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Synergism effect of iron nanoparticles with Ocimum Basilicum L. on breast cancer cell

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***Abstract*---**Breast cancer is the second leading cause of cancer deaths among women. The development of breast cancer is a multi-step process involving multiple cell types, and its prevention remains challenging in the world. Early diagnosis of breast cancer is one of the best approaches to prevent this disease. In some developed countries, the 5-year relative survival rate of breast cancer patients is above 80% due to early prevention. For people presenting without metastatic disease, therapeutic goals are tumor eradication and preventing recurrence. Ocimum basilicum L. (Basil) is a plant that has a place with the family Labiatae, also basil leave have activities intense cancer prevention agents, curbs aging, is an anticancer, antiviral, and has antimicrobial properties. This study aimed to determine effect of nanotechnology with basil as synergism effect on breast cancer cell also showing the side effect of treatment on normal cells. In this work breast cancer cells (MDA and MCF7) was treated with Iron nanoparticle that prepared by Cold Atmospheric Plasma (CAP) and Ocimum Basilcum L. (Basil) as synergism effect on the cells. The result of MCF7 cell growth inhibition percent by synergism treatment was (77.33%) after 72 hours of culturing, as well as MDA cells growth inhibition was (80.67%) after 72 hours of incubation. In other side, the results showing low inhibition of growth to normal cell (REF) that was (27.33%) in 72 hours period. The results showed basil and Iron nanoparticles have synergism efficacy on MCF7 and MDA cells also less side effect on REF cells and that positive indicate of basil as treatment of breast cancer.

***Keywords*---**breast cancer, cell line, basil, iron nanoparticle.

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Introduction

Breast cancer is the most common cancer among women and one of the most important causes of death among them.[1] It is a multifactorial disease[2] and various factors contribute to its occurrence. Although the disease occurs all over the world, its incidence, mortality, and survival rates vary considerably among different parts of the world, which could be due to many factors such as population structure, lifestyle, genetic factors, and environment.[3] Changes in risk factors have led to an increase in the prevalence of breast cancer, which is increasing every day.[4] Breast cancer is commonly handled with surgical treatment, which can be followed by way of chemotherapy or radiation therapy, or both. A multidisciplinary approach is prime.[5] Hormone receptor-effective cancers are often handled with hormone-blockading therapy over courses of several years. Monoclonal antibodies, or other immune-modulating remedies, can be administered in positive cases of metastatic and other advanced tiers of breast most cancers.[6]

In other side, Basil is an English name for *Ocimum basilicum L.*, while it is called Basilic, Basilikum and Albahaca, in French, German and Spanish, respectively. It is also called reihan and rehan in Persian and Arabic language, respectively.[7] Today, it is assessed that 80% of the total populace depends on plant arrangements as medication to meet their wellbeing needs [8]. *Ocimum basilicum L.* (Basil) is a plant that has a place with the family *Labiatae* and has demonstrated its capability to help deflect a few ailments in different nations.[9] The crucial oil from european basil contains excessive concentrations of linalool and methyl chavicol (estragole), in a ratio of about 3:1.[10] Different elements consist of: 1,8-cineole, eugenol, and myrcene, amongst others.[11] The clove heady scent of sweet basil is derived from eugenol.[12] The aroma profile of basil consists of 1,eight-cineole[13] and methyl eugenol.[12] In this species eugenol is synthesised from coniferyl acetate and NADPH.[14] Numerous examinations have built up that basil leave activities have intense cancer prevention agents, curbs aging, is an anticancer, antiviral, and has antimicrobial properties.[15] The aims of this study were the production of Iron nanoparticles with basil extract using cold atmosphere plasma (CAP) method, after which the mixture was applied to treat breast cancer by investigating the synergistic effect of the mixture on the growth ratio of cancer cells *in vitro* (MCF7 and MDA). In addition the side effect of Synergism on normal breast cells (REF) was examined.

