



# Petroleum ether extract of *Ophiorrhiza eriantha* Wight induces apoptosis in human breast cancer MCF-7 cell line

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## ABSTRACT

*Ophiorrhiza mungos* is a traditionally used anticancer plant and is a source of monoterpene indole alkaloid camptothecin. *Ophiorrhiza eriantha* Wight is a poorly studied allied species of *O. mungos*. In the present study, we compared the *in vitro* anticancer activity of different extracts of *O. eriantha* Wight with *O. mungos*. Petroleum ether extract of aerial part of *O. eriantha* Wight (OEAPE) was found to be the most active among the 12 extracts. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay on Michigan Cancer Foundation-7 (MCF-7) cell line was conducted and OEAPE shows significant activity. OEAPE induced apoptosis and an in-depth examination of the apoptotic process was carried out. After a statistical data analysis of results of Acridine orange assay, Western Blot analysis of p53, Bcl-2, Bax, caspase-7, caspase-9, Flow cytometry, and DNA fragmentation assay, it is evident that OEAPE induces apoptosis in breast cancer cell line (MCF-7).

## INTRODUCTION

Carcinoma of the breast is the second leading cause of cancer and is the fifth cause of death from cancer (Ferlay *et al.*, 2015; Momenimovahed and Salehiniya, 2019). Breast cancer is a collective term used to describe different types of neoplastic diseases affecting the mammary gland (Sainsbury *et al.*, 2000). Clinically relevant breast cancer typically derives from epithelial cells and thus termed as carcinoma (Makki, 2015). ER+ (estrogen receptor alpha positive) and PR+ (progesterone receptor alpha positive) breast cancer accounts for 60%–70% of human breast cancer. Less common cancer like human epidermal growth factor receptor 2 and triple-negative cancers are more aggressive and difficult to treat (Yersal and Barutca, 2014). Since many drugs used to treat cancer are obtained from plants, searching for new

molecules which can battle cancer cells in a better way is a goal of researchers in the field

The root of *Ophiorrhiza mungos* is traditionally used for cancer treatment in Ayurveda (Warrier and Nambiar, 1994). The whole plant of *O. mungos* is a source of monoterpene indole alkaloid camptothecin having a potent anticancer activity (Gharpure *et al.*, 2010). The presence of forty-seven species and nine varieties of the genus *Ophiorrhiza* was reported in the Indian subcontinent (Deb and Mondal, 2001). Out of this, 16 species and three varieties are reported from Southern Western Ghats (Sasidharan, 2004). *Ophiorrhiza eriantha* Wight is an erect subshrub belonging to the family Rubiaceae. high-performance thin layer chromatography densitometry-based quantification of camptothecin in methanol extract of the whole plant of *O. eriantha* Wight is done by Satheeshkumar *et al.* (2012). Jose and Satheesh Kumar (2004) developed a method for micro-propagation of *O. eriantha* Wight through leaf explant cultures. After an extensive literature review, it was found that no other research works were conducted on *O. eriantha* Wight.

Apoptosis is the major defense strategy of the cells from being cancerous (Kerr *et al.*, 1972). Regulation of apoptosis is

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controlled by initiator caspases and executioner caspases (Olsson and Zhivotovskiy, 2011). In this study, we selected two plants *O. mungos* L. and *O. eriantha* Wight, roots and aerial parts were separated, and sequentially extracted with petroleum ether, ethyl acetate, and ethanol. A preliminary anticancer screening study of all the 12 extracts was done on daltons lymphoma ascites (DLA) cell lines. Among them, the petroleum ether fraction of the aerial part of the *O. eriantha* Wight shows the maximum activity. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay of petroleum ether fraction on Michigan Cancer Foundation-7 (MCF-7) cell lines was conducted and shows significant activity. AO/EB staining is used to study nuclear changes and apoptotic body formations and the results show significant activity. Analysis of Bcl-2, Bax, Caspase-7, Caspase-9, and p53 was performed using the western blot method and the petroleum ether fraction of the aerial part shows significant activity. DNA fragmentation assay and Flow cytometry analysis show that petroleum ether fraction of aerial part of *O. eriantha* Wight has significant apoptotic activity against breast cancer cell lines.

## MATERIALS AND METHODS

### Plant materials

The whole plant of *O. eriantha* Wight (voucher specimen number PARC/2020/4207) and the whole plant of *O. mungos* L. (voucher specimen number PARC/2018/4018) were collected from Palode, Trivandrum district of Kerala, India and authenticated by Prof. Jayaraman, Director, Plant anatomy research center, West Tambaram, Chennai. The roots and aerial parts were separated, shade dried, and powdered by using an electric blender.

