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The potential role of Na oxamate alone or in combination with raloxifene Hcl in prevention of DMBA induced breast cancer.

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Running title: preventing breast cancer using Na oxamate.
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Abstract:
Breast cancer (BC) is the most prevalent type of cancer seen in women accounting for 25% of all female cancers and being a second leading cause of cancer death. There are many chemopreventive drugs, however, they have several drawbacks like raloxifene Hcl (RAL).Sodium oxamate is a compound acting by inhibition of lactate dehydrogenase (LDH) enzymereducing the rate of ATP generation and the rate of malignant cell proliferation and metastasis. The aim of this work was to investigate the preventive role of sodium oxamatealone or in combination with RAL in 7,12-Dimethylbenz[a]anthracene (DMBA) induced breast cancer in mice. **Methods:** This study was performed on 48 female swiss albino mice divided randomly into 6 equal groups. (Group 1) a normal control group. (Group 2) received a vehicle of olive oil. (Group 3) BC group received DMBA. (Group 4) BC group treated by RAL. (Group 5) BC group treated by Na oxamate. (Group 6) BC group treated by both RAL & Na oxamate. The bodyweight of each mice was recorded weekly. After euthanization, breast tissue and all macroscopic mammary tumors were excised. The tumor volumes,

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histopathological changes, immunohistochemical expression of Bcl2, LDH, tumor necrosis factor alpha (TNFα), reduced glutathione (GSH) and malondialdehyde (MDA) levels were measured.**Results:** Combination of RAL and Na oxamate showed evident antioxidant, anti-inflammatory, and apoptotic effects. **Conclusion:** These results open the way for using Na oxamate alone or as an adjuvant drug during chemoprevention use of RAL in BC.

Keywords:
Breast cancer, Lactate dehydrogenase enzyme, Raloxifene Hcl, sodium oxamate.

Abbreviations:
BC: breast cancer
RAL: raloxifene Hcl
LDH-A: lactate dehydrogenase A
LDH: lactate dehydrogenase
DMBA: 7,12-Dimethylbenz[a]anthracene
TNFα: tumor necrosis factor alpha
GSH: reduced glutathione
MDA: malondialdehyde
PAH: polyaromatic hydrocarbon
SERM: Selective estrogen receptor modulators
FDA: Food and Drug Administration
PBS: phosphate-buffered saline
H&E: hematoxylin and eosin
ER: estrogen receptor
PR: progesterone receptor
ROS: reactive oxygen species
ELISA: Enzyme-linked immunosorbent assay
IHC: Immunohistochemical

1. Introduction:

Breast cancer (BC) is a multifactorial, debilitating disease with numerous causes. It is defined by the uncontrolled growth and dissemination of atypical breast cells(Mehdi et al., 2018). BC is the most common form of cancer in females globally**.** Per year, about two million new cases are diagnosed worldwide, accounting for 25% of all female cancerswith the highest rate of death from cancer in women (Varghese et al., 2020).

The synthetic polyaromatic hydrocarbon (PAH),DMBA induces cancers in rodents that resemble human BC concerning both histology and morphology (Saravanan, Baskaran, & Sakthisekaran, 2016)**.** The mechanism of carcinogenicity is by double-strand breaks of DNA, in addition to the oxidative stress mechanism (Karimi, Ashrafi, Shomali, & Yektaseresht, 2019; Liu et al., 2016)**.**

Raloxifene Hcl (RAL) is one of the Selective estrogen receptor modulators (SERM)**.** FDA has recommended it for the chemoprevention of BC in high-risk postmenopausal patients, lowering the prevalence of both invasive and non-invasive

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oestrogen receptor positive BC by about 66 % and 72 %, respectively, with a safer profile than tamoxifen (Maximov & Jordan, 2016)**.**

RAL therapy is associated with many adverse effects including thromboembolic disorders such as pulmonary embolism and deep vein thrombosis, flu-like syndrome, hot flushes, gall stones, leg cramps and edema of the extremities. These side effects prompted the use of complementary and alternative medicine(Kabel & Elkhoely, 2016; Khorsand et al., 2018).As the chemo preventive drugs of BC have side effects hindered their use for long periods, it necessities to search for other safe agents for long-term periods.

BC cells show also in aerobic environments, an improvement in glycolysis which differs from normal cells that depend on glycolysis only in anaerobic conditions which is known as the Warburg effect (El-Sisi, Sokar, Abu-Risha, & El-Mahrouk, 2017; Xintaropoulou et al., 2015)**.** Because cancer cells depend mainly on glycolysis a variety of promising therapies are available for research(Y. Zhao, Butler, & Tan, 2013)**.**

The enzyme lactate dehydrogenase-A (LDH-A) is needed for BCcell growth and maintenance which is one of LDH enzyme isoforms that presents in breast tissue. LDH-A promotes the reaction of reduction of pyruvate to lactate in order to regenerate NAD+, which is needed for cancer cell glycolysis(El-Sisi et al., 2017; Le et al., 2010)**.**

Sodium oxamate inhibits LDH-A enzyme leading to cell cycle arrest and apoptosis. Preventing the conversion of pyruvate to lactate stops glycolysis which cancer cells depend on mainly to produce energy leading to inhibition of Proliferation, metastasis, and immunosuppression of cancer cells (Miskimins et al., 2014; Zhai, Yang, Wan, Zhu, & Wu, 2013)**.** Lately, sodium oxamate is considered a potential anticancer agent (Z. Zhao, Han, Yang, Wu, & Zhan, 2015)**.**

LDH-A is widely considered a safe therapeutic target in humans due to the comparatively mild effects of exertional myopathy associated with an inherited 20-bp deletion of the LDH-A gene (Doherty, Cleveland, Doherty, & Cleveland, 2013).

The aim of this study was to study the potential role of sodium oxamate, as LDH inhibitor, alone or in combination with RALin the prevention of DMBA induced BC in mice.

2. Materials and Methods

2.1. Drugs and chemicals

7,12-Dimethylbenz[a]anthracene (DMBA), RALandNa oxamatewere purchased from Sigma Aldrich St. Louis, Missouri, USA. DMBA was dissolved in olive oil. RAL andNa oxamate were freshly dissolved in sterile normal saline.Tissue lactate dehydrogenase ***(***LDH), tissue necrosis factor alpha (TNFα) level were measured using commercial ELISA kitssupplied by Sun Red. Tissue Reduced glutathione(GSH)levelandTissue malondialdehyde (MDA) level were measured by kits supplied by Biodiagnostic.Bcl2 was purchased from Master Diagnostica.

