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Original Article

A Comparative Antibacterial Activity of Three Common Spices Extract and their Anti-Proliferative and Apoptotic Effectiveness against Human Breast Adenocarcinoma Cells

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ABSTRACT

Objective: The present study was performed to analyse the antibacterial potential as well as the anti-proliferative and apoptotic efficacy of three common spices *viz.* Cardamom (*Elettaria cardamomum*), Cinnamon (*Cinnamomum verum*) and Fennel (*Foeniculum vulgare*). **Methods:** Antibacterial activity was determined by well diffusion assay against selected bacterial strains. Anti-proliferative activity was evaluated by cell viability assay and the apoptotic effect was observed by nuclear fragmentation analysis in MCF-7 cells. **Results:** The antibacterial activity result revealed that Cinnamon extract (CIE) showed maximum antibacterial activity against selected test organism followed by Cardamom (CAE) and Fennel (FEE). The cell viability results revealed that FEE induces the highest cytotoxicity (IC_{50} 73.9 µg/ml) against MCF-7 cells, while CIE showed the lowest efficacy (IC_{50} 98.2 µg/ml) as compared to control. **Conclusion:** The findings revealed that CIE has the most potent antibacterial efficacy, whereas FEE was found to be a more potent anti-proliferative and apoptotic agent against human breast carcinoma MCF-7 cells.

Key words: Antibacterial, Anti-proliferative, Apoptotic, Cell viability, Nuclear fragmentation.

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INTRODUCTION

The plants are the imported natural resource and have been extensively studied to develop therapeutic natural therapies in the last few decades.[1] Spices are also the plant-derived products and extensively used in flavouring various foodstuffs. The pungent spices have been widely used in food safeguarding as well as in embalming in India and other Islands.[2,3] Spices contain the essential oil which has been recognized to possess preventive and therapeutic properties on human health. Spices have been recognized for a wide range of therapeutic effects *viz.* anti-oxidant, anti-microbial, anti-carcinogenic, anti-mutagenic, anti-inflammatory, and hypo-lipidemic roles.[4] Basically, phytochemicals are actually responsible for medicinal value of every potent medicinal plant and their pharmacological properties are mainly depends on the phytoconstituents they possess.[5] It has been reported that various spices retain anti-microbial properties.[6] Therefore, spices are usually used in warmer climate countries, where the chances of more infectious disease. Using herbs as a potent anti-microbial agent has played a key role in almost every culture on earth; including Asia, Europe, Africa, and America.[7] Approximately 80% of the prescription depends on traditional medicine in the healthcare system and almost all traditional medicine comprises of crude plant extracts or sometimes its pure bioactive components.[8] The increasing side effect of chemo-therapeutic agents and antibiotic drug resistance shown by various pathogenic microbes led to the transmission of many other promising natural compounds for antimicrobial effect and some natural products were found to have potent against various pathogenic micro-organisms.[9,10] The spices are the natural and common anti-microbial agents present in our diet that imparts flavour to the food as well also provides anti-microbial immunity against several pathogens.[11,12] The anti-microbial activities of several dietary spices are due to occurrence of numerous natural potent bioactive components.[13] The plant origin drugs are often considered to be least toxic or without adverse effects as compared to synthetic ones.[14] Several findings have reported the antioxidant, anti-inflammatory, and immuno-modulatory effects of spices that might be responsible for the prevention as well as therapeutic agent against several cancers, including breast, lung, cervix, and prostate cancers, etc.[15]

In this study, we have examined the anti-microbial activity of three common Indian spices extract CAE, CIE and FEE against selected bacterial strains along with anti-proliferative and apoptotic potential in MCF-7 cells of human breast adenocarcinoma.

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MATERIALS AND METHODS

Collection and identification of plant samples

The three common spices Cardamom (*Elettaria cardamomum*), Cinnamon (*Cinnamomum verum)* and Fennel (*Foeniculum vulgare*) were purchased from local grocery shop of Lucknow and were authenticated by Professor Y. K. Sharma, Botanist and former Head, Department of Botany, University of Lucknow. All spices samples were gently washed with distilled water, shed dried, ground into fine powder and collected into airtight containers for further up to extract preparation.