### Extraction

40 grams of powdered roots and aerial part of *O. eriantha* Wight and *O. mungos* were separately packed in Soxhlet extractor and extracted sequentially with petroleum ether (60–80), Ethyl acetate, and Ethanol. Petroleum ether (60–80) and Ethyl acetate were purchased from Merck and absolute alcohol was purchased from Travancore sugars and Chemicals, Thiruvalla, Kerala (distilled to remove impurities). All the 12 extracts were evaporated to dryness under reduced pressure and stored at 0°C–4°C until for further use. All the 12 extracts were given the following codes. *Ophiorrhiza eriantha* root petroleum ether, *O. eriantha* root ethyl acetate, *O. eriantha* root ethyl alcohol. *Ophiorrhiza O. eriantha* aerial petroleum ether (OEAPE), *O. eriantha* aerial ethyl acetate, *O. eriantha* aerial ethyl alcohol, *O. mungos* root petroleum ether, *O. mungos* root ethyl acetate, *O. mungos* root ethylalcohol. *O. mungos* aerial petroleum ether, *O. mungos* aerial ethyl acetate, *O. mungos* aerial ethyl alcohol.

### Short term *in vitro* cytotoxicity study using DLA cells

The Daltons Lymphoma Ascites cells were obtained from Amala Cancer Research Centre, Thrissur, Kerala, washed thrice with phosphate buffer solution. Trypan blue exclusion method is used for determining Cell viability (Kanagamani *et al.*, 2017). Cells were treated with various concentrations of all the 12 extracts of *O. eriantha* Wight and *O. mungos*.

$$(\%) \text{ cytotoxicity} = \frac{\text{Number of dead cells}}{\text{Number of live cells} + \text{Number of dead cells}} \times 100$$

### MTT assay

MCF-7 cell line (Passage number 30), was procured from National centre for Cell Sciences, Pune, India (Vijayarathna and Sasidharan, 2012). Stock cell was then cultured and maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS, 100 µg/ml streptomycin, 100 U/ml penicillin, and incubated at 37°C with 5% Co<sub>2</sub>. MTT assay is used to estimate cytotoxicity. In a 48 well plate, approximately 1 × 10<sup>5</sup>/ml cells were seeded, with complete growth media (DMEM) and allowed to attach. Added 5,10,25,50, and 100 µg/ml of OEAPE. After 48 hours of incubation, in fresh medium, 20 µl of 0.5% MTT were added, again incubated for 4 hours. By measuring absorbance at 570 nm, the percentage cell viability was calculated using the following formula

$$(\%) \text{ Cell death} = \frac{\text{Ab of control} - \text{Ab of sample}}{\text{Ab of control}} \times 100$$

### Acridine orange/ethidium bromide (AO/EB) double staining

AO/EB double staining is used to study characteristic features observed during apoptosis (Nath *et al.*, 2014). MCF-7 cells were treated with 5, 10, and 20 µg/ml of OEAPE. Acridine orange stains all cells (live and dead). Ethidium bromide stains only dead cells. The viable cells appear uniformly green. Cells in the early stages of apoptosis are observed light green and late stages appears in orange to red color.

### Western blotting, flow cytometry

MCF-7 cells were treated with 5, 10, and 20 µg of OEAPE for 48 hours. Cells solubilized with lysis buffer. The protein lysates were separated by 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Diaminobenzidine is used for developing color (Bai *et al.*, 2020). Flow cytometry is a method for studying the cell cycle. Cellular DNA content can be measured and it gives the correct picture of cells in different phases of the cell cycle (Mousavi *et al.*, 2009). MCF-7 cells were treated with 0, 5, 10, and 20 µg/ml of OEAPE for 48 hours. Cells were harvested and incubated. Cell death was quantified using a flow cytometer. Data were analyzed with CellQueste software.

### DNA fragmentation assay

DNA fragmentation is a characteristic feature of programmed cell death. Cleavage pattern of DNA due to cytotoxic effect of OEAPE was analyzed using agarose gel electrophoresis (Fakai, 2019) MCF-7 Cell line obtained from National Centre for Cell Science, Pune, India. It is then treated with control, 5, 10 and 20 µg/ml of OEAPE and incubated for 24hours.

## RESULT AND DISCUSSION

Figure 1 represents the results of short-term *in vitro* cytotoxicity using DLA cells. Among all the 12 extracts of aerial

and root part of *O. eriantha* Wight and *O. mungos*, petroleum ether fraction of aerial part of *O. eriantha* Wight is the most active fraction (IC<sub>50</sub> 21.954 µg/ml). Since *O. mungos* is a known anticancer plant and a source of camptothecin, better activity for *O. eriantha* Wight compared to *O. mungos* was surprising, and we selected OEAPE for further study. MTT assay of OEAPE were conducted on MCF-7 human breast cancer cell line and the activity compared with quercetin. Tables 1 and 2 shows the

percentage cell death by OEAPE and Quercetin on MCF-7 cell line. Figure 2 represents significant anti breast cancer activity by OEAPE on MCF-7 cell line (IC<sub>50</sub> 23.92 µg/ml).

After confirming the anti-breast cancer activity, acridine orange/ethidium bromide assay, western blot analysis of Bcl-2, BAX, P53, Caspase 7, Caspase-9 were conducted for studying the apoptotic process. AO/EB staining is an excellent method for studying nuclear changes happening during apoptosis.