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2.2. Animals

In this study, 48 female swiss albino mice weighing 20-25 g were used. Mice were obtained from Tanta University Animal House. Mice were kept in wire mesh cages with constrictive hygienic precautions in an animal laboratory space. They had access to a normal animal diet and water ad libitum, and 1 week was required for acclimatisation prior to the start of the experiment. The academic "Research Ethics Committee, REC", Faculty of Medicine, Tanta University, Egypt, approved the use of animals and all laboratory procedures (Approval no. #33231/07/19).

2.3. Study design

The animals were divided randomly into 6 equal groups (8 mice for each) as follows: Group 1: was served as a normal control group received daily i.p injection of 0.2 ml sterile saline.Group 2: received a vehicle of olive oil 0.2 ml /week orally +daily i.p injection of 0.2 ml sterile saline.BC was induced in the other four groups using DMBA in a dose of1.0 mg /week for 6 weeks by oral gavage(Currier et al., 2005). Group 3: untreated BC(DMBAgroup) + daily i.p injection of 0.2 ml sterile saline.Group 4: BC group was treated by RALi.p. injection in a dose of 3mg/kg/day (Chang et al., 2017; Pinilla, Barreiro, Gonzalez, Tena-Sempere, & Aguilar, 2002; Turner, Sato, & Bryant, 1994).Group 5: BC group was treated by Na oxamatei.p injection in a dose of 300 mg/kg/day (El-Sisi et al., 2017).Group 6: BC group was treated by both RAL& Na oxamatein the same dosage regimen mentioned before. Treatment protocol started from 1st day of induction until the end of the experiment on day 42nd.

2.4. breast sampling

At the end of the experiment (after 6 weeks), the mice will be euthanized with pentobarbital sodium (150 mg/kg, i.p.) and lidocaine (10 mg/kg, i.p.)21, followed by dissection of the breast tissue and washing with phosphate-buffered saline (PBS) solution, pH 7.4, to dissolve all red blood cells or clots, The abdominal mammary glands were fixed in 10% formalin and processed for histopathological analysis and Bcl2 immunohistochemistry expression while the remaining glands were stored at -80 ºC until prepared for the assessment of the following tissue parameters: LDH levels, Tumor necrosis factor alpha (TNFα) levels, reduced glutathione (GSH) levels and malondialdehyde (MDA) levels.

2.5. Change in body weight

The bodyweight of each mice was recorded weekly until the end of the experimental period. The percentage of change of the body weight between the first week and the last week for each mice was calculated as follow:

$$\frac{body\ weight\ in\ last\ week - body\ weight\ in\ first\ week}{body\ weight\ in\ first\ week} \times 100$$

2.6. breast mass volume

After euthanization on the proper day, All macroscopic mammary tumors were excised, and The tumor volumes were measured (length x width) using vernier

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calipers and were calculated according to the following formula: Tumor volume ($mm^{3)}$ = 1/2 (length x $width^2$) (Alessandra-Perini et al., 2018).

2.7. Biochemical analysis

2.7.1. Tissue homogenization

The mammary glands were measured and homogenized in a tissue homogenizer in 5 ml PBS, pH 7.4 per gramme tissue. Centrifuge the homogenate at 4.000 rpm for 15 minutes at 4ºC. The supernatant was obtained for analysis and frozen at -80 ºC for no more than one month, preventing repetitive freeze-thaw periods.

2.7.2.Enzyme-linked immunosorbent assay (ELISA) of tissue Lactate dehydrogenase (LDH) level

The tissue samples of mammary gland were quantitatively assayed using mouse ELISA kits (Cat No. 201-02-0101) for mouse Lactate dehydrogenase (LDH); following the manufacturer's protocol. The values were expressed as (u/l).

2.7.3. Enzyme-linked immunosorbent assay (ELISA) of tissue Tumor necrosis factor alpha (TNFα) level (ng/L)

The tissue samples of mammary gland were quantitatively assayed using mouse ELISA kits (Cat No. 201-02-0118) for mouse Tumor necrosis factor alpha (TNFα); following the manufacturer's protocol.

2.7.4. Determination of tissue reduced glutathione (GSH) level

The decreased glutathione (GSH) concentrations (mg/g.tissue) were determined using a Kit (Cat. No. GR 2511), which is based on the Beutler spectrophotometric process (BEUTLER, DURON, & KELLY, 1963).

2.7.5. Determination of tissue malondialdehyde (MDA) level

Lipid peroxidation was assayed by measuring the levels of malondialdehyde (MDA) (nmol/g.tissue) in mammary gland tissues. MDA level was determined by measuring thiobarbituric reactive species according to the method of Ohkawa(Ohkawa, Ohishi, & Yagi, 1979) using a kit (Cat. No MD 2529), where the thiobarbituric acid reactive substances react with thiobarbituric acid to produce a pink colored complex whose absorbance is read at 534 nm.

2.8. Histological examination of H&E-stained sections

The abdominal mammary glands were fixed in a 10 % formalin paraffin section were done (5 microns), stained with hematoxylin and eosin(H&E), and processed for the examination of histopathological changes by light microscope.

2.9. Immunohistochemical detection of Bcl-2

Immunohistochemical staining was per-formed according to the manufacturer's protocol of the kit. Bcl-2 staining intensity together with percentage of positively

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stained cells were measured(Linjawi, Kontogiannea, Halwani, Edwardes, & Meterissian, 2004).

2.10. Statistical Analysis

GraphPad Prism, version 5 for Windows, was used to do the statistical analysis. The Shapiro-Wilk test was used to determine the normality of quantitative data (Changes in body weight, breast mass volume, biochemical values, and Bcl2 colour intensity). Values were calculated as mean standard error of mean for data with a normal distribution. Comparing unpaired groups was accomplished using the one-way ANOVA test (followed by the post-hoc Tukey's multiple comparison test if significant). For qualitative data, (percent of the development of breast mass) the variables were summarized as frequencies (count and percentage). Fisher-Freeman-Halton exact test was used to examine association between two categorical variables. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Change in body weight (% from the initial weight)

DMBA group (group 3)showed a significant decreasein body weight as compared to vehicle received group (group 2). Na oxamate treated group (group 5)showed a significant increase in body weight as compared to DMBA group (group 3). Combined RAL & Na oxamate treated group (group 6) showed a significant increase in body weight as compared to bothDMBA group (group 3)and RAL treated group (group 4).Table 1

3.2. Comparison of the breast mass volume (mm^3)

DMBAgroup(group 3)showed a significant increase in breast mass volume as compared to vehicle received group (group 2). Na oxamate treated group (group 5)and Combined RAL & Na oxamate treated group (group 6) showed a significant decreasein breast mass volume as compared to DMBAgroup(group 3). Table 1

Table 1: Comparison of different parameters in different studied groups.