Materials and Methods

Extraction

Sample collection

In the current study Basil was collect freshly from Dyala Government/ Hebheb gardens/Iraq at last of October 2020. From collected Basil was extract the leave part only, then the leaves collected was dehydrate for 14 days before extraction using Soxhlet extraction method using Absolute Ethanol (99%) [16].After extraction the solvent is removed, typically by using a rotary evaporator, yielding the extracted compound (Estragole, Linalool, heptadecene-(8)-carbonic acid,

Trans-anethole and Methyleugenol); the non-soluble portion of the extracted solid remains in the thimble.[17]

GC-MS analysis

The GC-MS analysis of the plant extract (basil) was made in a Agilent 7890 A instrument under computer control at 70 eV. About 1 µl of the basil extract was injected into the GC-MS using a micro syringe and the scanning was done for 45 min. As the compounds were separated, they eluted from the column and entered a detector which was capable of creating an electronic signal whenever a compound was detected. The greater the concentration in the sample, bigger was the signal obtained which was then processed by a computer. The time from when the injection was made (Initial time) to when elution occurred is referred to as the retention time (RT). The M/Z (mass/charge) ratio obtained was calibrated from the graph obtained, which was called as the mass spectrum graph which is the fingerprint of a molecule. Before analyzing the extract using gas chromatography and mass spectroscopy, the temperature of the oven, the flow rate of the gas used and the electron gun were programmed initially. The temperature of the oven was maintained at 100°C. Helium gas was used as a carrier as well as an eluent. The flow rate of helium was set to 1 ml/min. The identity of the components in the extracts was assigned by the comparison of their retention indices and mass spectra fragmentation patterns with those stored on the computer library and also with published literatures. Compounds were identified by comparing their spectra to those of the Wiley and NIST/EPA/NIH mass spectral libraries.[18]

Nanoparticles formation:

Cold Atmospheric Plasma (CAP) method

Iron nitrate [$Fe(NO_3)_3$] nanoparticle was formed by use cold atmospheric plasma (CAP). Plasma generator used located in the University of Baghdad/ faculty of Sciences for females/department of physical science exactly in the thin membranes laboratory, as well as, the plasma was applied on the mixture of Iron for 10 second, Basil extract and Deionized water, in the fact, the mixture was calculated by the Molarity equation (equation 1),

... (1)
Where is the (WT) is sample weight by unit Gram, M is Molarity by unit Mole/Litter, (M.WT) is molecular weight by unit Gram/Mole and (V. (ml)) is Volume by unit Milliliter [19]

Cell line

In this part of study three type of cell line (MCF7, MDA, and REF) were in Technology Research Center/ Molecular and Medical Biotechnology department of Al-Nahrin University.

Viable Cells Count

Tissue culture flasks which contain cancer cells are received, and growth medium is decanted off, then the flasks are washed twice using 1mL of trypsin-versene EDTA (Ethylene Diamine Tetra Acetic) solution. After washing, 1mL of trypsin versene is added to the flask and incubated 2-5 min. for cells detachment, then 4-5mL of PRMI growth medium (contains 10% FBS) is added to the flask. Then the cells are counted using counting chamber by inverted microscope (400x) to detect the viable cells. The following formula is used to compute the count of cells per volume unit (cell/mL) [20]

... (2)

Where, *c* is the viable cells count (per mL), *n* is the number of counted viable cells, while is the dilution factor (equal to 10).

Real time polymerase chain reaction [RT-qPCR]

The genetic analysis was done for the treated cells using the primers, to detect the effect of proposed therapies, so that top result of gene expression in the current study(Iron nanoparticle with basil extract) applied to RT-qPCR on these genes. The process located in Technology Research Center/ Molecular and Medical Biotechnology department of Al-Nahrin University. The following steps was describe RT-qPCR technique.

RNA extraction

The RNA Mini Prep provides a streamlined method for the purification of up to 50 µg (per prep) of high-quality RNA directly from samples in TRI Reagent. Total RNA, including small RNAs (17-200 nt), is effectively isolated from a variety of sample sources (blood). Isolation of RNA by conventional phase separation was shown to selectively enrich for some species of miRNA, leading to bias in downstream analysis. The Direct-zol™ method assures unbiased recovery of small RNAs including miRNA.

The procedure is easy. Simply apply a prepared sample in TRI Reagent® directly to the Zymo-Spin™ IIC Column and then spin, wash, and elute the RNA. No phase separation, precipitation, or post-purification steps are necessary. The eluted RNA is high quality and suitable for subsequent molecular manipulation and analysis (including RT-qPCR).

