RESEARCH ARTICLE

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Phytochemical Analysis and Apoptosis-Inducing Anticancer Activity of *Buddleja polystachia* Leaf Extract against HeLa Cell Lines

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Abstract

Objective: Cervical cancer is the second leading cause of mortality in women globally, with its incidence increasing due to multidrug resistance (MDR) and adverse side effects associated with chemotherapeutic agents. Hence, this study was carried out to investigate the selective cytotoxicity and apoptotic induction potential of *Buddleja polystachya* leaf diethyl ether (BPL-DE) extract on cervical cancer HeLa cell lines. **Methods:** The selective cytotoxicity of leaf extracts of *B. polystachya* was assessed by the MTT assay. The apoptotic induction potential of BPL-DE extract at IC_{50} (20 µg/mL) was assessed by Acridine Orange/Ethidium Bromide (AO/EB) dual staining. Furthermore, qRT-PCR analysis was performed to study mRNA gene expression of pro-apoptotic (*Bax, p53*) and anti-apoptotic genes (*Bcl-2*, survivin), as well as *caspase-9* and *caspase-3* gene expression levels. **Results:** The BPL-DE extract showed the highest selective cytotoxic effect against HeLa cells, with an IC_{50} of 20 µg/mL, resulting in a selective index of 25.31. AO/EB dual-staining analysis revealed that the BPL-DE extract at IC_{50} (20 µg/mL) significantly induced late apoptosis ($p \leq 0.001$). qRT-PCR results showed that the mRNA expression levels of Bax and p53 increased by 3.96-fold ($p \leq 0.01$) and 5-fold ($p \leq 0.001$), respectively. In contrast, *Bcl-2* and survivin mRNA expression levels were significantly downregulated by 1.02-fold ($p \leq 0.01$) and 1.13-fold ($p \leq 0.001$), respectively, at two-fold IC_{50} (40 µg/mL). The BPL-DE extract also upregulated the mRNA expression of *caspase-9* and *caspase-3* by 32.07-fold ($p \leq 0.001$) and 15.06-fold ($p \leq 0.01$), respectively, at two-fold IC_{50}. **Conclusion:** The findings of this study demonstrate that the BPL-DE extract significantly induced the *p53*-mediated intrinsic apoptotic pathway in HeLa cell lines and provides a potential alternative therapeutic agent for cervical cancer treatment by minimizing damage to normal cells.

Keywords: Apoptosis- *Buddelja polystachia*- Cervical cancer- Cytotoxicity-Phytochemical analysis

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Introduction

Cervical cancer is the second leading cause of cancer-related mortality among women worldwide [1]. According to 2022 reports of World Health Organization (WHO) the International Agency for Research on Cancer (IARC) appraised that every year 661,021 cervical cancer cases are diagnosed worldwide, resulting in 348,189 deaths [2]. Oncogenic Human Papilloma virus (HPV) specifically types 16 and 18 viral proteins E6 and E7 inhibit the tumor suppressor function of *P53* and Protein Retinoblastoma (PRb) and promotes the up regulation of the expression of anti-apoptotic proteins (Bcl-2 and survivin), thus promoting cancer cell survival and inhibit apoptosis [3].Chemotherapeutic drugs such as cisplatin and carboplatin have been reported to induce apoptosis resistance in cervical cancer cells by downregulating the expression of pro-apoptotic genes such as *P53, Bax*, and Bad while up regulating anti-apoptotic genes such as *Bcl-2, Bcl-xl,* and *Mcl-1* through activation of Mitogen- protein kinase (MAPK) pathway [4]. Plant derived anticancer agents such as Taxol, Camptothecin, Combretastatin and Rhinacanthone used for the treatment of cervical cancer through induction of apoptosis by targeting various apoptotic molecular pathways by modulating mitochondrial intrinsic pathways, inhibition

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of telomerase activity, disruption of tubulin dynamics, and regulation of DNA topoisomerases [5]. These agents have several limitations such as multidrug resistance (MDR) in cancer cells and toxicity to normal cells. Overcoming these limitations researchers have focused on novel strategies for the development of effective, safe and selective anticancer agents for the treatment of cervical cancer.

Buddleja polystachya (*B. polystachya*) is belonging to the family Buddlejaceae, is used in Eastern Africa especially in Ethiopia as a traditional medicine for the treating inflammation, neurological disorders and infectious diseases [6]. The crushed leaves of *B. polystachya* are given orally to treat malaria [7]. Fawzy et al. [8] report that aerial parts of *B. polystachya* dichloromethane and ethyl acetate extracts have shown high selective cytotoxicity against breast (MCF-7), hepatocellular carcinoma (HepG-2) and colon (HCT-116) cancer cell lines. However, to the best of our knowledge the anticancer activities and molecular mechanisms involved in apoptotic induction potential of leaf extract of *B. polystachya* on cervical cancer HeLa cell lines have not been extensively studied and necessitate further examined.

