ISSN: 03875547
Volume 45, Issue 01, February, 2022

In vitro anticancer activity of RA extracts of peppermint leaves against human cancer breast and cervical cancer cells

Faten Essam Hussain Aldoghachi[1*], Ula Mohamad Noor Almousawei[1], Falah Hassan Shari[1]

Department of Pharmacognosy, College of Pharmacy, Basra University, Basra – Iraq[1]

Corresponding Author: 1*

Keywords:
Mentha piperita, isolation, RA, antiproliferative, cytotoxic activity

ABSTRACT

Natural product research provides a unique opportunity to test new anticancer agents while also learning about significant and relatively important modes of action. Aromatic rosmarinic acid (RA), which is an ester of caffeic acid and 3,4-dihydro-xyphenyllactic acid, has been claimed to have anti-inflammatory, anti-obesity and cancer prevention characteristics. It is found in the Lamiaceae family. The anticancer capacity of rosmarinic acid derived from (Peppermint) Mentha piperita L. was tested in vitro towards breast Cancer cells MCF7 cell line at range of concentrations 100,250,500,750,1000µg/ml and human Cervical cancer HeLa cell line at different concentrations of 100, 250, 500, and 1000 µg/ml. The growth of MCF-7 breast cancer cells was slowed down by RA at a concentration of 1000 g/ml, 48% and the growth of cervical cancer cells HeLa was inhibited by RA at a concentration of 1000 µg/ml only 1%.

1. INTRODUCTION

A primary cause of death worldwide is cancer, according to the World Health Organization. A most common types of from cancer are reproductive system cancer which include a breast, cervical cancer, and prostate cancer [1]. Present drug therapy, because of their negative effects on non-targeted organs, such as chemotherapy, these treatments can worsen human health conditions. Anticancer drugs that have minimal or no cytotoxic action on healthy cells are an attractive new approach to cancer therapy. This strategy can be utilized as a long-term treatment in conjunction with standard short-term cytotoxic anticancer medicines [2]. New chemical groups of anticancer drugs can be tested, as well as novel mechanisms of action, through natural product research. Anticancer chemicals that originate from plants are the most popular source for alternative cancer therapy.

In terms of secondary metabolism, polyphenols may be the most important because of their medicinal and pharmacological effects. Polyphenols can be used to fight cancer, among other things [3- 5]. As apoptosis-inducing polyphenols are thought to have anticancer characteristics, they can be used. They were found to have anti-cancer properties such as anti-oxidant capacity, cancer cell growth inhibition, apoptosis induction, target specificity, and cancer cell cytotoxicity [6- 8].

The ester of caffeic acid and 3,4-dihydroxyphenyllactic acid is rosmarinic acid (RA). RA is a poly phenolic compound found mainly in plants belonging to the Lamiaceae family. The presence of this component isolated from peppermint "Mentha piperita L." leaves was discovered due to the abundance of RA in such plants. Several interesting biological actions have been documented, including antibacterial, antiviral, anticancer, anti-inflammatory, and antioxidant properties [9].

Study objectives were to examine the biological characteristics of this substance of rosmarinic acid from methanolic extract of *Mentha piperita* L as Cytotoxic effects on human breast cancer (MCF7) and human cervix carcinoma (HeLa) cell lines as well as normal competent cells in vitro were tested.

2. Materials and methods

2.1 Chemicals and reagents

RPMI-1640 medium(Gibco, USA), Dimethyl Sulfoxide (DMSO), Trypsin/EDTA(Capricorn, USA), Fetal Bovine Serum(FBS) (Gibco, USA), DMF(Santa Cruz, USA), MTT Stain (Sigma USA), Hexane (BDH, England), Methanol(BDH England),Chloroform(HIMEDIA India) Ethyl acetate(HIMEDIA India),Formic acid (BDH,England), Acetonitrile HPLC grade(SDFCL,India),Trifluoroacetic acid(SDFCL,India),Methanol HPLC grade (BDH, England), Hydrochloric acid(BDH,England), Rosmarinic acid standard 98% (Sigma Aldrich, Germany), Silica gel 60-120 mesh (FLUKA, Switzerland), TLC Plates(Silica gel F254, 20X 20 cm thickness 2mm) (Merck, Germany).

