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ORIGINAL ARTICLE

# Anti-Proliferative and Apoptotic Effects of *Rheum emodi* on Human Breast Adenocarcinoma, MCF-7 Cells, and Antimicrobial Effectiveness against Selected Bacterial Strains

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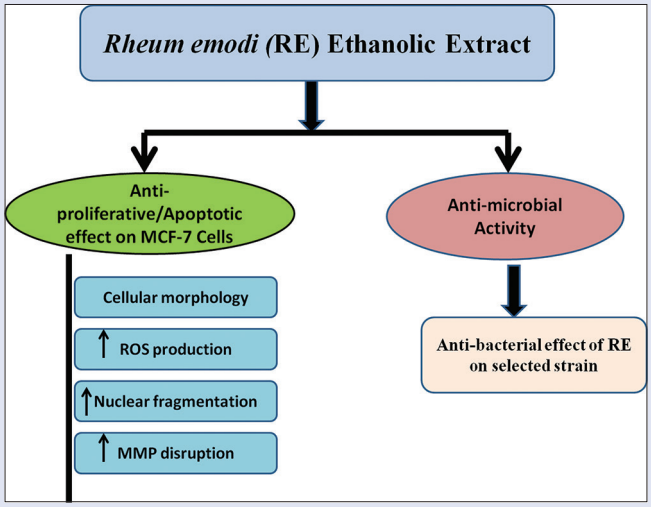
## ABSTRACT

**Background:** Breast cancer is the most common gynecological malignancy and one of the leading causes of death in women worldwide. Since antiquity to date, the saga of usage of plants as medicines has been the mainstay among the people. In Ayurvedic and Unani systems of medicine, *Rheum emodi* (RE) commonly known as Revand chini or Rhubarb is an important medicinal plant. It is a perennial herb belonging to family Polygonaceae and has antimicrobial, anticarcinogenic, and anti-inflammatory properties. The rhizomes of RE have anthraquinone derivatives such as emodin, aloe-emodin, rhein, emodin glycoside, and chrysophanol glycosides. **Objective:** In this study, we have investigated the anti-cancerous potentials of RE ethanolic extract on human breast adenocarcinoma cells MCF-7 and its antimicrobial effect against selected pathogenic bacterial strains. **Materials and Methods:** The apoptotic and anti-proliferative potentials of RE on human breast adenocarcinoma cells MCF-7 were observed through cell viability (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT]), reactive oxygen species (ROS) generation, nuclear fragmentation, and mitochondrial membrane potential (MMP) analysis. Antimicrobial activity of RE was screened through well-diffusion assay. **Results:** The results show that RE significantly inhibits the proliferation and induces apoptosis in MCF-7 cells of breast adenocarcinoma in a dose-dependent manner. The MTT cellular viability assay and morphological study reveal that RE significantly induces morphological alterations in MCF-7 cells in a dose-dependent manner and thus inhibits cell proliferation. The cytotoxic effect of RE through the induction of apoptosis is evident by the disruption of MMP, nuclear fragmentation, and ROS accumulation. In addition, we have found that RE is potent enough to decrease the microbial activity of selective bacterial strains. **Conclusion:** The findings demonstrated that RE promotes significant apoptosis in breast adenocarcinoma MCF-7 cells. Therefore, RE may be a potent candidate that aids for adjuvant cancer treatment though further studies are needed to elucidate the comprehensive mechanistic pathways.

**Key words:** Breast cancer, cell viability, mitochondrial membrane potential, nuclear fragmentation, reactive oxygen species, *Rheum emodi*

## SUMMARY

- In this study we investigated the anti-cancerous potentials of Indian medicinal herb *Rheum emodi* on human breast adenocarcinoma cells MCF-7 and anti-microbial activity on several pathogens. The results indicated that *Rheum emodi* ethanolic extract significantly inhibits cell proliferation in a dose dependent manner. Further RE induces apoptosis as evident by the disruption of MMP, nuclear fragmentation and ROS accumulation in MCF-7 cells. The anti-microbial activity exhibits that RE decreases the microbial growth of selective bacterial strains. Further *in vivo* and clinical studies are needed to validate its anti-cancer efficacy.




