The Anti-Proliferative Activity of D-Mannoheptulose Against Breast Cancer Cell Line Through Glycolysis Inhibition

Ahmed Ghdhban Al-Ziaydi[1,a)] Mohammed I. Hamzah[2,b)] Ahmed Majeed Al-Shammari[3, c)] Haider Sabah Kadhim[4,d)] and Majid Sakhi Jabir [5,e)]

[(1)] *Department of Medical Chemistry, College of Medicine, University of Al-Qadisiyah, Iraq*

[(2)] *College of Medicine, University of Al-Nahrain, Iraq*

[(3)] *Iraqi Center of Cancer and Medical Genetics Research, Mustansiriyah University, Iraq*

[(4)] *Department of Microbiology, College of Medicine, Al-Nahrain University, Iraq*

[(5)] *Department of Applied Sciences, Technology University, Iraq*

[a)] ahmed.alziaydi@qu.edu.iq
[b)] moh_alsafi75@colmed-alnahrain.edu.iq
[c)] Corresponding author: ahmed.alshammari@iccmgr.org
[d)] haider_kadhim@yahoo.com
[e)] msj_iraq@yahoo.com

Abstract. Cancer cells are mainly dependent on glycolysis to generate Adenosine Triphosphate (ATP) and intermediates required for cell growth and proliferation. Inhibition of glycolysis might be of therapeutic value in antitumor treatment. We investigated the effects of D-mannoheptulose (MH) as the inhibitor of hexokinase on glycolysis metabolism and apoptosis to prevent the proliferation and inhibit the growth of breast cancer cells (AMJ13 and MCF7) in vitro. Cancer cells were compared with a normal embryo fibroblast (REF) cell line via MTT cytotoxicity assay to determine the IC50 of different MH concentrations (13.125 - 1680 µg/ml) and treated with specific concentrations (250, 125, and 62.5 µg/ml) after incubation for 72h at 37 °C. Hexokinase activity, pyruvate, ATP concentration, and acidity were measured in treated and untreated breast cancer and normal cells after treatment with 62.5 µg /ml MH for 72 h at 37 °C. Our findings showed that MH inhibited the proliferation of breast cancer cell lines and induced killing by increasing cytotoxicity and apoptosis and decreasing hexokinase activity, pyruvate concentration, ATP, and acidity compared with the normal cell line. In addition, MH had low toxicity against normal cells. In conclusion, our results revealed that MH inhibits the glycolysis pathway by inhibiting hexokinase in breast cancer cells and their proliferation. Therefore, MH can be considered a promising treatment for breast cancer.

INTRODUCTION

Breast cancer is the most common cancer in women and the fifth leading cause of cancer deaths worldwide [1]. Breast cancer represents approximately 14.3% of all malignant diseases and one-third of registered cancers in females in Iraq. This increment in the incidence rate of breast cancer may be due to exposure to environmental pollution and many other factors such as life style, chemicals, and radioactive pollution, resulting from the use of depleted uranium during the Gulf War 1 and 2.[2]

Technologies and Materials for Renewable Energy, Environment and Sustainability
AIP Conf. Proc. 2307, 020023-1–020023-11; https://doi.org/10.1063/5.0032958
Published by AIP Publishing. 978-0-7354-4043-2/$30.00

Increased glycolysis is the main source of energy in cancer cells that use this metabolic pathway for ATP generation [3]. The breakdown of glucose via glycolysis yields two molecules of pyruvate and two net molecules of ATP [4]. Lactate dehydrogenase (LDH) converts lactate back into pyruvate to support citric acid cycle flux and gluconeogenesis. This entire process is called the Cori cycle [5]. Genetic disorders affect ATP generation in the mitochondria. Cancer cells mainly produce energy by an increased rate of glycolysis (200 times more than normal tissues of origin), followed by fermentation of lactate in the cytosol of the cell, even if oxygen is abundant [6]. Relative to normal cells, cancer cells are highly proliferative and thus require increased ATP to meet their metabolic demand [7]. The Warburg effect with aerobic glycolysis efficiently produces ATP and promotes cell proliferation by reprogramming metabolism to increase glucose uptake and stimulate lactate production [8]. Malignant cells are characterized by a high energy demand. Based on the "Warburg effect", tumor cells preferentially utilize glycolysis for glucose-dependent ATP generation [9].

Glucose enters the cell and generates pyruvate after a series of enzymatic reactions. Pyruvate then enters the mitochondria and splits into CO2 and water through the formation of ATP [10]. Under anaerobic conditions, pyruvate is fermented and converted into lactate by a reaction catalyzed by LDH. However, in cancer cells, pyruvate is preferentially converted into lactate even in the presence of oxygen [11; 12]. 3-Bromopyruvate and sodium citrate can inhibit glycolytic flux in human gastric cancer cell line MGC-803 by suppressing the activity of glycolytic key enzymes, resulting in ATP depletion and decreased lactate generation; these phenomena lead to insufficient energy to support mitosis, proliferation, and invasion of cancer cells [13]. The growth and proliferation of cancer cells are disrupted by the decreasing production of lactate [14]. Apoptosis or programmed cell death is an important control mechanism following DNA damage [15]. Reduced apoptosis or its resistance can modulate cancer cell growth [16]. Current strategies for cancer therapy are focused on apoptosis as a desirable target of cellular control in cancer [17]. Depletion of ATP by glycolytic inhibition potently induces apoptosis of multidrug-resistant cells [18]. Starvation induces apoptosis of breast cancer cells [19].This study aimed to induce breast cancer cell death via glycolysis inhibition and induction of apoptosis.

