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Gallic acid mitigates apoptotic cell death induced by the anticancer drug tamoxifen in female rats

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Abstract

Tamoxifen (TAM),the anti-estrogenic agentwas used for advanced breast cancer management. Despite its benefits, a number of clinical reports have demonstrated many side effects. Therefore, this study was designed to assess the possible hepatoprotective effects of Gallic acid (GA) against TAM-induced abnormalities in female rats. Fifty adult female *Sprague dawley*rats were divided into 5 groups. Group I: normal control, Group II: TAM group, Group III: GA group, Group IV:prophylactic group of GA, Group V:prophylactic group of silymarin. Blood and liver tissues were collected for biochemical, and histopathological studies. Group II; rats receiving TAM, showed significant increases in serum aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, E-cadherin, and Caspase-3 and decreases in albumin and Na^+/K^+ ATPase activity. The Prophylactic group of GA; group IV, significantly reduced the elevated levels of the previously mentioned biochemical markers, as well as increased the levels of serum albumin and liver Na^+/K^+ ATPase. Histopathology of liver and spleen also confirmed the protective effective of GA. The results confirm that administration of GA showed better results than that of the standard drug silymarin. Therefore, GA assumes to be a promising hepatoprotective agent that ameliorates the side effects induced by TAM therapy.

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Introduction

Despite persistent research effort to fight various diseases, cancer still remains as one of the biggest challenges for human beings. Worldwide, breast cancer is one of the most widespread carcinomas in females, resulting in more than 500,000 deaths with an estimated 1.6 million new cases every year. Two major approaches can be applied to overcome such problem. The first one is to develop new anticancer drugs and the second is to improve the efficacy of existing therapeutic agents. Therefore, various natural compounds have been extensively studied to prolong the half-life of drugs in circulation and minimize their side effects (Dorniani *et al.*, 2016). Estrogen receptor is considered as an important therapeutic target where more than 70% of breast cancer have a high expression level of the dominant form of estrogen receptors (ERs), (particularly ER-α and ER-β), and depend on the hormone to develop, therefore, they are considered as candidates for endocrine therapy. Diminishing the level of estrogen can directly moderate the development of cancer (Yamaguchi and Nakayama, 2017). These drugs are called selective estrogen receptor modulators (SERMs). SERMs are synthetic molecules which bind to estrogen receptors ER-α and ER-β that inhibit the activation of downstream genes and therefore, can modulate their transcriptional factors in various routes in diverse estrogen target tissues (Yang*et al.*, 2017b).

For almost three decades, tamoxifen (TAM), the pioneering selective ER modulator, has been used in routine clinical practice as the gold standard treatment for breast cancer and is proved to reduce the risk of breast cancer in high-risk women. As TAM is inexpensive and well-tolerated, it became the first line of adjuvant hormonal therapy (Davies *et al.*, 2013).TAM acts as an antagonist on ERs in breast tissue and blocks the effects of estrogen on proliferation of breast cancer cells by attaching to the estrogen receptors in breast cells. It shows ER agonist, antagonist or mixed activity in different tissues where it has estrogenic effects on bone, brain and liver, but antagonist activity on breast tissue (Wickramage *et al.*, 2017).

Although TAM therapy is clinically effective and has shown very good results in breast cancer treatment in the general population, more than 50% of advanced breast cancer can show insensitivity to the first-line TAM therapy. Also, approximately 30%-40% of the patients that receive TAM therapy experienced tumor relapse or progression (Chang *et al.*, 2011). Previous studies suggest that, five-year TAM therapy could improve the risk of its recurrence at least for 15 years, as well as decrease the mortality in 75%-80% in patients with ER positive breast cancer (Zembutsu *et al.*, 2017). Unfortunately, more than 30% of ER-positive breast cancer patients can frequently develop TAM-resistance after long-term treatment, and therefore, overcoming TAM-resistance with a novel therapeutic strategy is needed to treat breast cancer patients (Yang *et al.*, 2017a).

The recent studies have already reported the numerous long-term side effects of TAM, as patients are recommended to continue the treatment for more than 5 years. Several adverse effects such as hot flashes, night sweats, gynecological symptoms (vaginal dryness and vaginal discharge), depression, memory loss, sleep alterations, endometrial carcinoma, ocular problems, and thromboembolic disorders effects have been reported (Gao *et al.*, 2016).