Extract preparation

The aqueous extract of all three selected spices was prepared as per previously described protocol with slight modification.[16] Briefly, 100 g of each powdered spice was soaked in 500 ml double distilled water in a separate conical flask overnight, some drops of toluene were also added, to prevent any fungal infection. Afterwards, the flasks were placed on a rotary plate shaker for 48 h at 150 rpm. Subsequently, the solutions of spices were filtered with the help of Whatman filter paper No-1 and the extracts were concentrated on a rotary plate evaporator in vacuum at 37^{0}C.

Test micro-organisms used

Four potent bacterial strains were used for the determination of anti-microbial activity. *Escherichia coli* (NCIM 2065*), Bacillus pumilus* (NCIM 9369) and *Listeria monocytogenes* (NCIM 5279) were obtained from NCL Pune. *Enteropathogenic E. coli* E 2347 (EPEC) was obtained from KGMU, Lucknow.

Well diffusion method for anti-microbial activity

The anti-microbial effect of CAE, CIE and FEE were done by a previous standard method with some minor modifications.[17] Concisely, 20 ml sterile Mueller Hinton Agar (pH 7.0) was poured in 90 mm sterile Petri plates and then kept overnight for any contamination detection. Overnight cultures of mentioned selected bacterial strains having 0.5 OD were swabbed over sterile Agar plates. A cork borer having 6 mm diameter was used for wells formation and three wells were formed on plates, first for aqueous sample extract, second for positive control and third for negative control. Thereafter, 50 mg concentrated aqueous extract of each sample was solubilized in 1 ml of 0.5% Dimethyl sulfoxide (DMSO) and 40 µl of it poured in first well, 20 mg Streptomycin was solubilized in 1 ml of 0.5% DMSO and 40 µl of it poured in second well as positive control and 40 µl of 0.5% DMSO poured in third well as a negative control.

In vitro cell culture

The cell line of human breast carcinoma (MCF-7 cells) is extensively used for *in vitro* cytotoxicity assessment. The MCF-7 cells were obtained from NCCS, Pune and were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% Fetal bovine serum (FBS), 5% CO_2 and 95% humid air in a CO_2 incubator at 37°C.

SRB assay for cellular viability analysis

Sulforhodamine B (SRB) assay was performed to detect the anti-proliferative and apoptotic activity of CAE, CIE and FEE against MCF-7 cells based on protein content. Briefly, 1×10^4 MCF-7 cells per well were plated in 96 wells plate for overnight. After overnight incubation, the cells were treated with different concentrations *viz.* 25, 50, 75 and100 µg/ml of CAE, CIE and FEE for 24 h. Thereafter, cell monolayers were incubated with 10% trichloroacetic acid for 20 min and then SRB dye was added to each well for 30 min. The wells were gently washed with 1% acetic acid to remove excess dye. Afterwards, the protein-bound substrate was solubilized in tris base solution (10 mM) and absorbance was read with the help of ELISA microplate reader at 510 nm.

The percentage viability of MCF-7 cells was calculated by following formulae:

$$\% \text{ cell viability} = [1 - (\text{OD sample}/\text{OD control})] \times 100$$

The percent cell viability was plotted against the respective concentration of selected spice extracts. The 50% inhibition of viable cells (IC_{50}) was evaluated by plotting the graph of concentration of each spice extract against percent inhibition of MCF-7 cells.[18]

Cell morphology study

The cellular morphology of MCF-7 cells was analysed after treated with various concentrations of CAE, CIE and FEE.[19] Succinctly, MCF-7 cells were seeded at 1×10^4 cells in each well of 96 wells culture plate and then treated with 25, 50, 75 and100 µg/ml of CAE, CIE and FEE. After 24 h incubation, the morphological changes were examined by the use of an inverted phase contrast microscope (Nikon Ti-S, Japan).

Nuclear fragmentation analysis

The apoptotic activity of CAE, CIE and FEE was analysed by 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) dye on MCF-7 cells of breast carcinoma.[20] Briefly, the cells were seeded and treated with selected effective doses of CAE, CIE and FEE for 24 h. After the incubation period, the cells were fixed in PFA and then incubated for 10 min in permeabilizing buffer. Afterwards, 10 mM DAPI dye was added to each well and then photomicrographs were captured under Nikon fluorescent microscope.