Groups	**G1 (Control) (n = 8)**	**G2 (Vehicle) (n = 8)**	**G3 (DMBA) (n = 8)**	**G4 (RAL) (n = 8)**	**G5 (Na oxamate) (n = 8)**	**G6 (RAL+ Na oxamate) (n = 8)**
%of body weight change	12.8± 2.4	15.1 ± 3.6	-13.6 ± 4.8	-9.9 ± 4.1	4.3 ± 2.0	8.7 ± 2.0
	F = 12.36 P = 0.001***	p_1>0.05 (NS)	p_2<0.001***	p_3>0.05 (NS)	p_3<0.010** p_4>0.05 (NS)	p3<0.001*** p4<0.010** p5>0.05 (NS)
Breast mass volume (mm^3)	-----	----	376.9 ± 36.9	197.4 ± 22.3	204.2 ± 16.3	173.3 ± 15.4
	F = 7.175 P = 0.050*			p_3<0.050*	p_3<0.050* p_4>0.05 (NS)	p_3<0.05* p_4>0.05 (NS) p_5>0.05 (NS)
LDH (U/L)	393.9 ±	416.7	792.5 ±62.6	779.7 ± 61.8	298.4 ± 49.7	303.8 ± 43.7

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	23.8	±13.6				
	F = 24.352 P = <0.001***	p_1>0.050 (NS)	P2<0.001***	p_3>0.050 (NS)	p_3<0.001*** p_4<0.001***	p_3<0.001*** p_4<0.001*** p_5 >0.050 (NS)
TNFα (ng/L)	11.0 ± 0.4	10.7 ± 0.3	20.5 ± 1.4	16.0 ±0.4	16.0 ± 0.5	13.1 ± 0.5
	F = 29.555 P = <0.001***	p_1>0.050 (NS)	P2<0.001***	p_3<0.001***	p_3<0.001*** p_4>0.050 (NS)	p_3<0.001*** p_4<0.050* p_5 <0.050*
MDA (nmol/g.tissue)	26.8 ± 1.2	27.0 ± 1.1	54.1 ± 4.1	40.9 ±2.4	43.0 ± 1.7	31.5 ± 0.4
	F = 29.555 P = <0.001***	p_1>0.050 (NS)	P2<0.001***	p_3<0.010**	p_3<0.010** p_4>0.050 (NS)	p_3<0.001*** p_4<0.050* p_5 <0.050*
Reduced glutathione (mg/g.tissue)	60.8 ± 1.0	61.4 ± 1.4	35.0 ± 3.6	48.9 ±2.5	50.2 ± 3.5	60.6 ± 1.1
	F = 18.253 P = <0.001***	p_1>0.050 (NS)	P2<0.001***	p_3<0.010**	p_3<0.010** p_4>0.050 (NS)	p_3<0.001*** p_4<0.050* p_5 <0.050*
Bcl2 color intensity	0.0± 0.0	0.0± 0.0	3.87± 0.13	2.0± 0.27	1.75±0.16	1.37±0.18
	F = 24.836 P = <0.001***	p_1>0.050 (NS)	P2<0.001***	p_3<0.010**	p_3<0.010** p_4>0.050 (NS)	p_3<0.001*** p_4<0.050* p_5 <0.050*

— **values expressed as mean ± SEM -- F: Test statistic of one-way ANOVA, P= Comparison among all groups**

— **Tukey's test*:***

- *P1: Comparison of group 2 (vehicle received) vs group 1 (normal control).*
- *P2: Comparison of group 3 (DMBA treated) vs group 2 (vehicle received).*
- *P3: Comparison of group 4 (RAL treated), group 5 (Na oxamate treated) and group 6 (RAL & Na oxamate treated) Vs group 3 (DMBA treated).*
- *P4: Comparison of group 5 (Na oxamate treated) and group 6 (RAL & Na oxamate treated) Vs group 4 (RAL treated)*
- *P5: Comparison of group 6 (RAL & Na oxamate treated) Vs group 5 (NA oxamate treated).*

— **n= number ---- Significance: * = p <0.05, **= p<0.01, ***= p<0.001, NS= non-significant ----RAL= raloxifene Hcl**

3.3.Percent of the development of breast mass

DMBAgroup (group 3)showed a significant increase in Percent of the development of breast mass as compared to vehicle received group (group 2). Na oxamate treated group (group 5)and Combined RAL & Na oxamate treated group (group 6) showed a significant decrease in Percent of the development of breast mass as compared to DMBAgroup (group 3). Fig.1

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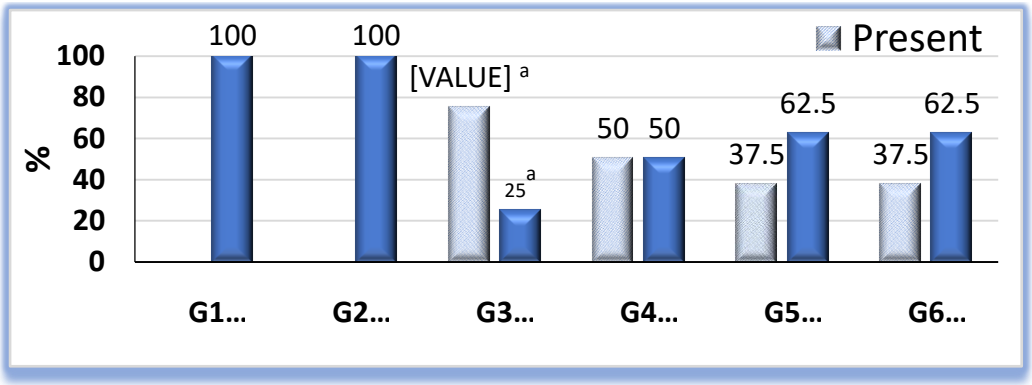

Fig.1. Percent of the development of breast mass in different studied groups.
— Fisher-Freeman-Halton exact test, n= number, NS=non-significant.
— a: significant difference from the other groups by examining adjusted residuals.