Several studies have documented that TAM-induced hepatotoxicity. Some instances have been severe with signs of hepatic failure, but most cases are self-limited. Long term TAM therapy has been associated with clinically apparent liver injury, development of liver steatosis, cirrhosis, and rare instances of clinically apparent acute liver injury. These side effects are typically arising within the first six months of treatment and having variable presentations with cholestatic and elevation of liver enzymes (Suddek, 2014). The fatty liver is one of the most common side effects that found in the majority of the patients on TAM-based therapy within the first 2 years of starting TAM therapy as shown by routine imaging using computerized tomography but is usually not accompanied by symptoms, although serum transaminase levels may be elevated in up to half of patients.

These elevated levels of liver enzymes usually improve within 6 months after stopping TAM therapy, but the improvement may be slow and in some cases, symptoms of portal hypertension persist. So, monitoring of serum liver enzymes during long term TAM therapy is often recommended (Rabinowich and Shibolet, 2015).Several cases of liver cirrhosis have been described after therapy with TAM for 3 to 5 years. Long term TAM therapy has also been linked to several cases of hepatocellular carcinoma in women with no other risk factors for this tumor (Gao *et al.*, 2016).

Anticancer agents originated from natural sources represent a promising alternative to be used in the treatment of many types of cancers. Phytochemicals with antioxidant properties are frequently used in combination with conventional therapies because they are considered as natural and safe for the prevention and treatment of many diseases. Numerous active ingredients of herbal extracts have been found to be potent modulators of drug metabolism and the effects on human health may rely on concentrations of each individual component. Herb-drug interactions can occur via drug metabolizing enzymes and drug efflux transporters (Athukuri and Neerati, 2016).

Phenolic compounds are secondary metabolites of plants that widely distributed in fruits, vegetables and medicinal plants and have been suggested as potential therapeutic agents with different properties for numerous chronic diseases (Aglan *et al.*, 2017). Several studies have shown that natural compounds with antioxidant activity are effective in protecting the liver from oxidative damages besides up regulating a group of cytoprotective genes (Perazzoli *et al.*, 2017).

Gallic acid (3,4,5-trihydroxy benzoic acid, GA), a water soluble polyhydroxyl phenolic acid, is one of the most putative botanical phenolic compound, which is widely distributed in grapes, green tea, berries, mango and pomegranate. GA is found both free and a part of tannins as one of the major active compounds in galatotanin which has been shown a wide range of pharmacological activities.

Apart from its therapeutic characteristics, it also has a variety of applications in food industry as substrate for the chemical synthesis of food preservatives such as pyrogallol and gallates, dye synthesis, textile and leather industry and a chelating agent in cosmetics (Umakumar *et al.*, 2012).

In view of the above mentioned beneficial effects of GA, the aim of this study, therefore, was designed to evaluate the possible hepatoprotective effects of GA against TAM-induced hepatotoxicity and its anti-inflammatory potential on the liver of *Sprague dawley* female rats. As well as, to determine whether the administration of GA in combination with TAM is a better choice over TAM monotherapy to overcome its side effects.

Materials and methods

Chemicals

TAM and GA were purchased from Sigma-Aldrich Company (Los Angeles, USA). All the therapeutic agents were refrigerated in a desiccator to avoid oxidation and thermal decomposition. All other chemicals used in this study were of high-quality analytical grade. All commercial kits used in this study were purchased from Diamond diagnostics kits (Hannover, Germany), Gamma trade (San Antonio, Texas USA), CUSABIO (Baltimore, USA) and MyBioSource (San Diego, USA) companies.

Animals

Fifty adult female *Sprague dawley* rats weighing 160-180 g of the same age (6-8 weeks) obtained from the breeding unit of Misr University Science and Technology (Research unit of pharmacology and chemistry, Cairo, Egypt) were certified and used throughout this study. Animals were maintained under standard conditions of ventilation, temperature (25±2°C), humidity (50-70%) and light/dark condition (12/12hrs). The rats were housed maximum five in polycarbonate cages with wire mesh floors on wood-chip bedding in the breeding unit of the Medical Research Center (Faculty of Medicine, Ain Shams University, Cairo, Egypt).Animals provided with free access to drinking tap water that passed through activated charcoal filter and sterile standard

diet (purchased from the Egyptian company of oil and soaps) of standard composition containing all macro and micro nutrients. All animal experiments were performed according to the protocols approved by the local institutional animal ethics committee of Ain Shams University.