Statistical analysis

All the experiments were performed in triplicates and data were implied as means ± SEM. The data were analyzed with the help of GraphPad Prism software. The ANOVA (one-way analysis of variance) was applied and a *P* value less than 0.05 were deliberated as statistically significant.

RESULTS

Anti-microbial effect

The anti-microbial activity result revealed that all tested spices possess potent antibacterial effect against selected bacterial strains (Table 1, Figure 3). The CIE showed significant antibacterial effect against all selected strains of bacteria with diverse degree of sensitivity, maximum activity was observed against *E. coli* (23.83 ± 0.57) followed by *Listeria monocytogenes* (23.3 ± 0.92) and least against *Bacillus pumilus* (20.37 ± 0.61). Whereas, CAE showed moderate activity and its maximum activity was seen on *Enteropathogenic E.* coli (21.62 ±0.93) and least for *E. coli* (17.5 ± 0.69); however, FEE showed least anti-bacterial activity and its maximum activity was found against *Listeria monocytogenes* (17.37 ± 0.43) and least against *E. coli* (11.43 ± 0.27).

Cell morphology analysis

Cell morphology study reveals that all selected spices CAE, CIE and FEE induce the cellular alterations in the morphology of breast carcinoma MCF-7 cells in a concentration-dependent manner (Figure 1A, B and C). The typical morphological features of apoptosis were observed in breast carcinoma cells *viz.* alteration in cellular morphology, cells acquired round shape detached from the surface, as compared to the elongated and spindle-shaped untreated MCF-7 cells suggests the cellular apoptosis. As evident from the photomicrographs, these selected spices extract at higher doses *i.e.,* 75 and 100 µg/ml concentrations induced more cell death whereas, on another hand, at lower concentration (25 and 50 µg/ml) induces lesser cytotoxicity as compared to control. As compared to all selected spices, the FEE induces more cytotoxicity in MCF-7 cells as compared to CAE and CIE.

Table 1: Anti-microbial activity of Cardamom (*Elettaria cardamomum*), Cinnamon (*Cinnamomum verum*) and Fennel (*Foeniculum vulgare*) extract (CAE, CIE and FEE) on selected bacterial strains measured by well diffusion assay. The values are expressed as mean ± SD of three replicates.

S. No.	Microorganism used	Diameter of zone of inhibition for test compound CAE (in mm)	Diameter of zone of inhibition for test compound CIE (in mm)	Diameter of zone of inhibition for test compound FEE (in mm)	Diameter of zone of inhibition for positive control i.e., Streptomycin (in mm)	Diameter of zone of inhibition for negative control i.e., DMSO (in mm)
1.	*Escherichia coli*	17.5 ± 0.69	23.8 ± 0.58	11.4 ± 0.27	25.3 ± 0.88	NI
2.	*Enteropathogenic E. coli* (EPEC)	21.6 ± 0.93	23.4 ± 0.54	15.7 ± 0.95	27.4 ± 0.33	NI
3.	*Bacillus pumilus*	18.1 ± 0.83	20.4 ± 0.61	12.4 ± 0.49	23.1 ± 0.66	NI
4.	*Listeria monocytogenes*	18.6 ± 0.92	23.3 ± 0.93	17.4 ± 0.44	20.9 ± 0.333	NI

Results are given as mean ± SD of zone of inhibition in mm (n=3)
NI- No Inhibition (0 mm value was used for statistical purpose)

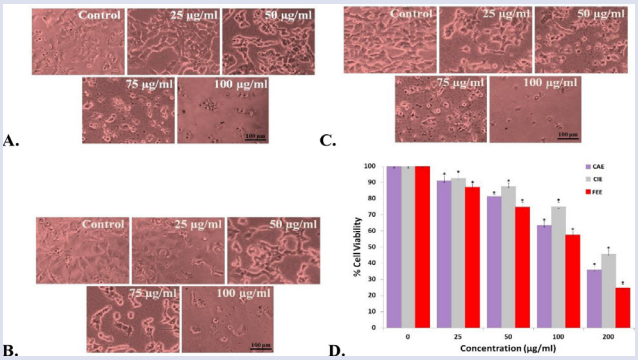

Figure 1: Anti-proliferative effect of Cardamom (*Elettaria cardamomum*), Cinnamon (*Cinnamomum verum*) and Fennel (*Foeniculum vulgare*) extract (CAE, CIE and FEE) *via* alterations in the cellular viability of MCF-7, breast carcinoma cells. (A, B and C) Morphological alterations in MCF-7 cells after treated with CAE, CIE and FEE at 25, 50, 75 and 100 µg/ml concentrations. (D) The percent cell viability of MCF-7 cells was measured after the exposure of CAE, CIE and FEE by SRB assay. The three independent experiments were performed and the values are represented as means ± SEM, $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared with the control.