Based on documented anticancer activities against different cancer cell lines, this study hypothesized that leaf extract of *B. polystachya* possessesanticancer effects on HeLa cell lines. Therefore, the aim of the present study was under taken to investigate the Ethno medicinal use of *B. polystachya* for the treatment of cervical cancer by screening the in vitro selective cytotoxicity of different solvent extracts and the most effective extract was further evaluated on HeLa cell lines to elucidate the possible mechanism of apoptotic induction to assess its potential use to develop in to a possible candidate for novel and effective anticervical cancer drug.

Materials and Methods

Plant Collection and Preparation of Extracts

Healthy leaves of *B. polystachya* were collected from Axum city, Ethiopia, during the month of April 2021,a botanist Mr. Melaku Wendaferash from the National Herbarium of Ethiopia, authenticated the plant with voucher specimen AM-001. The leaves were cleaned, with distilled water, shade dried and grounded into a fine powder. A method developed by Wado et al. [9], about 25 grams of powdered leaves material was extracted with 250 ml (1:10 w/v) of diethyl ether, chloroform, ethyl acetate, and ethanol with increasing polarity. The extraction has been carried out using a Soxhlet apparatus with continuous reflux for 12 hours at 50-60°C. After extraction, extracts filtered through Whatman number-1 filter paper, and filtrates were concentrated at 50-600Cusing a rotary evaporator under reduced pressure [9]. The percentage yield of each extract was calculated based on the initial weight of the plant material using the following formula:

$$\text{Percentageyield} = \frac{\text{Extractweightoftheplant (g)}}{\text{Dryweightoftheplant (g)}} \times 100$$

Phytochemical screening

Qualitative phytochemical analysis of leaf extracts of *B. polystachya* was performed according to established and modified methods of [10, 11].

Cell lines cultures

HeLa and normal mouse fibroblast (L929) cell lines were purchased from the American Type Culture Collection (ATCC).The cells were cultured in T-25 cell culture flasks containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS),4mM L-arginine, and 1X Antibiotic-Antimycotic Solution (100 U/ml penicillin and 100 U/ml streptomycin). These cell lines were incubated at 37°C in a humidified atmosphere of 95%with 5% CO_2 until they reached 80% confluence [12].

In vitro cytotoxicity assay

The cytotoxic effect of crude leaf extract of *B.polystachya* against HeLa and L929 cell lines were determined by MTT (3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide dye) assay [13].The cell lines were seeded with a density 1×10^4 cell/well in a 96 micro well plates with in complete DMEM media and incubated for 24 hours at 37°C in a humidified atmosphere of 95% with 5% CO_2. Subsequently the cells were treated with different concentrations of extracts 25, 50,100, 250 and 500 µg/ml andpositive control cisplatin (2.5, 5, 10, 20, 40 µg/ml)into each well in triplicates except to the control wells where nutrient medium (DMEM) was added and incubated for 24 hours. After treatment, 20 µl of MTT (5mg/ml) solution was added and allowed for 4 hours of incubation in dark 5% CO_2 incubator at 37°C.The insoluble formazan products were solubilized with 100µl of 10% Sodium Dodecyl Sulfide (SDS) then plates gently shaken simultaneously, and the solubilized formazans were measured spectrophotometrically usingmicro-ELISA plate-reader at 570 nm. The cytotoxicity was determined using following formula:

$$Cytotoxicity\ (\%) = 100 - \frac{OD\ of\ test}{OD\ of\ control} \times 100$$

IC_{50} values and selective index (SI) Selective cytotoxicity of the tested extracts were calculated according to the given formula:

$$Selective\ Index(SI) = \frac{IC50\ of\ normal\ cell\ lines}{IC50\ of\ cancer\ cell\ lines} \times 100$$

Cellular Morphological Analysis

To assess the apoptotic cellular morphological changes induced by diethyl ether leaf extract of *B.polystachya* (BPL-DE) on HeLa andL929 cell lines, 5×10^5 cell/well were seeded in to 12 well plates. After 24 hours of treatment with BPL-DE extract at IC_{50} (20 µg/ml), $2\times IC_{50}$ (40 µg/ml) and $4\times IC_{50}$ (80 µg/ml), the cellular morphological changes were observed using an inverted phase contrast microscope at 1000X magnification [14].

Acridine Orange/Ethidium Bromide dual staining assay

HeLa cells were seeded with density of 5×10^5 cells/well in 12-well plates in DMEM and treated with BPL-DE extract at IC_{50}(20 µg/ml)for 24 hours in 5% CO_2 incubator. After treatment, cells were centrifuged at 300×g for 10 minutes, the supernatant was discarded and the cells were washed twice with phosphate buffer saline

(PBS). The cells were stained using 1 µl of AO/EtBr solution (1µg/ml of PBS) [15] washed twice with PBS, and observed under inverted fluorescent phase contrast microscope at 1000X.AO/EtBr staining distinguishes between viable cells, early apoptotic, late apoptotic or necrotic cell based on florescence emission. Live cells appeared green, early apoptotic cells showed bright green, orange color were seen in late apoptotic and red necrotic cells [16].