Experimental design

After one week of acclimatization, animals were randomly divided into 5 groups of 10 rats each. Group I (Control group): animals served as normal control. Group II (TAM group): animals treated with TAM (45 mg/kg b.wt./day; I.P.) dissolved in saline for 7 successive days (Hard *et al.*, 1993; El-Beshbishy, 2005). Group III (GA group): animals received GA (100 mg/kg b.wt./day; orally by gastric gavage) for 14 days (Yeh and Yen, 2006).

Group IV (Prophylactic group of GA): animals administered TAM (45 mg/kg b.wt./day; I.P.) dissolved in saline for 7 successive days parallel to GA (100 mg/kg b.wt./day; orally by gastric gavage) for 14 days.Group V (Prophylactic group of silymarin): animals administered TAM (45 mg/kg b.wt./day; I.P.) dissolved in saline for 7 successive days parallel to silymarin (100 mg/kg b.wt./day; orally by gastric gavage) (Raj *et al.*, 2010) for 14 days.

Blood and tissue collection

At the end of the experimental period, animals were sacrificed by cervical decapitation. Blood collected from all rats by heart punctured method. Blood was allowed to clot and then centrifuged. The separated serum was stored at -20°C for biochemical analysis. At necropsy, one part of livers and spleens were excised immediately, rinsed from blood thoroughly in ice-cold isotonic saline.

These parts were cut with a surgical blade, and fixed in 10% phosphate-buffered formalin for histological examination. The second part of liver tissues was dissected out immediately, rinsed with ice-cold phosphate buffer and kept for determination of ATPase activity.

Biochemical parameters

All biochemical analyses were quantified using the commercial kits. Serum alanine aminotransferase (ALT; EC 2.6.1.2), aspartate aminotransferase (AST; EC 2.6.1.1) and alkaline phosphatase (ALP; EC 3.1.3.1) activities were assayed by the colorimetric methods of Reitman and Frankel (1957) and Belfield and Goldberg (1971), respectively. Total protein contents (Gornall *et al.*, 1949) and albumin (Doumas *et al.*, 1971) were also determined.

Cell death markers

To investigate the apoptotic changes in TAM-induced hepatotoxicity, CUSABIO (Baltimore, USA) rat epithelial-cadherin ELISA kit (Cat. No: CSB-E07308R) was used to measure E-cadherin levels. As well as, CUSABIO rat Caspase-3 ELISA kit (Cat. No: CSB-E08857r) was used to measure Caspase-3 levels as per manufacturer's instructions. Quantification of sodium-potassium adenosine triphosphatase (Na^+/K^+ ATPase) activity in liver tissues was assayed using My BioSource (San Diego, USA) Na^+/K^+ ATPase Microplate Assay Kit (Cat. No: MBS8243226).

Histological examination

Fixed liver and spleen samples were embedded in paraffin cubes, sliced into serial 4 µm-thick sections, stained with hematoxylin and eosin (H&E), examined and photographed under a light microscope (Bancroft and Stevens, 1990).

Statistical analysis

Statistical analysis of the obtained data was carried out and analyzed by Statistical Package for Social Science (SPSS), version 23.0 for Windows (SPSS® Chicago, IL, USA) software program. All data were expressed descriptively as the mean ± standard deviation (SD) of replicate determinations for quantitative parametric data. Statistical analysis was performed using analysis of variance (ANOVA) to test for differences in means of variables between groups. The significance of results were ascertained at $P<0.05$.

Results

No mortality was seen in animals during the study. The dose of GA did not initiate any side effects for the animals. Whereas some clinical observations were noticed in animals treated with TAM, such as body weight loss and general weakness during the experimental period with the appearance of some histological changes at the necropsy such as small cyst with fatty hard mass in liver, dark liver margins and enlarged spleen (Fig. 1 & 2).

Table 1. Liver function tests among the studied groups.