Conversion of RNA to cDNA

PrimeScriptTM RT reagent Kit is designed to perform the reverse transcription optimized for RT-qPCR. It uses PrimeScriptTM RTase, which features excellent extendibility and makes fast, efficient cDNA template synthesis for Real Time PCR possible. The step experimental procedure is simple and suitable for high throughput analysis. This kit can be used in combination with Real Time PCR reagent, SYBR® Premix.

Gene Expression Fold Change Analysis

RT-qPCR is used for quantification of the levels of gene expression. The measured CT values during thermal reaction are recorded to compute the following measurements [21]

Results and Discussion

The current study was give more than important result as following sequence

GC-MS analysis

GC-MS analysis for the dry leave extract using Absolute Ethanol (99%) as a solvent was carried out , many compounds were found , these compounds with their main benefits were summarized in Tables (1).

Table 1: GC-MS results of the main compounds found in the dry basil leaves extract using ethanol (99%) with its benefit

#	RT	Area%	Compound Name	Main Benefit
1	22.602	11.76	Estragole	Anticancer, antioxidant and antimicrobial activities[22][23]
2	52.459	22.22	Linalool	antibacterial and anticancer activity[23] [24]
3	49.195	3.36	Methyleugenol	Antioxidant activity and hepatoprotective effect[25]
4	49.830	3.00	Trans-anethole	antimicrobial, anti-inflammatory, anticancer and antioxidant properties[26]
5	48.361	4.98	n-Hexadecanoic acid Octadecanoic acid	essential for the synthesis of various hormones[27]
6	56.894	2.26	heptadecene-(8)-carbonic acid	Antioxidant[28]
7	57.677	4.75	Cycloheptane	hydrocarbon pneumonitis[29]
8	58.014	23.35	Oleic Acid	reduce the cardiovascular risk[30]
9	58.168	16.65	2-octyl-Cyclopropaneoctanal	immune boosters[31]
10	58.729	7.66	1,8-Cineole	Induce apoptosis [26]

As mentioned above, basil extract showed many contents that act anti-oxidant and anti-proliferative such as [Estragole (11.76%), Linalool (22.22%), heptadecene-(8)-carbonic acid (2.26%), Trans-anethole(3.00%) and Methyleugenol (3.36%)], The chemical components of sweet basil have been described in several reports and main ingredient of basil present in this study was compatible with several recent studies.[32]

Atif B.A.Mohammed study showed higher activity observed from basil as anti-proliferative activity towards the three cell lines,[33] also Renan Gianoti Torres

study was approved that basil extract is efficient against the human breast cancer cell line compared with other extract**,**[34]

Nanoparticles

In this study period (10 sec.) of atmospheric cold plasm were applied to Iron in order to form nanoparticles, that identified by AFM, SEM, FTIR and Xrd as the following Figure 1(A,B,and,C, respectively)

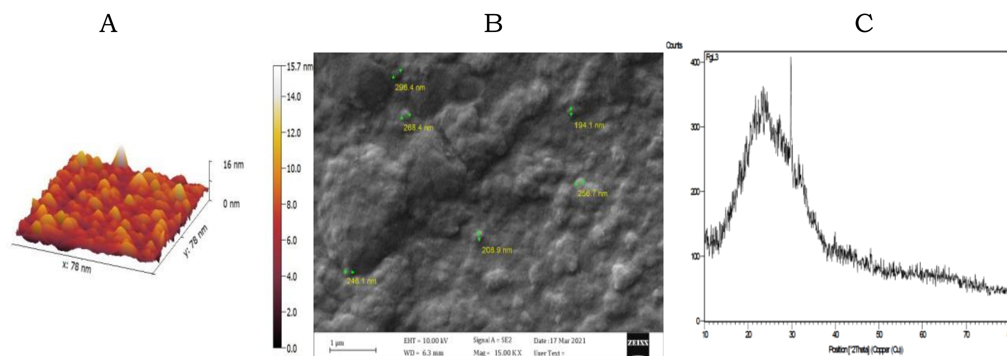

Figure 1: Identifications of nanoparticle by (A: AFM, B: SEM and C: Xrd)

The FT-IR chart was represented in the following Figure (2)