MATERIALS AND METHODS

Cell line and cell culture

Human breast cancer lines (MCF7 and AMJ13) and REF cell line were provided by the Cell Bank Unit of the Experimental Therapy Department of Iraqi Center of Cancer and Medical Genetics Research. These cell lines were cultured in RPMI-1640 medium (US Biological, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin–streptomycin (both from Capricorn-Scientific, Germany) and incubated in a humidified atmosphere with 5% CO2 at 37 °C. Exponentially growing cells were used for the experiments. Morphological changes in MCF7 and AMJ13 cells were observed using inverted and fluorescent microscopes [20].

MTT cytotoxicity assay

This study used two human breast cancer lines (AMJ13 and MCF7) and normal REF cell line. For cell culture, 1×10^4 cells were seeded into a 96-well microplate and incubated at 37 ℃ for 72 h until a monolayer was achieved as observed by inverted microscope. Cytotoxicity was investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were exposed to different diluted concentrations of MH (13.125, 26.25, 52.5, 105, 210, 840, and 1680 μg/ml) to determine IC50 then treated with specific concentrations of D- Mannoheptulose (Santa Cruz, USA) (250, 125, and 62.5 μg/ml). The cells were incubated at 37 °C for 72 h. Each well was added with 50 μl of each serum-free medium and MTT dye solution (2 mg/ml) and incubated for 3 h. Dimethyl sulfoxide (100 μl) was added into each well. The plate was wrapped with Parafilm, shaken, and incubated for 15 min. Optical density (OD) values of treated and untreated cells were measured at 492 nm by using an enzyme-linked immunosorbent assay plate reader [21].

Hexokinase assay

Hexokinase (HK) assay was conducted through colorimetric method using a hexokinase assay kit (ElabScience, USA). Treated and untreated cells samples were prepared through cell lysis. Spectrophotometer was preheated for 30 min, and the wavelength was adjusted to 340 nm and set to zero with distilled water. The prepared working solution was incubated at 37 °C for10 min before detection. The cells sample (50 µl) and working solutions (960 µl) from hexokinase assay kit were mixed, and the time was recorded immediately. The samples were incubated at 37 °C, and absorbance was recorded at 340 nm according to the manufacturer's recommendation.

Pyruvate assay

Pyruvate assay was conducted through colorimetric method using pyruvate assay kit (ElabScience, USA). Treated and untreated cells and blank tubes were used. Double-distilled water (0.1 ml) and Reagent 2 (0.5 ml) were added. In the standard tube, 0.1 ml of 0.2 µmol/ml sodium pyruvate standard solution and 0.5 ml of Reagent 2 were added. In the sample tube, 0.1 ml of sample and 0.5 ml of Reagent 2 were added. All tubes were mixed fully in a vortex mixer for 5 s and incubated at 37 °C for 10 min. Reagent 3 (2.5 ml) was added into each tube, mixed fully in a vortex mixer for 5 s, and incubated at RT for 5 min. The device was set to zero with double-distilled water. The OD value of each tube was measured at 505 nm in a 1 cm cuvette (according to manufacturer's recommendation).

ATP assay

ATP assay was conducted through colorimetric method using an ATP assay kit according to manufacturer's recommendation (ElabScience, USA). Treated and untreated cells were collected and centrifuged at 1000–1500 r/min for 10 min. The supernatant was removed, and cell sediment (about 10^6 cells/ml) was kept. Boiled double-distilled water (0.3–0.5 ml) was added to prepare cell suspension. The cell suspension was placed in a boiling water bath for 10 min, blended, and extracted for 1 min. The samples were centrifuged at 3500 r/min for 10 min, and the supernatant was taken for detection. Each tube was mixed and immersed in a water bath for 30 min at 37°C. The supernatant was obtained to measure OD at 636 nm wavelength.

pH measurement

Cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS and 1% (v/v) penicillin–streptomycin and incubated overnight in a humidified atmosphere with 5% CO2 at 37°C. Exponentially growing cells were used in the experiments. Cancer cells (1×10^4) were seeded in a 96-well microplate and incubated at 37 °C for 24-48 h until a monolayer was achieved as observed using inverted microscope then the media was discarded. Cells were exposed to MH (62.5 µg/ml) and incubated at 37 °C for 72 h. Media was discarded, PBS 200 µl was added to each well. Cells was detached by pipetting and was collected into centrifuge tube, then centrifuge at 3200 rpm for 5 min at 4°C. Supernatant was discarded and 1 ml ice -cold cell lysis buffer was added and mix well. Incubation ice for 40 min with vortex for (4-6) time during incubation ice. Cell suspension was transferred to centrifuge tube then speed centrifuge for 20 min at 4°C. Supernatant (cell suspension) was collected and saved in eppendorf tubes in deep freeze. Remove the eppendorf tubes containing the frozen cells from deep freeze and immediately place them into a 37°C water bath. Quickly was thawed the cells (< 1 minute) by gently swirling the eppendorf tube in the 37°C water bath until there is just a small bit of ice left in it. Drops of cell suspension were added to litmus paper to determine pH values which express acidity for breast and normal cell lines with comparison to the control [20].

Statistical analysis

All results were presented as standard deviation (SD) and mean ± standard error of the mean (SEM). Two-tailed t-test was conducted using Excel version 10, GraphPad prism version 7 (USA), and Isobologram version 1 to determine differences between groups under different conditions. The level of significance was set at $P<0.05$.

RESULTS

Cytotoxicity ratio (CT%) of MH on normal and breast cancer cell lines

The cytotoxicity levels of different diluted concentrations of MH (13.125, 26.25, 52.5, 105, 210, 840, and 1680 µg/ml) on normal (REF) and breast cancer cell lines (AMJI3 and MCF7) were evaluated by MTT assay. The percentages of cytotoxicity (CT%) of MH in REF were 1.67%, 3.68%, 6.15%, 8.78%, 10.63%, 14.79%, 18.58%, and 24.72% (Figure 1A). The CT% values of MH for AMJ13 were 27.29%, 31.96%, 35.78%, 40.4%, 44.15%, 47.61%, 50.82%, and 58.64% (Figure 1B). The CT% values of MH in MCF7 were 26.26%, 29.71%, 32.44%, 41.56%, 46.16%, 51.86%, 52.65%, and 60% (Figure 1C). The IC50 values of MH were 486.9 in REF, 124.7 in AMJ13, and 122.6 in MCF7 (Figures 1D–F).