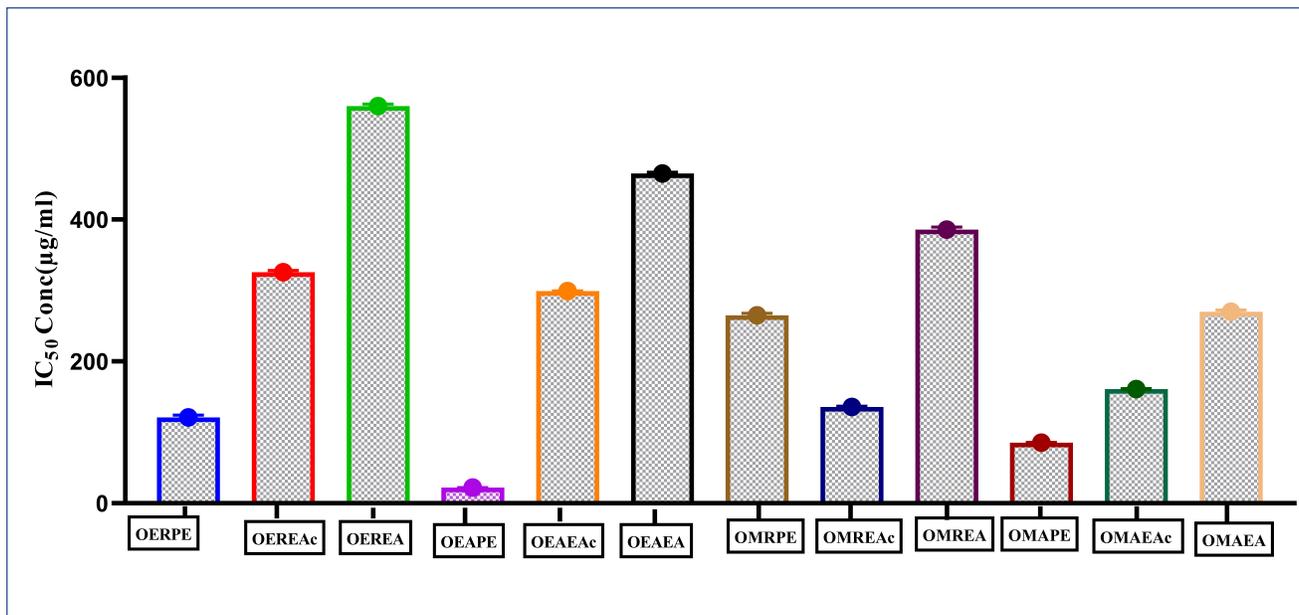


Figure 1. Short term *in vitro* cytotoxicity study using daltons lymphoma ascites (DLA) cells, of various extracts of *O. eriantha* Wight and *O. mungos* L. Data analysis was performed using Graph pad prism 8, values were denoted by mean ± SD. Statistical difference was found by One way analysis of variance (ANOVA) followed by Dunnet's Test among groups.

Table 1. MTT assay on MCF-7 cell line. Percentage cell death by OEAPE.

Concentration (µg/ml) OEAPE	OD 1	OD 2	OD 3	% of cell death			Mean	SD	SEM	% Live cell
100	0.384	0.389	0.382	60.93	60.34	60.94	60.74	0.3416	0.1972	39.26
50	0.426	0.428	0.432	56.66	56.37	55.82	56.28	0.4237	0.2446	43.72
25	0.462	0.465	0.468	53.00	52.59	52.14	52.58	0.4271	0.2466	47.42
10	0.581	0.586	0.590	40.89	40.26	39.67	40.27	0.6113	0.3529	59.73
5	0.603	0.604	0.608	38.65	38.43	37.83	38.30	0.4260	0.2460	61.70
Control	0.983	0.981	0.978	0	0	0	0.980	0.0025	0.0014	100
IC <sub>50</sub>	23.92									

Table 2. MTT assay on MCF-7 cell line. Percentage cell death by Quercetin.

Concentration (µg/ml) Quercetin	OD 1	OD 2	OD 3	% of cell death			Mean	SD	SEM	% Live cell
100	0.210	0.210	0.215	78.63	78.59	78.01	78.41	0.3463	0.1999	21.59
50	0.229	0.238	0.233	76.70	75.73	76.17	76.20	0.4831	0.2789	23.80
25	0.295	0.288	0.290	69.98	70.64	70.34	70.32	0.3266	0.1886	29.68
10	0.336	0.340	0.333	65.81	65.34	65.95	65.70	0.3206	0.1851	34.30
5	0.451	0.458	0.455	54.12	53.31	53.47	53.63	0.4266	0.2463	46.37
Control	0.983	0.981	0.978	0	0	0	0.980	0.0025	0.0014	100
sIC <sub>50</sub>	2.10									

