MiR30a suppresses prostate cancer cells by increasing apoptosis and regulation of MMPs

Azhar A. Ameen 1,2Department of Biology, College of Science, Basrah University azhar.ameen@uobasrah.edu.iq

Dhamia K.Suker 1,2Department of Biology, College of Science, Basrah University

ABSTRACT

MiR 30a has important association with a wide range of human cancers in control the main features of tumor cells such as proliferation and invasion, metastasis, clinical prognosis ,and treatment response. In the current study effects of the miR30a in induce apoptosis and regulation expression of Matrix Metalloproteinase (MMP2 and MMP9) in PC3 cell line in comparison to Vero cell line was investigated. The effect of miR30a on cell viability was observed by MTT- assay, to assess morphological changes due to apoptosis, cells have been fluorescently stained with acridine orange and ethidium bromide(AO- EB) and followed by qRT-PCR to reveal the levels of Caspase-8 and MMP2,9 in PC3 and Vero cell lines . The results showed that the viability of transfected cells was reduced significantly when compared to those in paired untreated and negative control treated cells as a result of apoptosis , transfected tumor cells showed more apoptotic and necrosis features compared with normal cells , and necrotic features , this was associated with upregulation of caspase8, and down regulation in the expression levels of MMP2 and MMP9 .These results suggested that the miR30a may have a potential role in cancer suppression and thereby.

Keywords: MiR30a, Prostate cancer,MMP2,MMP9,Caspase8,Apoptosis

Introduction

Prostate cancer is the most common male malignancy, despite advances in diagnosis and treatment, it is still a great cause of mortality and morbidity around the world(1) miRNAs are small non coding RNAs consisting of (19-25) nucleotides that have the ability to control several gene targets ,so play a role in the regulation of various biological and pathological pathway , such as formation and progression cancer(2). MiRNA studies showed that miRNAs can control gene transcription either alone or in combination with other transcription factors, resulting in disrupted cellular processes in prostate cancer, they also play an essential role in cancer development, with many miRNAs influencing cancer cell transformation, metastasis and treatment resistance. several studies have found that special miRNAs were upregulated as well as downregulated parallel in many types of human cancer and were frequently associated with specific cytogenetic abnormalities, there was a deregulation of several miRNAs in prostate cancer, which may play as oncogenes or tumor inhibition(3).

The miR30 family, which consist of five different premature miRNAs (miR30a, -30 b, -30c, -30d, -30e), was demonstrated to have various roles in regulating important steps of the tumorigenesis, metastasis, clinical prognosis and chemo resistance in many types of human cancers, further suggests referred to

that many members of miR30 family have important role in the progression of the prostate cancer by controling crucial signalling pathway molecules (4). MiR30a -5p changes cell proliferation in the PC-3 cell line and prostate cancer samples, it could lead to the discovery of new prostate cancer therapeutic targets (5). There was decrease in breast cancer cell line proliferation when treated with other type of microRNA(miR Let-7) (6)

Matrix metalloproteinases are a class of the extracellular calcium and zinc -dependent endopeptidases with enzymatic effect that can analyze extracellular matrix or basement membrane (7)and (8) demonstrated that there was a Positive correlation between increase in the expression level of MMP2 and MMP9 and malignant aggressiveness of breast cancer.(9) showed that the gene expression level of MMP12 was increased in some tissue of mice as an indicator for the inflammatory response .

Other investigation recorded that their expression levels could be depend as potential biomarker which and predict tumor behavior ,progression and prognosis in colon and Prostate cancer (10).

There was great attention in MMPs post transcriptional regulation by miRNAs, a complex correlation between: different MMPs , their regulating miRNAs, and the ways by which these interactions affect development of cancer, such as growth, invasion ,angiogenesis, and metastasis.(11) . The significance of miRNAs, which have been found to influence progression and metastasis of cancer via direct, and indirect interactions with matrix metalloproteinase (12).