RT-q PCR analysis

The m-RNA expression of apoptosis-related genes *P53*, *Bax*, *survivin, Bcl-2, caspases - 3* and 9 were assessed by RT-q PCR. HeLa cell lines with a 5×10^5 cells density were seeded in 12-well plates and treated with IC_{50} (20µg/ml) and $2\times IC_{50}$ (40µg/ml)of BPL-DE extract and incubated for 24 hours in 5% CO_2 incubator [17, 18]. Total RNA of treated and untreated HeLa cell lines were extracted using TRIzol method (Ambion, USA) according to the manufacturers protocol. The purity and concentration of RNA was measured by Nanodrop 2000 spectroscopy (thermo scientific).Then cDNA was prepared by using Prime Script™ cDNA Synthesis Kit, according to the manufacturer's instructions. The mRNAgene expression of target genes were analyzed by Quantitative real-time PCR (RT-qPCR) withGoTaq® qPCR Master Mix (Promega, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was usedas an internal control. Gene expression levels were calculated using the cyclic threshold method (ct method). The mean of the ct values from the triplicate was used to calculate the expression level of the target gene by using $2^{(-\Delta\Delta ct)}$ formula.

Statistical analysis

The results were expressed as the Mean ± Standard deviation (SD) of three independent experiments. The statistical difference among the test and control groups were evaluated by one-way analysis of variance (ANOVA) by Graph pad prism 9.0 software and followed by a Tukey-test. $p\leq0.05$, $p\leq0.01$, $p\leq0.001$ represents a significant difference between the control with the test group.

Results

The percentage yield

As shown in Table 1, we studied the color, consistency, and percentage yields of the crude extracts of *B. polystachya* leaves in the current study. When *B. polystachya* leaves were extracted using various organic solvents, the percentage yields of the leaves varied, yielding extract residues that ranged from 2.8 to 8.9 g per 25 g of *B. polystachya* leaves. It was found that ethanolic and ethyl acetate leaf extracts of *B. polystachya* have shown the highest percentage yields of 8.9 and 5.7 g per 25 g, respectively, while chloroform and diethyl ether gave the lowest percentage yields of 3.9 and 2.8 g per 25g, respectively.

Phytochemical screening

As shown in Table 2, the qualitative phytochemical analysis revealed that chloroform, ethyl acetate, and ethanolic leaf extracts of *B. polystachya* had shown the maximum presence of phenolic compounds and flavonoids and moderate levels of alkaloids, glycosides, and steroids. Whereas diethyl ether leaf extracts of *B. polystachya* were found to contain moderate levels.

In vitro selective cytotoxicity activities

As shown in Figure 1 and Table 3, BPL-DE, BPL-CH, BPL-EA, and BPL-ET extracts exhibited a notable dose-dependent selective cytotoxicity on HeLa cell lines. After 24 hours of treatment at higher concentration 500 µg/ml, BPL-DE and BPL-CH extracts were found to be more potentially inhibited the growth of HeLa cell lines, with inhibition of 95.32% and 92.99%, respectively (***$p\leq0.001$). Similarly, BPL-EA and BPL-ET extracts have shown significant inhibitory effects on HeLa cell lines with inhibition of 78.17% and 82.53%, respectively (**$p\leq0.01$). Cisplatin at 40 µg/ml, showed a notable cytotoxicity of 87.56% in comparison to the leaf crude extracts of *B. polystachya*.

These results indicated that BPL-DE, BPL-CH, BPL-EA, BPL-ET extracts, and cisplatin, exhibited substantial cytotoxic effects on HeLa cells, by outperform their effects on L929 cell lines. The IC_{50} values for the extracts were19.75, 29.85, 60.67, 55.15 and 9.53 µg/ml respectively. The selective cytotoxic activity of *B. polystachya* leaf extracts on HeLa cell lines were observed in the following order:

BPL-DE > BPL-CH > BPL-ET > BPL-EA.

Morphological changes

As shown in Figures 2a, under phase contrast microscopy, HeLa cells treated with IC_{50} (20 µg), $2IC_{50}$ (40 µg), and $4IC_{50}$ (80 µg) of BPL-DE for 24 hours showed cell shrinkage, irregular bulges in the cell membrane, cell rounding, decreased cell confluence and cell aggregation, as indicated by the red arrows in Figure 2a, compared to the control cell lines. When L929 cell lines were treated with various concentrations IC_{50} (20 µg), $2IC_{50}$ (40µg), and 4 IC_{50} (80µg) of BPL-DE extract, as illustrated in Figures 2b, have no significant morphological changes were observed.