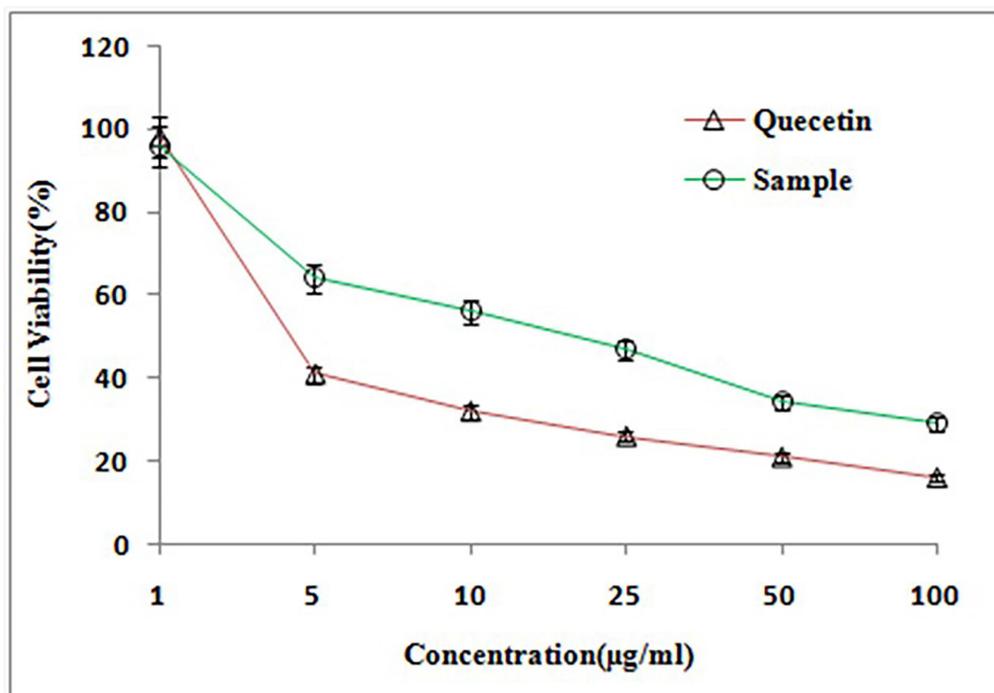


Figure 2. MTT Assay on MCF-7 cell line.

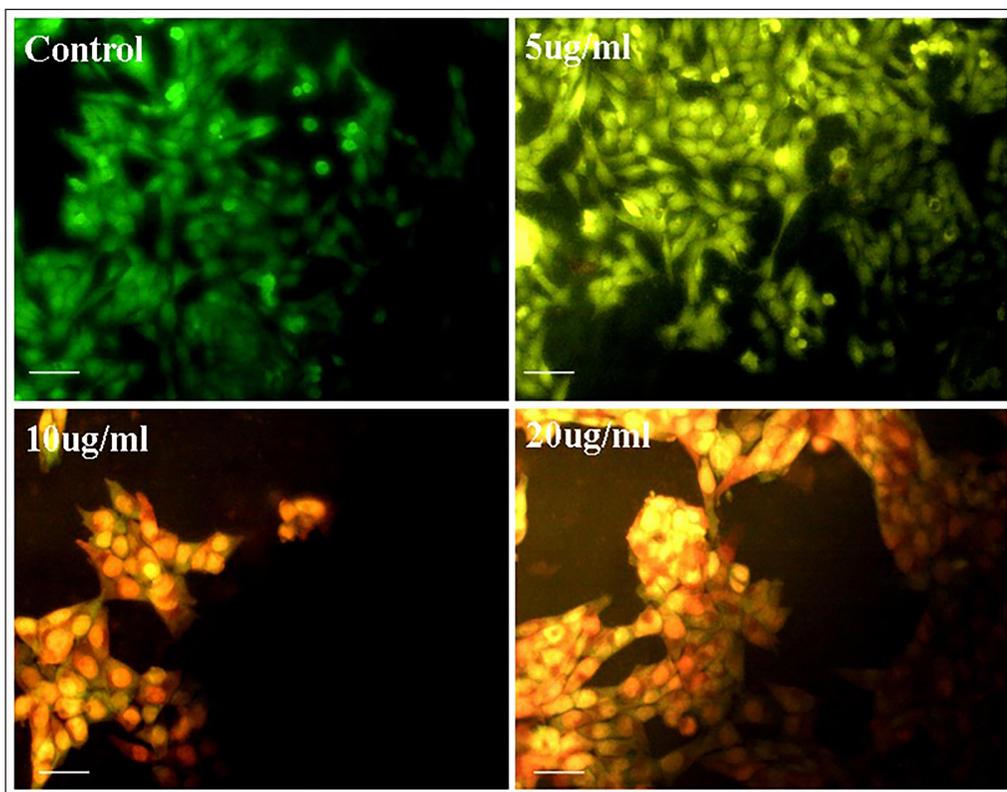
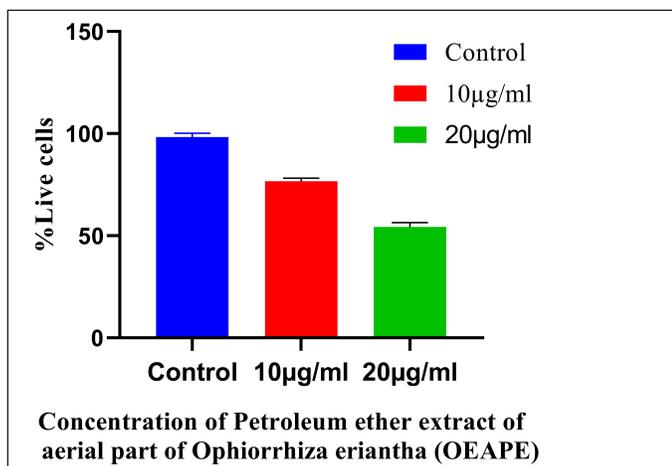