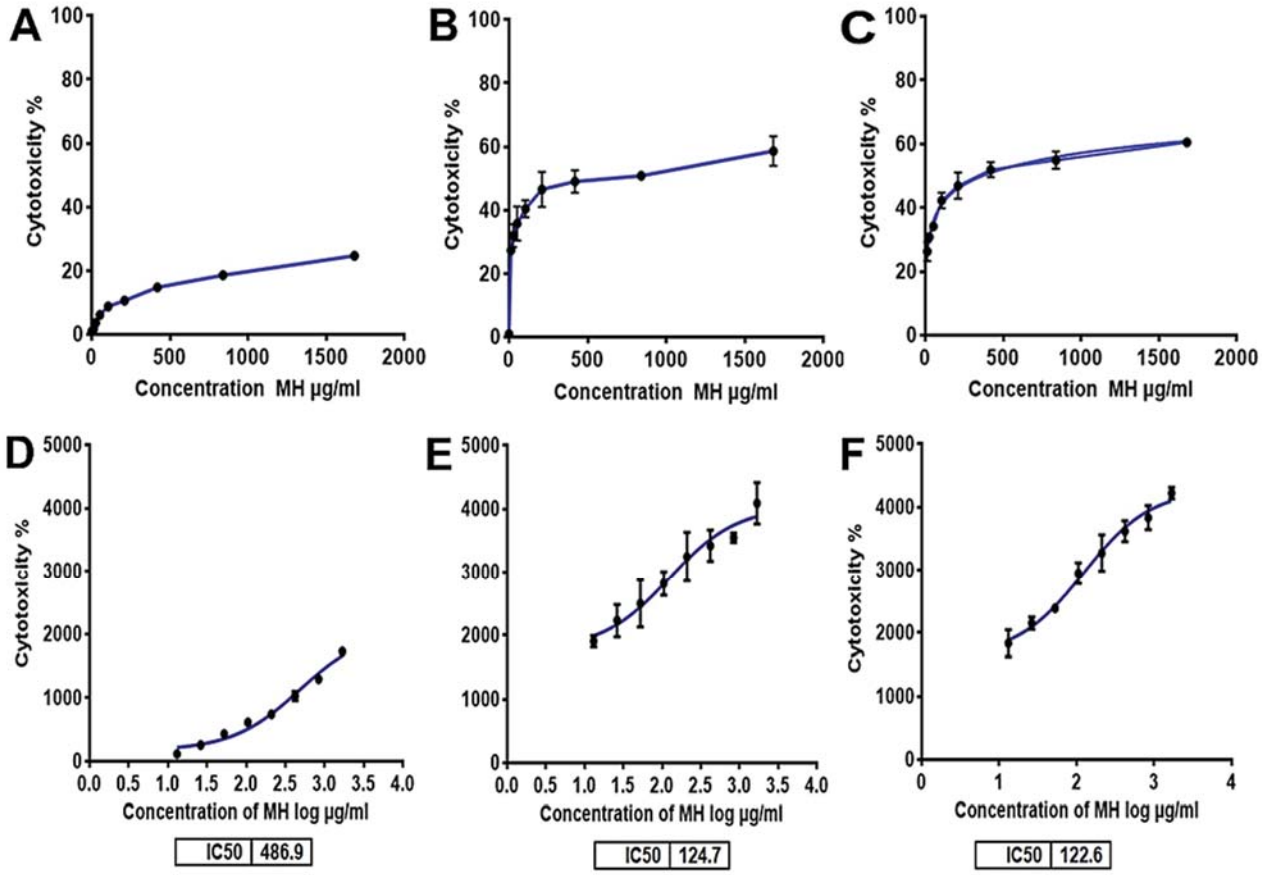

FIGURE 1. Cytotoxicity (%) of different concentrations of D-mannoheptulose (MH) to A- REF, B- AMJ13, and C- MCF7 cells. IC50 of MH on D- REF, E- AMJ13, and F- MCF7 cells as computed by the GraphPad Prism program.

To evaluate the effect of MH on cell proliferation, we investigated the cytotoxicity of REF, AMJ13, and MCF7 cell lines after MH treatment by using the MTT cytotoxicity assay with the specific of concentrations of MH based on IC50 (250, 125, and 62.5µg/ml) and incubation at 37 °C for 72 hr. The CT% of the three concentrations was 12.60%, 9.38%, and 7.10% for REF (Figure 2-A); 56.86%, 49.75%, and 42.58% for AMJ13 (Figure 2-B); and 52.82%, 46.30%, and 39.71% for MCF7 (Figure 2-C). Cytotoxicity% of the AMJ13 and MCF7 cell lines increased directly when the inhibitor (MH) dose was increased (Figure 2-B and C). The cytotoxicity assay revealed that the effects of MH on the breast cancer cell lines have a significant difference when compared with the normal REF cell line. The CT% in the

REF cell line was reduced after MH treatment relative to that after the breast cancer cell lines treatment (Figure 2) and (Figure 3-B). The inhibitor induced the cytotoxicity ratio significantly in the breast cancer cells when compared with normal REF cell line. In the present study was identified a significant elevation in the cytotoxicity in the breast cancer cells but not in the normal cells (Figure 3-B). In REF cell line, the IC50 (Figure 3A) increased and CT% (Figure 3B) decreased compared with those in cancer cell lines. A significant difference in IC50 after treated with MH in the breast cancer cell lines compared with the normal REF cell line (Figure 3-A).