3.4.Change in tissue Lactate dehydrogenase enzyme (LDH) levels

DMBAgroup (group 3)showed a significant increase in LDH level in breast tissue as compared to the vehicle received group (group 2). Na oxamate treated group (group 5) and Combined RAL& Na oxamate treated group (group 6) showed a significant decrease in LDH level as compared to both DMBAgroup (group 3)and RAL treated group (group 4). Table 1

3.5.Change in tissueTumor necrosis factor alpha (TNFα) levels

DMBAgroup (group 3)showed a significant increase in TNFα level as compared to the vehicle received group (group 2). Na oxamate treated group (group 5) showed a significant decrease in TNFα level as compared to DMBAgroup (group 3). Combined RAL& Na oxamate treated group (group 6) showed a significant decrease in TNFα level as compared to the DMBAgroup (group 3), RAL treated group (group 4) and Na oxamate treated group (group 5). Table 1

3.6. Change in reduced glutathione (GSH) and malondialdehyde (MDA) levels (markers of oxidative stress)

DMBAgroup (group 3) showed a significant decrease in GSH level and significant increase in MDA level as compared to the vehicle received group (group 2). Na oxamate treated group (group 5) showed a significant increase in GSH level and significant decrease in MDA level as compared to DMBAgroup (group 3). Combined RAL& Na oxamate treated group (group 6) showed a significant increase in GSH level and significant decrease in MDA level as compared to DMBAgroup (group 3),RAL treated group (group4) and Na oxamate treated group (group 5).Table 1

3.7. Light microscope histopathological examinationof stained sections (H&E)

Group 3(DMBAgroup) showed sheets of malignant ductal cells surrounded by desmoplastic reaction. Higher magnification showed malignant ductal cells exhibiting pleomorphism, hyperchromatisa, and abnormal mitotic figures.Group 5 (Na oxamate treated group) showed residual malignant cells with a focal area of necrosisand large areas of hyalinizationwith some lymphocytes (a sign of good prognosis). Group 6

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(combined RAL & Na oxamate treated group)showed focal residual malignant cells with massive necrosisand wide areas of hyalinization and calcificationwith many lymphocytes (a sign of good prognosis). Fig.2

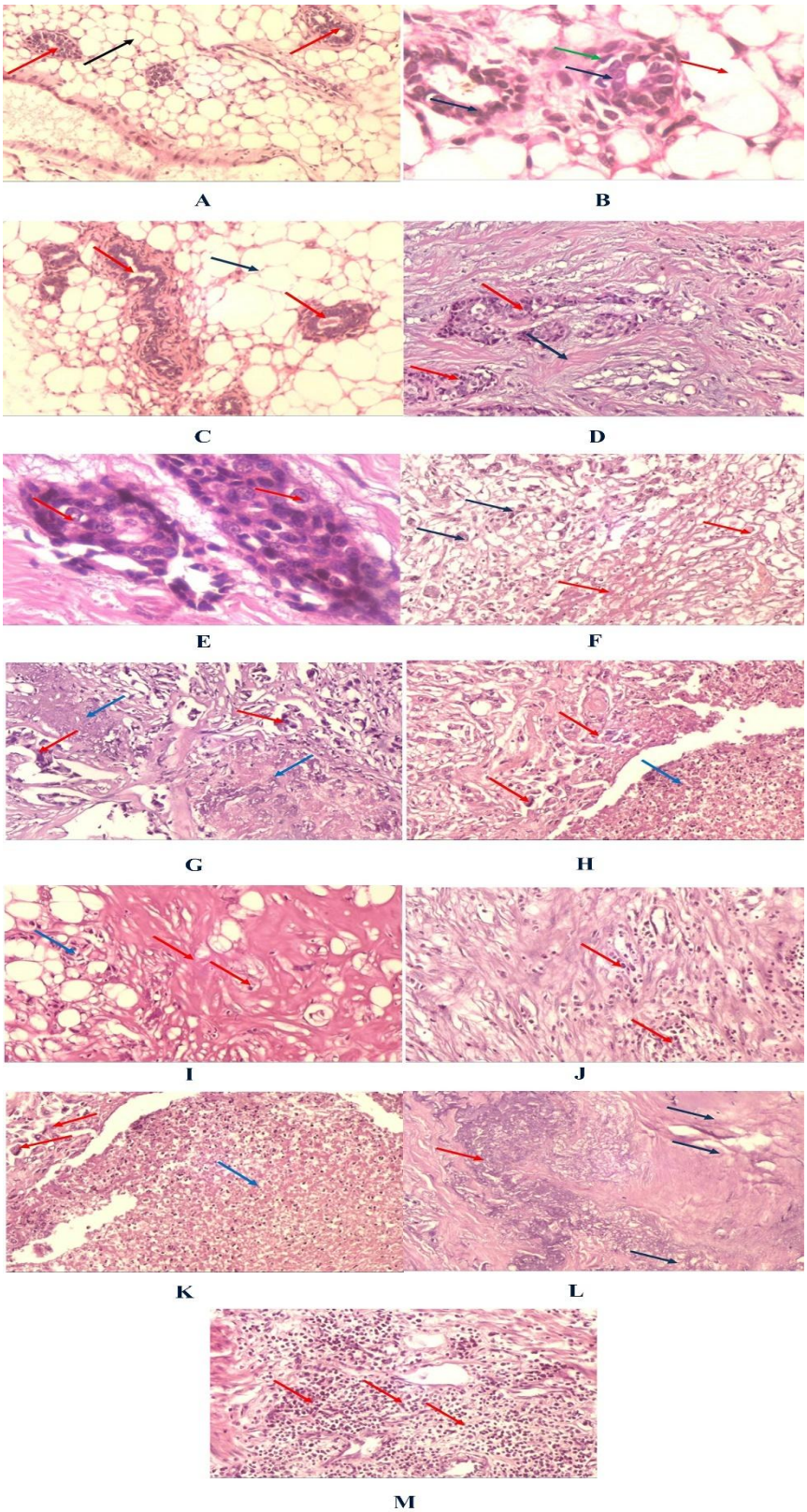

Fig.2.(A) Group 1 (normal control group) breast showed normal breast acini and ducts (red arrows) surrounded by fibrofatty tissue (black arrow) (H & E X 100). (B)

