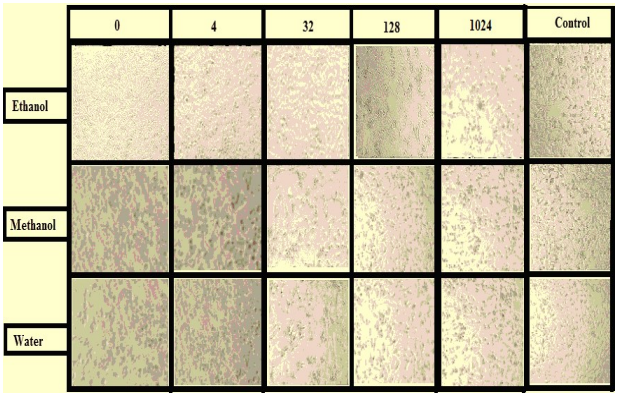


Fig. 2: Microscopic observation (40X magnification) of Vero-Cells after treatment with *C. longa* (Turmeric).

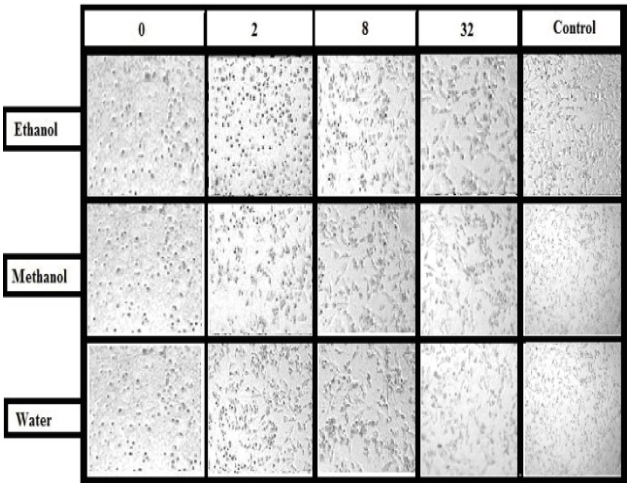


Fig. 3: Microscopic observation (40X magnification) of MDA-MB 231 cells treated with *E. officinalis* (Amla). Control: Uninduced MDA-MB 231 cells.

Morphological analysis

The cells were observed under an inverted microscope to analyze the morphological changes very well with the subsequent increase in the dilution of extracts. Overall, IC50 values of different plant extracts showed different inhibitory effects on cancer cell growth, however as plant extract concentration is decreased by increasing dilution gradually then the growth of cancerous cells is increased. Minor changes were observed in the shape of Vero cells, both in the control well along with the experimental wells. Such changes were mainly because of the uptake of the plant extract prepared with three different solvents into the cell membranes. In addition, no contamination occurred during experiments and resulted in some viable cancerous cells getting strongly attached to the bottom of the wells despite being washed by PBS. Dense growth of cells was present in the control wells which were incubated having a constant cell number along with the respective solvent.

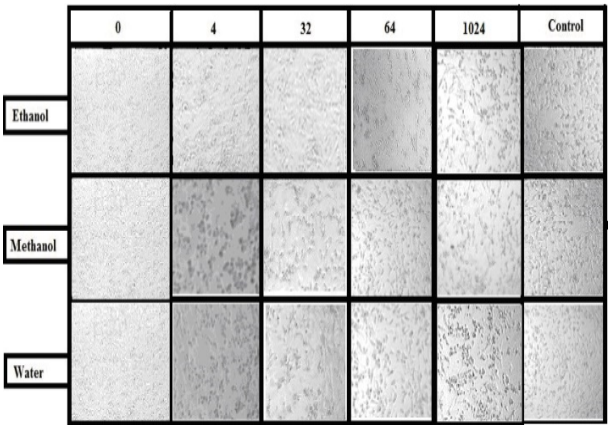


Fig. 4: Microscopic observation (40X magnification) of MDA-MB 231 cells treated with *C. longa* (Turmeric). Control: Uninduced MDA-MB 231 cells.