**Abbreviations used:** RE: *Rheum emodi*; MTT: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS: Reactive Oxygen Species; MMP: Mitochondrial Membrane Potential; DAPI: 4′,6-diamidino-2-phenylindole dihydrochloride; DCFH-DA: 2′,7′-dichlorodihydrofluorescein diacetate.

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## INTRODUCTION

Breast cancer is the most frequently diagnosed malignancy and one of the leading causes of death in women worldwide.[1] Being a heterogeneous disease, breast cancer's subtypes can be distinguished on the basis of tumor grade and the presence of various hormone receptors, namely estrogen receptor, human epidermal growth factor receptor-2, and progesterone receptor. From centuries, plants have served humankind as food and

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medicine. The therapeutic uses of plants and their metabolites have recently attracted scientists to work on them to find possible remedies for several diseases among which cancers rank on top.[2] *Rheum emodi* (RE) belongs to the family Polygonaceae and is commonly known as Indian Rhubarb.[3] Its growth is restricted to temperate and subtropical regions of the Himalayas.[4] Because of its extensive medicinal uses, Rhubarb is often known as "the wondrous drug."[5] Approximately 60 perennial species of genus *Rheum* are distributed globally, and about 10 species occur in India.[6,7] In the rhizomes of RE the anthraquinone derivatives are abundantly present, namely emodin, aloe emodin, chrysophanol, chrysophanol glycosides rhein, emodin glycoside, and physcion.[3,8] A growing number of studies have shown that RE possesses anticancerous, anti-oxidative, antidiabetic, antifungal along with hepatoprotective, and nephroprotective properties. This study aimed to investigate the anti-proliferative and apoptotic effects of RE ethanolic extract on MCF-7 cells of human breast adenocarcinoma and its antimicrobial efficacy on selected bacterial strains. In this study, we investigated the anticancerous efficacy of RE through cell viability, excessive ROS generation, disruption of mitochondrial membrane potential (MMP), and nuclear condensation assays. Further, antimicrobial potentials of RE was screened through well diffusion assay.

## MATERIALS AND METHODS

### Collection and preparation of *Rheum emodi* extract

The RE dried rhizome was collected from the Hamdard Unani medicinal shop, Sardar Unani Dawakhana. The specimen was authenticated as a dried rhizome of RE and given a reference number RC/05/16 by Hakim M. Qadeer. The dried rhizome was again shade-dried and then pulverized by the mechanical grinder. The 95% ethanolic extract was made using the Soxhlet apparatus.

### Culturing of cell line MCF-7

Human breast adenocarcinoma (MCF-7) cell line was obtained from the cell repository, National Centre for Cell Sciences, Pune, India. The cells were maintained in RPMI medium supplemented with 10% (v/v) fetal bovine serum (Himedia), 2.0 mM L-glutamine, 1.5 g/l $NaHCO_3$, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and 1% antibiotic solution. The cells were maintained at 37°C, 5% $CO_2$ in a humidified air.

### Morphological analysis of *Rheum emodi* treated MCF-7 cells

The effect of RE was analyzed for morphological changes in the cultured MCF-7 cells. About 10,000 cells were seeded per well in 96 wells culture plate and treated with different concentrations of RE, i.e., 10 μg/ml, 25 μg/ml, 50 μg/ml, 100 μg/ml, and 200 μg/ml.[9] After incubation of 24 h, the cells were observed for the cellular morphological alterations by inverted phase contrast microscope (Nikon ECLIPSE Ti-S, Japan).

### Cell viability assay in MCF-7 cells through 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide dye

The antiproliferative effects of RE on human breast adenocarcinoma MCF-7 cells were analyzed using enzymatic reduction assay of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).[10] The decrease of cell viability in MCF-7 cells was observed on treating it with different concentrations of RE ranging from 10 to 200 μg/ml. After 24 h and exposure, from 5 mg/ml stock solution of MTT dye, 10 μl was added in each well incubated for about 4 h at 37°C. The developed formazan blue crystals were solubilized by adding 100 μl dimethyl sulfoxide (DMSO) in each well and were then kept for 10 min at 37°C. The optical density was observed at 540 nm through microplate reader (BIORAD-680), and relative percentage cell viability was evaluated.