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Group 1 (normal control group) Higher magnification of the breast ducts and acini showing lining with inner low columnar cells (blue arrows) and outer myoepithelial cells (green arrow) surrounded by fibrofatty tissue (red arrow) (H & E X 400). (C) Group 2 (vehicle received group) breast showed normal breast lobules containing acini and ducts (red arrows) surrounded by fibrofatty tissue (blue arrow). (H & E X 100). (D) Group 3 (DMBA group) breast showed sheets of malignant ductal cells (red arrows) surrounded by desmoplastic reaction (blue arrow). (H&E X 100). (E) Group 3 (DMBA group) Higher magnification of the previous figure showing malignant ductal cells exhibiting pleomorphism, hyperchromatisa, and abnormal mitotic figures (red arrows). (H & E X 400). (F) Group 4 (RAL treated group) breast showed residual malignant cells (blue arrows) with individual cell necrosis (red arrows). (H and E X 100). (G) Group 4 (RAL treated group) breast showed residual malignant cells (red arrows) with areas of hyalinization (blue arrows). (H and E X 100). (H) Group 5 (Na oxamate treated group) breast showed residual malignant cells (red arrows) with a focal area of necrosis (blue arrow). (H and E X 100). (I) Group 5 (Na oxamate treated group) breast showed residual focal malignant cells (blue arrows) with large areas of hyalinization (red arrows). (H & E X 100). (J) Group 5 (Na oxamate treated group) breast showed some lymphocytes (a sign of good prognosis) (red arrows). (H & E X 100). (K) Group 6 (combinedRal& Na oxamate treated group) breast showed focal residual malignant cells (red arrows) with massive necrosis (blue arrow). (H and E X 100). (L) Group 6 (combined Ral& Na oxamate treated group) breast showed wide areas of hyalinization (blue arrows) and calcification (red arrows).(H & E X 100). (M) Group 6 (combinedRal& Na oxamate treated group) breast showed many lymphocytes (a sign of good prognosis) (red arrows).(H & E X 100)

3.8. Immunohistochemical (IHC) expression of Bcl2 estimated by the color intensity

DMBAgroup (group 3) revealed a significant increasein Bcl2 estimated color intensityas compared to the vehicle received group (group2). Na oxamate treated group (group 5) and Combined RAL& Na oxamate treated group (group 6) revealed a significant decrease in Bcl2 estimated color intensity as compared to DMBAgroup (group 3). Table 1

Group 3 (DMBAgroup) showed sheets of malignant ductal cells exhibiting strong Bcl2 positive staining. Groups 4, 5 and 6 showed residual malignant ductal cells exhibiting moderate, mild and weak positive Bcl2 stainingrespectively.Fig.3

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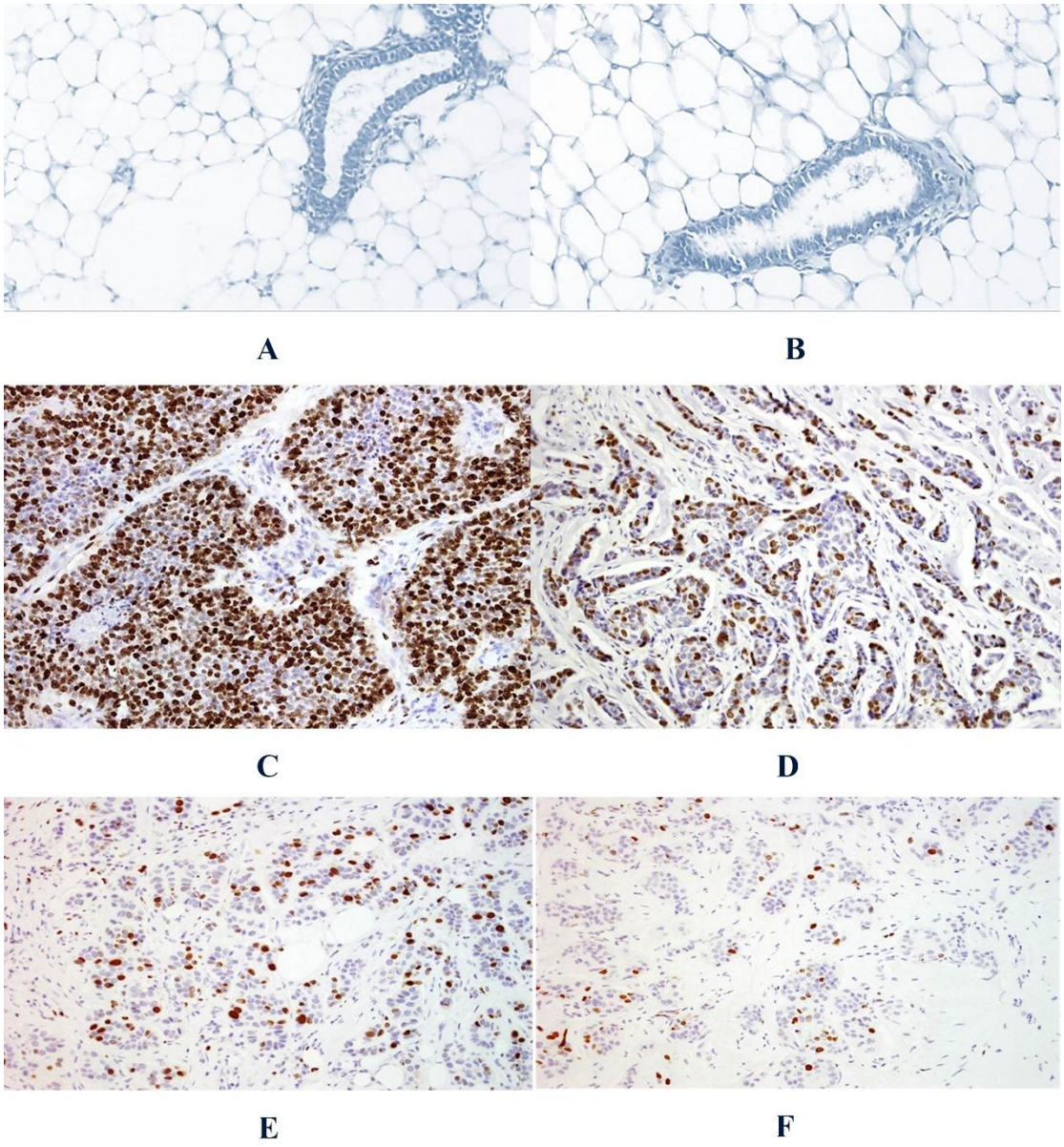