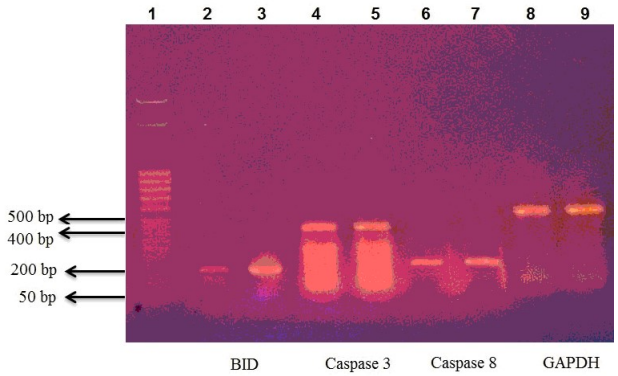


Fig. 5: Expression Profile of BID gene, Caspase-3, Caspase-8, and GAPDH genes in MDA-MB 231 cell-line induced with methanolic extract of turmeric. The untreated cells were considered as control while methanolic extract-treated cells were considered experimental. **Key:** 1st well: DNA Ladder, 2nd well: Untreated, 3rd well: Treated, 4th well: untreated, 5th well: treated, 6th well: untreated, 7th well: treated, 8th well: untreated, 9th well: treated.

Observation of cell morphology and IC_{50} of Vero-cells

The morphological alteration of Vero-Cells lines was observed from an inverted microscope as they were exposed to Emblica officinalis (Amla) and Curcuma longa (Turmeric) extracts. The cells showed prominent cytotoxic effects when exposed to these extracts. According to analysis, the number of dead cells increased significantly with the extracts of Curcuma longa (Turmeric) whereas, An increase in viable Vero-Cells was observed from the treatments of E. officinalis (Amla) and C. longa (Turmeric) extracts at dilution 32 and 128 (figs. 1, 2). The IC_{50} values are shown in table 2. According to the analysis, the effective plant extract was ethanolic C. longa (Turmeric).

Observation of cell morphology and IC_{50} of MDA-MB 231 cells

The morphological alteration of MDA-MB 231 cell lines was observed under an inverted microscope as they were exposed to *Emblica officinalis* (Amla) and *Curcuma longa* (Turmeric) extracts. The cells showed prominent cytotoxic effects after being exposed to these extracts. According to the analysis, the number of dead cells increased significantly with the extracts of *Curcuma longa* (Turmeric). An increase in viable Vero-Cells was observed from the treatments of *E. officinalis* (Amla) and *C. longa* (Turmeric) extracts at dilutions 8 & 32 and 32 & 64 respectively (figs. 3, 4). The relative number of viable cells as a percentage of control was calculated by measuring absorbance at 540 nm. The IC_{50} values are shown in table 3. According to the analysis, the effective plant extract was methanolic *C. longa* (Turmeric). In general, the *Curcuma longa* (Turmeric) ethanolic extract showed IC_{50} at 1024-fold dilution for the Vero cell line and the methanolic extract represented IC_{50} at 1024-fold dilution for MDA-MB 231 cell line. It was observed that the expression of BID was upregulated in methanolic turmeric extract-induced MDA-MB 231 cells, while Caspase-3 and Caspase-8 expressions were unchanged as compared to uninduced MDA-MB 231 cells. The activated BID cleaved into tBID and activated the intrinsic pathway which caused death in methanolic turmeric extract-induced cancerous cells. A 1024-fold dilution was discovered in antiproliferative bioassays of *Curcuma longa* (Turmeric) methanolic and ethanolic extracts. As BID gene expression is more in treated cells than in untreated cells therefore more BID production leads to more tBID production and more tBID leads to more activation of the intrinsic pathway of cell death. How BID is converted into tBID and how tBID regulates the intrinsic pathway of cell death is mentioned in Flow Diagram A.

Expression analysis of apoptotic genes

It is known that the family of Cysteinyl proteases i.e., Caspases are significantly involved in initiating a process of apoptotic cell death. In this regard, Caspases are synthesized initially as an inactive zymogen, but later on, they convert into an active complex comprised of several heterodimeric subunits. To confirm the apoptosis-inducing potential of *C. longa* (Turmeric) extract, the activation of BID, Caspase-3, and Caspase-8 was analyzed by checking their expression in RT-PCR. However, before cDNA synthesis for PCR, the RNA quantity from both treated as well as normal cells was determined with the help of Nanodrop. The RNA quantity was 690.5ng/µl from treated cells and 717.3ng/µl from normal cells. According to the results of RT-PCR, the BID gene in treated cells extract was found over-expressed when compared to untreated cells. The product size of 200 base-pair (bp) was found on gel electrophoresis. Secondly, the expression of Caspase-3 was found more enhanced in treated cell extract than in untreated cells and showed a product size of 427 bp. This product size was found both in treated and untreated cells. Lastly, the expression of Caspase-8 was also found enhanced in treated extract cells than in untreated and showed a product size of 213 bp. This product size was found both in treated and untreated cells. Both over-expression and enhanced gene expressions have shown apoptosis triggering which leads to cell death (fig. 5).

