Apoptosis as a therapeutic strategy for breast cancer: the role of Thymax, a gross thymic extract, in modulating cell death pathways

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Background
Breast cancer is a prevalent disease in women and a leading cause of cancer-related health issues. Thymax, a thymic extract, has shown potential for inducing breast cancer cell apoptosis *in vitro*.
Objective
This study aims to investigate how Thymax induces apoptosis and inhibits breast cancer growth and metastasis *in vivo*.
Materials and methods
Thymax treatment was divided into five groups: the first group (negative control) – normal rats without tumors. In the second group (positive control), rats were injected subcutaneously in the mammary gland with a single dose of 50 mg/kg b.w. of 7,12-Dimethylbenz(a)anthracene (in 2 ml of corn oil) and allowed to develop tumors for 120 days. Group 3: Thymax was orally administered 6 days a week to tumor-bearing rats (0.4 mg/rat) and continued for 5 weeks. Tumor-bearing rats in group 4 (Thymax injection) received 0.1 ml of Thymax solution through intraperitoneal injection twice weekly for 5 weeks. The last group was Thymax mix (oral and injection); tumor-bearing rats received Thymax solution by dual routes: orally with 0.4 ml six times per week and intraperitoneally with 0.1 ml twice weekly for 5 weeks. Thymax treatment, beginning after 120 days of tumor induction, continued for 5 weeks.
Results and conclusion
Thymax- induced apoptosis in breast cancer cells by increasing cytochrome c, tumor necrosis factor receptor type 1-associated death domain protein (TRADD), and Fas associated death domain (FADD) levels. It also activated the mitochondrial-dependent pathway with up-regulation of tumor protein gene (P53) expression and cysteine-dependent, aspartate-specific peptidase (caspase-8) activation. Thymax restored normal renal and hepatic cell function and enhanced the immune system by improving total antioxidant levels and inhibiting malondialdehyde levels in treated animals. Histopathological results showed a significant apoptotic effect in the group receiving Thymax injections, demonstrating its capability to induce apoptosis without tumors or atypia in mammary glands.
Our findings indicate that Thymax has a significant effect on enhancing tumor cell death and inducing apoptosis *in vivo*. Thymax may also modulate proapoptotic and antiapoptotic protein expression and activity, regulate the penetrability of the mitochondrial membrane, and release cytochrome c. Furthermore, our findings show that the injection route of Thymax is the fastest and most efficient method to deliver the extract to the tumor site and exert its antitumor effects. These results suggest that Thymax has the potential to be a novel adjuvant in the treatment of breast cancer, as it can enhance the efficacy of conventional therapies and reduce the risk of recurrence and metastasis.

Keywords:
apoptosis, breast cancer, caspase-8, *in vivo*, oxidative stress, Thymax

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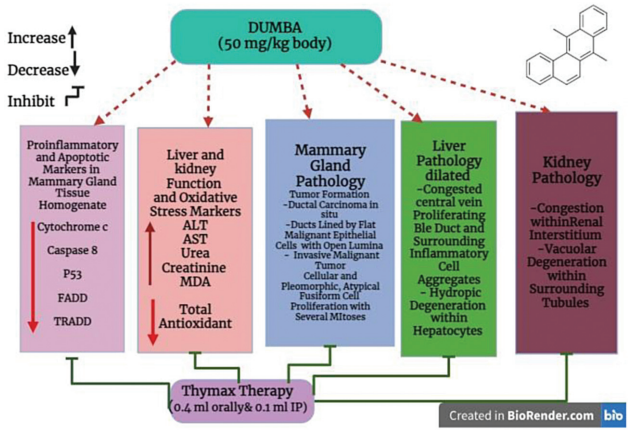
Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; Bcl-2, B cell lymphoma 2; caspase-8, cysteine-dependent, aspartate-specific peptidase; DCs, monocyte-derived dendritic cells; DMBA, 7,12-Dimethylbenz(a)anthracene; FADD, Fas-associated death domain; IL, interleukin; IM, inner membrane; LPO/MDA, lipid peroxidation/ malondialdehyde; MMP, mitochondrial membrane potential; NF-κB, nuclear factor kappa B; ROS, reactive oxygen species; TNFR1, tumor necrosis



factor receptor 1; TNF-α, tumor necrosis factor-alpha; TRADD, tumor necrosis factor receptor type 1-associated death domain protein.

Introduction

Cancer was the world's second most common cause of death in 2018, claiming more than 9.5 million lives that year [1,2]. All income levels continue to see high cancer incidence rates, with low-income and middle-income nations having the greatest fatality rates [3]. Certainly! breast cancer is a significant global health issue, affecting women and causing morbidity. It originates from mammary cells and can spread if left untreated. Early diagnosis plays a crucial role in determining treatment options such as radiotherapy or chemotherapy [4–8].

Breast cancer research is a top priority due to its significant impact on health. It is the most common cancer in women globally, with increasing mortality and incidence rates expected [9]. Recent studies highlight its prominence as a leading cause of cancer-related deaths among women under 45, particularly in young women [10]. In developing countries, the incidence and mortality of breast cancer are projected to rise significantly in the next two decades [11]. Breast cancer poses challenges like early metastasis, aggressive invasion, resistance to existing treatments, and high mortality rates. Understanding apoptosis is vital for maintaining balance in multicellular organisms, supporting healthy growth, and combating cancer. Alterations in apoptosis can lead to abnormal cell formation, uncontrolled division, and mutation accumulation. Thus, controlling apoptosis is key in treating cancer [12].

So far, there have been very few ways to treat the disease when it reaches advanced stages. Because cancer spreading and treatment are mainly analgesic and reduce survival ratios, it is not easy to find an apoptotic agent with slight side effects for metastatic breast cancer cells [13]. Traditional therapy options such as chemotherapy, radiotherapy, and surgical treatment have severe effects and negatively impact patients' quality of life [14,15]. Due to the metastatic nature of breast cancer, treatment options for advanced stages are limited. Finding an apoptotic agent with minimal side effects for metastatic breast cancer cells is challenging [13]. By 2040, an alarming increase in new cancer cases is expected globally. There is a crucial need for alternative therapies to enhance patient well-being with fewer adverse effects [16].

The thymus gland is an important part of the immune system, similar to the tonsils and adenoids. The soul was believed to reside in this area due to its association with the word 'thymus' in Greek [17]. The thymus shrinks as we age, increasing in size until around 2–3 years old and then decreasing during adolescence due to hormonal changes [16]. In recent times, there has been growing interest in utilizing thymic preparations, both natural and synthetic, to enhance and boost the responsiveness of the host immune system [18]. This approach has shown promising results in treating various disorders, including cancer. By leveraging thymic preparations, medical professionals aim to strengthen the immune system's ability to combat diseases more effectively while minimizing adverse effects [17]. Understanding the intricate relationship between childhood immune development, thymus function, and harnessing thymic preparations opens new avenues for improving treatments and enhancing patient outcomes in various disease contexts [19,20]. The thymus has been utilized for over a century as a promising treatment to enhance health. It plays a crucial role in the production of immunologically competent T-cells and other important biological processes related to immune reactivity. The thymus generates progenitor cells that undergo growth and differentiation, eventually maturing into T-cells. These mature T-cells, once they leave the thymus, play a significant role in coordinating various functions of the adaptive immune system [21]. This intricate process highlights the importance of the thymus in maintaining a forceful immune response [22,23]. The decline of the thymus has been implicated in the reduced ability of the immune system to recover from significant injury. Elderly individuals may experience increased mortality and morbidity due to immunosuppression caused by treatments like chemotherapy, radiation, or exposure to diseases such as HIV and hepatitis C [24]. Researchers have been focusing on finding effective and safe methods for

thymus regeneration in clinical settings [25]. Thymic tissue transplantation and the use of thymic hormones have shown promise in partially reversing thymic involution or reducing T lymphocyte proliferation [17]. The production of self-hormones by the thymus, such as thymus humoral factor, thymopentin, thymulin, and thymosin, plays a role in controlling immune cell transformation and selection [19].