2.2 Plant Materials

Peppermint plants were cultivated in a private orchard at Abu Al- Khaseeb district, Basra, and authenticated by Asst. Prof. Dr.Ula Al- Mosawy, Pharmacognosy Department, Pharmacy College, Basra University. For future usage, fresh peppermint leaves were harvested in September (2020) from the plant, cleaned and rinsed, dried in the shade until all water molecules had evaporated, then processed into fine powder using a mechanical blender.

2.3 Extraction procedure

Powdered of the dried leaves of peppermint defatted with hexane by Soxhlet extractor equipment for 24 hours the resultant extract allowed to stand for 24 hours to evaporate all hexane used then the sample then extracted with 90% methanol (1:10) under reflux condenser for 45 minutes and the resultant extract filtered by using filter paper and solvent was evaporate by using rotary evaporator .The resultant extract stored in dark glass container at 4^{o} c, then HPLC and TLC discovery and purification of rosmarinic acid by column chromatography.

2.4 Chromatographic analysis for the detection of rosmarinic acid

2.4.1 Thin Layer Chromatographic

TLC examination of the extract in relation to the rosmarinic acid standard was performed using a mobile phase of chloroform: ethyl acetate: formic acid (5: 4: 1, v/v/v). A compounds were identified using UV detection at 254nm after the standard solution and sample were spotted on silica gel 60 F_{254} as stationary phase.

2.4.2 High Performance Liquid Chromatography HPLC -UV analysis of rosmarinic acid

For the detection and estimation of rosmarinic acid in the peppermint extract (1g/10 mL) of crude extract, HPLC analysis was done. The (HPLC) method with UV detection was used to analyze the extract of peppermint leaves. Prior to injecting aliquots of 5 mL of each sample into the HPLC system, the mixture was

run through a 0.45 m disposable filter and then through a prominence HPLC system (shimadzu) with a degasser (DGU-20A). The HPLC system was set to 45°C and used five micro C18 columns. Elution with a mixture was used to detect the samples. Solution A contains 0.5 ml of trifluoroacetic acid in 1 liter of acetonitrile, which must be degassed for at least 10 minutes before moving on to Solution B (20:80 percent). Another option would be to degas for at least 10 minutes in 0.5 ml of trifluoroacetic acid in 1 liter of water in Solution B. A flow rate was set at1.0 ml / min, detected by UV at 230 nm. Run time duplicate the retention time of test (about 30 min) in the sample solution The rosmarinic acid was detected according to retention time of the standard rosmarinic acid which is prepared by Weighing about 20 mg of rosmarinic acid standard in 100 ml volumetric flask add 70 ml 0f methanol and Shake vigorously about 5min, then volume completed by the same solvent and mix then inject. (rosmarinic acid conc 0.2 mg/ml) as standard solution.

2.4.3 Gas chromatography–mass spectrometry GC-MASS analysis

Screening of methanolic extracts done by using a mass spectrometer Agilent gas chromatograph equipped and coupled to a mass detector Agilent 5977A spectrometer with a HP- 5MS (5% Phenyl methyl siloxen), 30m ×0.25mm × 0.25 mm ID of capillary column .The temperature of injector was 290C . the oven temp started with 40c maintained for 5 min then raised gradually to 300 0C at rate of increment 10\min, helium gas 99.99% used as mobile phase at flow rate of 1ml\min. 1 uL is the injection volume. It took 4 minutes for the solvent to cool down and the GC-MS to run for the entire time that the mass spectra were obtained. (Split mode) injections were used for the samples (50:1) It was determined that the mass spectral scan range was 45 to 650 (m/z).

2.4.4 Isolation and Purification of Rosmarinic acid by Column Chromatography

A column chromatography method was utilized to separate rosmarinic acid from the crude extract of M. piperita. The percentages of solvent systems and their volumes, the height of the packed bed column, and the stability of the absorbent were all adjusted in order to produce a rosmarinic acid-rich extract with minimal solvent use. The crude extract was fractionated using a 3-16 cm column chromatography packed with 50 g of silica gel 60 (60–200 mesh) that was employed. chloroform as a solvent system: To separate rosmarinic acid by column chromatography, TLC studies yielded ethyl acetate: formic acid (5: 4: 1, v/v/v) as mobile. phase.

0.1 grams (10mL) of crude extract were put into the packed bed column. At a flow rate of 2 mL/min, the chosen solvent solution was gently introduced and passed through the packed column.

The plant fraction was collected from every 10mL of solvent that ran out of the packed column.