### Reactive oxygen species generation assay

The 2′,7′-dichlorodihydrofluorescein diacetate fluorescent probe is commonly employed to evaluate the ROS generation. To study ROS generation in MCF-7 cells, cells were exposed to different concentrations of RE.[11] The MCF-7 cells ($1 \times 10^4$ per well) were seeded as described above for the MTT assay and were then exposed to selective doses of RE, namely 50 μg/ml, 100 μg/ml, and 200 μg/ml for 12 h. After the treatment, the cells were incubated with 10 mM concentration of DCFH-DA dye for 30 min at 37°C. After incubation, the reaction mixture was replaced by 200 μl phosphate-buffered saline (PBS), and then, the plate was kept in the dark on plate shaker for 10 min at room temperature. The fluorescence ROS images were captured with the help of inverted fluorescent microscope (Nikon ECLIPSE Ti-S, Japan) and the ROS intensity was observed and quantified with the help of freely accessible IMAGE J software (imagej.nih.gov/ij/).

### 4′,6-Diamidino-2-phenylindole dihydrochloride staining for nuclear condensation

By using fluorescent nuclear dye 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), the apoptotic effect of RE was analyzed.[12] The MCF-7 cells were seeded and were given treatment as it was given before for ROS assay, i.e., 50 μg/ml, 100 μg/ml, and 200 μg/ml for 24 h. Cells were then washed with PBS and were fixed in 4% paraformaldehyde for 10 min. Then with permeabilizing buffer (3% paraformaldehyde and 0.5% Triton X-100), the cells were permeabilized and then stained with 20 mM concentration of DAPI dye for 20 min. The nuclear fragmentation was observed by using a fluorescent microscope (Nikon ECLIPSE Ti-S, Japan) and the images were captured and a number of apoptotic cells were quantified.

### Assessment of mitochondrial membrane potential

Disruption of MMP is a potent marker for apoptosis, and we used Rhodamine 123 dye to detect a decrease in MMP of MCF-7 cells treated with RE.[13] To study the loss of MMP, the cells were seeded as before for ROS assay and then given selective doses of RE, namely 50 μg/ml, 100 μg/ml, and 200 μg/ml. After 24 h of exposure cells were stained by 5 mM concentration of Rhodamine 123 dye for 30 min. The wells were gently washed with PBS and images were then taken with the help of fluorescent microscope (Nikon ECLIPSE Ti-S, Japan).

### Antimicrobial assay

#### *Micro-organisms used in the study*

The antimicrobial activity of RE was carried out by well diffusion method against few selected bacterial strains that are as follows:

a. From the National Chemical Laboratory (Pune), we obtained the strains of *Escherichia coli* (NCIM 2065), *Staphylococcus aureus* (NCIM 5021), and *Bacillus pumilus* (NCIM 9369)
b. From King George's Medical University, Lucknow, we obtained the strains of *Listeria monocytogenes* (NCIM 5279) and Enteropathogenic *E. coli* (EPEC) strain (E 2347).

#### *Well diffusion technique*

To test the antibacterial activity of RE, well diffusion technique was performed.[14] In the sterile Petri plates, 20 ml of sterile Mueller–Hinton Agar was poured and allowed to rest until it gets solidified. Sterile cotton was used to swab the broth culture of the above-mentioned test organisms/strains over the solidified agar Petri plates. With the help

of sterile cork borer, three wells were made, first for positive control, second for negative control, and third for the sample loading on the Petri plates. Afterward, 20 µl RE was then added into the sample well and Streptomycin was used as positive control and DMSO was used as a negative control for each bacterial strains. The plates were then incubated at 37°C for 24 h. The measurement of the zone of inhibition was quantified for the antibacterial activity of RE.

### Statistical analysis

The antimicrobial activity results were expressed as a mean ± standard deviation and tests were performed in triplicates. For significant difference and comparison of variance and means (where $P \leq 0.05$ differences were considered as statistically significant), the One-way analysis of variance (ANOVA) was done, followed by Tukey's test using SPSS Software Version 16.0 (IBM corp., New York, USA), USA. For anticancerous activity, the experiments were performed thrice, and data were expressed as the mean ± standard error of the mean. The analysis was performed using ANOVA, followed by the Dunnett's test using Graph Pad Prism software (Version 5.01, California, USA) and the value of $P \leq 0.05$ were considered as statistically significant.