Thymosin 1 (T1) has shown promising potential as a chemopreventive drug in animal models, demonstrating its ability to reduce lung adenomas, prevent mammary carcinogenesis, and improve animal survival rates [26]. Furthermore, T1 has been utilized in the treatment of various cancers, such as lung, kidney, and melanoma [27]. Thymic extracts, including thymosin fraction 5 (TF5), have also been found to possess immunomodulatory effects [28]. These extracts stimulate and enhance the maturation of T-cell differentiation while activating dendritic cells (DCs) and natural killer cells [29]. TF5, a protein derived from the bovine thymus, consists of multiple hormonal-like factors. Its inhibitory properties have been observed in hematopoietic leukemia cell lines and neuroendocrine tumor cells [30–33]. In addition to inhibiting cell growth, TF5 has been shown to increase lymphocyte cyclic nucleotide levels and enhance lymphocyte movement [34,35]. Moreover it produces an inhibitory factor that acts on various T-cell subpopulations [36]. These findings suggest that thymic factors may directly limit tumor cell proliferation while indirectly modifying immune cell activities to exert their anticancer and antimetastatic effects. Thus, exploring the therapeutic potential of these thymic factors may pave the way for novel approaches to cancer treatment [37].

Thymax, a new thymic product, has shown potential for activating human monocyte-derived DCs and reversing age-related functional decline in immune cells [38–41]. Stimulation of DCs by Thymax leads to the release of cytokines like interleukin (IL)-12 and IL-6 while reducing IL-10 production [42]. This activation may contribute to an increase in clusters of differentiation 4+T-cells and the activation of type 1 T helper cells [43]. Additionally, Thymax *in vitro* has been found to trigger cell death in human breast cancer cells (Michigan Cancer Foundation-7) by activating cysteine-dependent, aspartate-specific peptidase (caspases) 8 and 9 and reducing mitochondrial polarization [17]. This study aims to investigate how Thymax inhibits cell cycle progression and activates the mitochondrial mechanism of apoptosis, thereby producing anticancer effects *in vivo*.

Materials and methods

Experimental animals

This study used female rats of the Sprague-Dawley type that weighed 120–150 g. The rats were purchased from the animal house at the National Research Centre in Dokki, Giza, Egypt. Before the experiment, the rats were housed in plastic cages for 1 week and given time to adapt to their surroundings under regular conditions with a 12-h light–dark cycle. During the experiment, the rats had unrestricted access to purified water and commercialized food. The environmental conditions, such as temperature, humidity, and light levels, were carefully controlled and standardized throughout the study. All procedures involving animals followed the guidelines set by the National Health and Medical Research Council and were approved by the Institutional Animal Ethics Committee of the National Research Centre in Giza, Egypt (No. 19-204).

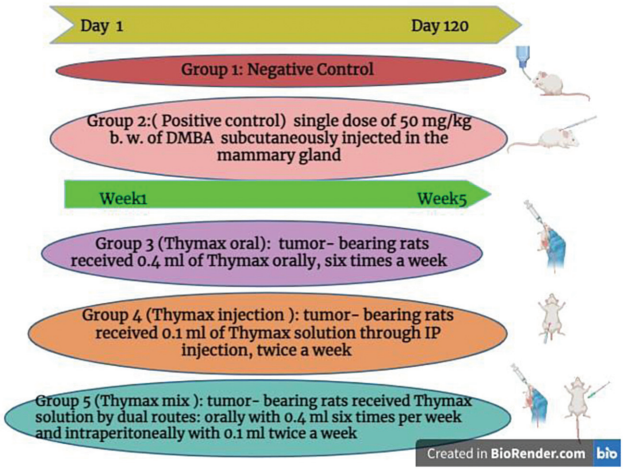
Tumor induction

After 1 week of adaptation, the rats were injected with 7, 12-dimethylbenz [α] anthracene (DMBA) purchased from Sigma Chemicals Company, USA (St. Louis and Burlington, MA). A single dose of 50 mg/kg body weight in 2 ml of corn oil was subcutaneously injected into the mammary gland. The healthy rats had an average weight of 120–150 g. They were then monitored for tumor development for 120 days [44].

Experimental protocol and drug administration

The different treatment groups were used to assess the effects of Thymax on tumor growth and development in the rats (Figure 1).

Figure 1



Schematic illustrations of the animal grouping and experimental design.

- Group 1 (negative control): normal rats without tumors.
- Group 2 (positive control): rat injected subcutaneously with a single dose of 50 mg/kg b.w. of DMBA in 2 ml of corn oil in the mammary gland [44] and allowed to develop tumors for 120 days.
- Group 3 (Thymax oral): tumor-bearing rats received 0.4 ml of Thymax orally six times a week for 5 weeks.
- Group 4 (Thymax injection): tumor-bearing rats received 0.1 ml of Thymax solution through intraperitoneal injection twice a week for 5 weeks [45].
- Group 5 (Thymax mix): tumor-bearing rats received Thymax solution by dual routes: orally with 0.4 ml six times per week and intraperitoneally with 0.1 ml twice a week for 5 weeks.

Sampling

At the end of the induction period, rats in groups 1 and 2 were sacrificed. Rats in groups 3, 4, and 5 were sacrificed at the end of the fifth week of the experiment posttreatment. From the retro-orbital plexus of rats while under diethyl ether anesthesia, samples of blood were obtained in clean tubes. The first blood sample was taken at the end of the induction period for untreated tumor-bearing rats and normal control rats without tumors. When the experiment was over, a second blood sample was collected from the treated groups. Blood samples were collected and allowed to coagulate. They were then cool-centrifuged using a Hettich centrifuge (Newtown, Connecticut, USA) at 3000 rpm for 10 min. For examination of biochemical assays, the resulting sera were preserved at −80°C after separation.

Preparation of tissue homogenates

Tissue homogenates were prepared as follows: rats with and without treatment, bearing tumors, were sacrificed. Breast tissue samples (5 μm) were cut and weighed. Breast tissues were rinsed with ice-cold phosphate-buffered saline (0.01 M, pH=7.4), weighed, minced, and homogenized (SONICS homogenizer, France, Taguig City 1634, Metro Manila, Philippines) in phosphate-buffered saline (9 ml/g of tissue) using a glass homogenizer on ice. The homogenates were then centrifuged at 3000 rpm for 10 min. Supernatants were collected and frozen at −80°C for further analysis.