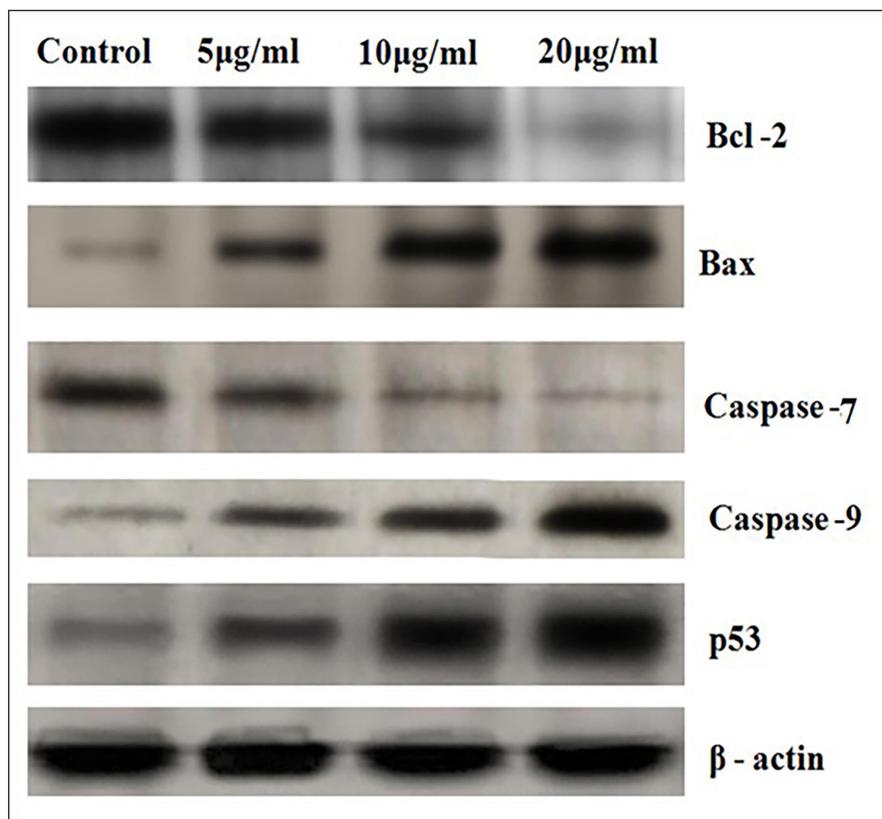
Figure 3. Images of acridine/ethidium bromide staining.



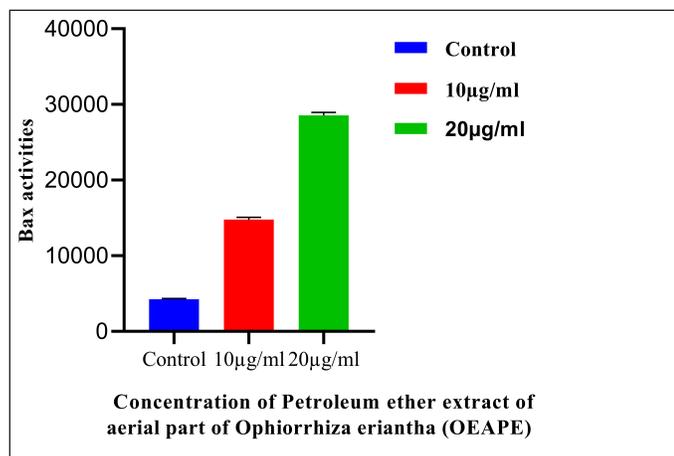
**Figure 4.** Acridine orange/ethidium bromide staining. Concentration of OEAPE. Data analysis was performed using Graphpad prism 8, values were denoted by mean  $\pm$  SD. Statistical difference was found by One way ANOVA followed by Dunnet's Test among groups. The values were compared with control group (\* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.001$ ). As compared with control group 20 µg/ml of OEAPE showed decreased percentage of live cells ( $p < 0.001$ ).

Figure 3 represents the effect of control, 5, 10, and 20 µg/ml OEAPE on MCF-7 human breast cancer cells. Cells treated with 20 µg/ml of extract show the decreased percentage of live cells when compared to control cells. Figure 4 represents the percentage live cells against different concentration of OEAPE. Live cells and apoptotic cells can be easily distinguished by the percentage uptake of AO/EB. Live cells appear green in color due to permeation of acridine orange. Ethidium bromide permeates only when cells lose their membrane integrity, and hence dead cells appear red and the apoptotic nucleus stains orange.