2.5 Preliminary study for the cytotoxic activity of rosmarinic acid

2.5.1 Maintenance of cell cultures

HBL100 HeLa PC3 cells were received from the IRAQ Biotech Cell Bank Unit in Basrah and maintained in RPMI-1640 supplemented with 10% Fetal bovine, 100 units/mL penicillin, and 100 g/mL streptomycin. This cell line was used to study normal and cancer cells. Reseeded twice a week at 50 percent confluence with Trypsin-EDTA, cells were kept at 37 °C and 5 percent CO2 [10].

2.5.2 Combination Cytotoxicity Assays

Combination 96-well plates were used for the cytotoxicity experiment. Each well contained 1 x 104 cells from each cell line. It was only after 24 hours or after the cells had reached a confluent monolayer that the measured drug concentrations were applied. To test cell viability after 72 hours of treatment, the medium was removed, 28 L of 2 mg/mL MTT was added, and the cells were incubated for 2 h at 37° C. The residual crystals in the wells were solubilized by adding 100 L of DMSO (Dimethyl Sulphoxide) and then incubating

at 37 °C for 15 minutes with shaking after the MTT solution was removed [11]. The absorbency was determined on a microplate reader at 620 nm (test wavelength); the assay was performed in triplicate. The inhibition rate of cell growth (the percentage of cytotoxicity) was calculated as the following equation:

If A is the mean optical density in untreated wells and B is the mean optical density in treated ones, then PR=B/A*100 and IR=100-BPR [12].

3. Results and Discussion

3.1 Extraction, and detection of rosmarinic acid

The reflux methanolic extract of the peppermint leaves showed extraction yields of 0.9%. This extracts was obtained in 45 min at 70 o c showing that reflux is fast extraction method.

Using HPLC, rosmarinic acid was identified by comparing its retention time to rosmarinic acid's standard. The standard solution of rosmarinic acid has a retention time of 3.287 minutes. " Peppermint leaves extract may be seen in a chromatogram (Fig.1 and Tab.1), which indicates the existence of this component in the reflux methanolic extract of the peppermint leaves, as shown in (Fig.2, Tab.2).

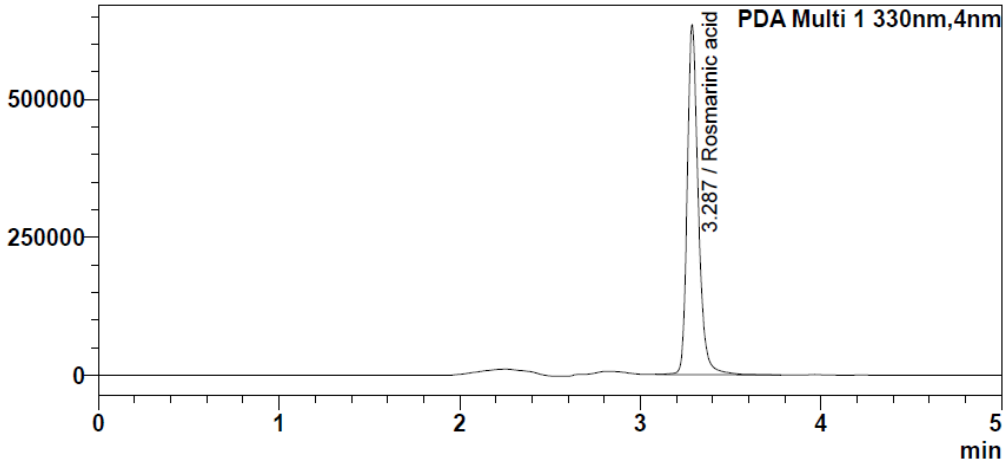

Figure (1): HPLC chromatogram of standard rosmarinic acid

Table (1): Retention time and peak area of HPLC analysis for rosmarinic acid standard

ID#	Name	Ret. Time	Area	Tailing Factor	Resolution
1	Rosmarinic acid	3.287	2705192	1.259	--
Total			2705192		

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Volume 45, Issue 01, February, 2022