Biochemical analyses

Spectrophotometrically (MY 1345003 spectrophotometer, China) and using kits purchased from Reactivos GPL (Barcelona, España), serum alkaline phosphatase activity was estimated kinetically according to the method described by Kind and King [46]. Alanine aminotransferase and aspartate aminotransferase (ALT and AST) activities were estimated as described by Reitman and Frankel's [47] method; urea and creatinine levels were estimated according to the methods of Patton and Crouch [48] and Bowers and Wong [49], respectively. Total antioxidants and lipid peroxidation/malondialdehyde (LPO/MDA) levels were measured using Elabscience (Biochemical Assay Kit) according to Smith *et al.* [50] and Ohkawa *et al.* [51], respectively.

Breast tissue homogenate (tumor with or without treatment) levels of cytochrome c (Cyt c), tumor necrosis factor receptor type 1-associated death domain protein (TRADD), Fas-associated death domain (FADD), tumor necrosis factor receptor 1 (TNFR1), caspase-8, and tumor protein gene (P53) were measured using ELISA technique (UV-2401; Shimadzu, Japan) and rats ELISA reagent kits purchased from SinoGeneClon Biotech Co. Ltd, China.

Histopathological preparation

At the end of the experiment, animals were sacrificed. Mammary gland tissue, liver, and kidney samples were dissected and fixed in 10% buffered formalin for 48 h. The tissues were then processed, cleared, and prepared into paraffin blocks. Serial sections, each 5 μm thick, were made from each block and stained with hematoxylin and eosin for histopathologic study [52]. The sections were examined using an Olympus CX41 research microscope. To avoid bias, an experienced observer interpreted the histopathologic examination by blinding the sample identity. Digital photomicrographic sections were taken at various magnifications using a CCD digital camera (Olympus SC100) attached to the microscope.

Statistical analysis

Using one-way analysis of variance with a paired sample test method and post-hoc analysis, statistical comparisons were performed.

Results and discussion

Biochemical assessment of the apoptotic signaling

The impact of cytochrome c

Thymax is a crude extract from the thymus gland that can inhibit cancer, and its metastasis has been studied in various in-vitro studies. One of the mechanisms by which Thymax exerts its anticancer effects in human breast cancer cells is by inducing in-vitro programmed

cell death, or apoptosis. Apoptosis can be triggered by various signals, such as DNA damage, oxidative stress, or immune system activation. Our findings (Table 1) showed that the untreated group (DMBA) had much lower levels ($P \leq 0.01$) of cytochrome c in their homogenates than the normal rats. Thymax treatment resulted in a notable elevation in cytochrome c levels ($P \leq 0.01$). Thymax-injected animals led to a fourfold rise ($P \leq 0.001$) in cytochrome c level, which is more prominent than the increase observed in the oral and mix groups ($P \leq 0.05$). Our results are consistent with other studies that revealed that Thymax exerts its anticancer effects by disrupting the balance between proapoptotic and antiapoptotic factors in breast cancer cells, leading to mitochondrial damage, and caspase activation. This results in the activation of the intrinsic apoptotic pathway, which culminates in DNA fragmentation and cell death [53].

The main ways that cells die by apoptosis are the extrinsic way and the intrinsic way, which is also called the mitochondrial way [54–57]. The extrinsic pathway starts when death receptors (like CD95 and TNFR1) on the cell surface are activated in the plasma membrane [57,58]. The intrinsic pathway happens when drugs, radiation, or other stresses damage the mitochondria and prevent their integrity [59,60]. In the intrinsic pathway of apoptosis, mitochondria play a crucial role, proceeding through the involvement of mitochondrial membrane potential (MMP) [61]. The matrix and the intermembrane space are the two distinct parts of mitochondria: the inner membrane (IM) surrounds the matrix, and the outer membrane surrounds the intermembrane space. The IM contains different kinds of molecules, such as ATP synthase, the electron transport chain, and the adenine nucleotide translocator. These molecules help the respiratory chain create a difference in electric charge and concentration, which is also called MMP, when the conditions are normal in the body – perturbation of the IM results in alterations in the MMP [62]. B cell lymphoma 2 (Bcl-2), situated on the IM, is believed to be pivotal in maintaining MMP. Cytochrome c, specific procaspases, and the apoptosis-inducing factor are found in the space between the membranes. The disruption of mitochondrial membranes leads to the release of proapoptotic molecules into the cytosol. The liberation of cytochrome c triggers the assembly of apoptotic protease-activating factor-1 and procaspase-9, culminating in the formation of an apoptosome [55,61]. Procaspase-9 undergoes dimerization and activation, subsequently activating executioner caspases to orchestrate apoptosis. In the cytosol, cytochrome c binds to apoptotic protease-activating factor-1, a protein that forms the apoptosome, a complex that activates caspase-9, an enzyme that initiates the apoptotic cascade [62]. Cytochrome c and the mitochondria are essential for apoptosis, which is triggered by various signals that indicate something is wrong with the cell, such as DNA damage, infection, or a lack of growth factors [63–65]. Thymax's anticancer effect is supported by other studies that demonstrated its therapeutic effects *in vitro* and *in vivo* [66]. Thymax induced apoptosis through a mitochondrial pathway *in vitro* in human breast cancer cells and activated [67] human monocyte-derived DCs, and *in vivo*, it restored the age-related decline of immune function in mice [68].

The impact of caspase 8

In the current study, a reduction in the quantities of caspases 8 in tumor homogenate was observed in the untreated rats in comparison to the control group. Rats administered with Thymax exhibited elevated quantities of caspases 8, specifically in the injected rats ($P \leq 0.05$, 0.01) in contrast with the tumor rats

Table 1 Cytochrome c and caspase 8 concentrations in breast tissue homogenate in normal and tumor-bearing animals and the therapeutic effect of Thymax

Groups	Parameters					
	Cytochrome c (pg/ml)	Significance relative to normal	Significance relative to tumor	Caspase 8 (ng/ml)	Significance relative to normal	Significance relative to tumor
G1 (normal) Negative control	4.63±0.63	–	###	2.30±0.22	–	NS
G2 (tumor) Positive control	2.30±0.22	***	–	1.90±0.12	NS	–
G3 Thymax oral	5.46±0.35	*	#	3.12±0.32	***	#
G4 Thymax injection	7.90±0.26	*	###	4.02±0.38	*	##
G5 Thymax mix (oral and injection)	5.67±0.43	*	##	3.17±0.32	NS	#

Data are presented as mean±SEM (N=8). Significance relative to normal: *P value less than or equal to 0.05, **P value less than or equal to 0.01, ***P value less than or equal to 0.001; significance relative to tumor, #P value less than or equal to 0.05, ##P value less than or equal to 0.01, ###P value less than or equal to 0.001, NS: P value more than 0.05.

(Table 1). Caspase-8, a protein that initiates apoptosis in response to various signals received by the cell, plays a crucial role in eliminating damaged or unwanted cells and preventing the formation of cancer. Recent studies conducted by Elwakkad *et al.* [69] have illustrated that the administration of Thymax, a substance derived from the thymus, leads to a reduction in the MMP, activation of caspase-3, and an increase in the Bax/Bcl-2 ratio within neoplastic cells. These findings indicate that through an intrinsic pathway, Thymax induces apoptosis [70]. The death receptor pathway for apoptosis was activated, specifically; the FADD protein binds to a comparable domain in procaspase 8 via its death effector domain motif. Upon FADD recruitment of procaspase 8, the resulting complex of Fas, FADD, and procaspase 8 is referred to as the death-inducing signaling complex. Activation of procaspase 8 occurs through self-cleavage upon cluster formation [71]. Subsequently, active caspase 8 triggers the activation of downstream caspases 3 and 7, culminating in cellular apoptosis. Earlier in vitro studies have also shown that Thymax makes caspases 8 and 9 active in human breast cancer cells of the Michigan Cancer Foundation-7 type, suggesting that Thymax-induced apoptosis may involve both caspase-dependent and caspase-independent pathways [72].