Methods:

Cell cultures and transfections

The prostate cancer (PC3) cell line and normal cells (Vero)cell line, were commercially obtained from the Babilon medical college cell culture unite\Iraq and cultured in Dulbeccos Modified Eagle Medium (DMEM) supplemented with (10%) fetal bovine serum, L- Glutamin, (100) µg\ ml and Streptomycin (100) µg \ml Penicillin, at 37°C in a humidified atmosphere with (5%) CO2. All mimics of miR30a , their negative control and Transfection reagent (lipofectamine) were purchased from ABM.good (Kanada) ,for the transfection of: mimics mir30a, and their negative control, the transfection reagent was used as recommended by the manufacturers.

MTT assay

Cells seeded in 96- wells plate before one day of transfected with mimics miR30a and their negative control ,then added 10 ul of MTT and 90 ul of medium to each well ,the cell incubated for 2-4 h After incubation, media was discarded and 100 ul of DMSO was added to each transfected well to solubilize the formazan crystal, then incubated in the dark for 20 minutes., when purple color was appeared , reading at 490 nm to get the optical density of each well by Eliza reader,(5) wells for every treatment group were analyzed, and all independent treatments were done in triplicate .

Fluorescence staining

Morphological evaluation to changes of cells viability and apoptosis due to transfected with miR30a were performed using acridine orange-ethidium bromide(AO&EB) staining techniques, after transfected cells slide chamber (8wells)for 48h , Dual fluorescent staining solution 1 µ containing (100) µg/ml AO and (100) µg/ml EB was added to each chamber of a slide , incubated for 25seconds, then covered with a coverslip and examined under that fluorescent microscope (13).

Gene expression

After miR30a transfection, total RNA was isolated using the Geneaid Total RNA Kit (korea). The quality and quantity of all RNAs samples were assessed by using a Nano-Drop spectrophotometer. For each sample, about 1 µg RNA was used for synthesis of cDNA (Bioner RT PreMix-Korea) from total RNA according to the manufacturer's instructions then, quantitative reverse transcription- polymerase chain reaction (qRT-PCR) was done by using Gene Expression Assays (SyberGreen™)(Bioneer, Korya). Gene expression levels were normalized to (GAPH) expression, then the expression levels of Caspase8,MMP2 and MMP9 were calculated by 2-ΔΔCq method (14) ,the primers

sequences that used in this study were reveals in Table (I)

Table (1): The primers sequences used in the current study

Primers	sequences, 5'-3'
Caspase-8	F: GATGAGGCAGACTTTCTGCT R: CATAGTTCACGCCAGTCAGGAT
MMP2	F: GCT ACG ATG GAG GCCCTAATG R: TCT CCT TGG GGC AGC CAT
MMP9	F:TTGACAGCGACAAGAAGTGG R:GCCATTCACGTCGTCCTTAT
GAPH	F: GAC AGT CAG CCG CAT CTT CT R:TTA AAA GCA GCC CTG GTG AC

Statistical Analysis

All statistical analysis were performed by using SPSS software version 23, (P0.05) was considered to demonstrate a statistically significant difference, data in this study were obtained from at least three independent experiments and displayed as mean standard deviation.

Results

The results indicated that PC3 cells transfected with miR30a were showed a concentration -dependent inhibition manners of cell viability , so these effects began to emerge until a high concentration of mimic miR30a, and represented by a decrease in the cell population and apoptotic morphological changes in transfected cells compared to untreated and negative control-treated cells which appeared fewer effects.

Significant inhibition in a rate of viability of cells was found between PC3 transfected with different concentrations of mimic miR30a (P <0.01), for (48h) in comparison to untreated group and negative- control treated cells, figure (1), the highest percentage of PC3 cell viability was (83.716) in concentration (5nm) and the lowest percent of viability was (40.690 nm) in concentration (100 nm)

Vero cells transfected with miR30a showed no evident different in cell population in comparison to control and negative control group , so changes in cell viability between transfected ,control and negative control group didn't quite reach to significant (p>0.05) figure (1.B) ,the highest viability was (87.893) in concentration (5 nm), while the lowest was (56.080) in concentration (100 nm), The rat of the viability of cells was higher in transfected Vero cells than in transfected PC3 cells in different concentrations of mimic miR30a (p<0.01).