## RESULTS

### Effect of *Rheum emodi* in MCF-7 cells

By using MTT assay and cell morphology assessment, we have found the experimental doses of RE on human breast carcinoma cells MCF-7. Photomicrographs clearly reveal the morphological changes that occur in the cells, i.e., the shape of the cells were drastically changed from normal to rounded off, distorted and at higher concentrations of RE. Morphological studies suggest that as the dose of RE increases there occurs a change in cellular morphology, thereby inducing cell death in MCF-7 cells [Figure 1a]. The 50–200 µg/ml doses of RE induce significant cell death in MCF-7 cells. The cell viability data showed that at the doses 50 µg/ml, 100 µg/ml, and 200 µg/ml of RE the percentage cell viability was 74.24%, 58.14%, and 38.18%, respectively, i.e., the cell death was statistically significant at higher concentration of RE as compared to the lower doses of RE [Figure 1b]. We have selected three effective doses (50 µg/ml, 100 µg/ml and 200 µg/ml) for further studies as the last three doses are more effective.

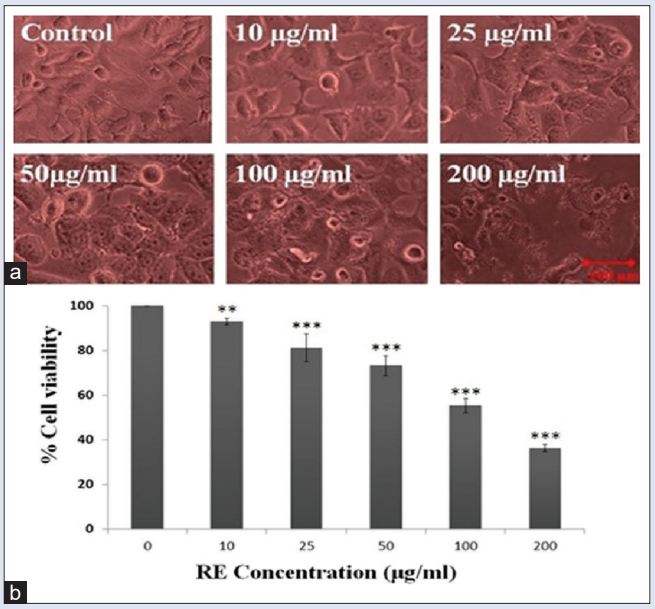


**Figure 1:** (a) Photomicrographs shows cells morphology treated with *Rheum emodi* extract after 24 h upon incubation with MCF-7 cells. (b) Shows percentage cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cells were treated with 10–200 µg/ml of *Rheum emodi* extract for the period of 24 h. $P < 0.05$ is considered as statistically significant

### *Rheum emodi* induces excessive reactive oxygen species in MCF-7 cells

The fluorescent photomicrograph shows RE increases intracellular ROS production in human breast carcinoma cells MCF-7 in a dose-dependent manner. The ROS intensity in RE treated MCF-7 cells gets elevated as compared to control depending on dose [Figure 2a]. At 50 µg/ml concentration, the significant increase in ROS production in RE treated MCF-7 cells was approximately 8.73% as compared to control. The elevated ROS production was found at 100 µg/ml and 200 µg/ml concentrations of RE which were recorded approximately 16.38% and 48.72%, respectively [Figure 2b]. Thus, the RE induces ROS production within MCF-7 cells, thereby inducing cell death through apoptotic pathways.

### *Rheum emodi* induces apoptosis in MCF-7 cells via nuclear condensation

By using fluorescent DAPI dye, the nuclear apoptosis, i.e., condensation and fragmentation of nuclei were observed in MCF-7 cells. The photomicrograph shows that at higher concentration of RE maximum chromatin condensation takes place [Figure 3a]. At the optimum doses, namely 50 µg/ml, 100 µg/ml, and 200 µg/ml of RE, the apoptotic cells were observed 16.00%, 27.30%, and 44.20%, respectively [Figure 3b]. Thus, the RE induces nuclear condensation within MCF-7 cells in a dose-dependent manner.