Fig.3.(A) Group 1 (normal control group) breast showed normal breast lobule negative for Bcl2 expression. (X 100) (B) Group 2 (vehicle received group) breast showed normal breast lobule negative for Bcl2 expression. (X 100) (C) Group 3 (DMBA group) breast showed sheets of malignant ductal cells showing apparent strong Bcl2 positive staining. (X 100) (D) Group 4 (RAL treated group) breast showed residual malignant ductal cells showing apparent moderate positive Bcl2 staining. (X 100) (E) group 5 (Na oxamate treated group) breast showed residual malignant ductal cells showing apparent mild positive Bcl2 staining. (X 100) (F) Group 6 (combinedRal& Na oxamate treated group) breast showed residual malignant ductal cells showing apparent weak positive Bcl2 staining. (X 100)

3.9.Correlation between breast mass volume and different tissue parameterslevels in untreated BC (DMBA group)

There was positive significant correlation between breast mass volume and tissue LDH level, tissue TNFα level, tissue MDA level and Bcl2 color intensity, While there

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was negative significant correlation between breast mass volume and tissue GSH level. Fig.4

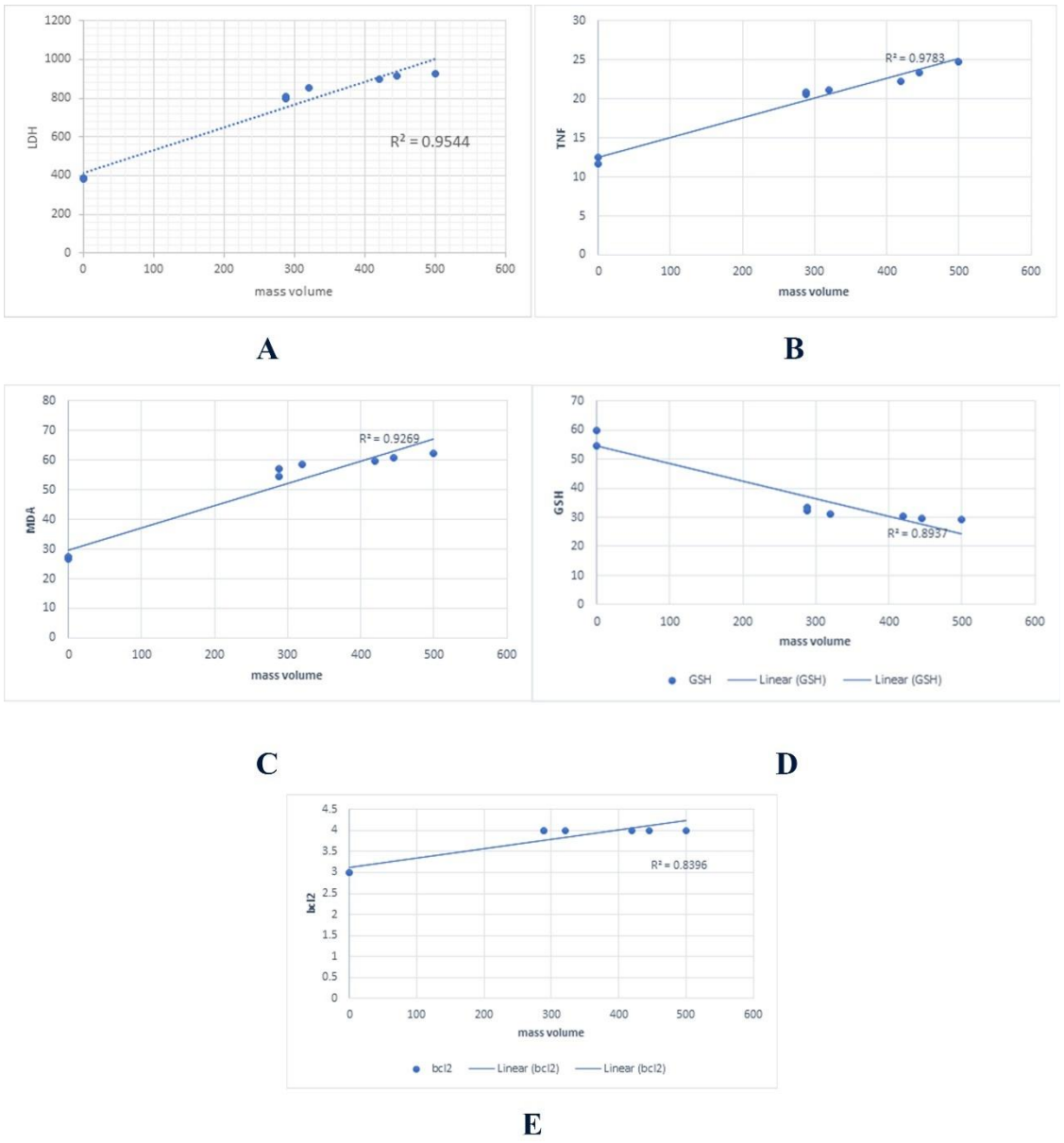

Fig.4.(A) Correlation between breast mass volume and tissue LDH level in Group 3 (DMBA group). (B) Correlation between breast mass volume and tissue TNFα level in Group 3 (DMBA group). (C) Correlation between breast mass volume and tissue GSH level in Group 3 (DMBA group). (D) Correlation between breast mass volume and tissue MDA level in Group 3 (DMBA group). (E) Correlation between breast mass volume and Bcl2 color intensity in Group 3 (DMBA group).

4. Discussion

BC is considered the most prevalent form of cancer in women worldwide(Akram, Iqbal, Daniyal, & Khan, 2017)**.** It affects approximately one out of four women (Bray et al., 2018)**.** Several risk factors such as genetics, hormones, the environment, sociobiology, and physiology predispose to BC(Ferlay et al., 2015)**.**DMBA, a synthetic PAH, initiates the generation of free radicals that cause

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carcinogenesis in rodents that morphologically and histologically resemble human cancers(Sakthisekaran, Baskaran, & Saravanan, 2016).The cytochrome p450 enzymes activate DMBA metabolically, resulting in the formation of adducts with DNA that are carcinogenic and mutagenic(Chen et al., 2019).

For these reasons, DMBA was chosen in this study for induction of the BC model to find the agents that can protect against the cascade leading to the ultimate BC. The mechanism of DMBA carcinogenesis entails the destruction and interruption of the tissue's redox equilibrium, resulting in oxidative stress(Akhouri, Kumari, & Kumar, 2020), introduction of chronic inflammation (Anbalagan, Raju, & Shanmugam, 2017) and imbalance of cell apoptosis by inhibiting apoptosis and activating antiapoptotic protein (Chen et al., 2019).