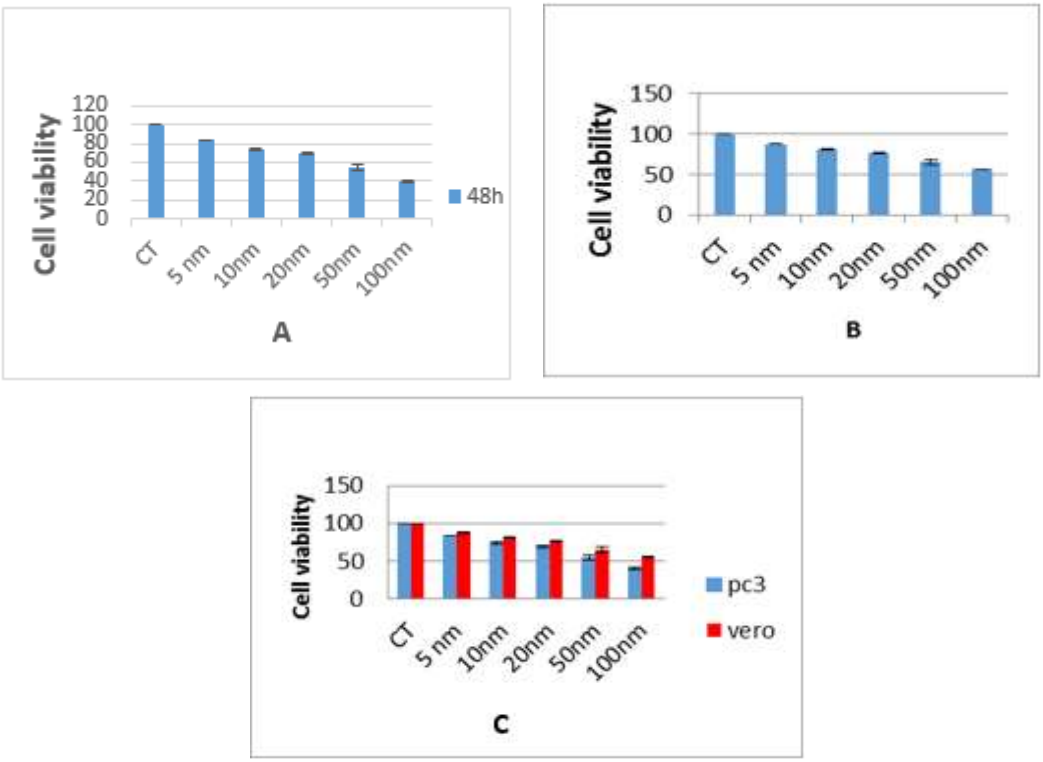


Figure (1) The viability of cells as determined by MTT assay ,after transfected with different concentration of miR30a for 48h A: PC3,B :Vero ,($p<0.01$) one way a nova ,C: comparison between transfected PC3 and Vero cell, , Two way Anova ($p<0.01$)

Acridine orange/ethidium bromide double staining

The morphological characteristics of cellular death in transfected cells were revealed by (AOEB) double staining, allowing a clear distinction between normal cells and those that have undergone apoptosis, intact cell membrane and healthy appearance in living cells , early apoptotic and late apoptotic .PC3 cells transfected with 50 nm&100 nm of miR30a were found to appear early apoptosis with a bright-green nucleus referred as chromatin condensation (white arrow), a secondary necrotic cell which can be noted as an intact nucleus which stained orange (blue arrowhead) and late apoptosis showed nuclear margination and cellular blebbing , whereas Vero cells transfected with the same concentration of miR30a had less apoptotic signs as shown in figure(2)