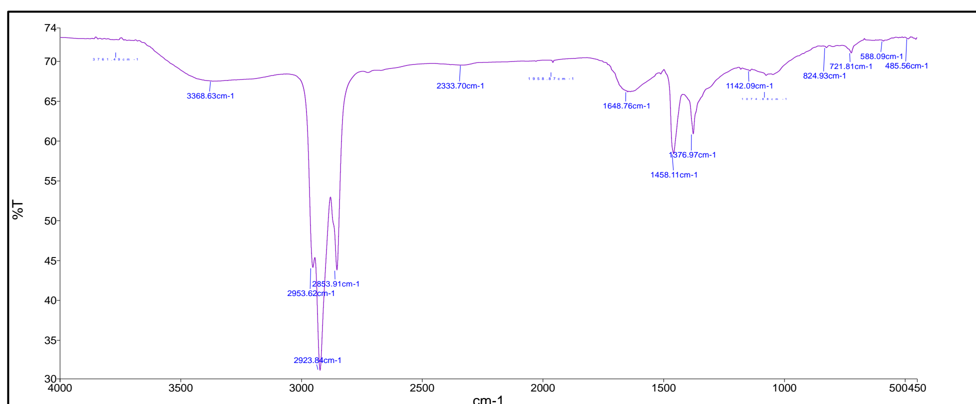

Figure 2: FTIR of Iron nanoparticle prepared by CAP

Cell line

In the current study, three cell lines [MCF7, MDA and REF] were treated with two groups of nanoparticles Iron nanoparticle (FeNp) and Iron (Fe) nanoparticle with extract (E) as combination, also detect the cytotoxicity for each cell line and gene expression was identified for combination group only because of this group give high result of growth inhibition as showing below. The cytotoxicity was calculated for breast cancer cell lines, MCF7 and MDA under different doses of FeNp alone 100ppm, 50ppm, and 25 ppm, and the combinations of three doses of FeNp and E also were assay, at Fe100E5= 100ppm+5µg, Fe50E5= 50 ppm+5µg, Fe25E5= 25 ppm+5µg, Fe100E10= 100 ppm+10µg, Fe50E10= 50 ppm+10µg, Fe25E10= 25

ppm+10µg, Fe100E20= 100 ppm+20µg, Fe50E20= 50 ppm+20µg, and Fe25E20= 25 ppm +20µg to be compared with the impact of these treatments on the REF normal breast tissue cell line. The potential of effectiveness of combined treatments was evaluate. As following:

MCF7

After 72hrs incubation, the maximum growth inhibition percent (GI) was 78% in case of Fe100E10 as shown in Figure (3) and Table (2). also all the combination showed synergism effect as appear in Figure (4).

Table 2: Growth inhibition (GI%) and standard division (SD) for Iron (after incubation 72hr) nanoparticle and combination

Iron Nanoparticle			Combination		
Dose	GI%	±SD	Dose	GI%	±SD
25 ppm	67.66	3.8	Fe25E5	63.66	5.5
			Fe50E5	69	1
			Fe100E5	71	1
50 ppm	68.33	3.2	Fe25E10	69.33	1.2
			Fe50E10	74	3.6
			Fe100E10	78	1
100 ppm	71	2.7	Fe25E20	71	1.7
			Fe50E20	75	2
			Fe100E20	77.33	2.1

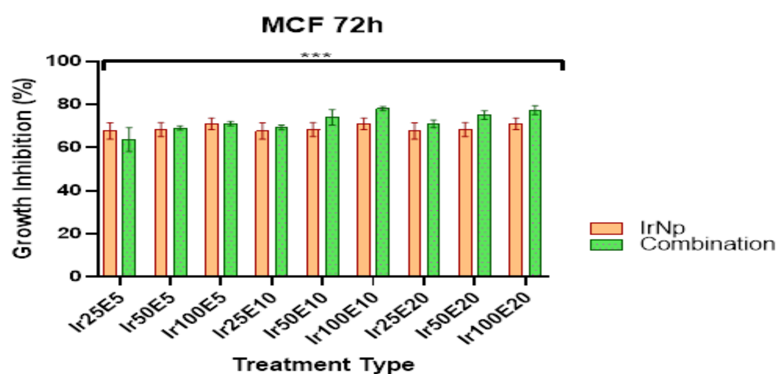

Figure 3: Growth inhibition rate MCF7 cytotoxicity (72hrs incubation) [***: (P value = 0.0001) highly significant]