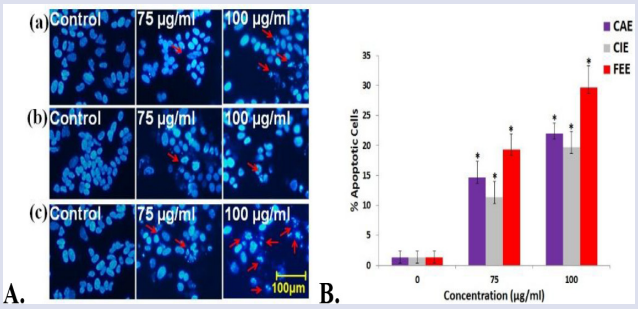

Figure 2: Nuclear apoptosis in CAE, CIE and FEE treated MCF-7 cells stained with DAPI dye. (A) The fluorescent photomicrographs showing the fragmented and condensed nuclei (indicated by red arrows) of MCF-7 cells treated with 75 µg/ml and 100 µg/ml concentrations of CAE, CIE and FEE. (B) The statistical data express percent of apoptotic cells at different concentrations of CAE, CIE and FEE with respect to untreated cells. As a minimum, three independent experiments were performed and the values are expressed as means ± SEM, $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared to the control.

Cell viability analysis

The result of % cell viability exhibits that CAE, CIE, and FEE decrease the cell viability of breast carcinoma cells in a concentration-dependent manner, as depicted in Table 2. The SRB assay also supports the morphological analysis data and FEE was found to be the most cytotoxic and the 50% inhibitory concentration (IC_{50}) calculated as 73.9 µg/ml against MCF-7 cell line followed by CAE 86.8 µg/ml and CIE (98.2 µg/ml) respectively (Figure 1D).

Nuclear Condensation assay

The result of nuclear condensation revealed the apoptotic cell death in MCF-7 cells of human breast carcinoma. As depicted in Figure 2A, showing condensed and fragmented nuclear apoptotic bodies in MCF-7 cells after treated with selected doses of CAE, CIE and FEE advocates the cellular apoptosis. The quantitative fragmented and condensed nuclei exhibited that CAE induced 14.66% and 22.00%, whereas, CIE induced 11.33% and 19.66% at 75 and 100 µg/ml concentrations, with respect to the untreated control cells (Figure 2B). The FEE induces the highest cytotoxicity and induces about 19.33% and 29.66% apoptotic cells at 75 and 100 µg/ml concentrations. Induction of condensed and fragmented apoptotic nuclei in human breast carcinoma MCF-7 cells, suggests these spices extract encouraged apoptotic cell death.

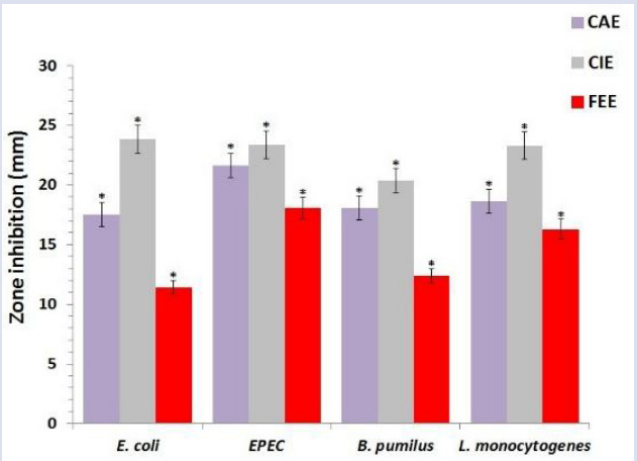

Figure 3: Anti-microbial effect of Cardamom (*Elettaria cardamomum*), Cinnamon (*Cinnamomum verum*) and Fennel (*Foeniculum vulgare*) extract (CAE, CIE and FEE) by the comparative length of Zone of Inhibition against selected bacterial strains. The values are expressed as mean ± SD of three replicates.