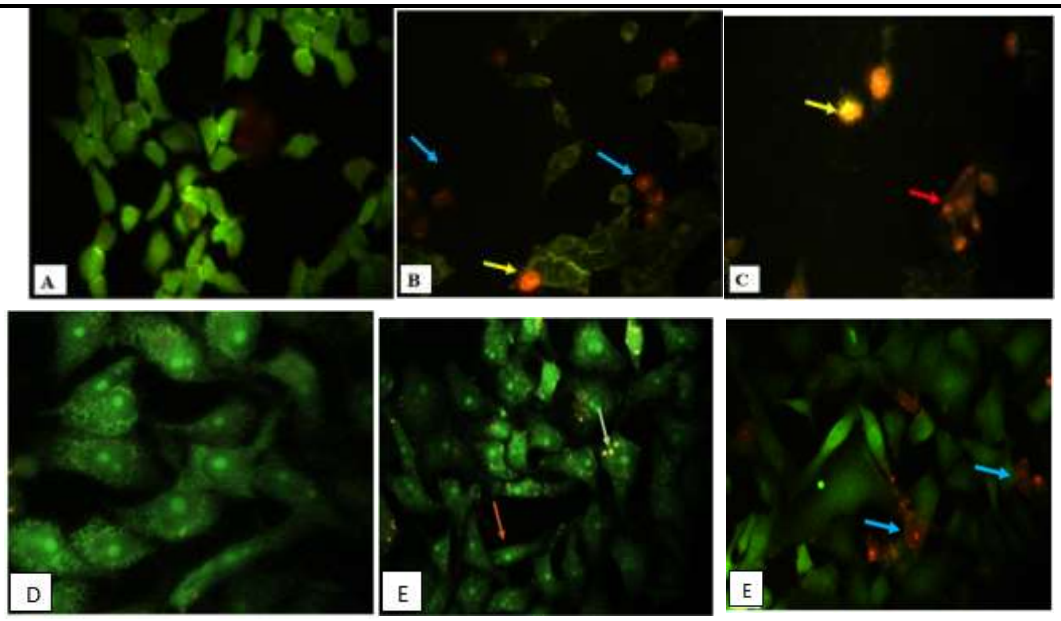
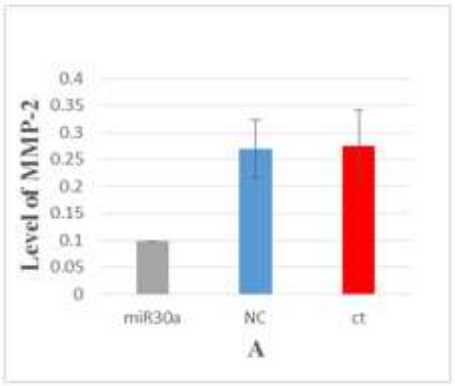


Figure (2) : Morphological features of apoptosis were demonstrated by (AO/EB) double staining with a fluorescent microscope in PC3 transfected with miR30a: ,(A)control group,(B) PC3 cells transfected with 50nm of miR30a ,and PC3 cells transfected with 100nm of miR30a , and transfected Vero cells: ,(D)control group,(E) Vero cells transfected with 50nm of miR30a ,and (F)Vero cells transfected with 100nm of miR30a, :(red arrow) represent apoptotic body,(yellow arrow) represent late apoptotic,(white arrow)represent early apoptotic ,(bule arrow) represent necrosis cell, ,(green arrow) represented early necrosis , (orang arrow) represented healthy cell,400x.

Regulation of MMP2

The expression level of MMP2 were decreased significantly (P<0.05)in PC3 transfected with miR30a for (**48** h) compared with the control in concentration dependent manner , while there was no differences in the level of MMP2 between control and negative groups treated cells., figure(3,A)The expression level of MMP-2 in PC3 cell transfected with miRNA 30a was decreased by (0.725826) fold, compared to untreated cell

Compared to control and negative control the expression level of MMP-2 was no significantly different (P> 0.05) in Vero cells transfected with miRNA 30a for (48h) , As showed in figure (3,B). It was decreased by (. 0.418350) in transfected Vero cell ,in compared to control cells, the level of MMP2 was higher in PC3 than in Vero cells .