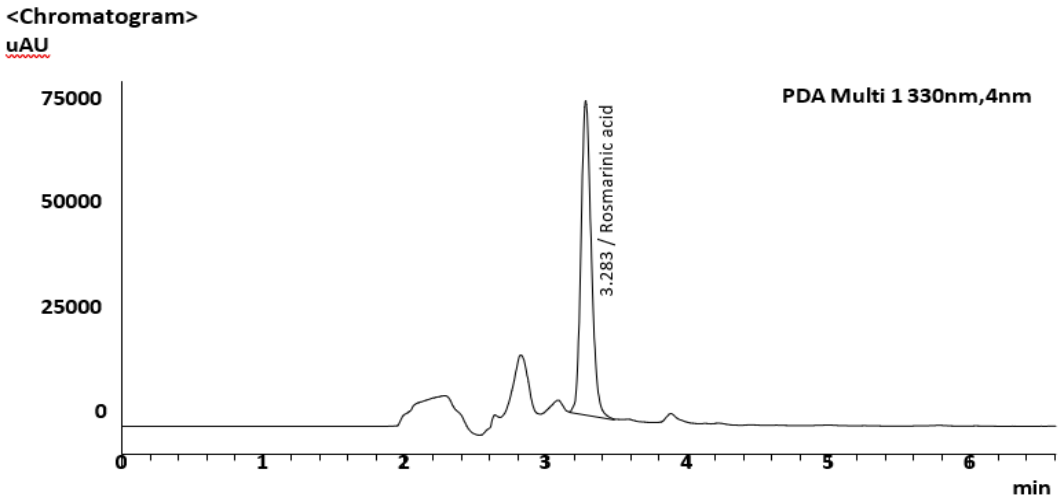

Figure (2): HPLC chromatogram of reflux extraction peppermint with methanol

Table (2): Retention time and peak area of HPLC analysis for rosmarinic acid extracted from peppermint leaves

ID#	Name	Ret. Time	Area	Tailing Factor	Resolution
1	Rosmarinic acid	3.283	360942	1.188	--
Total			360942		

Identification of the compound was performed by comparing its mass spectra and retention time R_t with the available rosmarinic acid references as shown in figure (3). The chromatogram of crude extracts shows that rosmarinic acid peaks represent at R_t 14.107.

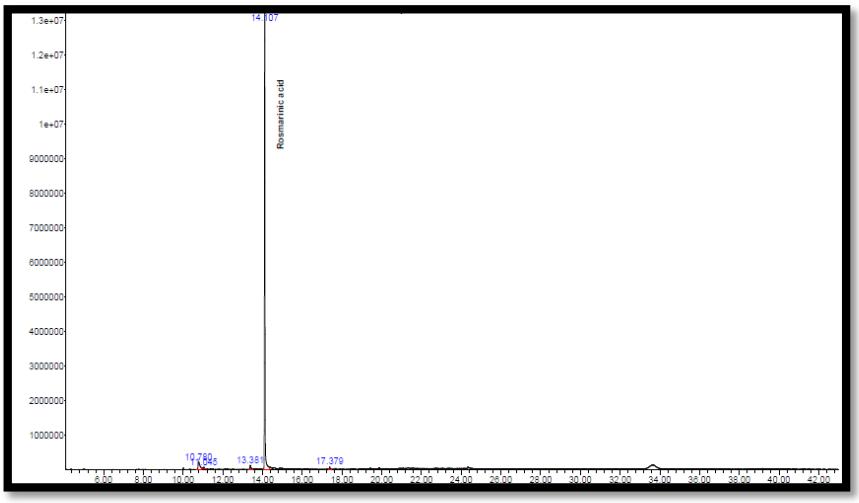

Figure (3): The GC-MASS chromatograms of Rosmarinic acid standard.

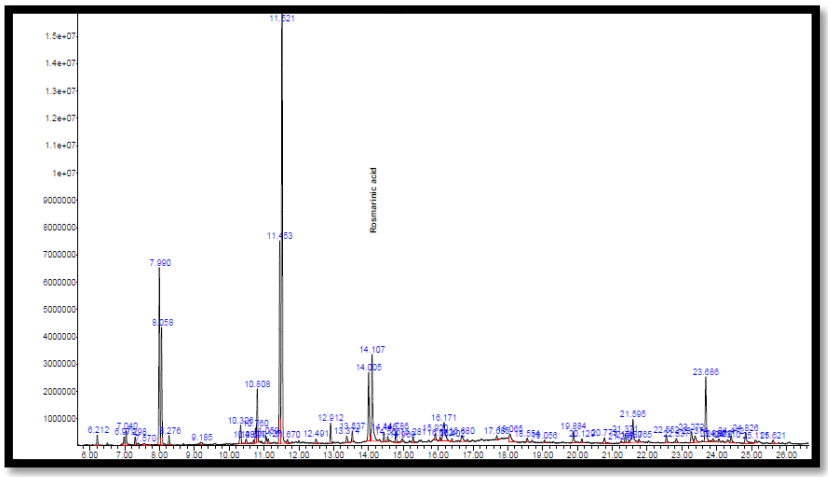

Figure (4): The GC-MASS chromatograms of *M. piperita* crude extract.