The impact of P53

In the existing findings, the apoptotic P53 of untreated tumor-bearing rats exhibits a lower level compared to the normal control. Conversely, in tumor-bearing treated animals, there is a significant increase in P53 expression following Thymax therapy ($P \leq 0.05$), which is observed in both the injection and mix groups (Table 2). Results align with others who illustrated that P53 is a factor that controls the expression of genes and starts important processes to control the damage and prevent abnormal cell growth when there is damage to the DNA, activation of oncogenes, low oxygen levels, or loss of normal cell contacts [73,74]. It regulates cellular growth by inducing senescence, cell cycle arrest (at G1 and/or G2 phase), or apoptosis [75]. The decision is influenced by several factors, such as how much p53 is expressed, what kind of stress signal is there, what kind of cell it is, and what is the situation of the cell when it is exposed to stress [76]. Through apoptosis, p53 have the ability to eliminate excessive, damaged, or infected cells, which is crucial for the proper regulation of cell proliferation in multicellular organisms [77,78]. P53 is activated by both external and internal stress signals, leading to its accumulation in the nucleus in an active form. Consequently, p53 induces either arrest of viable cell growth or apoptosis, which plays a crucial role in tumor suppression. The growth inhibitory actions of p53 prevent the proliferation of cells with damaged DNA or with a potential for neoplastic transformation. Additionally, p53 contributes to cellular processes such as differentiation, DNA repair, and angiogenesis, which also seem important for tumor suppression [79].

The impact of tumor necrosis factor receptor 1

Returning to our results, the amount of TNFR1 in the homogenate of rats with tumor is significantly lower than that of the normal rats, as shown by Table 3 ($P \leq 0.01$, 0.001). In contrast, following Thymax therapy, there is a notable increase in TNFR1 expression when compared to the positive control group ($P \leq 0.05$, 0.01, and 0.001). These findings corroborated our results, which revealed that TNFR1 is a cell surface receptor that binds to tumor necrosis factor-alpha (TNF-α), a proinflammatory cytokine that has diverse effects on cell survival and death. Through the extrinsic and the intrinsic pathways, TNFR1 can trigger apoptosis [80]. Various factors can change both pathways, such as Bcl-2 family proteins, nuclear factor kappa B (NF-κB), and

Table 2 Fas-associated death domain and P53 concentrations in breast tissue homogenate in normal and tumor-bearing animals and the therapeutic effect of Thymax

Groups	Parameters					
	FADD (ng/ml)	Significance relative to normal	Significance relative to tumor	P53 (μg/l)	Significance relative to normal	Significance relative to tumor
G1 (normal) Negative control	0.633±0.05	–	NS	306.5±0.36	–	NS
G2 (tumor) Positive control	0.518±0.03	NS	–	290.0±16.0	NS	–
G3 Thymax oral	1.49±0.13	**	##	303.9±2.9	NS	NS
G4 Thymax injection	2.90±0.21	***	###	328.0±1.8	***	#
G5 Thymax mix (oral and injection)	2.16±0.12	***	###	317.5±1.2	***	#

FADD, Fas-associated death domain. Significance relative to normal: *P value less than or equal to 0.05, **P value less than or equal to 0.01, and***P value less than or equal to 0.001; significance relative to tumor: #P value less than or equal to 0.05, ##P value less than or equal to 0.01, and ###P value less than or equal to 0.001 and NS: P value more than 0.05.

Table 3 Tumor necrosis factor receptor 11 and tumor necrosis factor receptor type 1-associated death domain protein concentrations in breast tissue homogenate in normal and tumor- bearing animals and the therapeutic effect of Thymax

Groups	Parameters					
	TNFR1 (ng/ml)	Significance relative to normal	Significance relative to tumor	TRADD (pg/ml)	Significance relative to normal	Significance relative to tumor
G1 (normal) Negative control	179.7±8.3	–	NS	103.8±1.1	–	NS
G2 (tumor) Positive control	157.5±15.5	NS	–	100.7±0.6	NS	–
G3 Thymax oral	167.2±7.7	NS	NS	125.0±3.4	**	#
G4 Thymax injection	309.2±23.6	*	#	183.0±3.4	***	###
G5 Thymax mix (oral and injection)	238.1±6.8	*	#	141.3±5.9	**	##

Data are presented as mean±SEM (*N*=8). TNFR1, tumor necrosis factor receptor 1; TRADD, tumor necrosis factor receptor type 1-associated death domain protein. Significance relative to normal: **P* value less than or equal to 0.05, ***P* value less than or equal to 0.01, and****P* value less than or equal to 0.001; significance relative to tumor: #*P* value less than or equal to 0.05, ##*P* value less than or equal to 0.01, and ###*P* value less than or equal to 0.001 and NS: *P* value more than 0.05.

reactive oxygen species (ROS) [81]. Thymax and theophylline have been shown to upregulate TNFR1 expression and sensitize breast cancer cells to TNF-α-mediated apoptosis [82,83]. On the other hand, TNFR1 can also promote cell survival and proliferation in breast cancer cells by activating NF-κB and other signaling pathways. Therefore, TNFR1 is a potential target for breast cancer therapy, as modulating its expression or activity may enhance the efficacy of conventional treatments or reduce the risk of recurrence and metastasis [84,85].

The impact of FADD and TRADD

Table 2 demonstrates that the level of FADD in the homogenized tumor tissue of rats with only a tumor is lower than that in the homogenized normal control tissue. In contrast to animals with tumors, the expression of FADD significantly increased up to sixfold following Thymax therapy ($P \leq 0.05$) and was clearly observed in both the injected and mixed groups. The current results agree with other studies that found that TRADD, also known as tumor necrosis factor receptor superfamily member 1A-associated death domain protein, assumes a pivotal role in the regulation of apoptosis by participating in both the extrinsic and intrinsic pathways of cell death [86]. In the context of breast cancer, TRADD has been implicated in the promotion of cell survival and resistance to apoptosis. In breast cancer cells, the overexpression of TRADD may contribute to the progression of tumors and the resistance to chemotherapy, thereby fostering increased cell proliferation, diminished apoptosis, and heightened cell survival [87]. Consequently, the targeting of TRADD signaling pathways has emerged as a prospective therapeutic strategy for breast cancer, since the inhibition of TRADD has the potential to sensitize breast cancer cells to apoptosis-inducing agents and augment the response to treatment [58,88–90]. TRADD functions as a protein that facilitates the transmission of signals from the TNFR1, known as a death receptor, which in turn triggers the process of apoptosis in response to the presence of TNF-α. The role of TRADD (Table 3) in breast cancer cell apoptosis is twofold; contingent upon its specific subcellular localization and the partners with whom it interacts with Hsu *et al.* [91]. On the one hand, TRADD is capable of promoting apoptosis by recruiting FADD and caspase-8 to TNFR1, thereby forming a complex that activates the extrinsic apoptotic