### *Rheum emodi* disrupts mitochondrial membrane potential in MCF-7 cells

The change in MMP was measured by a decrease in fluorescent intensity of red fluorescence caused by Rhodamine 123 dye. The photomicrograph shows a decrease in red fluorescence in the cells treated with 50 µg/ml, 100 µg/ml, and 200 µg/ml of RE [Figure 4a]. The quantitative data reveals that the red-florescence in RE treated cells was found to be 87.48%, 56.15%, and 33.40% at 50 µg/ml, 100 µg/ml, and 200 µg/ml of RE treatment, respectively [Figure 4b]. The loss of MMP in RE treated MCF-7 cells indicates more cell deaths.

### Antimicrobial assay

An antimicrobial study of RE was performed by well diffusion method, and the results are summarized in Table 1. RE has a varying degree of sensitivity against selected test micro-organisms. The study suggested that RE was the most sensitive against *L. monocytogenes* (28 ± 1.52 mm) and *E. coli* (26 ± 0.57 mm), followed by EPEC (24 ± 1.15 mm) and *B. pumilus* (24 ± 0.57 mm), whereas, the strains of *S. aureus* (22 ± 0.57 mm) was found to be less sensitive to RE [Figure 5].

## DISCUSSIONS

One of the leading causes of death among women is breast cancer.[1] Phytochemicals have significantly demonstrated cytotoxicity to various cancer cells by inhibiting proliferation or inducing apoptosis without harming the normal cells, thus making phytochemicals ideal anticancer therapeutic agents.[15] Flavonoids and polyphenolic compounds halt many microbial infections.[16] Many studies have shown that the roots of RE have antibacterial and antifungal activities.[17,18] We have

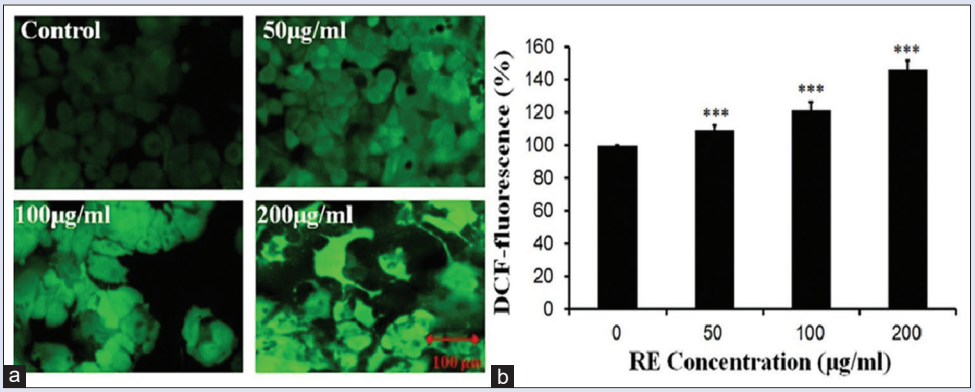


**Figure 2:** (a) Photomicrographs shows intracellular reactive oxygen species accumulation in MCF-7 cells treated with *Rheum emodi* extract (50–200 µg/ml) at 12h incubation. (b) Effect of *Rheum emodi* extract (50–200 µg/ml) on intracellular reactive oxygen species production in MCF-7 cells. Values expressed as the percentage of fluorescence intensity relative to the control. $P < 0.05$ is considered as statistically significant

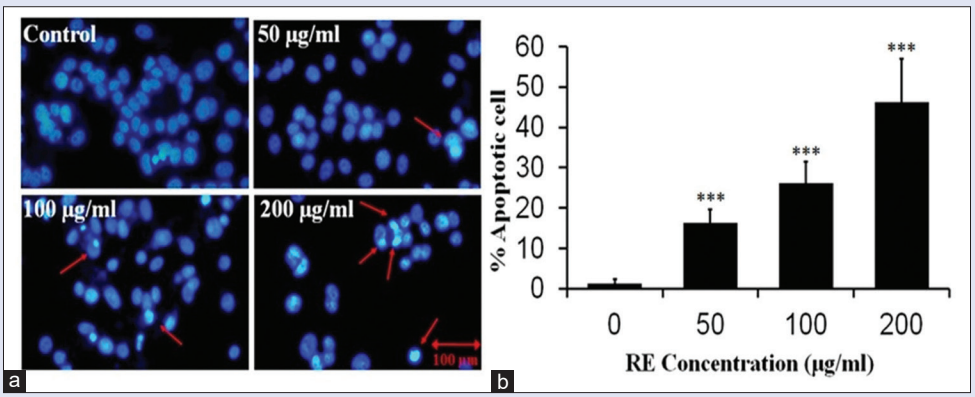


**Figure 3:** (a) Photomicrographs shows nuclear condensation treated with *Rheum emodi* extract after 24 h incubation in MCF-7 cells. The red arrows show condensed and fragmented cells. (b) Shows the values expressed as the percentage of apoptotic cells relative to the control when treated with *Rheum emodi*. $P < 0.05$ is considered as statistically significant