DISCUSSION

The extracts of *Emblica officinalis* (Amla) and *Curcuma longa* (Turmeric) were examined to determine their potential antiproliferative effect by Neutral Red-Dye uptake assay on Vero i.e., kidney epithelial cell-line derived from African green monkey and MDA-MB 231 i.e., human breast adenocarcinoma cell-line. The cells showed prominent cytotoxic effects when exposed to these extracts. The effective plant extract was ethanolic *C.*

longa (Turmeric) as per morphological alteration of both Vero-Cells lines and MDA-MB 231 cell lines. According to the results of RT-PCR, the BID gene in treated cells extract was found over-expressed when compared to untreated cells. The expression of Caspase-3 was found more enhanced in treated cell extract than in untreated cells and showed a product size of 427 base pairs. Lastly, the expression of Caspase-8 was also found enhanced in treated extract cells than in untreated and showed a product size of 213 bp. We found the expression levels of Caspase-3, Caspase-8, and BID genes in treated and untreated breast cancer cells and evaluated the most effective plant extract against breast cancer cells (MDA-MB-231). In general, we found that the *Curcuma longa* (Turmeric) ethanolic extract showed IC_{50} at 1024-fold dilution for the Vero cell line and methanolic extract represented IC_{50} at 1024-fold dilution for MDA-MB 231 cell line. The activated BID cleaved into tBID and activated the intrinsic pathway which caused death in methanolic turmeric extract-induced cancerous cells. The current research proposed that the methanolic extract of *C. longa* (Turmeric) can trigger the death receptor pathway by an enhanced expression of the BID gene, resulting in an up-regulated intrinsic pathway-mediated cell death, while the extrinsic pathway is not up-regulated as expressions of Caspase-8 and Caspase-3 were same in treated and untreated cells. The extrinsic pathway is induced by direct interaction between "death-ligands" and "death receptors", in which Caspase-3, Caspase-8, and BID are activated (Debatin *et al*., 2004 a&b) and Caspase-8 recruits by FADD during apoptosis from ligands interacting with the death receptors (Ashkenazi and Dixit, 1999).

The current study also reported cytotoxicity as a percentage of cell viability estimated from the antiproliferative test in both Vero and MDA-MB 231 cell lines in a dose-dependent manner after 48 hours of incubation with each plant extract. The hiring of initiator Caspases 8,10 promotes the pathways of induced receptors, which takes them to a "death-inducing signaling complex" (DISC). These activated Caspases then procure effector Caspases to degrade cellular targets. Caspase-8 can do cleaving of BH3-only protein Bid. The culminated truncated Bid (tBid) then shifts to mitochondria and generates cytochrome c release for the activation of Caspase-9,3. Caspase-3 is an effect of Caspase and activates intrinsic and extrinsic pathways of cell death as it also plays a major role in apoptosis (Ghavami *et al*., 2009). Soung *et al*., (2005) observed that Caspase-8 was mutated in gastric cancer. The same is the case with Caspase-3, many studies discovered mutations in it in different cancers. For example, the Caspase-3 mutation was reported in the MCF-7 breast cancer cell line (Ghavami *et al*., 2009). The ligation of various death receptors, such as CD95, TRAIL, and tumor TNF-Rs induces activation of Caspase-8, cytochrome *C* and the bcl-2 family. Both nuclear damage as well as endoplasmatic reticulum stress help in increasing the activity of Caspase-9. Hence, both these pathways converge specifically at proteolytic activation of the effect of Caspases 3, 6, and 7 (Svejda *et al*., 2010). We can suggest that *C. longa* (Turmeric) may have induced the oligomerization of Caspase-8. Apoptosis induced from death receptors is used to settle through FADD and that is why it is suggested that apoptosis from *C. longa* (Turmeric) occurs from death receptors. Our results indicate that *C. longa* (Turmeric) induced the cleavage of proapoptotic protein BID leading to the formation of t-BID and triggering the intrinsic pathway (Sutton *et al*., 2000). Thus, it is quite likely that methanolic *C. longa* (Turmeric) activated both checkpoints, i.e., BID, which leads to cleavage of BID (into tBID) and hence, triggers an intrinsic pathway of apoptosis (Gross *et al*., 1999). It also activated Caspase-8 and Caspase-3 leading extrinsic pathways (Abdel-Hameed *et al*., 2012). The results of the present study showed potent cytotoxic effects of ethanolic *C. longa* (Turmeric) extracts on Vero and methanolic *C. longa* (Turmeric) extracts on MDA-MB 231 cells. The IC_{50} value of the extract was found to be much lower than the specified value proposed by NCI for the categorization of a highly pure compound that can be an anticancer agent. Reduction in the viable cell number was very evident after 24 to 48 hours of treatment with the plant extract. Further, the morphological changes were also very more prominent in methanolic extract-treated cells, which showed extensive blebbing along with disruption which suggested induction of apoptotic mechanism for cell death.