The concentration of identified compound estimate theoretically by GCMSS as shown in table (3)

Table (3): concentration of rosmarinic acid in *M. piperita* crude extracts by GC Mass

Crude sample	Retention time	Response	Concentration ppm
M.piperita	14.107	2215594	24.00

3.2 Purification of Rosmarinic acid by column chromatography

Column Chromatography was conducted on 1 g of crude extract corresponding to 35 g of the total extract to give 0.026 g of rosmarinic acid (0.9% w/w).

The difference in adsorption intensity between the solute molecules in the mobile phase and the stationary phase determines the separation of solutes. Molecular attachment to absorption sites on the stationary phase will be contested by both the mobile and the solute phases. Higher absorption strength molecules will remain in the column, whereas lower absorption strength molecules will be eluted out of the column by the mobile phase [13]. To get better separation and resolution, you need increase the length of the packed column. Flow rate should be kept at a medium level to avoid spreading zones and poor separation, which can both be caused by low flow rates. Depending on the solvent's polarity, different chemicals elute at different rates through the column. It is easier for polar molecules to flow through the column because the solvent is more effective at displacing them from an adsorbent's surface. Formic acid was also found to be beneficial in the separation of components without tailing effects, at a concentration of 1 to 3% [16]. To prevent board peak formation during separation, the acid contributes protons to the silica-solute hydrogen bond. If you'd like to increase the rosmarinic acid content, you'll need to use the silica gel column once more, this time with chloroform and formic acid as solvents [17]. For column chromatography in the fractionation of plant crude extract, the choice of eluting solvent system is critical.

The isolated compound from column chromatography purification method was subjected to different identification methods: analytical TLC, HPLC analysis in comparison with RA standard to confirm that the isolated compound is rosmarinic acid.

ISSN: 03875547
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An isolated Rosmarinic acid that purified from column chromatography was detected by analytical TLC under UV light at 254 nm, with reference to rosmarinic acid standard, as shown in Figure (5) and Table (4) and the presence of one spot with same R_f value as compare with rosmarinic acid standard indicate that purified compound is rosmarinic acid and with a good purity.

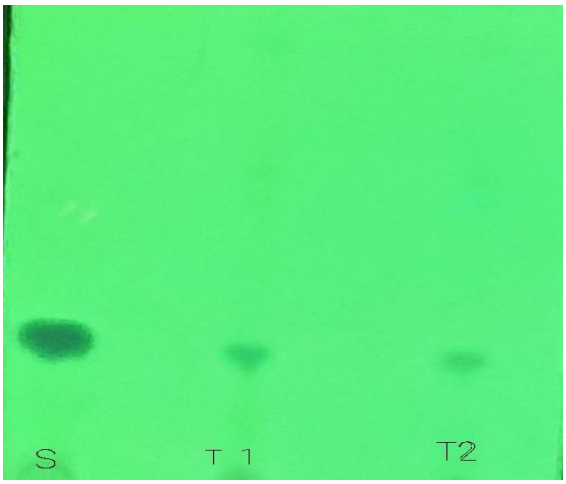

Figure (5): Analytical TLC for isolated rosmarinic acid under UV light at 254nm T=test sample S= standard.

Table (4): Rf of Rosmarinic acid standard and Rosmarinic acid in the crude extract of peppermint leaves in TLC

Solvent system	R_f Value of RA standard	R_f Value of RA in peppermint
Chloroform: ethyl acetate: formic acid (5:4:1)	0.34	0.32

Rosmarinic acid that was purified from column chromatography was detected by HPLC with reference to rosmarinic acid standard, as shown in Figures (6) (7) and the presence of single peak with same Rt of reference standard indicate good purity.