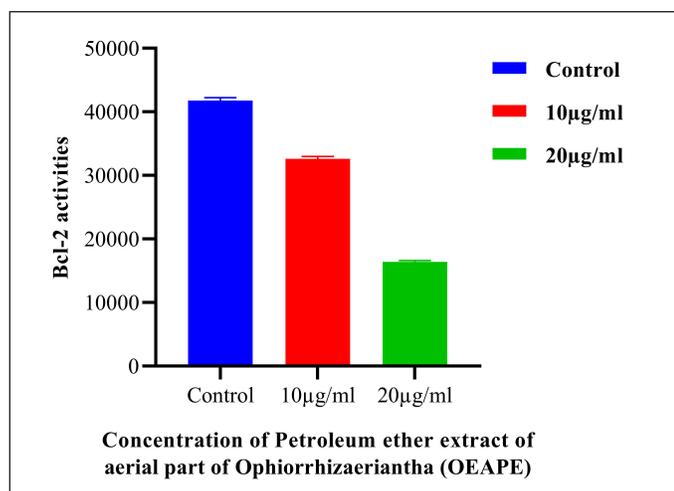
The intrinsic mechanism of apoptosis which occurs in mitochondria are controlled and regulated by proteins of Bcl-2 family (Cory and Adams, 2002). Bcl-2 proteins plays major role in regulation of permeability of the mitochondrial membrane. Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w, and BAG are anti-apoptotic in nature and Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk are pro-apoptotic (Siddiqui *et al.*, 2015). p53 has an important role in regulation of the Bcl-2 family of proteins. Bax proteins induce the release of cytochrome c from the mitochondrial intermembrane space into the cytosol which triggers caspase cascade (Gogvadze *et al.*, 2006). Figure 5 represents results of western blot analysis.



**Figure 5.** Western Blot analysis.

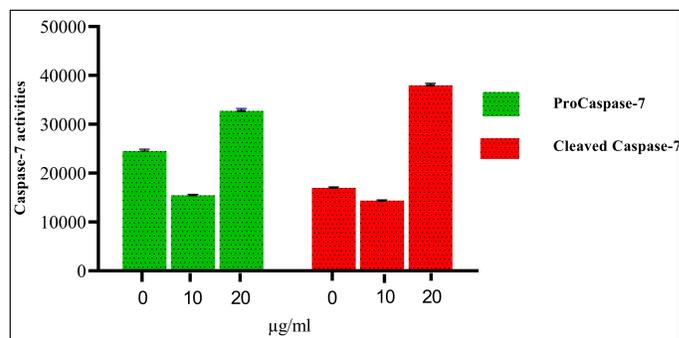


**Figure 6.** Western blot analysis of Bax protein. Concentration of OEAPE. Data analysis was performed using Graph pad prism 8, values were denoted by mean  $\pm$  SD. Statistical difference was found by one way ANOVA followed by Dunnet's test among groups. The values were compared with control group ( $*p < 0.05$ ,  $**p < 0.005$ ,  $***p < 0.001$ ). As compared with control group 20  $\mu\text{g/ml}$  of OEAPE showed significant increase in Bax Activity ( $p < 0.001$ ).

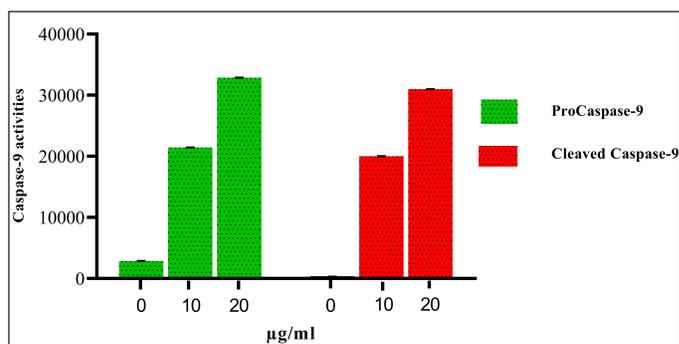


**Figure 7.** Western Blot analysis of Bcl-2 protein. Concentration of OEAPE. Data analysis was performed using Graph pad prism 8, values were denoted by mean  $\pm$  SD. Statistical difference was found by One way ANOVA followed by Dunnet's Test among groups. The values were compared with control group ( $*p < 0.05$ ,  $**p < 0.005$ ,  $***p < 0.001$ ). As compared with control group 20  $\mu\text{g/ml}$  of OEAPE showed significant reduction in Bcl-2 activity ( $p < 0.001$ ).