Table 1. Colour, Consistency and Percentage Yield (w/w)

Plant	Solvent	Colour	Consistency	Percentage yield (g/25g)
B. polystachya	Dietheyl Ether	Light Brown	Sticky	2.89
	Chloroform	Green	Sticky	3.95
	Ethyl Acetate	Reddish	Non – Sticky	5.79
	Ethanol	Orange	Non – Sticky	8.97

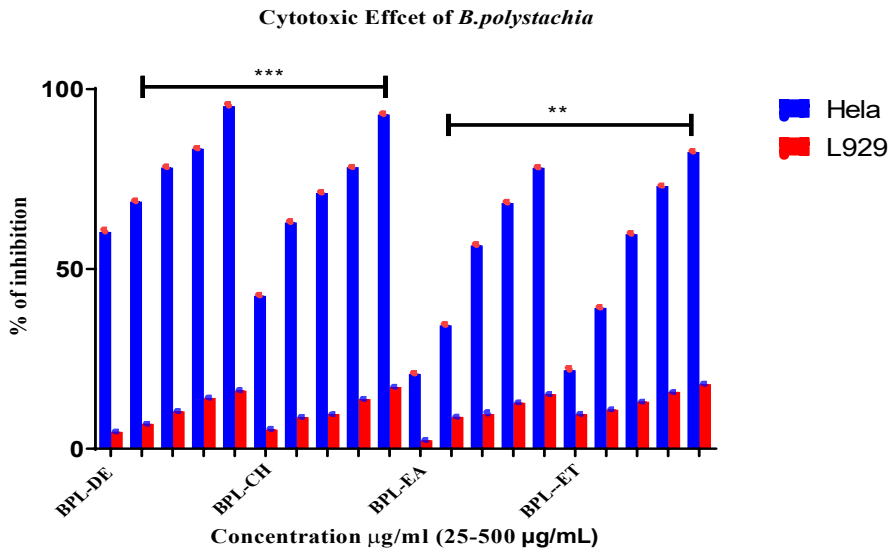

Figure 1. Cytotoxic Effect of Leaf Crude Extracts of *B. polystachya* at Different Concentrations (25-500 µg/ml) on HeLa and L929 Cell Lines.

Acridine orange / Ethidium bromide (AO/EB) staining

As presented in Table 4 after 24 hours of treatment with BPL-DE extract at IC_{50} (20 µg/mL) significantly decreased the viable HeLa cells from 94.43% to 10.03% and substantially increased early apoptotic, late apoptotic, and necrotic cells from 5.57 % to 36 % (**p≤0.01), 3.35% to 44.3% (***p≤0.001) and 1.7 % to 9.62% (*p≤0.05), respectively compared with untreated cells (Figure 3b). As shown in Figure 3a (A), florescent microscopy observation revealed that control HeLa cells are characterized by a regular monolayer appearance (indicated by a yellow circle with a red arrow). In contrast, Figure 3a (B) showed that after treatment, the cells become rounded and disconnected from each other (indicated by a yellow circle with a red arrow). As shown in Figure 3a(C) control HeLa cells exhibited a uniform green color, indicated by the red rounded circle with an orange arrow.

Pro-apoptotic and anti-apoptotic gene expression

RT –qPCR m-RNA gene expression studies of pro-apoptotic gene *Bax* revealed that BPL-DE extract treated HeLa cells was significantly (**p≤0.01) increased m-RNA expression by 0.83 and 3.96 at IC_{50} (20 µg/mL) and $2IC_{50}$ (40 µg/mL) respectively while compared to control, as shown in Figure 4 and Table 4. In addition,the anti-apoptotic *Bcl-2* m-RNA gene expression revealed that BPL-DE extract treated HeLa cells as shown in Figure 4 significantly (**p≤0.001) down regulated by 0.76 and 1.02 at IC_{50} (20 µg/mL) and $2IC_{50}$ (40 µg/mL) respectively while compared to control.

As shown in Figure 4, from the RT –qPCR m-RNA gene expression results of p53revealed thatBPL-DE extract treated HeLa cells were significantly (***p≤0.001) up regulates m-RNA expression of 1.1 and 5 at IC_{50} (20 µg/mL) and $2IC_{50}$(40 µg/mL) respectively compared to control in addition m-RNA gene expression of survivin results revealed that BPL-DE treated HeLa cells were significantly (***p≤0.001) down regulated by 0.66 and 1.13 folds respectively at IC_{50} (20 µg/mL) and $2IC_{50}$(40 µg/mL) while compared to control, which indicated that a significant inhibition of this protein normally inhibits apoptosis and promotes cell survival.

Caspase-9&3 gene expression

As shown in Figure 5, *caspase-9* gene expression increased steadily in comparison to control, the RT-qPCR

Table 2. Preliminary Phytochemical Screening

	Buddleja polystachya			
Phytochemicals	Diethyl ether	Chloroform	Ethyl acetate	Ethanol
Alkaloids	+	++	++	++
Phenolic compounds	++	+++	+++	+++
Flavonoids	++	+++	+++	+++
Tannin	++	++	+++	+++
Glycosides	++	++	++	++
Terpenoids	-	-	++	++
Saponin	_	_	_	+
Steroids	++	++	++	++
Anthraquinones	--	--	++	++

"-", Absence; "+", Minor presence; "++", Moderate presence; "+++", Indicates the maximum presence of secondary metabolites.