Table 2: Percentage of cell viability Inhibition in MCF-7 cells treated with various concentrations (25-100 µg/ml) of Cardamom (*Elettaria cardamomum*), Cinnamon (*Cinnamomum verum*) and Fennel (*Foeniculum vulgare*) extract (CAE, CIE and FEE) by SRB assay. The three independent experiments were performed and the values are represented as means ± SEM as compared to control.

S. No.	Concentration (µg/ml)	Percent viability inhibition of MCF-7 cells by		
		CAE	CIE	FEE
1	0	100	100	100
2	25	91.063	92.658	87.245
3	50	81.366	83.299	74.809
4	75	63.544	71.736	56.273
5	100	35.963	43.379	23.817

DISCUSSION

There is an increasing need for naturally derived bioactive compounds for pharmacological application because synthetic treatments have their own side effect. The established therapies cause severe side effects and hence strong alternative therapies are required.[21] The plant-derived compounds provide a vast prospect in the production of safe therapeutic drugs, that can cure various diseases without or least side effect.[22] Plant products or phytochemicals have the potency to encourage various biological activities because of the capability to diffuse through the cell membrane. Spices are the plant-derived product and contain various bioactive phytoconstituents such as flavones, flavonoids, terpenoids, and alkaloids etc.[23] Presence of these phytochemicals in these spices made them a promising agent for various pharmacological activities. The various therapeutic effects of spices have been reported which includes antibacterial, antifungal, anti-emetic, anti-ulcer, antiplatelet, anti-pyretic, anti-inflammatory, antioxidant and cytotoxic properties.[24] Various previous studies have also displaying the antibacterial and bactericidal effect of some potent spices and herbs towards various pathogenic bacteria.[3] In this study, we have used three spices cardamom (*Elettaria cardamomum*), cinnamon (*Cinnamomum verum*) and fennel (*Foeniculum vulgare*) extract (CAE, CIE and FEE) for their anti-microbial and cytotoxic activity. The result of antimicrobial activity reveals that these spices significantly inhibit the growth of all the strains of bacteria in a dose-dependent manner. Among these three selected spices, CIE was found to be the most effective as compared to CAE and FEE. Recently, it has been reported that cinnamon, common household spice has been known to possess many medicinal properties. In a previous study, it was found that the cinnamon extract was found to be the most anti-bacterial effect and inhibits five pathogenic bacteria (*S. aureus, L. monocytogenes, Campylobacter jejuni, Salmonella enteritidis, E. coli*), having a bacteriostatic concentration of 0.075% or less against all selected spices.[25] Another study also suggests cinnamon as a potent antibacterial activity against acne bacteria.[26] A previous study reported anti-microbial activity of essential oils of cinnamon bark against *Porphyromonas gingivalis*.[27] The major constituent of cinnamon is cinnamaldehyde, known for potent anti-microbial activity.[28] The results obtained from the experiment also support the hypothesis that spices have an inhibitory effect on diseased causing bacteria. In the Previous study, it has been shown that antimicrobial activity of fourteen spices including cardamom, cinnamon, and fennel by agar diffusion method and it was found that cinnamon exhibits stronger anti-microbial properties than cardamom and fennel in well diffusion assay which is in agreement with results of our experiments.[29]

Spices are not only conveying aroma, colour, taste to food preparations and sometimes curb undesirable odours but also afford various health benefits to human health. Spices can be allied in the prevention and remedy of various chronic diseases *viz.* diabetes, cancer and heart diseases deprived of apprehension of severe side effects as caused by various synthetic drugs.[30] 'Ayurveda' the original native Indian medicine system, uses various herbs and spices in blends as potent preventive and curative agents.[31] But detailed comparative studies regarding the anti-microbial and cytotoxic activity of cinnamon, cardamom, and fennel are not available. Therefore, we have selected three potent spices cardamom, cinnamon and fennel for their comparative anti-microbial and apoptotic effect against human breast carcinoma cells.