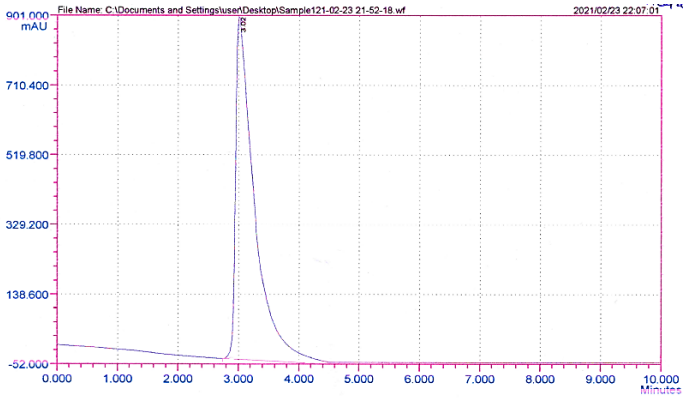

Figure (6): Analytical HPLC for RA standard.

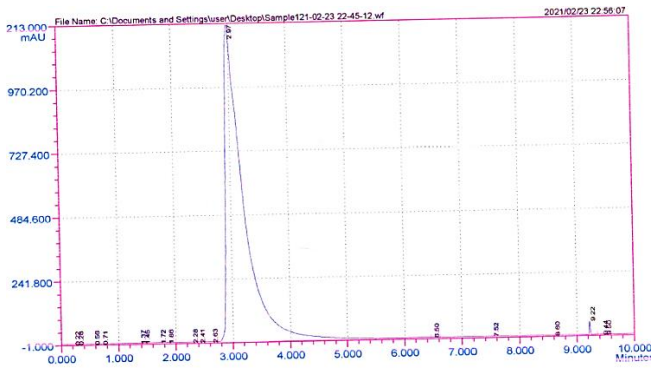

Figure. (7): Analytical HPLC for isolated RA

3.3 Role of Rosmarinic acid as anticancer agent

3.3.1 Breast cancer cell line

In breast cancer cells MCF-7inhibited cell proliferation at concentration of 1000µl/ ml of RA and decrease viability % approximately to the half in cancer cell (Tab5) (fig8). while in normal cell (HBL) RA have very simple effect (4.1%) which can be negligible (Table6) (figure 9) when compare with cell line without treatment with RA.

This give us idea that RA can act as selective cytotoxic compound which can reduce viability of cancer cell without considerable effect on healthy cells

Tab (5): Anticancer effects of RA *In vitro* study breast cancer cell (MCF-7).

Concentration µg/ml	0	1000
mean absorbance	0.3528	0.1706
Viability %	99.98866	48.35053

Tab (6): Anticancer effects of RA *In vitro* study in normal cell (HBL).

Concentration µg/ml	0	1000
mean absorbance	0.4104	0.3938
Viability %	100	95.95517

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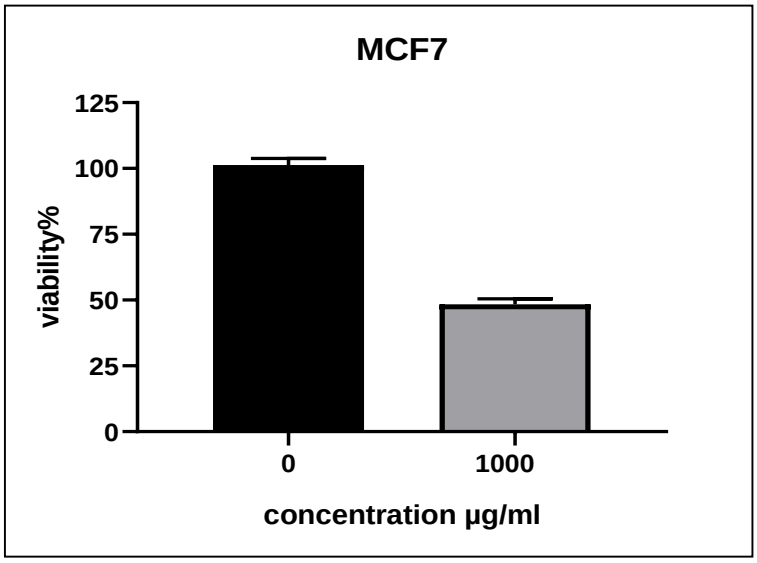


Figure (8): Antiproliferative activity of RA against breast carcinoma cells.