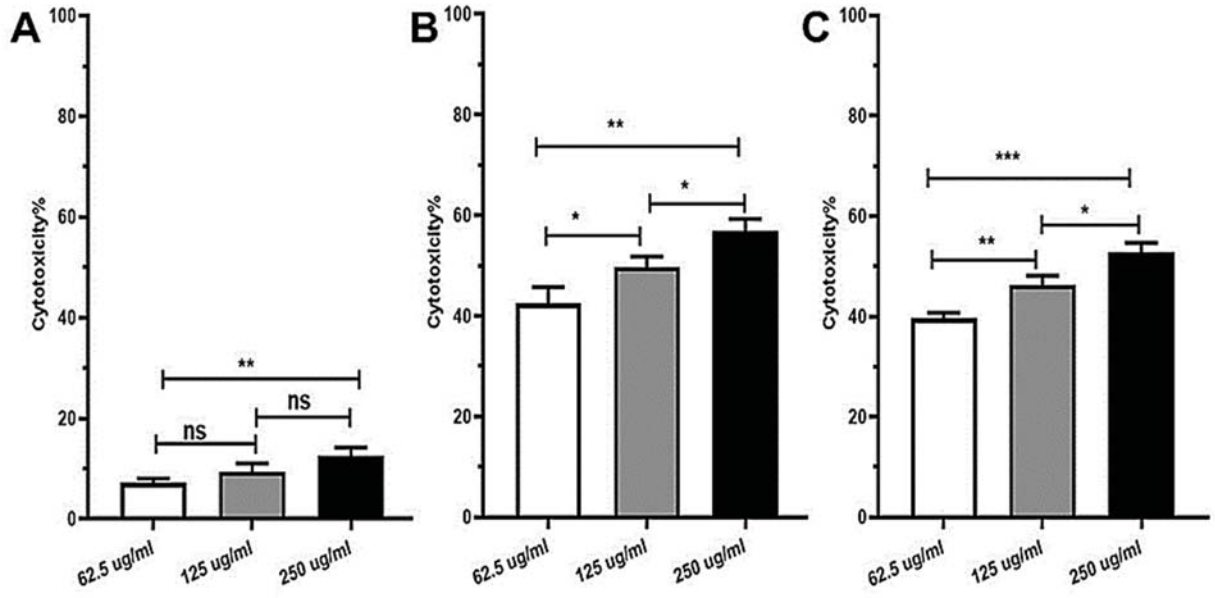

FIGURE 2. Cytotoxicity percentage of three concentrations of MH (250, 125, and 62.5 µg/ml) against normal REF and breast cancer MCF7 and AMJ13 cells. The concentration of 250 µg/ml induced the best effect. A- REF, B- AMJ13, and C- MCF7. The values represent the mean ± SD *P<0.05, **P<0.01, ***P<0.001

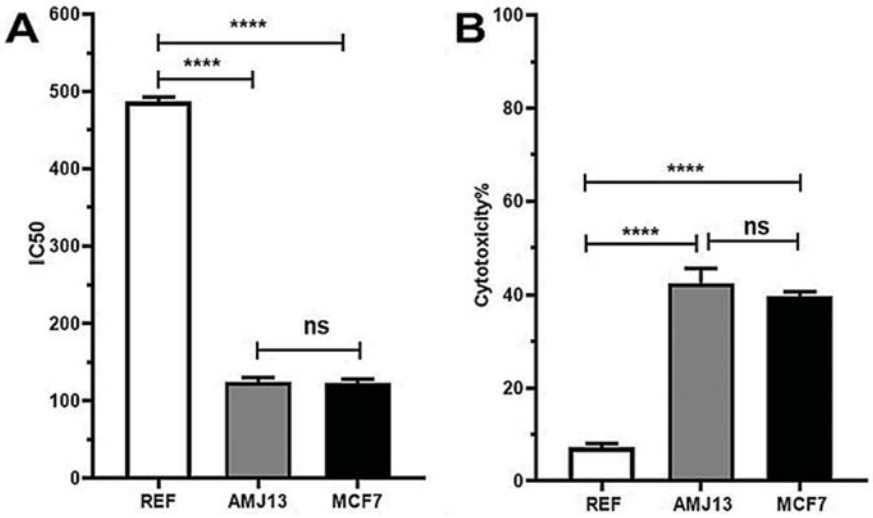

FIGURE 3. Comparison between REF, AMJ13, and MCF7 cells after treatment with MH for 72 h; A- IC50 and B- CT% of MH (62.5 µg/ml)

Glycolysis inhibition and results of HK, pyruvate, and ATP assays

HK assay

The HK enzyme was evaluated for the comparison of treated and untreated cells at 72 hr. MH induced hexokinase inhibition, thus HK activity was decreased (Figure 4). The HK activity slightly decreased in REF cells after treatment with MH compared with control. The effect of the inhibitor and control on REF cells had a non-significant difference (Figure 4-A). The HK activity in AMJ13 and MCF7 cell lines decreased after treatment with 62.5 µg/ml MH for 72 h due to the inhibition of HK enzyme compared with that in the control. MH inhibited the HK activity significantly in the breast cancer cells when compared with control. In the present study was identified a significant reduction in the HK enzyme activity in the breast cancer cells but not in the normal cells (Figure 4).