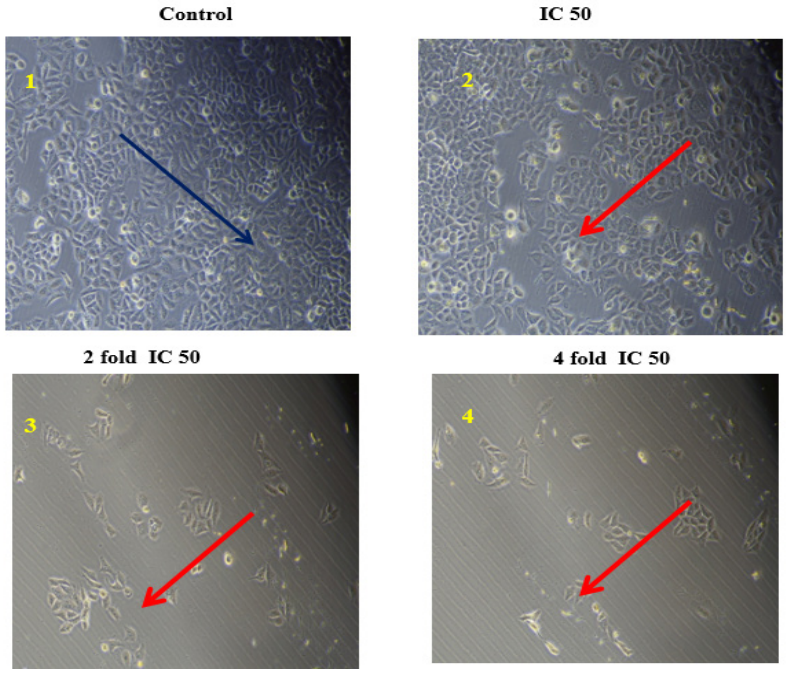

Figure 2 (a). Morphological changes of HeLa cell lines observed under phase contrast microscope (1000X) (1 (Control), 2, 3 and 4) HeLa cell lines treated with BPL-DE at IC_{50} (20 µg/mL), $2IC_{50}$ (40 µg/mL), and $4IC_{50}$ (80 µg/mL) respectively. Morphological changes were indicated in red arrows.

analysis revealed that enhanced levels of *caspase -9* was 15.87 folds at IC_{50} (20 µg/mL) of BPL-DE (**p≤0.01) and 32.07 folds at $2IC_{50}$ (40 µg/mL) (***p≤0.001). Similarly, *caspase-3* mRNA expression up regulation increasedby 5.94-foldat IC_{50}(20 µg/mL) (**p≤0.01) and 15.06-fold at $2IC_{50}$ (40 µg/mL) (**p≤0.01) compared to the control. Collectively these findings indicated that BPL-DE significantly up regulates the m-RNA gene expression of the *caspase-9* and *caspase-3* genes, which are considered as hall marks of induction of apoptosis in HeLa cell lines.

Discussion

The percentage yields of the results were in line with earlier findings of Haile et al. [19], reported that the n-hexane, ethanol, and methanol solvents have shown

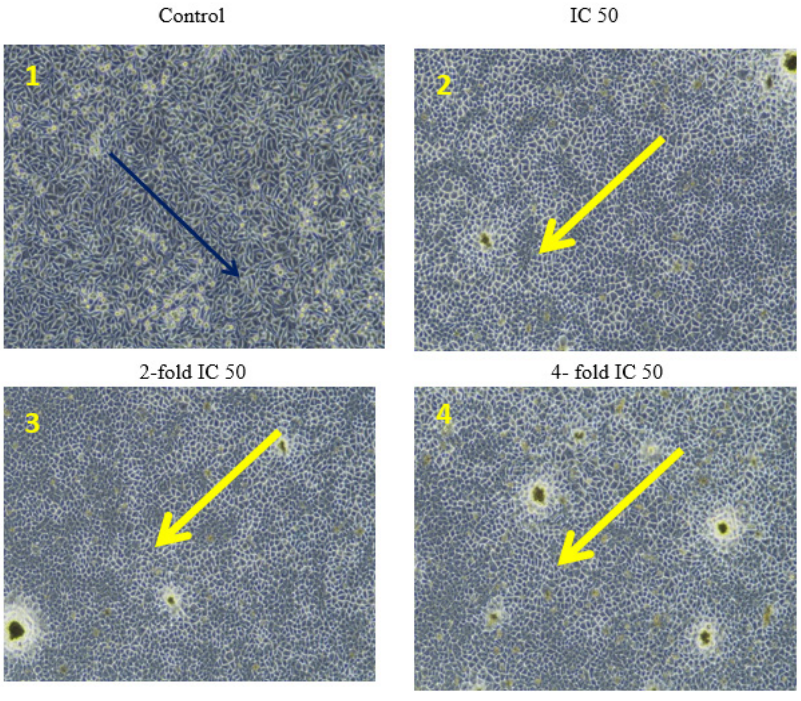

Figure 2(b). Morphological changes of cell lines (L929) observed under phase contrast microscope (1000X) (1 (Control) 2,3and 4) L929 cell lines treated with BPL-DE at IC_{50} (20µg/mL), 2 fold IC_{50} (40µg/mL), and four fold IC 50 (80µg/mL) respectively. Morphological changes were indicated in yellow arrows.