Table 4 Serum alanine aminotransferase and aspartate aminotransferase activities in normal and tumor-bearing animals and the therapeutic effect of Thymax

Groups	Parameters					
	ALT (U/l)	Significance relative to normal	Significance relative to tumor	AST (U/l)	Significance relative to normal	Significance relative to tumor
G1 (normal) Negative control	246.3±19.8	–	###	284.9±2.3	–	###
G2 (tumor) Positive control	406.3±32.6	***	–	404.6±2.7	***	–
G3 Thymax oral	244.5±34.3	NS	NS	213.3±3.3	*	###
G4 Thymax injection	149.5±14.2	*	##	191.6±3.7	***	###
G5 Thymax mix (oral and injection)	180.8±38.7	NS	#	201.8±1.7	***	###

Data are presented as mean±SEM (*N*=8). Significance relative to normal: **P* value less than or equal to 0.05, ***P* value less than or equal to 0.01, and****P* value less than or equal to 0.001; significance relative to tumor: #*P* value less than or equal to 0.05, ##*P* value less than or equal to 0.01, and ###*P* value less than or equal to 0.001 and NS: *P* value more than 0.05.

Table 5 Serum urea and creatinine concentrations in normal and tumor- bearing animals and the therapeutic effect of Thymax

Parameters Groups	Urea (mg/dl)	Significance relative to normal	Significance relative to tumor	Creatinine (mg/dl)	Significance relative to normal	Significance relative to tumor
G1 (normal) Negative control	48.92±1.0	–	###	0.166±0.04	–	#
G2 (tumor) Positive control	69.82±1.1	***	–	0.524±0.07	*	–
G3 Thymax oral	51.5±0.5	*	###	0.262±0.02	NS	#
G4 Thymax injection	48.9±1.0	*	###	0.102±0.04	NS	###
G5 Thymax mix (oral and injection)	56.9±0.7	*	###	0.192±0.03	NS	##

Data are presented as mean±SEM (N=8). Significance relative to normal: *P value less than or equal to 0.05, **P value less than or equal to 0.01, and***P value less than or equal to 0.001; significance relative to tumor: #P value less than or equal to 0.05, ##P value less than or equal to 0.01, and ###P value less than or equal to 0.001 and NS: P value more than 0.05.

pathway. On the other hand, TRADD can impede apoptosis by binding to TRAF2 and receptor-interacting protein, proteins that activate the NF-κB pathway and confer resistance to TNF-induced cell death [92]. Furthermore, TRADD can traverse between the cytoplasm and the nucleus, with its nuclear localization being correlated with heightened sensitivity to TNF-α and augmented apoptosis. Additionally, nuclear TRADD can establish interactions with p53, a tumor suppressor protein that oversees the intrinsic apoptotic pathway. Hence, TRADD emerges as a pivotal regulator of apoptosis in breast cancer cells, and manipulating its activity may have therapeutic implications [93].

Thymax reduces oxidative stress and protects liver and kidney functions

We did tests to see if Thymax therapy had any bad effects on internal organs (liver and kidney) and the balance of oxidation and reduction in the animals. In Tables 4 and 5 the levels of serum ALT, AST, urea, and creatinine were considerably elevated (P≤0.001) in animals with tumors in comparison to normal or untreated animals. In animals treated with Thymax, the parameters pertaining to liver and kidney function, which are associated with animals with tumors, were restored. Animals injected with Thymax demonstrated the most impressive efficacy in significantly reducing the levels of serum ALT, AST, urea, and creatinine (P≤0.001). In comparison to the normal control group, animals with tumors exhibited a significant increase in MDA levels (P≤0.001). The administration of Thymax to these animals effectively ameliorated and reduced MDA levels (P≤0.001) in a noticeable manner when compared to the tumor-bearing animals. Conversely animals with breast cancer displayed a remarkable decrease in total antioxidant levels, thus compromising their immune system (P≤0.05) relative to the normal rats. However, Thymax therapy resulted in a significant enhancement of the immune system, leading to a notable elevation (P≤0.05) in the total antioxidant levels when compared to the animals with breast cancer or the positive control group (Table 6). Our results corroborated other findings that revealed that the harmful effects of DMBA on hepatic cells and kidneys are consistent with Hendi *et al.* [94] and Chen *et al.* [95]. DMBA enzymatic activity induces oxidative stress by generating ROS and enhancing the amount of lipid peroxidation, and this oxidative stress is important in carcinogenesis [96–98], explaining the depletion of

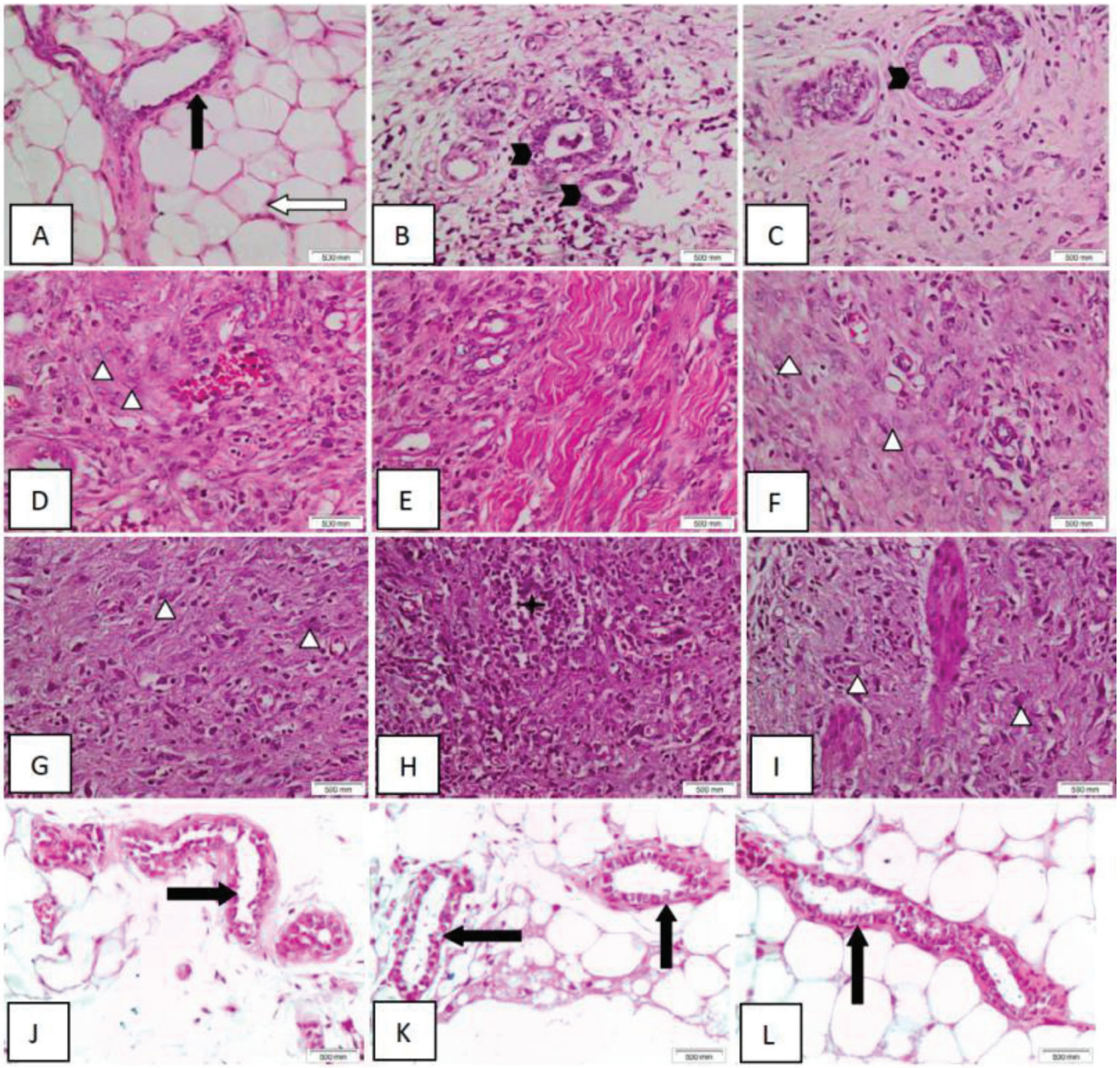
Table 6 Malondialdehyde and total antioxidant levels in breast tissue homogenate in normal and tumor-bearing animals and the therapeutic effect of Thymax