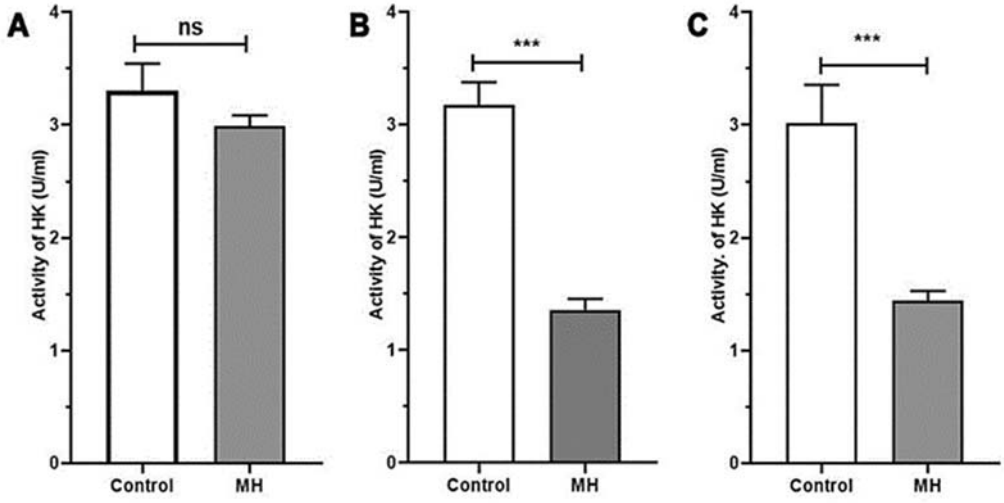

FIGURE 4. Activity of HK (U/ml) in REF, AMJ13, and MCF7 cells exposed to MH compared with controls. The values represent mean ± SD **P<0.01, ***P<0.001

Pyruvate assay

The pyruvate concentration was assessed for the comparison of treated and untreated cells at 72 hr. MH induced glycolysis pathway products inhibition, thus pyruvate concentration was decreased (Figure 5). The pyruvate concentration slightly reduced in REF cells after treatment with MH compared with control. The effect of the MH and control on REF cells had an insignificant difference (Figure 5-A). The pyruvate concentration in breast cancer cell lines decreased after treated with 62.5 µg/ml MH for 72 h due to the inhibition of the HK enzyme by MH compared with that in the control, thus led to the inhibition of glycolysis (Figure 5-B, C). MH inhibited the pyruvate significantly in the breast cancer cells when compared with control. In the present study was noticed a significant reduction in the pyruvate concentration in the breast cancer cells but not in the normal cells (Figure 5).

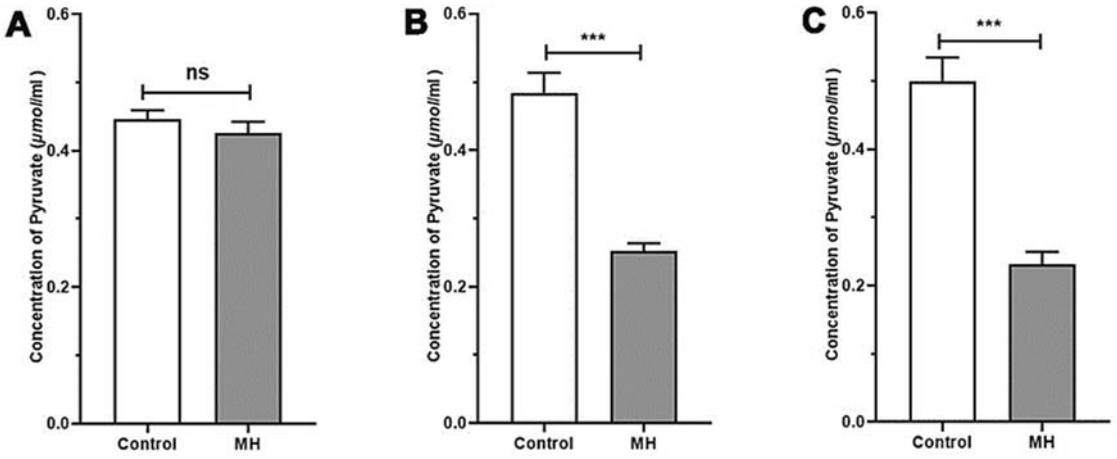

FIGURE 5. Pyruvate concentration (in µmol/ml) in A- REF, B- AMJ13, and C- MCF7 cells treated with MH compared with controls. The values represent the mean ± SD **P<0.01, ***P<0.001

ATP assay

The effect of MH on ATP production was assessed in treated and untreated cells at 72 hr. MH induced inhibition of HK enzyme, pyruvate, thus ATP concentration was decreased (Figure 6). The ATP concentration slightly deficiency in REF cells after treatment with 62.5 µg/ml MH compared with control. The effect of the MH and control on normal REF cells had an insignificant difference (Figure 6-B). The ATP levels decreased in all MH-treated breast cancer cells relative to controls, due to the inhibition of the glycolysis by MH (Figure 6-C, D). MH inhibited the ATP concentration significantly in the breast cancer cells when compared with control. Normalization measurement of ATP had a significant difference between REF and (AMJ13, MCF7) cell lines and a nonsignificant between AMJ13 and MCF7 cells after treated with MH Figure 6-A). The study noticed a significant difference in the ATP concentration in the breast cancer cells but not in the normal cells (Figure 6).