The Bax/Bcl2 ratio plays a major role in the regulation of the mitochondrial apoptotic pathway (Salakou *et al.*, 2007). Increase in the ratio of Bax (pro-apoptotic protein) expression to Bcl-2 (antiapoptotic protein) expression indicates apoptosis. Western blot analysis of pro-apoptotic Bax protein shows significant



**Figure 8.** Western Blot analysis of Caspase-7 activities petroleum ether extract of OEAPE. Concentration of petroleum ether extract of OEAPE. Data analysis was performed using Graph pad prism 8, values were denoted by mean  $\pm$  SD. Statistical difference was found by One way ANOVA followed by Dunnet's Test among groups. The values were compared with control group ( $*p < 0.05$ ,  $**p < 0.005$ ,  $***p < 0.001$ ). As compared with control group 20  $\mu\text{g/ml}$  of OEAPE showed increased caspase-9 activity ( $p < 0.001$ ).



**Figure 9.** Western Blot analysis of Caspase-9 activities OEAPE. Concentration of OEAPE. Data analysis was performed using Graph pad prism 8, values were denoted by mean  $\pm$  SD. Statistical difference was found by One way ANOVA followed by Dunnet's Test among groups. The values were compared with control group ( $*p < 0.05$ ,  $**p < 0.005$ ,  $***p < 0.001$ ). As compared with control group 20  $\mu\text{g/ml}$  of OEAPE showed increased caspase-9 activity ( $p < 0.001$ ).

elevation after treatment with 20  $\mu\text{g}$  OEAPE (Fig. 6). Bcl-2 concentration is significantly reduced upon treatment with 20  $\mu\text{g}$  OEAPE (Fig. 7). There was significant difference in Bax /Bcl-2 ratio between control and OEAPE-treated cells.

Caspases are a family of cysteine aspartate proteases. On treatment with different concentrations of OEAPE, at 20  $\mu\text{g/ml}$  of OEAPE shows a significant increase in caspase-7 and caspase-9 activities. Figures 8 and 9 shows the concentrations of pro caspase and cleaved caspase at control, 10 and 20  $\mu\text{g/ml}$  OEAPE.

Flow cytometry is a method for studying the cell cycle. Cellular DNA content can be measured and it gives correct picture of cells in different phases of the cell cycle. Different stages of cell cycle are stoichiometrically stained with Propidium Iodide which