Groups	Parameters					
	MDA (nmol/g)	Significance relative to normal	Significance relative to tumor	Total antioxidant (U/ml)	Significance relative to normal	Significance relative to tumor
G1 (normal) Negative control	6.80±0.04	–	###	11.56±0.4	–	##
G2 (tumor) Positive control	16.5±0.05	***	–	7.92±0.2	**	–
G3 Thymax oral	7.04±0.4	NS	###	10.6±0.3	NS	###
G4 Thymax injection	4.25±0.5	*	###	12.44±0.2	NS	###
G5 Thymax mix (oral and injection)	5.67±0.6	NS	###	11.4±0.3	NS	###

Data are presented as mean±SEM (N=8). MDA, malondialdehyde. Significance relative to normal: *P value less than or equal to 0.05, **P value less than or equal to 0.01, and***P value less than or equal to 0.001; significance relative to tumor: #P value less than or equal to 0.05, ##P value less than or equal to 0.01, and ###P value less than or equal to 0.001 and NS: P value more than 0.05.

total antioxidants in tumor-bearing animals. While there was a considerable increase in LPO in rats with breast cancer, antioxidants may protect against cancer by directly or indirectly scavenging free radicals and ROS [99,100]. Liver toxicity can be caused by many substances, such as alcohol, carbon tetrachloride, chemotherapeutic agents, and acetaminophen [101–104]. These substances can trigger oxidative stress, which produces ROS. ROS are very harmful and can damage cell components like lipids, proteins, DNA, and RNA. The body has a cellular antioxidant system that protects cells from ROS damage. This system consists of low-molecular-mass antioxidants [105], such as glutathione, α-tocopherol, and ascorbic acid, and the main antioxidant enzymes, such as copper-containing and zinc-containing superoxide dismutase (CuZn-SOD) and catalase. When there are more oxidants than antioxidants in

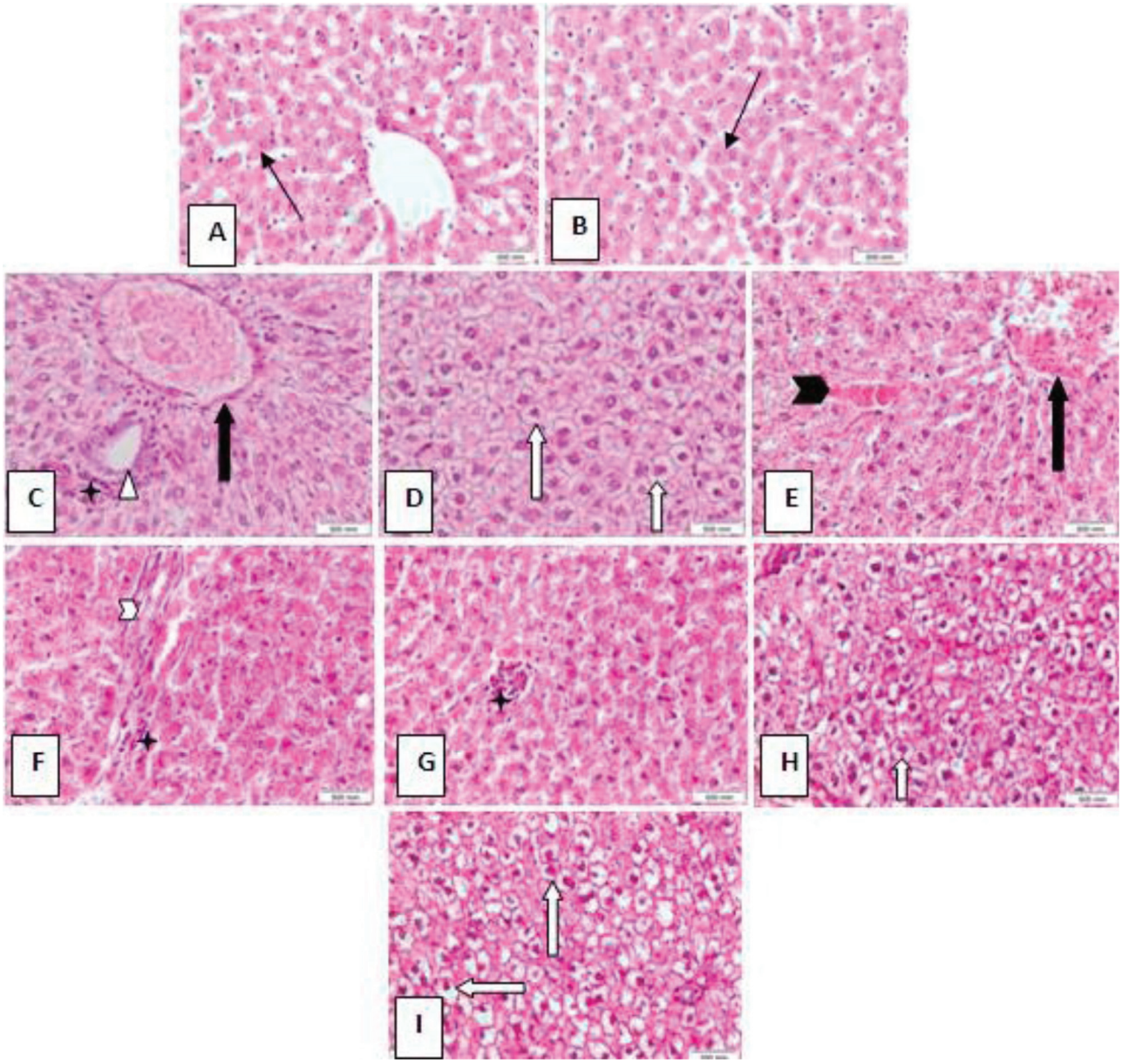
Figure 2



Mammary gland tissue of (a) control female rats showing ordinary benign mammary duct lined by benign double epithelium and myoepithelium (thick black arrow), within background of fat cells (white arrow). (b–f) DMBA-injected rats showing: (b, c) DCIS (black arrowheads). (d, f) Pleomorphic, fusiform tumorous cell proliferation with atypical nuclei (white triangles). (e) Fusiform tumorous cell proliferation at left half of the picture and invading in between muscle bundles at the right half. (g–i) Thymax oral treated rats showing: (g) residual tumorous proliferation formed of fusiform cells with pleomorphic, atypical nuclei (white triangles). (h)Aggregates of inflammatory cells amidst residual tumor cells (black star). (i) Residual tumor cells invading in between muscle fibers (white triangles). (j) Thymax injected rats showing benign mammary ducts (thick black arrow). No tumor. (k, l) Thymax oral and injected rats showing no tumor with mammary ducts lined by benign, double epithelial, and myoepithelial cells (thick black arrows) approximating control (hematoxylin and eosin, ×200). DCIS, ductal carcinoma in situ; DMBA, 7,12-Dimethylbenz(a)anthracene.