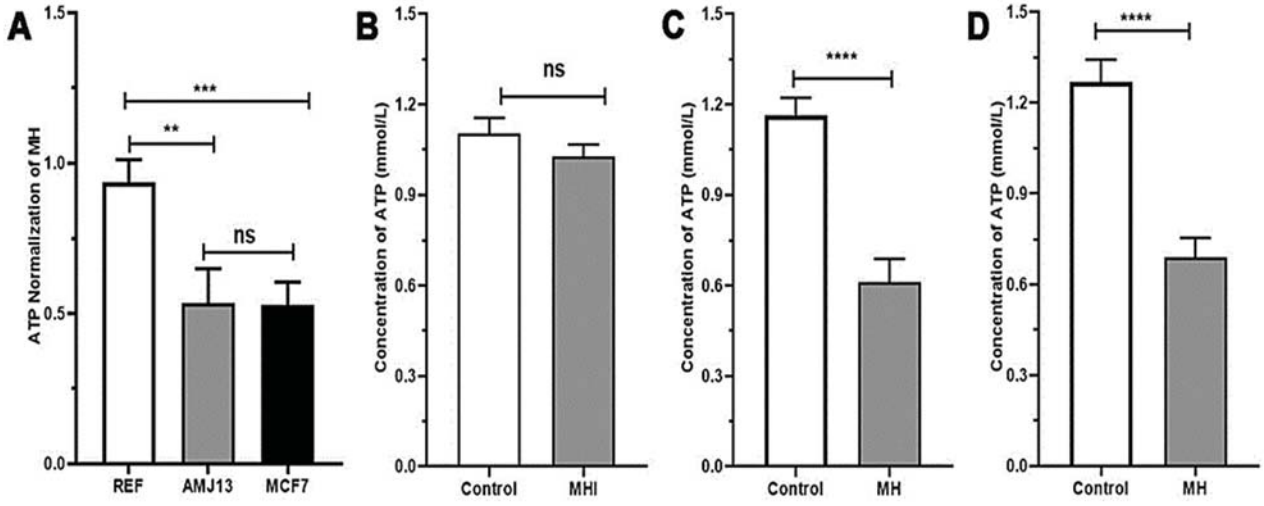

FIGURE 6. ATP concentration (in mmol/L) in REF, AMJ13, and MCF7 cells exposed to MH compared with controls. A- Normalization of cell lines, B- REF, C-AMJ13, and D-MCF7. The values represent mean ± SD **P<0.01, ***P<0.001

pH measurements (acidity)

The effect of MH on the acidity (pH measurement) was assessed in treated and untreated cells at 72 hr. The pH of the three cell lines (REF, AMJ13 and MCF7) increased after treatment with 62.5 µg/ml MH for 72 h compared with the control. This finding could be due to the inhibition of acidity (represent lactic acid) because of reduced lactate concentration compared with the control. The MH inhibited the acidity significantly in the breast cancer cell lines (Figure 7-C, D) but was an insignificantly in REF cell line (Figure 7-B) when compared with control. Normalization measurement of acidity had a significant difference between REF and breast cancer cell lines and a nonsignificant difference between MH-treated AMJ13 and MCF7 cell lines (Figure 7-A). MH promoted proliferation inhibition and reduced the level of acidity in both breast cancer cell lines but not in normal REF cell line. In the current study was detected a significant difference in the acidity in the AMJ13 and MCF7 cells but not in the normal REF cells (Figure 7).

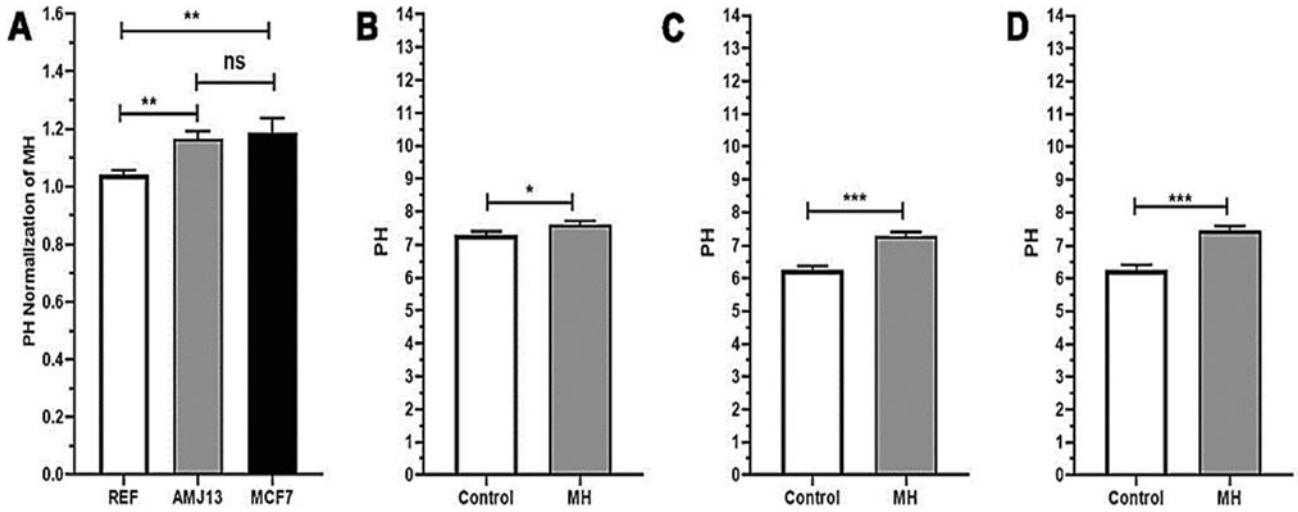

FIGURE 7. Effect of MH on the acidity of cell lines detected through pH measurements. (A) Normalization of MH–cell lines and (B) MH–REF, (C) MH–AMJ13, and (D) MH–MCF7 cell lines in comparison with controls.

DISCUSSION

The in vitro results of this study revealed that increased concentrations of MH led to elevated cytotoxicity and increased antiproliferation effect on breast cancer cell lines due to inhibited glycolysis. This effect on the glycolysis pathway can reduce the concentrations of HK, pyruvate, and ATP and increase killing in AMJ13 and MCF7 cells. Cytotoxicity was reduced depending on the concentration gradient of MH for treatment of REF cells. The cytotoxicity of MH to normal REF cells slightly increased compared with that to cancer cells. The difference in the cytotoxicity of MH to normal and cancer cells may be attributed to differences in receptors, cell environment, and pH. The effect of MH on the inhibition of cell growth was less on normal REF cells compared with AMJ13 and MCF7 cells. Our results also support the findings [22], in which MH increased the glycolysis of tumor cells in comparison with normal cells. Our results revealed that MH has a powerful effect on inducing the killing of breast cancer cells by decreasing the levels of HK, pyruvate, ATP and the acidity. Our results are consistent with those reported by [23] and [24].