Curcumin exhibits anti-inflammatory, immunomodulatory, anti-angiogenic, and anticancer properties. It takes Curcumin has shown anti-proliferative activity in different cancers; it is an inhibitor of NF-κB which also down-regulates the "gene products including Bcl-2, c-myc, NOS, COX-2, TNF-α, Cyclin D1, MMP-9 and interleukins (IL-1, IL-2, IL-6, IL-8, IL-12)" (Wilken *et al*., 2011). Curcumin has been proven to be an anticancer agent in the bladder (Tian *et al*., 2008) and prostate (Mendonça *et al*., 2009) cancer cell lines (Bar-Sela *et al*., 2010). Curcumin can promptly reduce mitochondrial membrane activity and the secretion of cytochrome *c* can activate Caspase-9, 3 for apoptotic cell death (Jana *et al*., 2004). Clinical trials have been conducted on specific doses (e.g., 1-8 g) of curcumin on different cancer patients of the bladder, colorectal, pancreas, skin cancer, colon, and breast. The histological improvements and antitumor effects were noted (Hsieh, 2001). Another study by Lin and Karin (2003) also indicated that curcumin as an antitumorigenic and chemopreventive in combination with chemotherapy drugs can treat ovarian cancer. It was also mentioned by Sharma *et al*. (2005) that curcumin inhibited proliferation and induced apoptosis in different malignant cell lines

derived from malignant tumors of the kidney, breast, colon, lymphoid, melanoma, hepatocellular, leukemia, prostate, and intestine. Zheng *et al*., (2016) indicated that curcumin can treat different cancers. In a clinical trial, oral curcumin (6.0g/day) was used as a treatment in cancer radiotherapy to lessen side effects in breast cancer patients (Ryan *et al*., 2013). A dose of curcumin (i.e., 6000mg/day) for three weeks has also been selected to treat metastatic breast cancer patients along with a standard dose of docetaxel (Bayet-Robert *et al*., 2010). Amla promotes apoptosis and blocks cell proliferation through its immunomodulatory properties, this has been affirmed in many cancer "cell lines including T-lymphoid Jurkat, human erythromyeloid K562, erythroleukemic HEL cell lines and B-lymphoid Raji" (Khan *et al*., 2002). Amla can also use as adjuvant therapy in cancer patients with cyclophosphamide treatment (Madhuri *et al*., 2011). A study by De *et al*., (2013) indicated that amla can be opted for as a treatment either alone or in combination with chemotherapeutic drugs for ovarian cancer through its activation potential of autophagy and inhibition of angiogenesis. The anticancer impacts of aqueous extract of amla were observed in several human cancer cell lines: " HeLa (cervical), A549 (lung), MDA-MB-231 (breast), HepG2 (liver) and SW620 (Colorectal) and SKOV3 (ovarian)" (Ngamkitidechakul *et al*., 2010). It is inferred from previous studies that amla can inhibit various tumor growth in "stomach, liver, breast, uterus and pancreas cancers". It can also minimize the side effects of radiotherapy or chemotherapy (Madhuri *et al*., 2011).

CONCLUSION

The cell-based bioassay and gene expression analysis of cells induced with methanolic extract of *C. longa* (Turmeric) showed activation of apoptosis via. converting BID into t-BID (intrinsic pathway) and activating Caspase-3 along Caspase-8 (extrinsic pathway). The cell growth inhibition and apoptosis induction in cancer cells may make Turmeric an attractive natural source of new anti-cancerous compounds.

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