In the present study, the results of Group 3 (DMBAgroup) when compared to vehicle received group (group 2) showed significant increase in tumor development percent, breast mass volume, tissue LDH level, tissue TNFα level, tissue MDA level and Bcl2 expression while there was a significant decrease in tissue GSH level and percentage of body weight change.These results were in harmony with histopathological changes in the breast tissue and the results of previous studies as Baltaci et al. (2018),de Alencar et al. (2019)and Sahin et al. (2020)(Baltaci et al., 2018; de Alencar et al., 2019b; Sahin et al., 2020).

In this study, the percentage of body weight change decreased significantlyof Group 3 (DMBAgroup). These result were in accordance with Gan et al. (2019), Karimi et al. (2019), Arokia Vijaya Anand et al. (2019) and Rajendran et al. (2019)(Arokia Vijaya Anand et al., 2019; Gan et al., 2019; Karimi et al., 2019; Rajendran et al., 2019). They suggested that the total body weight was reduced due to the alterations in energy metabolism due to the continuous demand of energy for cancer cells make the energy reserves depleted so lead to considerable weight loss, also it has been suggested that the liver is involved in metabolic alterations causing weight loss in cancer.

In this study there was significant increase in percentage of tumor development and tumor mass volume in Group 3 (DMBAgroup). These result were in accordance with Abd El-Rahman et al. (2017), Alessandra-Perini et al. (2018)(Abd El-Rahman et al., 2017; Alessandra-Perini et al., 2018).

Cancer cells show an increase in level of LDH due to enhanced rate of glycolysis even in conditions of good oxygen supply. This process is known as the Warburg effect(Al Bawab, Zihlif, Jarrar, & Saleh, 2020; Rani & Kumar, 2016).As regards to tissue LDH level of Group 3 (DMBAgroup), it was found that significant elevation in tissue LDH level. These results agreed with Valvona and Fillmore. (2018)and Metibemu et al. (2020)(Metibemu et al., 2020; Valvona & Fillmore, 2018). They proposed that the increased LDH activity in cancer cells could be due to the mechanism of increased cell proliferation, migration, or invasion relative to normal cells, as a large cancer cell population needs a more rapid energy supply. To satisfy this enormous and rapidly increasing energy need, cancer cells use LDH action, which is beneficial for metabolic requirements and aerobic glycolysis in malignant cells. Other potential explanations for elevated LDH activity in cancer cells include

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increased LDH gene expression in cancer cells relative to normal cells and the Warburg effect.

Animals in Group 3 (DMBAgroup) presented a state of inflammation as represented by significant increase in tissue TNFα level. TNF is a critical proinflammatory cytokine secreted by macrophages that plays a critical role in the production of cancer. This result agreed with Zhang et al. (2017) andMartínez-Reza et al. (2017)(Martínez-Reza et al., 2017; Zhang et al., 2017).

DMBAgroup (group 3) presented a state of oxidative stress as represented by a significant decrease in reduced glutathione content (GSH) and significant increase in MDA. These results agreed with Gan et al. (2019) and Zeweil et al. (2019)(Gan et al., 2019; Zeweil et al., 2019).

In the present study, Group 3 (DMBAgroup) showed decreased apoptosis as indicated by upregulating Bcl2 immunohistochemical expression (apoptotic marker) in the breast tissue. This proved that the BC occurs via inhibition of programmed cell death "apoptosis". these results agreed with the previous findings ofDeng and Letai. (2013), Honma et al. (2015)(Deng & Letai, 2013; Honma et al., 2015).

RAL is an FDA approved oral second generation, selective estrogen receptor modulator (SERM). Raloxifene exhibits partial agonist and antagonist activity on estrogen receptors (ER)estrogenic effects on bone and lipid metabolism, antiestrogenic effects in the breast, and a neutral effect on the endometrium, depending on the tissue type(Provinciali, Suen, Dunn, & DeCensi, 2016). RAL has anti-inflammatory, antioxidant and apoptotic effects (Kabel & Elkhoely, 2016). Mammary carcinomas caused by DMBA express both the ER and progesterone receptors. (PR) (Karimi et al., 2019).

For similar reasons, RAL was used in this study to control BC.

As the chemo preventive drugs of BC have side effects hindered their use for long periods, it necessities to search for other safe agents for long-term periods.

In this study, there was a significant increase in the percentage of body weight of Na oxamate treated group5. These were in accordance with An et al. (2017)(An et al., 2017). Whilethere was decrease in the percentage of body weight of RAL treated group (group 4) as a result of body fat loss by its estrogenic effects and the physiological regulation of the energy balance. These results were in accordance with Kubatka et al. (2002), Jamdade et al. (2016) and Tatar et al. (2019)(Jamdade et al., 2016; Kubatka et al., 2002; Tatar et al., 2019)

There was significant decrease in percent of tumor development and tumor mass volume in Na oxamate treated group (group5). These results were in accordance with Figueroa-González et al. (2016) and Kusumawati et al. (2018)(Figueroa-González et al., 2016; Kusumawati et al., 2018). They suggested that oxamate inhibited tumor growth, induced cancer cell apoptosis, and reduced tumor size in other organs as colorectal, melanoma, prostate, and lung cancer.

In this study, Na oxamate treated group (group 5) showed significant decrease in the levels of tissue LDH as it is a structural analogue of pyruvate, which competes with pyruvate to inhibit LDH-A activity. These results agreed with Valvona and Fillmore. (2018) and Das et al. (2019)(Das et al., 2019; Valvona & Fillmore, 2018).

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Na oxamate treated group 5declined significantly in the levels of tissue TNFα, which suggested that Na oxamate had an anti-inflammatory effect. These results agreed with Ye et al. (2016)and Wu et al. (2017)(Wu et al., 2017; Ye et al., 2016).

In the present study, the high levels of MDA have been found in after treatment with sodium oxamate, a peroxidation inhibitor, to demonstrate that the decrease in oxidative stress and significant increase in GSH level. These results agreed with El-Sisi et al. (2017)(El-Sisi et al., 2017). as oxamate inhibit production of ROS in human cancerous cellslead to apoptosis and G2/M arrest also LDH-A inhibition induced G0/G1 arrest (Yang et al., 2014).