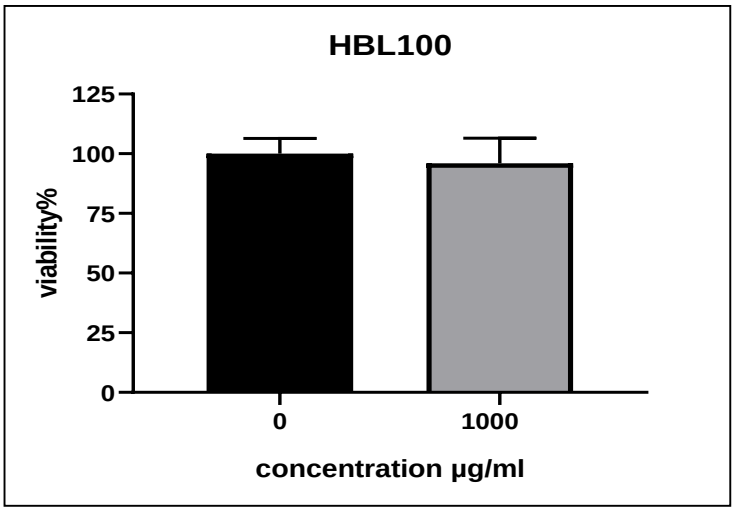


Figure (9): Antiproliferative activity of RA in normal cell HBL.

For more details further experiment carried out using different concentrations starting from 100mg/ml to 1000 mg /ml to get idea about RA behavior against cancer cell in different concentrations as in (table7) (figure 10). And the results show that rosmarinic acid activity against cancer cell appear only in high dose while in low dose have no effect on cancer cells.

Tab (7): Anticancer effects of RA *In vitro* study breast cancer cell (MCF-7).

Concentration µg/ml	**100**	**250**	**500**	**750**	**1000**
Viability %	98.453	100.743	97.0916	102.599	50.351

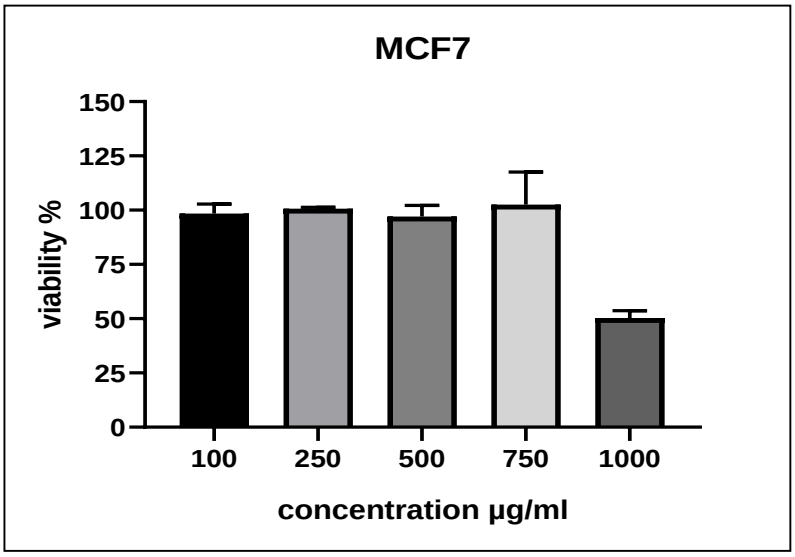


Figure (10): Antiproliferative activity of RA against breast carcinoma cells.

3.3.2 Cervical cancer

In cervical cancer cells line HeLa inhibited cell proliferation at concentration of 1000µl/ ml of RA and decrease viability % consider negligible (Table8) (figure 9).

Tab (8): Anticancer effects of RA *In vitro* study in HELA

Concentration µg/ml	**100**	**250**	**500**	**750**	**1000**
Viability %	**102.083**	**103.055**	**131.147**	**178.274**	**99.0124**