Table 3. *In vitro* Cytotoxicity Effect of *B. polystachya* Leave Extracts

Extracts/Standard	Concentration (µg/ml)	% Cytotoxicity		IC50 (µg/ml)	Selective Index (SI)
		Hela Cells	L929	Hela Cells	
BPL-DE	25	60.33 ±066	4.69±0.15	19.75	
	50	68.74±0.32	6..95±0.02		25.31
	100	78.19±0.33	10.42±0.11		
	250	83.42±0.33	14.08±0.21		
	500	95.32±0.66	16.18±0.12		
	25	42.53±0.33	5.34±0.30		
BPL-CH	50	62.95±0.39	8.8±0.10	29.85	16.75
	100	71.14±0.34	9.64±0.04		
	250	78.31±0.10	138±0.15		
	500	92.99±0.33	17.19±0.12		
BPL-EA	25	20.83±0.31	2.34±0.16		
	50	34.34±0.38	8.85±0.14	60.67	8.24
	100	56.56±0.42	9.64±0.53		
	250	68.34±0.40	12.8±0.12		
	500	78.17±0.26	15.2±0.03		
	25	21.85±0.66	9.64±0.14	55.15	9.06
BPL-ET	50	39.15±0.33	10.85±0.12		
	100	59.66±0.42	13.09±0.03		
	250	73.08±0.15	15.8±0.04		

Table 4. Apoptotic Induction Effect of BPL-DE at IC_{50} on HeLa Cells and L929

Test	Number of Apoptotic cells /100			
	Live cells (Green)	Early Apoptotic (Green &yellow)	Late Apoptotic (Orange & light red)	Necrosis (Red)
Control	94.43±0.15	5.57± 0.78	3.35 ± 0.25	1.7± 0.19
BPL-DE (IC50 20 µg)	10.03 ±066	36 ±0.57		9.62±0.3

the percentage yields of 2.56 g, 45.37 g, and 30.96 g, per 100 g, respectively from leaf extracts of *B. polystachya* by using maceration process and these reports render support to the current finding. Similarly, the percentage yields obtained in various solvents are consistent with the findings of Getahun et al. [20], found that the yields of the leaf extract of *B. polystachya* varied depending on the solvent used: chloroform yielded 21.3 g per 80 g of leaves, whilst ethyl acetate yielded 32.6 g per 80 g. Our findings coincide with Tesfamaryam et al. [21], who found that 80% methanolic leaf extracts of *B. polystachya* yielded 19.5g/100g by maceration. The obtained extracts from leaves of *B. polystachya* are considered adequate for subsequent experimental activities such as phytochemical analysis, evaluation of in vitro selective cytotoxic activities and induction of apoptotic potential in HeLa cervical cancer cell lines.

Our phytochemical analysis results similar with previous reports of Mohammed et al. [22], reported that hydro alcoholic leaf extracts of *B. polystachya* has shown the presence of flavonoids, phenolic compounds, tannins, saponins, steroids, and anthraquinones.

Table 5. Modulation Effect of BPL-DE at IC_{50} on Pro-Apoptotic and Anti-Apoptotic Gene Profile on HeLa Cell Lines

S.No	Gene	Control	*B. polystachya* leaves diethyl ether extract Fold of up regulation	
			IC_{50}	2 fold IC_{50}
1	*Bax*	1.3	2.13	5.26
2	*P53*	1.3	2.4	6.3
3	*Bcl-2*	1.3	-	-
4	*Survivin*	1.3	-	-
5	*Capases-9*	1.75	17.62	33.82
6	*Capases-3*	1.75	7.69	16.81