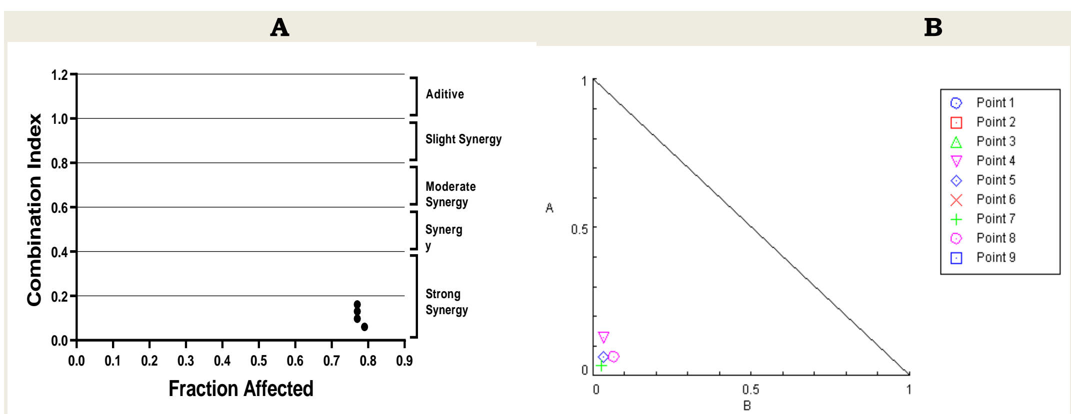

Figure 4: Synergic effect in MCF7 (72hrs incubation) (A) combination Indexes (B) isobologram analysis

The result of 72 hours incubation for MCF7 cell line and also treated with Iron nanoparticle and combination with extract was increase the rate of GI with concentration and that meaning the efficacy and death rate of Iron, Extract and combination nanoparticles was increase with concentration, also cytotoxicity of treatments was highly significant effect on MCF7 at 72hr of incubation as illustrated in Figure (4).

MDA

In MDA cell line 72hr incubation, maximum cytotoxicity was 77.33% in case of Fe100E20, as shown in Figure (5) and Table (3). All combinations showed strong synergism effect, as illustrated in Figure (6)

Table 3: Growth inhibition (GI%) and standard division (SD) for Iron (after incubation 72hr) and combination nanoparticle

Iron Nanoparticle			Combination		
Dose	GI%	±SD	Dose	GI%	±SD
25 ppm	74.67	2.3	Fe25E5	77	2
			Fe50E5	78.67	3.2
			Fe100E5	80.67	2.1
50 ppm	75.33	2.1	Fe25E10	75	2
			Fe50E10	78.67	1.5
			Fe100E10	81.67	1.5
100 ppm	77.33	0.6	Fe25E20	71.33	1.5
			Fe50E20	78.67	3.2
			Fe100E20	80.67	2.1

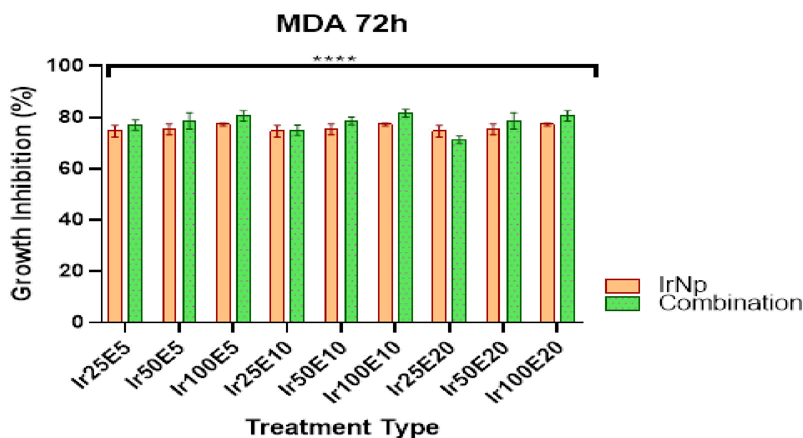

Figure 5: Growth inhibition rate MDA cytotoxicity (72hrs incubation)[****: (P value <0.0001) highly significant]