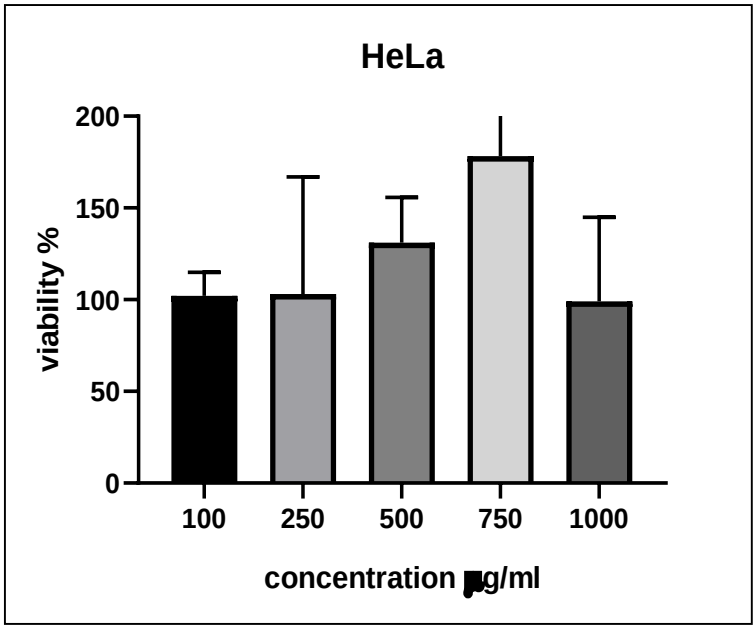
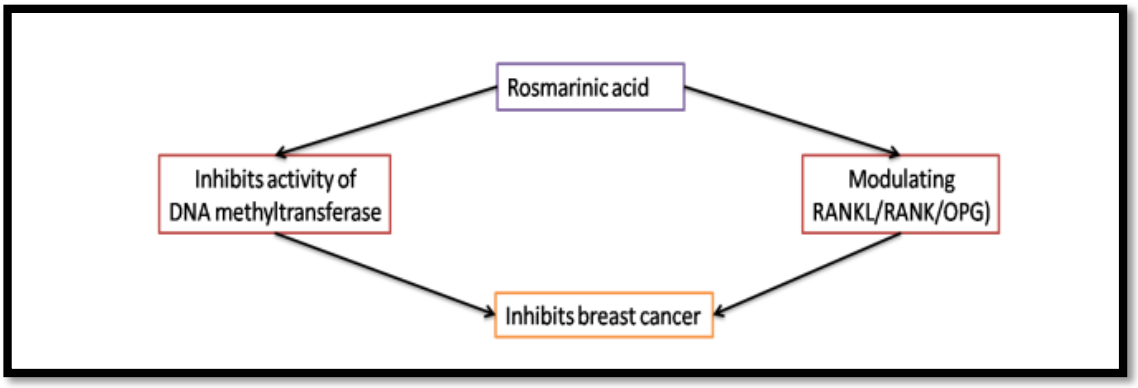


Figure (11): Antiproliferative activity of RA in cervical cancer

DNA methyltransferase activity in human breast cancer MCF7 cells was allegedly reduced by RA [18]. In cancer, abnormal alterations in the DNA methylation pattern lead to promoter hypermethylation and

transcriptional silence in a wide range of tumor suppressor genes, resulting in tumor formation. This impact can be reversed by inhibiting DNA methyltransferase, making it a promising therapeutic option for the treatment of cancer. To prevent bone metastasis in breast cancer, RA has been demonstrated to suppress the migration of MDA-MB-231BO human breast cancer cells, which can be dose-dependently reduced. RA may prevent bone metastases from breast cancer via the RANKL/RANK/osteoprotegerin (OPG)/interleukin-8 (IL-8) pathway and at the same time suppress the expression of IL-8 (IL-8) [19]. Osteoprotegerin is a pro-angiogenic factor, and inhibition of OPG can be of benefit in inhibiting metastasis of cancer cells [20]. Elevated levels of IL-8 expression by breast cancer cells has been implicated in the osteolysis associated with metastatic breast cancer [21]. RA also showed toxicity Two breast cancer cell lines, Adriamycin-resistant MCF-7/Adr and wild-type MCF-7/wt, were tested [22]. Mechanisms of rosmarinic acid to prevent breast cancer following different routes are presented in scheme (1)



Scheme. (1) Inhibition of Cancer Growth by Rosmarinic Acid Breast Cancer [24]

According to preliminary research, RA may exert its anti-inflammatory and antioxidant activities as well as preventing the growth and migration of cancer cells, as well as causing the selective death of cancer cells. Antiangiogenic properties have also been proven by the compound's ability to inhibit human umbilical vein endothelial cell proliferation; migration; adhesion and tube formation, [23] which can be beneficial in preventing tumor growth and metastasis. RA has also been found to reverse multi-drug resistance in SGC7901/Adr cells and increase the intracellular accumulation of Adriamycin and rhodamine and decrease the transcription of MDR1 gene and the expression of P-gp in SGC7901/Adr cells [24].

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