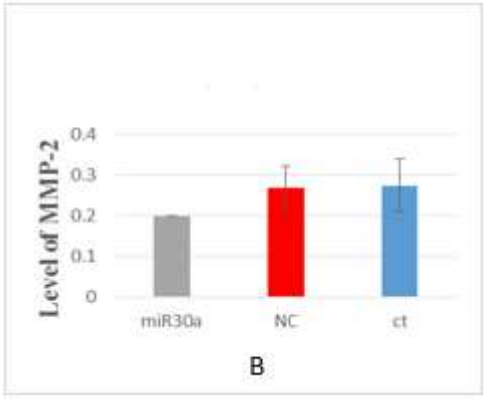


Figure (3): Regulation expression of MMP-2 in cells transfected with mimic miR30a for 48 h ,A:PC3 cells (P<0.05) ,B:Vero cells (P>0.05) as determined by un-paired t-test.

Regulation of MMP-9

Results showed Significant downregulate in the level of MMP-9 was noted in PC3 cells transfected with miR30a for (48) compared with the control and negative control group (P<0.05), and there was no difference in MMP9 level between negative control and control , figure (4) , this downregulation was more evident with increasing concentration of miR30a. It was reduced by (0.5309) fold in transfected cells incompared to control cells.

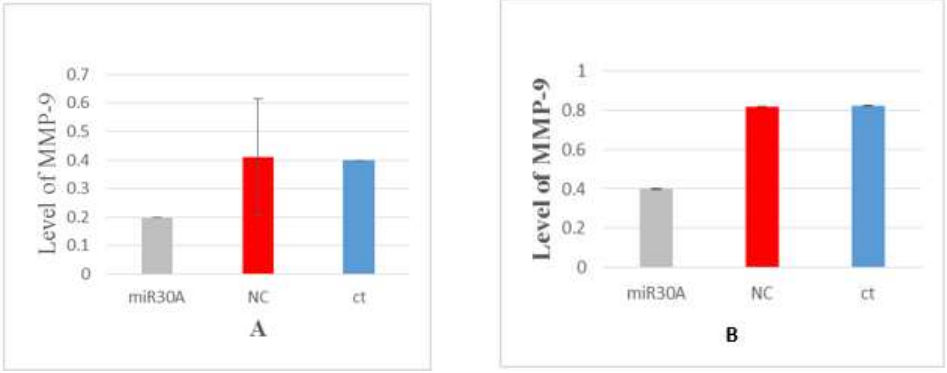


Figure (4) MMP-9 expression level cells transfected with mimic miRNA30a for 48h, A: PC3 cells(P<0.05) ,B:Vero cells (P>0.05) as determined by un-paired t-test.

No significant difference in the level of MMP-9 was observed between Vero cells transfected with miR30a for 48 h and control cells (P > 0.05), or between control and negative control group figure (3.B) .

The expression level of MMP-9 was decreased by(0.4952) fold in transfected Vero cells for (48h) compared to control cell .also The result indicated that the downregulation of MMP-9 was higher in in transfected PC3 than in the transfected Vero cells (P<0.05)

Activation of caspase8

The results showed that miR30a significantly reduced the level of caspase8 (P<0.05) in transfected PC3 compared to the control group ,concentration - dependent manner whereas there was no statistically differences between the control and negative control group, figure (5:A) , the relative expression level of caspase8 was increased by (12.27) fold in the transfected cell for (48h) compared to the control cell.

The result showed Significant different in expression level of caspas8 between Vero cell incubated with miR 30a for (48h) compared to the control group (P<0.05) , figure(5:B) , and no significant difference in caspase8 level between control and negative control group , it was increased by (6.825246) fold in transfected cell for (48h) when compared to untreated PC3cell. The expression level of caspase8 was higher significant in PC3 than in Vero cells