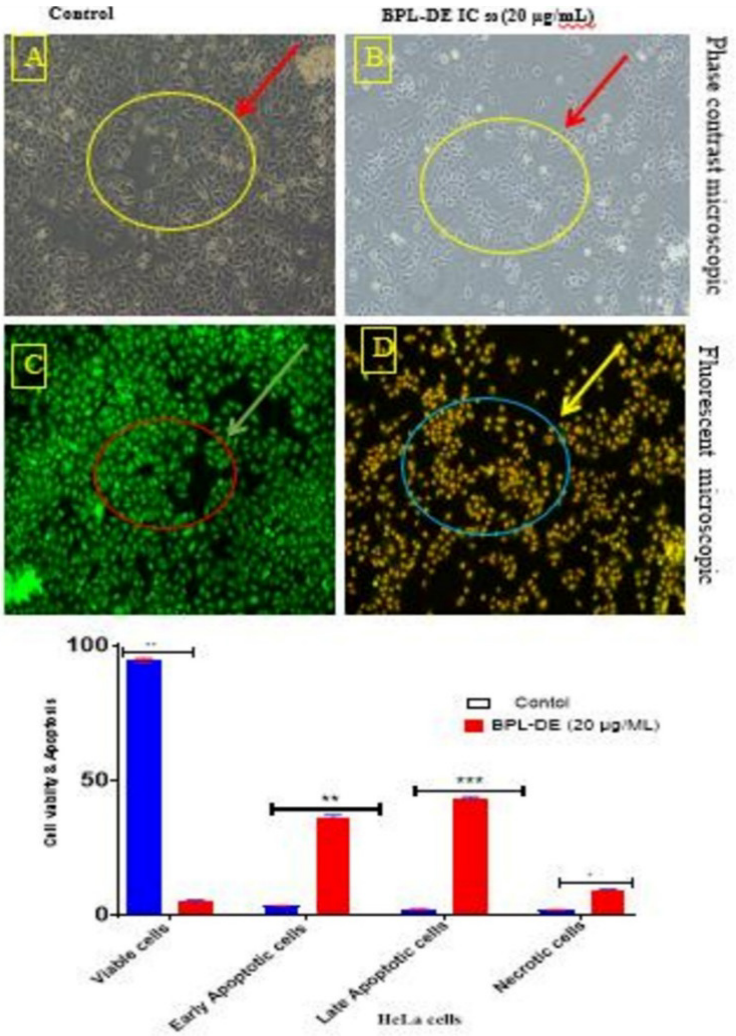

Figure 3. Nuclear Changes of HeLa Cell Lines Observed Untreated (A) Treated (B) under phase contrast microscope (1000X) and Untreated (C) Treated (D) observed under fluorescent microscopy using Acridine orange / Ethidium bromide dual staining (3a) and percentage of viable cells, early apoptotic, late apoptotic and necrotic cells presented as the mean of three replicates ± SD. (3b).

The in vitro selective cytotoxic activities of organic leaf extracts of *B. polystachya* against HeLa cervical cancer cell lines, are inconsistent with Fawzy et al. [23], reported dichloromethane, ethyl acetate, N-butanol, and

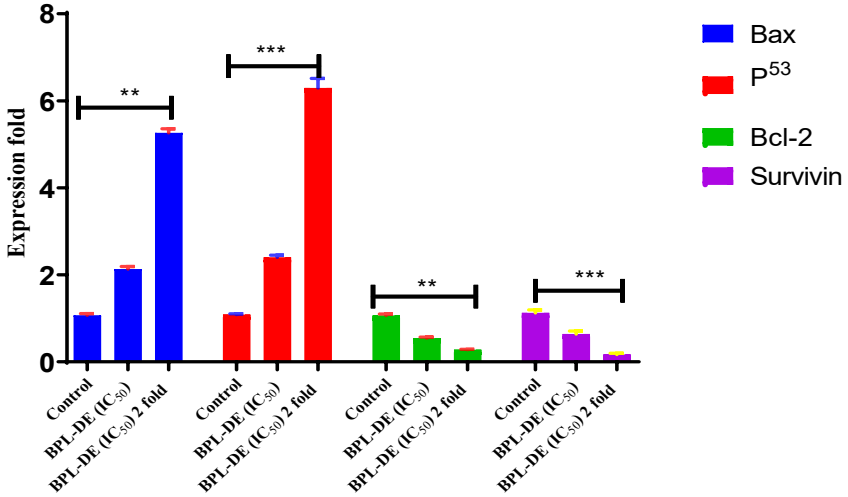

Figure 4. Apoptotic Effect of BPL-DE at IC_{50} (20 µg/mL) and $2IC_{50}$ (40µg/mL) on m-RNA Gene Expression Analysis of Pro-Apoptotic (*Bax & P53*) and Anti-Apoptotic (*Bcl2 & Survivin*) in HeLa Cell Lines for 24 hrs. qualitative real times by PCR method

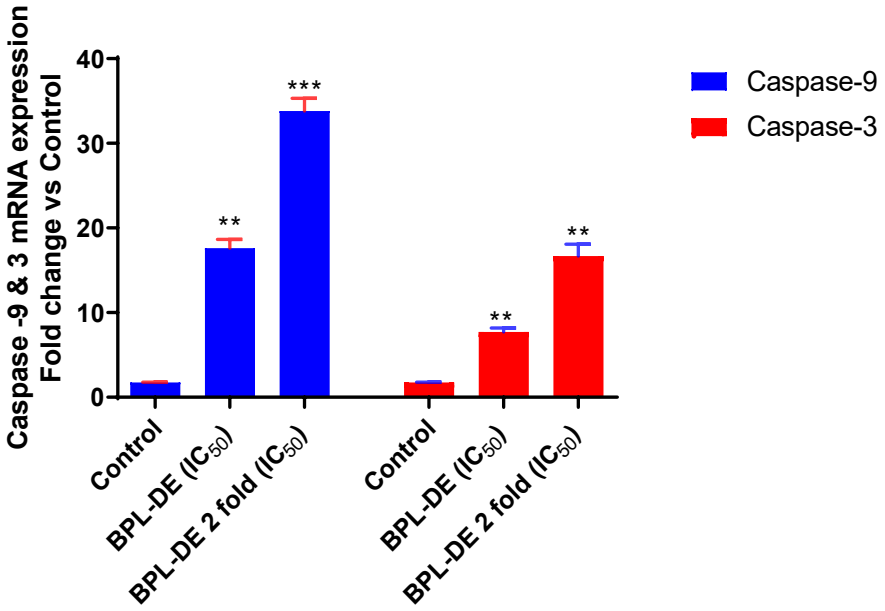

Figure 5. Apoptotic Effect of BPL-DE at IC_{50} (20µg/mL) and 2 fold (40µg/mL) on m-RNA Gene Expression Analysis of *caspase 9* & 3 in HeLa cell lines for 24 hrs. qualitative real time by PCR method. Values are presented as the mean of three replicates ± SD. Significant differences (*p≤0.05, **p≤0.01, ***p≤0.001) between B. polystachya leaf extracts.