Groups / Mean ± SD	Group I (Control)	Group II (TAM)	Group III (GA)	Group IV (GA + TAM)	Group V (Silymarin + TAM)
ALT (units/ml)	15.50 ± 1.91	33.50 ± 9.81[a]	19.50 ± 6.35[b]	15.00 ± 2.58[b]	18.25 ± 5.32[b]
AST (units/ml)	30.00 ± 1.63	79.00 ± 17.32[a]	32.00 ± 1.63[b]	36.50 ± 7.50[bc]	80.00 ± 17.36[a]
ALP (IU/L)	102.00 ± 1.63	351.00 ± 64.14[a]	127.50 ± 10.97[ab]	103.75 ± 14.54[bc]	253.50 ± 14.43[ab]
Total Proteins (g/dL)	8.30 ± 0.16	8.90 ± 0.69	8.50 ± 0.34	8.78 ± 0.35	8.60 ± 0.23
Albumin (g/dL)	5.03 ± 0.26	3.93 ± 0.13[a]	4.95 ± 0.33[b]	4.73 ± 0.31[b]	5.05 ± 0.06[b]

ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase.

Data are expressed as mean ± SD. *P* values were statistically measured by ANOVA test, a: *P* value < 0.05 when compared to control, b: *P* value < 0.05 when compared to group TAM group, c: *P* value < 0.05 when prophylactic group of GA compared to prophylactic group to silymarin.

Liver function tests

The data summarized in Table 1indicated that, AST, ALT and ALP activities were significantly increased ($P<0.001$) in rats receiving TAM when compared to the control group. For GA, the same parameters showed normal levels with non-significant changes in the activity of ALT and AST but a significant increase ($P<0.05$) in ALP were reported when compared to control.

Comparing to the severe liver damage that observed and statistically documented in TAM group, the liver enzymatic activities of prophylactic group treated with GA were significantly lowered (AST and ALT $P<0.05$ while ALP $P<0.001$). In addition, a marked significant recovery in AST ($P<0.05$) and ALP ($P<0.001$) enzymatic levels were observed in prophylactic group treated with GA when compared to the group protected with silymarin.

Table 2. Levels of some cell death parameters among the studied groups.

Groups / Mean ± SD	Group I (Control)	Group II (TAM)	Group III (GA)	Group IV (GA + TAM)	Group V (Silymarin + TAM)
E-Cadherin (ng/ml)	13.58 ± 0.81	30.18 ± 2.50[a]	12.93 ± 0.21[b]	20.11 ± 0.42[abc]	23.42 ± 1.17[ab]
Caspase-3 (ng/ml)	2.57 ± 0.02	7.49 ±0.41[a]	3.25 ± 0.23[ab]	4.31 ± 0.02[abc]	4.99 ± 0.38[ab]
ATPase (U/mg protein)	41.45 ± 0.82	28.59 ± 1.32[a]	39.38 ± 1.45[ab]	36.09 ± 0.48[abc]	34.02 ± 0.99[ab]

Data are expressed as mean ± SD. *P* values were statistically measured by ANOVA test, **a**: *P* value < 0.05 when compared to control, b: *P* value < 0.05 when compared to group TAM group, **c**: *P* value < 0.05 when prophylactic group of GA compared to prophylactic group to silymarin.

Furthermore, the hepatic total proteins content showed no significant changes between all studied groups. Meanwhile, the albumin levels were significantly decreased ($P<0.001$) in rats treated with TAM while GA showed a normal level when compared to control.

The alleviating effect of GA was noticed in prophylactic group through the significant increase ($P<0.001$) level of albumin when compared to TAM group. The same results were recorded for the prophylactic group of silymarin.

Cell death markers

Determination of serum E-cadherin

The results represented in Table 2 and Fig. 3revealed that the serum level of soluble E-cadherin was significantly increased (*P<0.001*) in rats receiving TAM either alone or combined with other therapeutic formulations (GA or silymarin) when compared to control. On contrary, E-cadherin level was significantly decreased in prophylactic groups (*P<0.001* for GA prophylactic group and *P<0.05* for silymarin prophylactic group) when compared to TAM group. A significant decrease (*P<0.05*) was observed in prophylactic group of GA when compared to that treated with silymarin. On the other hand, GA showed a significant decrease (*P<0.001*) of E-cadherin level when compared to TAM group.

Table 3. The severity of histopathological alterations in liver and spleen tissues of the studied groups.