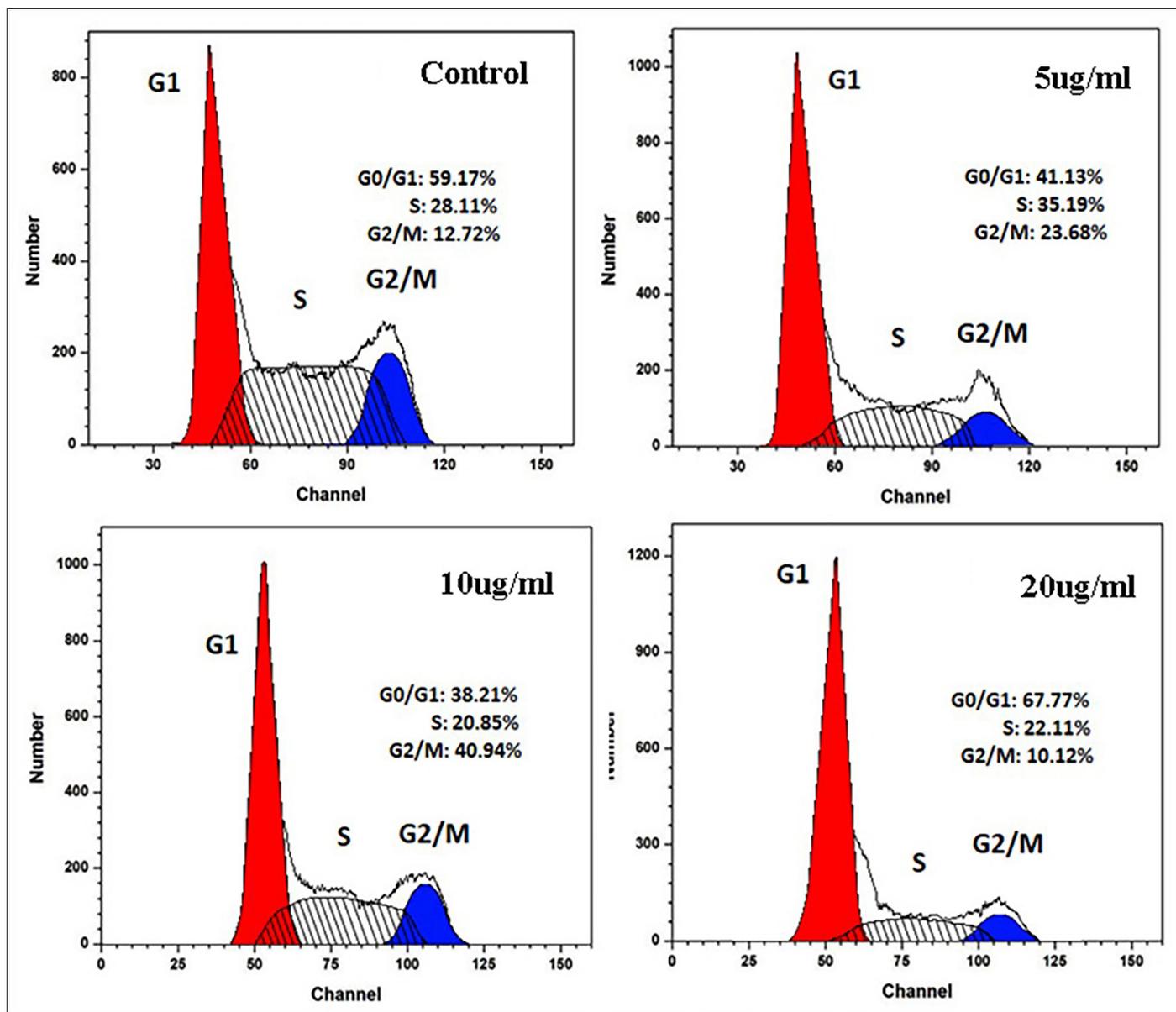
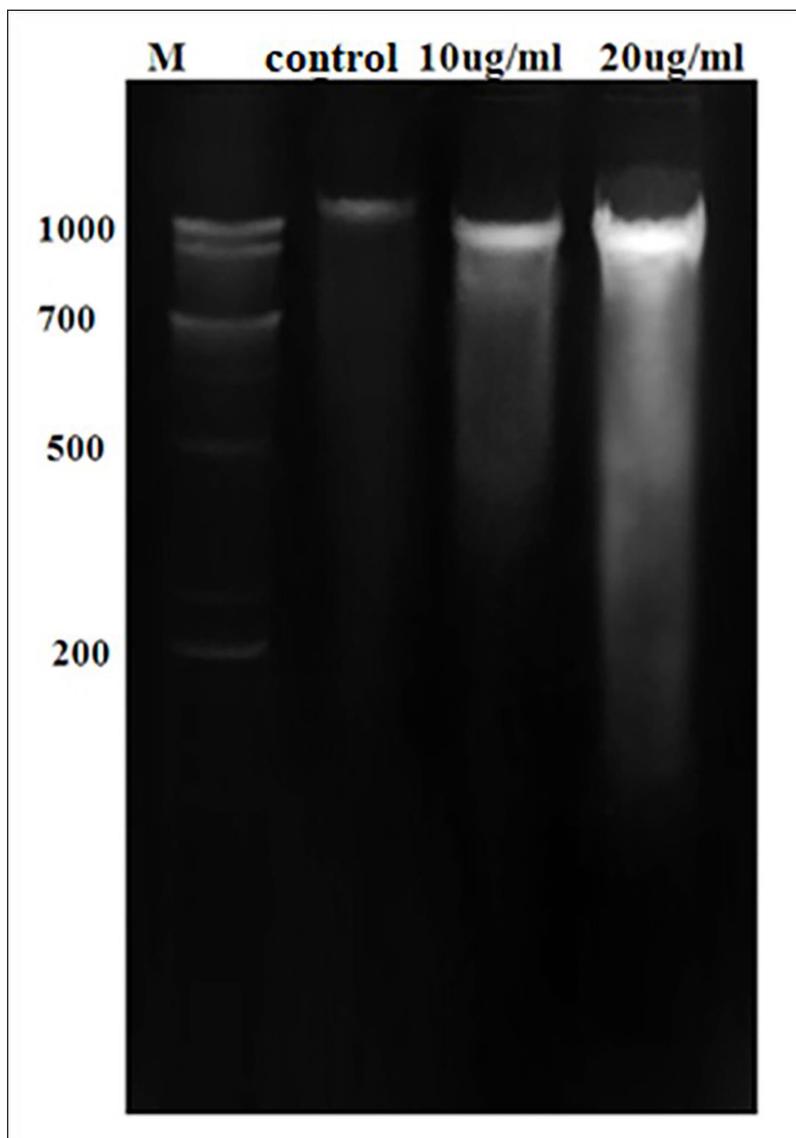


Figure 10. Flow cytometry analysis.

allows differentiation cells in G1, S, and G2/M phase. MCF-7 Cells were incubated with 0, 5, 10, and 20 µg/ml of OEAPE. The flow cytometry analysis (Fig. 10) shows that the 10 and 20 µg/ml of OEAPE arrested the cell cycle in G2/M phase after 24 hours.

Natural DNA fragmentation is a landmark for apoptosis. The DNA isolated from MCF-7 cells upon treatment with various

concentrations of OEAPE for 24 hours, a typical ladder pattern of inter nucleosomal fragmentation was observed (Fig. 11). When MCF-7 cells were treated with 20 µg/l, OEAPE shows significant action on DNA. These results indicate that the OEAPE caused DNA fragmentation in MCF-7 cells.



**Figure 11.** DNA fragmentation analysis.

## CONCLUSION

In the present study, we compared the anticancer activity of *O. eriantha* Wight with *O. mungos*. It was a surprise that the OEAPE showed better activity compared to the known anticancer plant *O. mungos*. The analysis of the results clearly indicates that the OEAPE induce apoptosis on breast cancer MCF-7 cell line.

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## CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

## AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors

are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

#### ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

#### DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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#### CONFLICT OF INTEREST

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