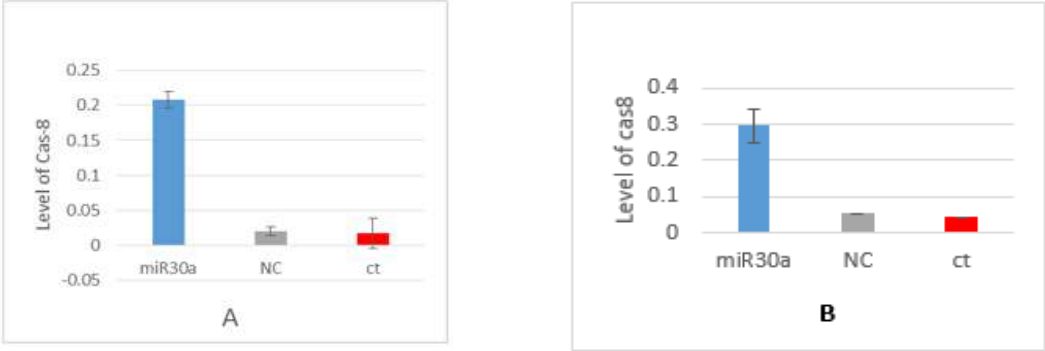


Figure (4) Expression level of Caspase8 in cells transfected with miR30a : A : PC3 B:Vero cells , the error bars display the standard deviation of mean, (P<0.05) as determined by un-paired t-test

Discussion

MiR30 family was frequently regulated and downregulated biological behavior's of tumor growth such as epithelial-mesenchymal transition, invasion and chemo-resistance, via targeting various downstream genes in several types of cancers(15).The current study revealed that the transfected PC3 cell line with miR-30a was resulted to reduce cell viability and induce apoptosis and necrotic features in concentrations- dependent manner so there was significant increase of Caspase 8 in experimental PC3 cells compared to control and negative control group, this results indicate a therapeutic potential of miR30a in prostate cancer growth in vivo ,this result resemble with (16) reported that prostate cancer cells transfected with miR30a revealed a higher rate of apoptosis and reduced invasion and migration of cells, and overexpression of miR30a-3p induced higher rates of cells death (5) also referred to that the expression level of miR30a-5p was decreased in PCa and this downregulation promotes the progression of PCa.

In other types of cancer, previous studies found In vitro and in vivo, miR-30a suppressed lung cancer cell proliferation, invasion, and promoted apoptosis(17) , (18) discovered that miR30a acts as a tumor inhibitor in a different of solid tumors ,and (19)indicated that miR30a-3p inhibits liver cancer cell proliferation by mediates signaling pathway of cell apoptosis, (19) observed that cell growth of hepatocellular carcinoma cells was suppressed by miR30a-3p primarily by inducing cell apoptosis, and ,(20) revealed that anticancer activity against MCF7 and SKG cell lines caused inhibition of viability and increased cell death.

(21) mentioned to Intra tumoral injection of mimic miR 29b inhibited growth of prostate cancer significantly in nude of mice ,Subsequent study revealed that the high expression of miR29b decrease proliferation of prostate cancer cell PC3 in a time- dependent manner and promote death of cell .(22) reported that miR-143 induced activation of Caspas3, thus inducing death in osteosarcoma cells .

(23) referred to that levels of Caspase 3 were increased in cells transfected with miRNAs mimics significantly, Consistently, caspase-8 levels were significantly increased upon transfections of miR 221-3p and miR125b-5p ,however, the level of caspase 9 was not markedly changed when transfections with miR221-3p , miR-125b-5p, and miR-145-5p mimic

The results showed the gene expression level of MMP2 &MMP9 in prostate cancer (PC3) cell line transfected with miR30a were remarkably decreased (P<0.05)in concentration dependent manner when compared with control and negative control group. (24) also noted that the protein expression levels of MMP2 and MMP9 were significantly reduced in esophageal carcinoma cells (EC109 and EC9706) transfected with miR30a-3p mimics when compared to the control . Decrease levels of miR30a were shown in Prostate Cancer cells, such as (DU145, C4-2, 22Rv1,and PC3), which reported that high growth and invasive phenotype , associated with increased expression levels of MMP2 and MMP9, and this character induces cell growth and invasion of PCa, this action was inverted

when the levels of miR30a were revived in DU145 and PC3 cells (25) .