Hence, the inhibition of glycolysis for specific inhibitors, namely, 3-bromopyruvate and 2-deoxyglucose, resulted in mitochondrial pathway-induced apoptosis. Furthermore, the overexpression of LDH-B by panepoxydone inhibitor in cancer cell lines led to enhanced apoptosis and mitochondrial damage [25]. Morphological changes in AMJ13 and MCF7 cells treated with MH for 72 h. As observed via an inverted microscope, MH had cytopathic effect on treated cells, in which a large number of cramped cells with granulation and shrinkage of cells were noticed compared with control. MH could inhibit the glycolytic pathway in MCF7 and AMJ13 cells by suppressing the activity of HK.

The HK activity decreased in breast cancer cell lines in comparison with control. However, MH inhibited the HK activity significantly in the breast cancer cells when compared with control. In the present study was identified a significant reduction in the HK enzyme activity in the breast cancer cells but not in the normal cells. Therefore, suppressing HK activity prevented the proliferation of cancer cells. This result is in agreement with the findings of [26], who mentioned that HK via its mitochondrial location suppresses the death of cancer cells. For this reason, targeting the key enzyme by inhibitors led to the inhibition of glycolysis and therefore suppressed the proliferation of cancer cells.

The effect of the MH and control on REF cells had an insignificant difference. The pyruvate concentration in breast cancer cell lines decreased after treated with MH due to the inhibition of the HK enzyme by MH compared with that in the control, thus led to the inhibition of glycolysis. MH inhibited the pyruvate significantly in the breast cancer cells when compared with control. In the present study was noticed a significant reduction in the pyruvate concentration in the breast cancer cells but not in the normal cells.

Pyruvate concentration depends on the action of HK in the glycolysis pathway 5; therefore, the decrease in HK led to the deficiency of pyruvate. Hence, MH could prominently suppress the generation of intercellular ATP by inhibition of glycolysis. Therefore, the ATP concentration was considerably reduced in AMJ13 and MCF7 cell lines treated with MH compared with the control. The study noticed a significant difference in the ATP concentration in the breast cancer cells but not in the normal cells. Another study reported that mitochondrial membrane potential ($\Delta\psi m$) is necessary for the activity of ATP synthase, which generates ATP; loss of $\Delta\psi m$ and mitochondrial damage inhibit the activity of ATP synthase, causing ATP deficiency 9. [27] and [28] showed that the decreased ATP level in MCF7 and MDA-MB-231 cells is effective in inhibiting energy metabolism in breast cancer cells. When glycolysis is inhibited, lactate production is completely stopped and intracellular ATP concentration is abruptly decreased [29]. Similarly, 14. Hence, ATP depletion could result in insufficient energy to support the mitosis, proliferation, and invasion of cancer cells. pH levels in breast cancer cell lines treated with MH increased (less acidity) in comparison with control. MH had lesser effect on the pH of normal REF cells than on the pH of cancer cell lines. The increase in pH, which was due to the reduced lactate concentration caused by the decrease in the pyruvate concentration and led to inhibit the growth and proliferation of cancer cells. MH induced proliferation inhibition and reduced the level of acidity in both breast cancer cell lines but not in normal REF cell line. In the current study was detected a significant difference in the acidity in the breast cancer cells but not in the normal REF cells. These results are compatible with the study of [25], who proved that deficiency in lactate concentration reduced the acidity of the cell environment and considered a favorite to prevent the proliferation of cancer cells because tumors grow in acidic environment.

CONCLUSION

D-Mannoheptulose could inhibit the proliferation of breast cancer cells by enhancing cytotoxicity through inhibiting the glycolysis pathway, represented by reduced concentrations of HK, pyruvate, and ATP. MH also reduced environmental acidity of breast cancer cells. Hence, MH has potent cytotoxicity against breast cancer cells and a less toxic effect on normal REF cells. MH can induce breast cancer death via glycolysis inhibition and apoptosis induction. The results provide new light in the field of developing therapeutic strategies for breast cancer in the near future. Our investigation proves the strong effect of MH therapy on cancerous cells in vitro. MH can be considered for future treatment of cancer cells.

REFERENCES

1 Sarah F Brennan, Marie M Cantwell, Chris R Cardwell, Louiza S Velentzis, and Jayne V Woodside, "Dietary patterns and breast cancer risk: a systematic review and meta-analysis," The American journal of clinical nutrition **91** (5), 1294-1302 (2010).

2 A. M. Al-Shammari, "Environmental pollutions associated to conflicts in Iraq and related health problems," Rev Environ Health **31** (2), 245-250 (2016).

3 Kheshwant S Gill, Philana Fernandes, Tracey R O'Donovan, Sharon L McKenna, Kishore K Doddakula, Derek G Power, Declan M Soden, and Patrick F Forde, "Glycolysis inhibition as a cancer treatment and its role in an anti-tumour immune response," Biochim Biophys Acta 1866 (1), 87-105 (2016).

4 Raheel Chaudhry and Matthew Varacallo, "Biochemistry, Glycolysis", in *StatPearls [Internet]* (StatPearls Publishing, 2018).