**Table 1:** Anti-microbial activity of *Rheum emodi* ethanolic extract by well diffusion method

| Bacteria strain name | Diameter of zone of inhibition for RE (mm) | Diameter of zone of inhibition for positive control i.e., streptomycin (mm) | Diameter of zone of inhibition for negative control, i.e., DMSO (mm) |
|---|---|---|---|
| *Bacillus pumilus* | 24±0.57 | 28±1.52 | No zone of inhibition |
| EPEC | 24±1.15 | 20±1.15 | No zone of inhibition |
| *E. coli* | 26±0.57 | 30±1.15 | No zone of inhibition |
| *Listeria monocytogenes* | 28±1.52 | 31±0.57 | No zone of inhibition |
| *Staphylococcus aureus* | 22±0.57 | 26±1.15 | No zone of inhibition |

Values are given as mean±SD of zone of inhibition from 3 independent experiments. RE: *Rheum emodi*; SD: Standard deviation; DMSO: Dimethyl sulfoxide; *E. coli: Escherichia coli*; EPEC: Enteropathogenic *E. coli*

investigated the antimicrobial efficacy of RE and found out that it is a potent candidate that hampers the growth of some selected bacterial strains. RE is a herb of enormous pharmacological significance and has been used in the treatment of numerous ailments, namely muscular pain, constipation, diarrhea, indigestion, skin problems, and menstrual disorder.[19]

A study has shown that both aqueous, and methanolic extracts of RE have promising anti-cancerous activity against Hep3B, PC-3, and MDA-MB-435S cell lines in a dose-dependent manner as they induce cellular apoptosis and halt the proliferation.[20] Here, we have worked on MCF-7 cells to see the therapeutic efficacy of RE on it. The cell viability assay reveals that RE significantly halts the abnormal growth of MCF-7 cells in a dose-dependent manner. The morphological analysis showed drastic changes in the shape of the cells, and they get detached from the substratum.

Excessive ROS produced in cancerous cells increases basal oxidative stress. The induction of ROS accumulation in cancer cells plays a key role in the pharmacological activity of several anticancerous compounds, thereby causing mutation, aging and death of tumor cells.[21-23] Excessive ROS disrupts the cytoskeleton and plasma membrane and thus leads to damage in chromosomes.[24] In both extrinsic and intrinsic pathways of cell survival and cell death, ROS plays an important role.[25] Phytochemicals used as anticancer compounds cause accumulation of ROS in cancerous cells ultimately leading to its death.[26] The qualitative