The expression of MMP2 and MMP9 were suppressed by upregulated level of miR30a-3p, induced cell apoptosis, as indicated by upregulation of Caspase3 expressions, so hypothesize that miR30a-3p has an inhibitory role on invasion and migration of MHCC-97H cell by modulating pathway of some genes such as the MMP2/9 (19).

The results indicated that miR30a had good cytotoxicity against PC-3 cells, but was less sensitive to normal cells ,so there was no significant different in cell viability, less influence on their appearance, in addition there was low significant differences in the expression levels of caspases , MMP2 and MMP9 , between treated and untreated vero cells.

The high sensitivity of malignant cells may indicate the differences in rates of growth between normal and malignant cells, the differences in sensitivity, its main ingredients in malignant and normal cells, may result to the presence of distinct cell surface receptors, differences in the uptake of some drugs and ,intracellular retention transport, (27).

Refrences

1. 1-Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. CA Cancer J Clin 2019;69:7-34.
2. 2- Si, W., Shen, J., Zheng, H., & Fan, W. (2019). The role and mechanisms of action of microRNAs in cancer drug resistance. *Clinical epigenetics*, *11*(1), 1-24.
3. 3- Vanacore, D., Boccellino, M., Rossetti, S., Cavaliere, C., D'Aniello, C., Di Franco, R., ... & Facchini, G. (2017). Micrornas in prostate cancer: an overview. *Oncotarget*, *8*(30), 50240.
4. 4- Liu, M. M., Li, Z., Han, X. D., Shi, J. H., Tu, D. Y., Song, W., ... & Zhen, L. L. (2017). MiR-30e inhibits tumor growth and chemoresistance via targeting IRS1 in Breast Cancer. *Scientific reports*, *7*(1), 1-10.
5. 5- Zhao, H., Zhang, W., Lai, X., Zhu, H., Zhang, S., Wu, W., ... & Tan, J. (2019). MiR-30a-5p frequently downregulated in prostate cancer inhibits cell proliferation via targeting PCLAF. *Artificial cells, nanomedicine, and biotechnology*, *47*(1), 278-289.
6. 6- Ali, S. F. (2021). Role Of Let-7ain Breast Tumor Proliferation By Target Of PIK3CA And AKT1 Expression. NVEO-NATURAL VOLATILES & ESSENTIAL OILS Journal| NVEO, 4574-4580.
7. 7- Jarrah ,R.H., Suker,D.K , Khairallah A S, Mohammed,M K and Abood,R.A.(2019)., DETERMINATION OF SERUM MATRIX METALLOPROTEINASE 2 AND 9 (MMP-2) (MMP-9) IN BREAST CANCER INVASION AND METASTASIS.Inter.J of Adv. Res. 7(1):806-810.
8. 8- Ameen, A. A., Suker, D. K., & Abood, R. (2021). Assessment Of Matrix Metalloproteinase-2 &Tissue Inhibitor-1 In Human Prostate Cancer. NVEO-NATURAL VOLATILES & ESSENTIAL OILS Journal| NVEO, 13165-13177.
9. 9- Ameen, A. A. J. (2021). The impact of ZnO Nanoparticles on the liver and lung tissues in mice. NVEO-NATURAL VOLATILES & ESSENTIAL OILS Journal| NVEO, 8953-8960.
10. 10 -Mahmood, N. A., Abdulghany, Z. S., & Al-Sudani, I. M. (2019). The Expression Levels of Matrix Metalloproteinases Inhibitors TIMP1, TIMP2 and Matrix Metalloproteinases MMP2, MMP9 in Iraqi Patients Diagnosed with Colon Cancer. Govaresh, 24(3), 172-178.
11. 11- Li, L., & Li, H. (2013). Role of microRNA-mediated MMP regulation in the treatment and diagnosis of malignant tumors. *Cancer biology & therapy*, *14*(9), 796-805.
12. 12- Abba, M., Patil, N., & Allgayer, H. (2014). MicroRNAs in the Regulation of MMPs and Metastasis. *Cancers*, *6*(2), 625-645.
13. 13- McCarthy, N. (1993). Apoptosis induced by cancer chemotherapeutic drugs and its genetic suppression. PhD. Thesis, Faculty of Medicine and Dentistry of University of Birmingham.