5 L. R. Gray, S. C. Tompkins, and E. B. Taylor, "Regulation of pyruvate metabolism and human disease," Cellular and molecular life sciences : CMLS **71** (14), 2577-2604 (2014).
6 K. O. Alfarouk, D. Verduzco, C. Rauch, A. K. Muddathir, H. H. Adil, G. O. Elhassan, M. E. Ibrahim, J. David Polo Orozco, R. A. Cardone, S. J. Reshkin, and S. Harguindey, "Glycolysis, tumor metabolism, cancer growth and dissemination. A new pH-based etiopathogenic perspective and therapeutic approach to an old cancer question," Oncoscience **1** (12), 777-802 (2014).
7 Rob A Cairns, Isaac S Harris, and Tak W Mak, "Regulation of cancer cell metabolism," Nat. Rev. Cancer **11** (2), 85 (2011).
8 O. Warburg, "On the origin of cancer cells," Science **123** (3191), 309-314 (1956).
9 M. G. Vander Heiden, L. C. Cantley, and C. B. Thompson, "Understanding the Warburg effect: the metabolic requirements of cell proliferation," Science (New York, N.Y.) **324** (5930), 1029-1033 (2009).
10 J. Lu, M. Tan, and Q. Cai, "The Warburg effect in tumor progression: mitochondrial oxidative metabolism as an anti-metastasis mechanism," Cancer letters **356** (2 Pt A), 156-164 (2015).
11 R. A. Gatenby and R. J. Gillies, "Why do cancers have high aerobic glycolysis?," Nature reviews. Cancer **4** (11), 891-899 (2004).
12 C. L. Markert, "Lactate Dehydrogenase Isozymes: Dissociation and Recombination of Subunits," Science (New York, N.Y.) **140** (3573), 1329-1330 (1963).
13 X. Guo, X. Zhang, T. Wang, S. Xian, and Y. Lu, "3-Bromopyruvate and sodium citrate induce apoptosis in human gastric cancer cell line MGC-803 by inhibiting glycolysis and promoting mitochondria-regulated apoptosis pathway," Biochemical and biophysical research communications **475** (1), 37-43 (2016).
14 Y. Lu, X. Zhang, H. Zhang, J. Lan, G. Huang, E. Varin, H. Lincet, L. Poulain, and P. Icard, "Citrate induces apoptotic cell death: a promising way to treat gastric carcinoma?," Anticancer research **31** (3), 797-805 (2011).
15 S. W. Lowe and A. W. Lin, "Apoptosis in cancer," Carcinogenesis **21** (3), 485-495 (2000).
16 Irene M Ghobrial, Thomas E Witzig, and Alex A Adjei, "Targeting apoptosis pathways in cancer therapy," CA: a cancer journal for clinicians **55** (3), 178-194 (2005).
17 Rebecca SY Wong, "Apoptosis in cancer: from pathogenesis to treatment," Journal of Experimental & Clinical Cancer Research **30** (1), 87 (2011).
18 R. H. Xu, H. Pelicano, Y. Zhou, J. S. Carew, L. Feng, K. N. Bhalla, M. J. Keating, and P. Huang, "Inhibition of glycolysis in cancer cells: a novel strategy to overcome drug resistance associated with mitochondrial respiratory defect and hypoxia," Cancer research **65** (2), 613-621 (2005).
19 Y. Fu, J. Li, and A. S. Lee, "GRP78/BiP inhibits endoplasmic reticulum BIK and protects human breast cancer cells against estrogen starvation-induced apoptosis," Cancer research **67** (8), 3734-3740 (2007).
20 Ahmed Majeed Al-Shammari, Worod Jawad Kadhim Allak, Mahfoodha Umran, Nahi Y Yaseen, and Ayman Hussien, "Angiogenesis Factors Associated with New Breast Cancer Cell Line AMJ13 Cultured in Vitro," Advances in Breast Cancer Research **4** (04), 100-108 (2015).
21 AMH Al-Shammari and NY Yaseen, "In Vitro Synergistic enhancement of Newcastle Disease Virus to Methotrexate cytotoxicity against tumor cells," Al-Anbar Journal of Veterinary Sciences **5** (2), 102-109 (2012).
22 Nina A Mikirova, JJ Casciari, Michael J Gonzalez, Jorge R Miranda-Massari, NH Riordan, and Jorge Duconge, "Bioenergetics of human cancer cells and normal cells during proliferation and differentiation," Cancer Ther OncolInt J **3**, 1-8 (2017).
23 M. Danos, W. A. Taylor, and G. M. Hatch, "Mitochondrial monolysocardiolipin acyltransferase is elevated in the surviving population of H9c2 cardiac myoblast cells exposed to 2-deoxyglucose-induced apoptosis," Biochemistry and cell biology = Biochimie et biologie cellulaire **86** (1), 11-20 (2008).
24 J. S. Kim, K. J. Ahn, J. A. Kim, H. M. Kim, J. D. Lee, J. M. Lee, S. J. Kim, and J. H. Park, "Role of reactive oxygen species-mediated mitochondrial dysregulation in 3-bromopyruvate induced cell death in hepatoma cells : ROS-mediated cell death by 3-BrPA," Journal of bioenergetics and biomembranes **40** (6), 607-618 (2008).
25 R. Arora, D. Schmitt, B. Karanam, M. Tan, C. Yates, and W. Dean-Colomb, "Inhibition of the Warburg effect with a natural compound reveals a novel measurement for determining the metastatic potential of breast cancers," Oncotarget **6** (2), 662-678 (2015).
26 S. P. Mathupala, Y. H. Ko, and P. L. Pedersen, "Hexokinase II: cancer's double-edged sword acting as both facilitator and gatekeeper of malignancy when bound to mitochondria," Oncogene **25** (34), 4777-4786 (2006).

27 G. Cheng, J. Zielonka, D. M. McAllister, A. C. Mackinnon, Jr., J. Joseph, M. B. Dwinell, and B. Kalyanaraman, "Mitochondria-targeted vitamin E analogs inhibit breast cancer cell energy metabolism and promote cell death," BMC cancer **13**, 285 (2013).

28 Y. Ding, Z. Liu, S. Desai, Y. Zhao, H. Liu, L. K. Pannell, H. Yi, E. R. Wright, L. B. Owen, W. Dean-Colomb, O. Fodstad, J. Lu, S. P. LeDoux, G. L. Wilson, and M. Tan, "Receptor tyrosine kinase ErbB2 translocates into mitochondria and regulates cellular metabolism," Nature communications **3**, 1271 (2012).

29 S Gonin-Giraud, AL Mathieu, S Diocou, M Tomkowiak, G Delorme, and J Marvel, "Decreased glycolytic metabolism contributes to but is not the inducer of apoptosis following IL-3-starvation," Cell death and differentiation **9** (10), 1147 (2002).