aqueous extracts of the aerial parts of *B. polystachya* have shown a dose-dependent cytotoxicity against breast (MCF-7), liver (HepG-2), and colon (HCT-116) cancer cell lines. Additionally, the SI values of BPL-DE, BPL-CH, BPL-EA, and BPL-ET extracts on HeLa cell lines compared with L929 cell lines were 25.3, 16.75, 8.24, and 9.06, respectively (Table 2). Our results have shown that BPL-DE had a half cytotoxic concentration (CC50) of >500 µg/ml against L929 cell lines, with an IC_{50} of 20 µg/ml against HeLa cell lines, resulting in a selective index of 25.31 (Table 3).

In accordance to the US National Cancer Institute plant screening program of Boik, crude extracts having cytotoxicity with IC_{50} value below 20 µg/ml against cancer cell lines are selected for validating its anticancer activities. Based on this criterion, the BPL-DE extract was selected for investigation of the apoptotic potential in HeLa cell lines by studying pro and anti-apoptotic gene expression using real-time PCR. According to [24], flavonoids and phenolic compounds in plant extracts supports their medicinal properties and therapeutic potential for cancer treatment.

These results suggested a selective cytotoxicity of BPL-DE towards HeLa cancer cells, highlighting its potential as a targeted therapeutic agent. Our results are in agreement with, Ozcan et al. [25] reported methanolic tuber extract of C. sanguicolle demonstrated effective cytotoxic effects on human cervical cancer (HeLa and C-4 I) cell lines, with IC_{50} values of 0.01 mg/ml and 0.001 mg/ml at 48 hours, respectively by inducing morphological changes, and the formation of apoptotic bodies, in a dose-dependent manner. In contrast, when HeLa cells were treated with IC_{50} (20 µg/ml) of BPL-DE extract the nuclear membrane changes resulted in the appearance of cells with four different colors: green, green mixed with orange, orange with light red, and red, due to nuclear changes such as chromatin condensation and DNA fragmentation [26]. The results of AO/EB dual staining confirmed that the BPL-DE extract significantly exhibits cytotoxic effects on HeLa cell lines through the induction of both late and early apoptosis.

Our results are in agreement with Twilley et al. [27], reported that the ethanolic leaf and stem bark extracts of Buddleja salinga shown potent cytotoxicity against melanoma skin cancer (UCT-MEL-01) cell lines, by inducing substantial apoptosis confirmed by hematoxylin and eosin staining. Our findings are consistent with Suh et al. [28] reported that ethanolic extract of green algae Chloromonas species was up regulate *caspase-3* and p53, while down regulating Bcl-2 in HeLa cells at 12.5 and 25 µg/mL for 24 hours. Prasad et al. [29] reported that an ethanolic extract of orchid Dendrobium chrysanthum up regulated *Bax* and *p53* while decreased *Bcl-2* in HeLa cell lines at 450 µg/ml for 24 hours. These previous reports have shown that the BPL-DE extract can effectively up regulates the m-RNA gene expression of *Bax* and down regulates the *Bcl-2* expression.

Our findings are in line with the previous findings of Le-Trung et al. [30], reported that the hexane fraction of the ethanolic fruit extract of Sphaerocoryne affinis exhibited strong anticancer activity by inducing apoptosis against HeLa cell lines by up regulating the intrinsic pathways of *caspase-3* and *caspase-9*. In other studies of Mohd-Salleh et al. [31] are similar with current results, who reported that ethyl acetate and hexane leaf extracts of Pereskiableo have induced apoptosis through the activation of *caspase -3* mediated intrinsic pathways and also up regulation of gene expression of *Bax*, *p53*, and down regulation of *Bcl-2*.

In conclusion the present study highlighted that

BPL-DE extract, had potent dose-dependent selective cytotoxicity against the HeLa cell line through nuclear changes by up regulating tumor suppressor *p53*, *Bax* and down regulated *Bcl-2* and survivin genes, via *caspase-9* and *caspase-3* mediated intrinsic apoptosis in HeLa cell lines. Based on the current finding, BPL-DE extract could be useful for the development of cost effective and safe therapeutic agent for the treatment of cervical cancer.

Author Contribution Statement

Amete Mehari A, Zenebe Teka M, Teklemicheal Tesfey and Guesh Mulaw conducted the experimental work and acquisition of all the data in Department of Biology and Chemistry. Neelima P, Kamalakararao K and Naveen Kumar A.Dinvolved in collection of review of literature, draftingmanuscript and editing. Krishna Chaithanya K performedstatistical analysis and organized manuscriptaccording to the submission guidelines of Journal. Allauthors reviewed and verified final version ofthe manuscript.

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Scientific Approval

It is a part of an approved student thesis

Ethical Declaration

There is no involvement of human participants or animals hence did not require ethical approval.

Data availability statement

All data t supporting the finding of this study areincluded within the article.

Conflict of interest

The authors declare have no conflict of interest.

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