In case of cytotoxicity, fennel showed the highest cytotoxicity on MCF-7 cell lines and lowest efficacy showed by cinnamon which is just reversed in the anti-microbial assay. In another study, it has been showed that there is no correlation between cytotoxicity and anti-microbial activity.[32] The apoptosis is categorized by the characteristic morphological changes like nuclear fragmentation, shrinkage and blabbing of cells as compared with the normal healthy cells.[33] In this study, untreated MCF-7 display spindle shape and elongated appearance, whereas at various concentrations of CAE, CIE and FEE exhibits spherical shapes and detached from substratum reveal the apoptotic and anti-proliferative efficiency of these promising spices. A previous study, the cellular morphological changes in human colon carcinoma cells revealed that isorhamnetin, a quercetin metabolite induces the apoptosis depending upon doses.[34] In present study, we have examined the anti-proliferative and apoptotic effect of CAE, CIE and FEE *in vitro* and found that FEE was the most cytotoxic against MCF-7 cells of human breast carcinoma, followed by CAE and CIE. A previous study revealed the strong cytotoxic effect of fennel oil against HeLa, CCRF-CEM, Caco-2, MCF-7 and CEM/ADR5000 cancer cells.[35] The main bioactive component present in fennel, anethole induces anti-cancer and anti-inflammatory properties *via* initiation of tumour necrosis factor-alpha (TNF-α) by transcription factor NF-kB. Studies revealed that anethole suppressing the tumour growth by modulation of these cytokines.[36] A previous study also described anti-hepatic and anti-breast carcinoma effect of methanolic seed extract of fennel by modulating antioxidant defence system.[37] Another study demonstrated that fennel ethanolic extract induces the apoptosis of leukaemia cells *in vitro*.[38] Interestingly, these findings also support our study and advocate the anti-proliferative and apoptotic effects of these selected spices in a concentration-dependent manner.

CONCLUSION

The overall evaluation of this study showed that these selected spices have good antibacterial potential and inhibitory effect on proliferation of breast carcinoma MCF-7 cells. The findings can revealed that these spices can be used as effective anti-microbial stuffs against selected bacterial strains and CIE has the best anti-bacterial activity in comparison of CAE and FEE. All these selected spices extract also possess anti-proliferative and apoptotic potential, FEE has the most cytotoxic potential to encouraged apoptosis in MCF-7 cells of human breast adenocarcinoma. Further, some more studies are also needed to validate their efficacy on *in vivo* and clinical trial level.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

IC_{50}: The concentration required for 50% inhibition; **g**: Gram; **ml:** Milliliter; **h:** Hours; **ºC:** Degree celsius; **DMSO:** Dimethyl sulfoxide; **µl:** Microlitre; **%:** Percentages; **CO_2:** Carbon dioxide; **SRB:** Sulforhodamine B; **µg/mL:** Microgram per milliliter; **µm:** Micrometer; **min:** minutes, **OD:** Optical densities; **SEM:** Standard error of mean.

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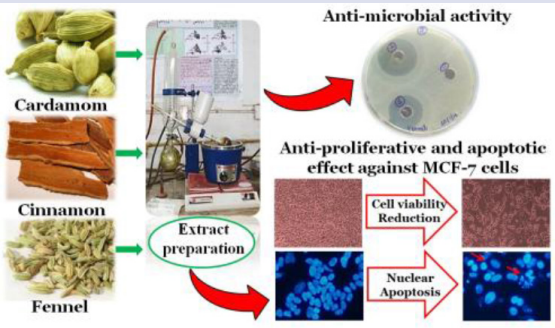
GRAPHICAL ABSTRACT



SUMMARY

- Three common spices viz. Cardamom (*Elettaria cardamomum*), Cinnamon (*Cinnamomum verum*) and Fennel (*Foeniculum vulgare*) were analyzed for their antibacterial as well as anti-proliferative and apoptotic effectiveness.
- The Cinnamon extract (CIE) showed maximum antibacterial activity against selected test organism followed by Cardamom (CAE) and Fennel (FEE).
- The anti-proliferative and apoptotic activity results revealed that FEE encourages highest cytotoxicity (IC_{50} 73.9 µg/ml) against human breast carcinoma MCF-7 cells, while CIE showed the lowest efficacy (IC_{50} 98.2 µg/ml) as compared to untreated control cells.

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