14. 14- Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *methods*, *25*(4), 402-408.
15. 15- Fan, M. J., Zhong, Y. H., Shen, W., Yuan, K. F., Zhao, G. H., Zhang, Y., & Wang, S. K. (2017). MiR-30 suppresses lung cancer cell 95D epithelial mesenchymal transition and invasion through targeted regulating Snail. *Eur Rev Med Pharmacol Sci*, *21*(11), 2642-2649.
16. 16 -Zhang, L., Zhang, X. W., Liu, C. H., Lu, K., Huang, Y. Q., Wang, Y. D., ... & Xu, B. (2016). miRNA-30a functions as a tumor suppressor by downregulating cyclin E2 expression in castration-resistant prostate cancer. *Molecular medicine reports*, *14*(3), 2077-2084.
17. 17- Guan, Y., Rao, Z., & Chen, C. (2018). miR-30a suppresses lung cancer progression by targeting SIRT1. *Oncotarget*, *9*(4), 4924.
18. 18- Li, X., Jiao, M., Hu, J., Qi, M., Zhang, J., Zhao, M., ... & Han, B. (2020). miR-30a inhibits androgen-independent growth of prostate cancer via targeting MYBL2, FOXD1, and SOX4. *The Prostate*, *80*(9), 674-686.
19. 19- Chen, Q., Gao, Y., Yu, Q., Tang, F., Zhao, P. W., Luo, S. K., ... & Mei, H. (2020). miR-30a-3p inhibits the proliferation of liver cancer cells by targeting DNMT3a through the PI3K/AKT signaling pathway. *Oncology letters*, *19*(1), 606-614.
20. 20- AlRazn, R. S., & AbdulHussein, Z. R. (2021). Biological Effect of Rhamnolipid as Antioxidant and Anticancer Agent. Annals of the Romanian Society for Cell Biology, 25(6), 1744-1758.
21. 21- Yang, X., Sun, J., Sun, H., Wen, B., Zhang, M., An, H., ... & He, S. (2021). MicroRNA-30a-3p acts as a tumor suppressor in MHCC-97H hepatocellular carcinoma cells by targeting COX-2. Journal of Cancer, 12(13), 3945.
22. 22-Sur, S., Steele, R., Shi, X., & Ray, R. B. (2019). miRNA-29b inhibits prostate tumor growth and induces apoptosis by increasing bim expression. *Cells*, *8*(11), 1455.
23. 23- Li, W. H., Wu, H. J., Li, Y. X., Pan, H. G., Meng, T., & Wang, X. (2016). MicroRNA-143 promotes apoptosis of osteosarcoma cells by caspase-3 activation via targeting Bcl-2. Biomedicine & pharmacotherapy, 80, 8-15.
24. 24_ Karadag, A., Ozen, A., Ozkurt, M., Can, C., Bozgeyik, I., Kabadere, S., & Uyar, R. (2021). Identification of miRNA signatures and their therapeutic potentials in prostate cancer. Molecular Biology Reports, 48(7), 5531-5539.
25. 25- Fan, Y., Bian, X., Qian, P., Wen, J., Yan, P., Luo, Y., ... & Zhang, Q. (2019). miRNA-30a-3p inhibits metastasis and enhances radiosensitivity in esophageal carcinoma by targeting insulin-like growth factor 1 receptor. *Molecular medicine reports*, *20*(1), 81-94.
26. 26- Zhu, Q., Li, H., Li, Y., & Jiang, L. (2017). MicroRNA-30a functions as tumor suppressor and inhibits the proliferation and invasion of prostate cancer cells by down-regulation of SIX1. *Human cell*, *30*(4), 290-299.
27. 27- Samarghandian, S., & Shabestari, M. M. (2013). DNA fragmentation and apoptosis induced by safranal in human prostate cancer cell line. *Indian journal of urology: IJU: journal of the Urological Society of India*, *29*(3), 177.