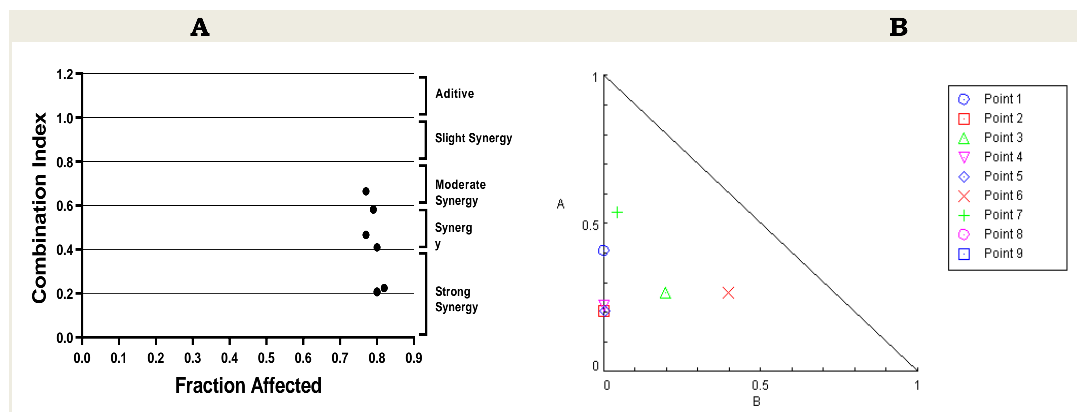

Figure 6: Synergic effect in MCF7 (72hrs incubation) (A) combination indexes (B) isobologram analysis

As showed, the result of 72 hours incubation for MDA cell line treated with Iron nanoparticle and combination was increase the rate of GI with concentration and that meaning the efficacy and death rate of Iron nanoparticle and combination was increase with concentration, also cytotoxicity of treatments was highly significant effect on MDA at 72hr of incubation as illustrated in Figure (6). In addition Ramanuj K Gupta study observed significant decrease in cell survival in (MCF-7, MDA-MB-468 and MDA-MB 231) cancer cell lines when treated with leaves of basil extracts. [35]

REF

After 72hrs incubation, did not showed any cytotoxicity, the maximum cytotoxicity was 48% in case of Fe25E5 as shown in Figure (7) and Table (4). All of combinations showed additive effect, except Fe100E20 showed synergism effect at all, Figure (8).

Table 4: Growth inhibition (GI%) and standard division (SD) for Iron (after incubation 72hr) and combination nanoparticle

Iron Nanoparticle			Combination		
Dose	GI%	±SD	Dose	GI%	±SD
25 ppm	37.33	2.1	Fe25E5	35.33	3.5
			Fe50E5	33.33	2.3
			Fe100E5	30.33	2.5
50 ppm	36.33	3.1	Fe25E10	37	2
			Fe50E10	32.67	2.9
			Fe100E10	31.33	3.5
100 ppm	34.33	3.1	Fe25E20	37	2
			Fe50E20	30.33	1.5
			Fe100E20	27.33	1.5

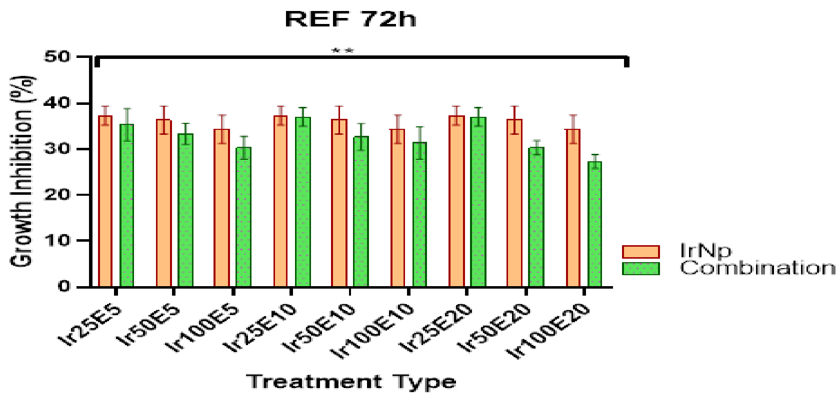

Figure 7: Growth inhibition rate REF cytotoxicity (72hrs incubation)[**:(P value= 0.0011) highly significant]