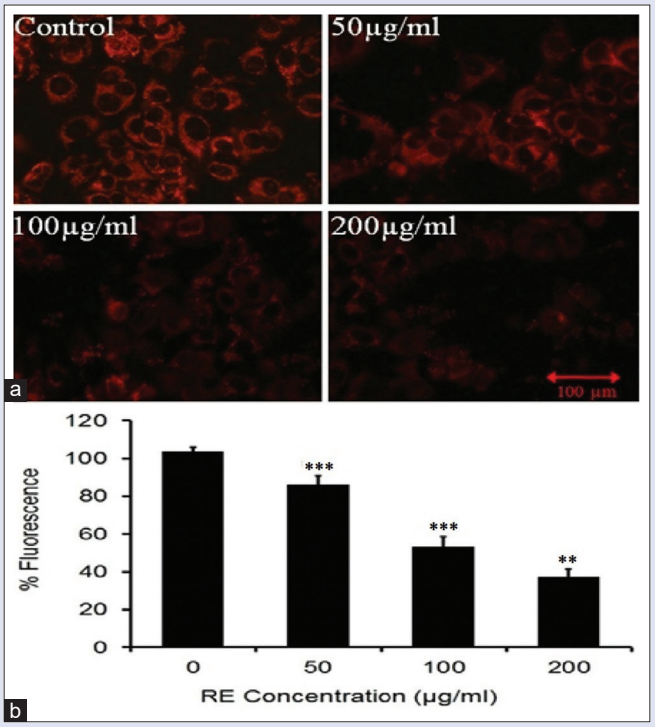


**Figure 4:** (a) Photomicrographs shows disruption of mitochondrial membrane potential treated with *Rheum emodi* extract after 24 h incubation in MCF-7 cells. (b) Values expressed as the percentage fluorescence relative to the control on treating MCF-7 cells with *Rheum emodi*. $P < 0.05$ is considered as statistically significant

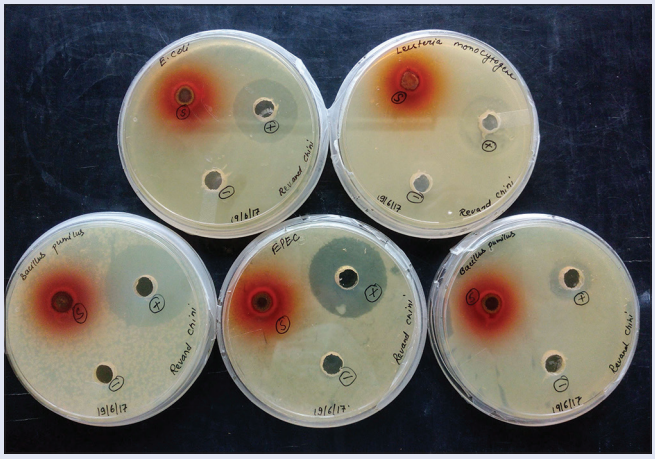

**Figure 5:** Anti-microbial activity of *Rheum emodi* by Agar well diffusion method using *Listeria monocytogenes*, *Escherichia coli*, Enteropathogenic *Escherichia coli*, *Bacillus pumilus* and *Staphylococcus aureus* as test microorganism

and quantitative results of ROS generation reveal that RE induces ROS-mediated cell death in MCF-7 cells in a dose-dependent manner. Therefore, increased intracellular ROS accumulation at higher doses of RE contribute to cellular apoptosis of MCF-7 cells.

One of the hallmarks of apoptosis is the fragmented and condensed nucleus in the cells.[27] Our findings suggest that RE is potent enough to induce apoptosis in MCF-7 cells in a dose-dependent manner as Figure 3a shows fragmented and condensed nuclei at higher doses of RE. The MCF-7 cells acquire spherical shape, lose membrane integrity and get detached from the surface at higher concentrations. These morphological alterations thus revealed that RE significantly induces apoptosis in MCF-7 cells.

Disruption of MMP is an early event in apoptosis.[28,29] Mitochondria play an important role in both the cell's survival and death by sending the death signals to different pathways. In apoptosis, the mitochondria lose their membrane potential and release cytochrome-c into the cytosol which then forms apoptosome and completes the intrinsic pathway of cell death.[30,31] The higher doses of RE show a decrease in the percentage red fluorescence which suggests that there is depolarization in the membrane potential and the mitochondria loses its membrane integrity. Thus, RE significantly disrupts MMP and induces apoptosis in a dose-dependent manner. Thus, our findings suggest that RE has the potential to halt proliferation and induce apoptosis in MCF-7 cells.

## CONCLUSION

Our findings suggest that RE has potent antiproliferative and apoptotic activity on human breast adenocarcinoma MCF-7 cells in a dose-dependent manner. The MTT cell viability assay reveals that RE inhibits cell proliferation and alters the cellular morphology of MCF-7 cells. The active phytoconstituents of RE also possess apoptotic properties, namely anti-proliferative effect, cytotoxicity, and cellular atrophy, thus making them to be a prospective candidate as future chemopreventive agents. Our findings also suggest that RE inhibits the growth of various bacteria and possesses a potent antimicrobial effect. Further studies are needed to validate its efficacy as being a promising candidate which can minimize harmful side effects of chemotherapeutics and will provide a better quality life.

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Nil.

## Conflicts of interest

There are no conflicts of interest.

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