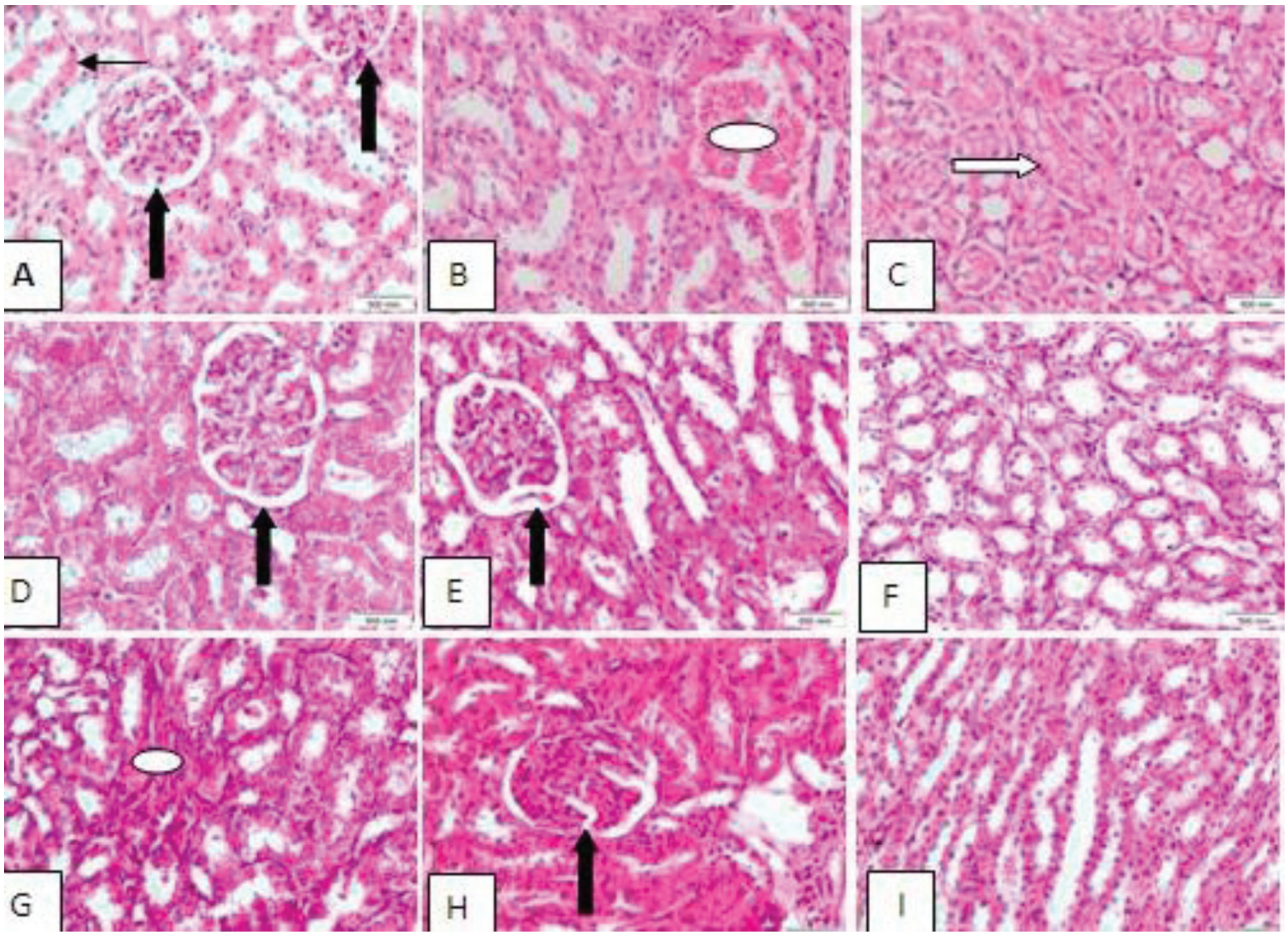
the liver, oxidative stress occurs. This can alter many antioxidant enzymes and nonenzymatic antioxidants [106–108]. GSH, the main nonenzymatic antioxidant defence, is regenerated by an NADPH-dependent reaction. GSH has a crucial role in attenuating oxidative stress by scavenging hydroxyl radicals, inhibiting LPO and eliminating H_2O_2 [109]. Moreover GSH depletion may impair cell defence against free radical-mediated cellular damage and cause cell death [110,111]. Lipid peroxide, which is related to free radicals [112], is one of them. MDA is a major product of lipid peroxide breakdown. The current study demonstrated that Thymax therapy coadministration increased antioxidant enzyme activities and GSH levels while decreasing LPO products. This suggests that Thymax enhanced the

Figure 3



Liver tissue sections of (a, b) control rat showing ordinary liver cells (thin black arrows) arranged in single cell thick plates, radiating from central vein. (c, d) DMBA-injected rats showed (c) dilated congested central vein (thick black arrow) with proliferating bile duct (white triangle) and surrounding inflammatory cell aggregates (black star). (d) Hydropic degeneration within hepatocytes (white arrows). (e–g) DMBA-injected rats treated with oral Thymax showed: (e) dilated congested central vein (thick black arrow) and congestion within sinusoids (black arrow head). (f) fibrotic bands within hepatic lobules (white arrow head) and adjacent inflammatory cell aggregates (black star). (g) Inflammatory cell aggregates within lobular hepatocytes (black star) together with numerous ordinary liver cells. (h) DMBA-injected rats treated with injected Thymax showed residual hydropic degeneration within hepatocytes (white arrow). (i) DMBA injected rats treated with combined oral and injection Thymax showed residual hydropic degeneration (white arrows) within liver cells (hematoxylin and eosin, ×200). DMBA, 7,12-Dimethylbenz(a)anthracene.

Figure 4



Kidney tissue sections of (a) control rat showing ordinary glomeruli with average cellularity (thick black arrows) surrounded by tubules lined by cuboidal epithelium (thin black arrows). (b, c) DMBA-injected rats showing: (b) congestion within interstitial tissues (white oval shapes) and (c) vacuolar degeneration within epithelium lining tubules (white arrows). (d) DMBA injected rats treated with oral Thymax showing ordinary glomerulus with average cellularity (thick black arrow) and surrounding ordinary tubules. (e–g) DMBA injected rats treated with injected Thymax showing: (e) averagely cellular glomerulus (thick black arrow), (f) ordinary tubules, and (g) congestion within renal interstitium (white oval shape). (h, i) DMBA injected rats treated with combined oral and injection Thymax showed: (h) averagely cellular glomerulus (thick black arrow) and (i) ordinary tubules (hematoxylin and eosin, ×200). DMBA, 7,12-Dimethylbenz(a)anthracene.

total antioxidant activity and prevented the peroxidation of membrane lipids by scavenging free radicals.