There is significant decrease in Bcl2 expression in Na oxamate treated group (group 5) which prove it's apoptotic effect. These results correlated withZhai et al. (2013),An et al. (2017) and Feng et al. (2018)(An et al., 2017; Feng et al., 2018; Zhai et al., 2013). Na oxamateinduced apoptosis in BC cells is usually connected with decreased levels of expression of anti-apoptotic factors of Bcl2(Kusumawati et al., 2018; Yang et al., 2014).

When Na oxamate treated group (group5) compared to RAL treated group (group 4), it revealed superiority in effect on glucose metabolism by inhibiting LDH so inhibiting glycolysis which is the major pathway that cancer cells rely on for energy production.But when Na oxamate treated group (group5) compared to RAL treated group 4 the difference was insignificant as regard to anti-inflammatory, antioxidant and apoptotic effects. This is proved by assessment of tissue TNFα as an inflammatory mediator, assessment of tissue MDA and reduced glutathione representing the oxidative and antioxidant effects and assessment of Bcl2 immunostaining which is an antiapoptotic marker.

The combined RAL& Na oxamate treated group (group 6) showed evident antioxidant, anti-inflammatory and apoptotic effects represented by significant decrease in tissue LDH level, tissue TNFα, tissue MDA level and Bcl2 expression and significant increase in tissue GSH level, showed significant increase in the percentage of body weight and there was significant decrease in percent of breast mass and breast mass volume developed as compared to the DMBA group 3.

When the combined RAL& Na oxamate treated group (group 6) was in comparison between both RAL group 4and Na oxamate treated group (group5), it exhibited superiority in anti-inflammatory, antioxidant and apoptotic effects.

5. Conclusion

These findings suggest that Na oxamate can protect the breast tissue against BC development and produce promising effects in DMBA induced BC which mimics BC in human as it decreases inflammation, oxidative stress, antiapoptotic effects and improve the histopathological picture that's all reflected as amelioration in disease activity represented by a decrease in the percent of tumor development and breast mass volume. However, the combination of RAL and Na oxamate provided an additional amelioration on the disease activity with exhibited additional effects as regard to the improvement of inflammation. In addition to the added antioxidant and apoptotic effects of Na oxamate, it antagonize oxidative stress and antiapoptosis induced by the disease itself so it provide more chemopreventive influence.**As a**

conclusion, these encouraging results support using Na oxamate alone or as an adjuvant therapy to RAL to reduce risk of cancer breast development which provide better results. This should be verified in further animal studies for a longer period and human clinical studies.

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Declaration of competing interest

This is a confirmation that there is no conflict of interest in this work.

Author contributions

All the named authors participated sufficiently in this research work according to the speciality and expert of each one. Hanan M. Elkhouly, performed most experiments and data analysis, participated in writing of the manuscript. Fleur Fathiand Dina Tahoondesigned the study and helped with writing. Heba Abd El-Galilwas involved inexperimental design, participated in performing experiments, data analysis, writing and revision. All authors read and approved the final manuscript.

The authors declare that all data were generated in-house and that no paper mill was used.

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Figure captions

Fig.1. Percent of the development of breast mass in different studied groups.

— *Fisher-Freeman-Halton exact test, n= number, NS=non-significant.*

— *a: significant difference from the other groups by examining adjusted residuals.*

Fig.2.(A) Group 1 (normal control group) breast showed normal breast acini and ducts (red arrows) surrounded by fibrofatty tissue (black arrow) (H & E X 100). (B) Group 1 (normal control group) Higher magnification of the breast ducts and acini showing lining with inner low columnar cells (blue arrows) and outer myoepithelial cells (green arrow) surrounded by fibrofatty tissue (red arrow) (H & E X 400). (C) Group 2 (vehicle received group) breast showed normal breast lobules containing acini and ducts (red arrows) surrounded by fibrofatty tissue (blue arrow). (H & E X 100). (D) Group 3 (DMBA group) breast showed sheets of malignant ductal cells (red arrows) surrounded by desmoplastic reaction (blue arrow). (H&E X 100). (E) Group 3 (DMBA group) Higher magnification of the previous figure showing malignant ductal cells exhibiting pleomorphism, hyperchromatisa, and abnormal mitotic figures (red arrows). (H & E X 400). (F) Group 4 (RAL treated group) breast showed residual malignant cells (blue arrows) with individual cell necrosis (red arrows). (H and E X 100). (G) Group 4 (RAL treated group) breast showed residual malignant cells (red arrows) with areas of hyalinization (blue arrows). (H and E X 100). (H) Group 5 (Na oxamate treated group) breast showed residual malignant cells (red arrows) with a focal area of necrosis (blue arrow). (H and E X 100). (I) Group 5 (Na oxamate treated group) breast showed residual focal malignant cells (blue arrows) with large areas of hyalinization (red arrows). (H & E X 100). (J) Group 5 (Na oxamate treated group) breast showed some lymphocytes (a sign of good prognosis) (red arrows). (H & E X 100). (K) Group 6 (combinedRal& Na oxamate treated group) breast showed focal residual malignant cells (red arrows) with massive necrosis (blue arrow). (H and E X 100). (L) Group 6 (combined Ral& Na oxamate treated group) breast showed wide areas of hyalinization (blue arrows) and calcification (red arrows).(H & E X 100). (M) Group 6 (combinedRal& Na oxamate treated group) breast showed many lymphocytes (a sign of good prognosis) (red arrows).(H & E X 100)

Fig.3.(A) Group 1 (normal control group) breast showed normal breast lobule negative for Bcl2 expression. (X 100) (B) Group 2 (vehicle received group) breast showed normal breast lobule negative for Bcl2 expression. (X 100) (C) Group 3 (DMBA group) breast showed sheets of malignant ductal cells showing apparent

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strong Bcl2 positive staining. (X 100) (D) Group 4 (RAL treated group) breast showed residual malignant ductal cells showing apparent moderate positive Bcl2 staining. (X 100) (E) group 5 (Na oxamate treated group) breast showed residual malignant ductal cells showing apparent mild positive Bcl2 staining. (X 100) (F) Group 6 (combinedRal& Na oxamate treated group) breast showed residual malignant ductal cells showing apparent weak positive Bcl2 staining. (X 100)

Fig.4.(A) Correlation between breast mass volume and tissue LDH level in Group 3 (DMBA group). (B) Correlation between breast mass volume and tissue TNFα level in Group 3 (DMBA group). (C) Correlation between breast mass volume and tissue GSH level in Group 3 (DMBA group). (D) Correlation between breast mass volume and tissue MDA level in Group 3 (DMBA group). (E) Correlation between breast mass volume and Bcl2 color intensity in Group 3 (DMBA group).