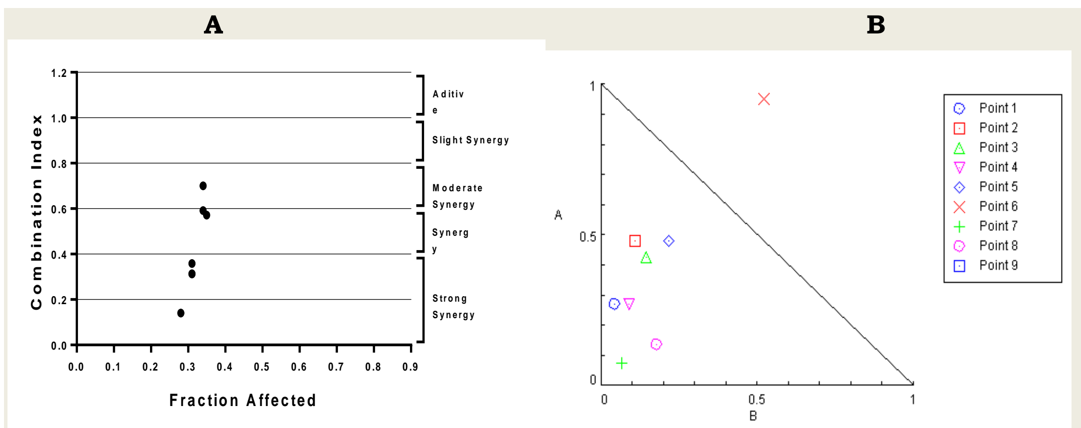

Figure 8: Synergic effect in MCF7 (72hrs incubation) (A) combination indexes (B) isobologram analysis

Gene expression analysis

Gene expression analysis was evaluated for different proteins by using Ct values of RT-qPCR analysis, P53 in human breast cancer cell lines MCF7 and MDA, using a combination of Iron nanoparticle and Basil extract with dose 100 ppm of Iron and 20 µg basil extract in MCF7 and MDA. In MCF7 cells, the P53 was upregulated after 72hr incubation, also in MDA cells, the P53was upregulated 72hr of incubation.

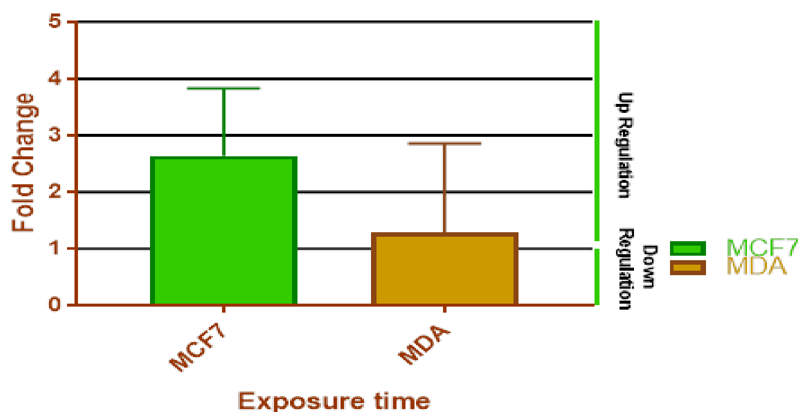

Figure 9: Gene expression analysis of MCF7 and MDA at 72h period

The result above showed the mixture was induced upregulation of p53 expression in MCF7 and MDA cells as showed in Figure (9), indicating these compounds are able to activate and stabilise p53 protein expression. That may be done by RNA damage and that lead to cell apoptosis.

Conclusion

Basil extract in dry form with Iron nanoparticle give high efficacy as anti-proliferative as appear above, MCF7 cells were inhibit when treated with combination of Iron nanoparticle and basil extract more than Iron nanoparticle alone, also the MDA cells were inhibit in same matter, also as showed the efficacy of combination on MDA cells more than MCF7, in addition the gene expression result appear upregulation of P53 gene in MCF7 and MDA that point to apoptosis, all that indicate the efficacy of basil extract. In other side the REF cells were less inhibit when treated with combination than Iron nanoparticle alone, so that give positive feedback of basil as less side effect breast cancer treatment.

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