Histopathological alterations

Tissue sections of control rat mammary gland showed mammary acini and ducts lined by benign double luminal epithelial and basal myoepithelial cell layers. Surrounding it, thin eosinophilic connective tissue was seen. The stroma consisted of mature fat cells (Figs 1 and 2a). Tissue sections of DMBA-injected rat mammary glands showed tumor formation in all rats in the group, varying between ductal carcinoma in situ and invasive malignant tumor. Ductal carcinoma in situ showed ducts lined by flat malignant epithelial cells (Fig. 2b and c) with open lumina (clinging pattern). An invasive malignant tumor showed markedly cellular and pleomorphic, atypical fusiform cell proliferation with several mitoses, some being abnormal (Fig. 2d–f). Atypical cells were seen infiltrating in between muscle fibers (Fig. 2e). Tissue sections from mammary glands of rats treated with oral Thymax showed residual tumor tissue formed of proliferating fusiform cells with pleomorphic and atypical nuclei invading in between muscle fibers (Fig. 2g–i). Tissue sections from mammary glands of rats injected with Thymax treatment showed no tumor tissue. Ordinary mammary ducts and acini were seen with benign double epithelial and myoepithelial cell lining, which approximated control (Fig. 2j). No tumor, no atypia. Mammary tissue sections of rats treated with combined oral and injected Thymax showed no tumor tissue. Benign mammary ducts and acini lined by benign regular inner epithelial and outer myoepithelial cells were observed, similar to control (Fig. 2k and l). No tumor, no atypia.

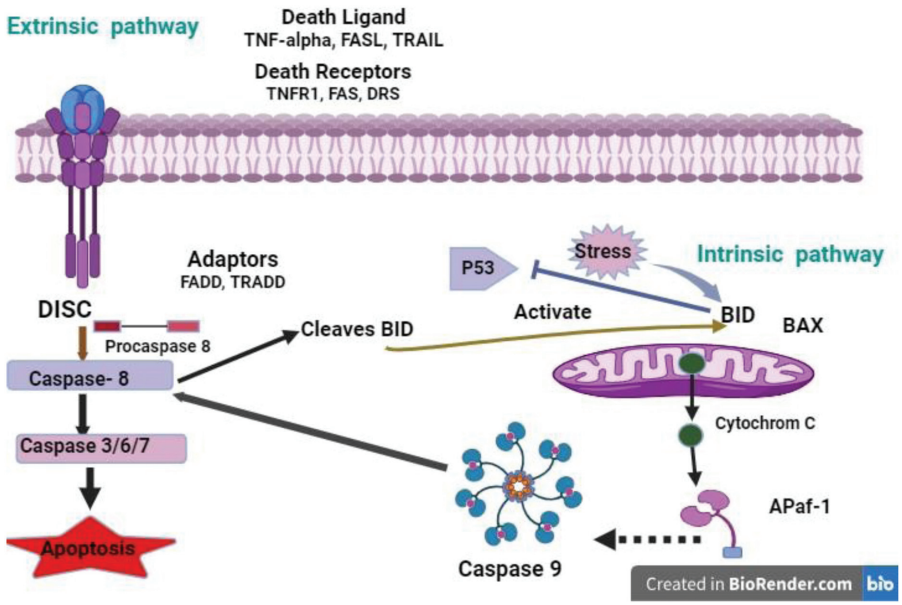
Liver tissue sections of the control rat (Fig. 3a and b) show ordinary liver cells arranged in single-cell thick plates, radiating from the central vein. DMBA-injected rats showed C: dilated, congested central vein with proliferating bile duct and surrounding inflammatory cell aggregates (Fig. 3c, d) and hydropic degeneration within hepatocytes (Fig. 3d). DMBA-injected rats treated with oral Thymax showed (Fig. 3e–g) dilated congested central veins and congestion within sinusoids. Fibrotic bands within hepatic lobules and adjacent inflammatory cell aggregates (Fig. 3f). Inflammatory cell aggregates within lobular hepatocytes, together with numerous ordinary liver cells (Fig. 3g). DMBA-injected rats treated with injected Thymax showed residual hydropic degeneration within hepatocytes (Fig. 3h). Residual hydropic degeneration within liver cells is shown in the Thymax mix group (Fig. 3i).

Kidney tissue sections of the control rat showed ordinary glomeruli with average cellularity surrounded by renal tubules with low cuboidal regular epithelial lining (Figs 4a and Fig. 55). DMBA-injected rats showed congestion within the renal interstitium (Fig. 3b) and vacuolar degeneration within surrounding tubules (Fig. 3c). Oral Thymax rats displayed a picture approximating control. Ordinary glomeruli with average cellularity were noticed. Surrounding ordinary tubules showed low cuboidal epithelial linings (Fig. 3d). Kidney tissue sections of Thymax-injected animals showed averagely cellular glomeruli and surrounding ordinary tubules, showing low cuboidal epithelial lining. Our results are in harmony with those of other researchers who found that DMBA is a carcinogenic and immunosuppressive hydrocarbon agent used to induce tumors. Its mechanism involves enhancing the formation of prostaglandin E2, which promotes breast cancer spread and leads to mutations and cellular antioxidant imbalance [113]. DMBA acts on cancer-causing genes, initiating mutations and causing breast cancer, and it also has immunosuppressive effects [69,114]. By generating ROS and peroxides, DMBA increases oxidative stress, a key factor in carcinogenesis [115]. The histopathological examination of the mammary gland tissue, liver, and kidney showed normal or near-normal congestion within the renal interstitium (Fig. 3e, f, and g). In DMBA-injected rats treated with combined oral and intravenous injection, Thymax tissue showed average cellular glomeruli surrounded by ordinary tubules (Fig. 3h and i). The present findings showed that the ability of Thymax to induce apoptosis is greater in the injected and mixed route compared with the oral one. Thymax and its impact on liver and kidney functions show its capability to mitigate the alterations induced by DMBA, according to our novel study.

Conclusion

Our study concluded that Thymax could be a novel type of adjuvant for breast cancer therapy, as it can trigger apoptosis in cancer cells without harming normal cells. However, more studies are required to

Figure 5



Extrinsic and intrinsic mechanisms of apoptosis.

verify the effectiveness and safety of Thymax in living organisms and in human trials.

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Authors' contributions: A.E. designed the study, participated in the practical part, and performed the statistical study and revised the manuscript in the final form for publication. A.A.G.E.D. performed the histopathological studies. H.A.S. is the corresponding author who prepared Thymax treatment, participated in samples collection, preparing the tissue homogenates, performing the tests for parameters, view the results, writing the manuscript, and prepared it in the final form for publication. M.H.A. implemented the induction of breast cancer in animals and followed up with the animals all over the treatment period, participated in sample collection, prepared the tissue homogenates, and performed the tests for parameters. All authors read and approved the submitted version.

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Conflicts of interest

There are no conflicts of interest.

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