Organ	Alterations \ Group	Group I (Control)	Group II (TAM)	Group III (GA)	Group IV (GA + TAM)	Group V (Silymarin + TAM)
Liver	Portal inflammatory reaction	-	+++	-	+	++
	Fibrosis in portal area	-	++	-	-	-
	Congestion	-	++	-	-	++
	Focal hemorrhage in parenchyma	-	++	-	+	-
	Focal necrosis	-	+	-	-	-
	Degeneration	-	++	-	++	+
Spleen	Congestion in red pulp	-	+++	-	++	-
	Lymphoid depletion in white pulp	-	+++	-	-	++

+++: Severe ++: Moderate +: Mild -: Nil

Determination of serum Caspase-3

The level of Caspase-3 was markedly increased *(P<0.001)* in all groups when compared to control, meanwhile co-administration of GA in prophylactic group showed an ameliorating effect by significantly decreasing the level of caspase-3 *(P<0.001)* when compared to TAM group. Additionally, administration of GA in TAM-intoxicated group showed powerful anti-apoptotic effect more than silymarin by significantly decreasing (*P<0.05)* the level of caspase-3 when compared to the group protected with silymarin (Table 2 & Fig. 3).

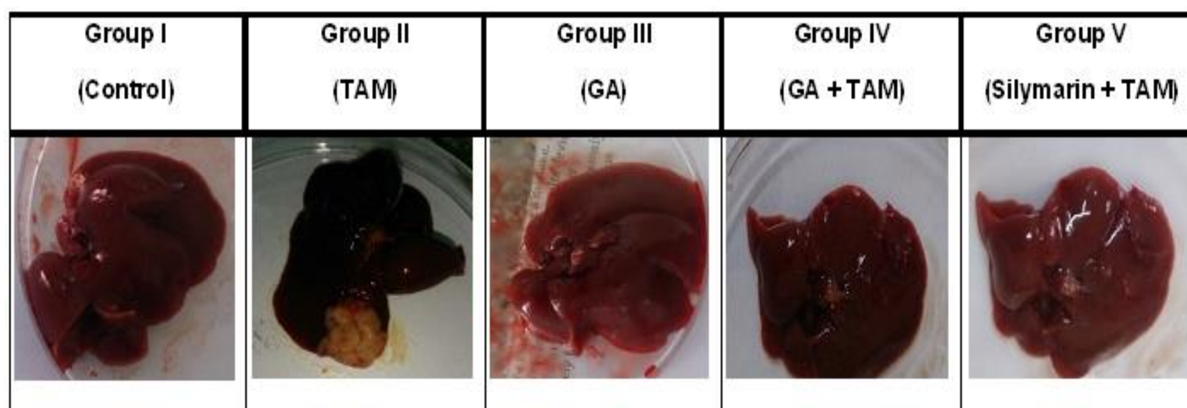


Fig. 1. Morphological alterations in liver among the studied groups.

Determination of Na^+/K^+ ATPase activity in liver tissues

Na^+/K^+ ATPase activity of liver tissues was significantly decreased *(P<0.001)* when TAM was administered either alone or combined with both prophylactic groups of GA and silymarin. However, the ATPase activity was significantly increased (*P<0.001)* in animals that were protected with GA or silymarin in prophylactic groups when compared to the TAM-intoxicated group.

Meanwhile, the prophylactic group of GA showed a significant increase (*P<0.05*) in the activity of ATPase enzyme when compared to the prophylactic group of silymarin. Conversely, the administration of GA alone showed significant higher level (*P<0.001)* of ATPase activity when compared to TAM group (Table 2 & Fig. 3).

Fig. 2. Morphological alterations in TAM spleen versus control.

Histopathological studies

Table 3 and Fig. 4 & 5 represent the alterations in liver and spleen histology among different groups. The liver sections of control animals showed no histopathological alteration with normal structure of the central vein and surrounding hepatocytes in the parenchyma (Fig. 4A & 4B).

The liver sections of TAM-treated animals showed sever congestion in the portal vein associated with inflammatory cells infiltration and fibrosis mainly surrounding the bile ducts. Focal necrosis with inflammatory cells infiltration was detected in the parenchyma (Fig. 4C). Also, there was focal hemorrhage in the parenchyma in association with fatty change in some individual hepatocytes (Fig. 4D). Liver of animals treated with GA showed normal hepatic architecture with no histopathological alteration and well-preserved cytoplasm, prominent nucleus with normal histological structure of the central vein and surrounding hepatocytes in the parenchyma (Fig. 4E & 4F).

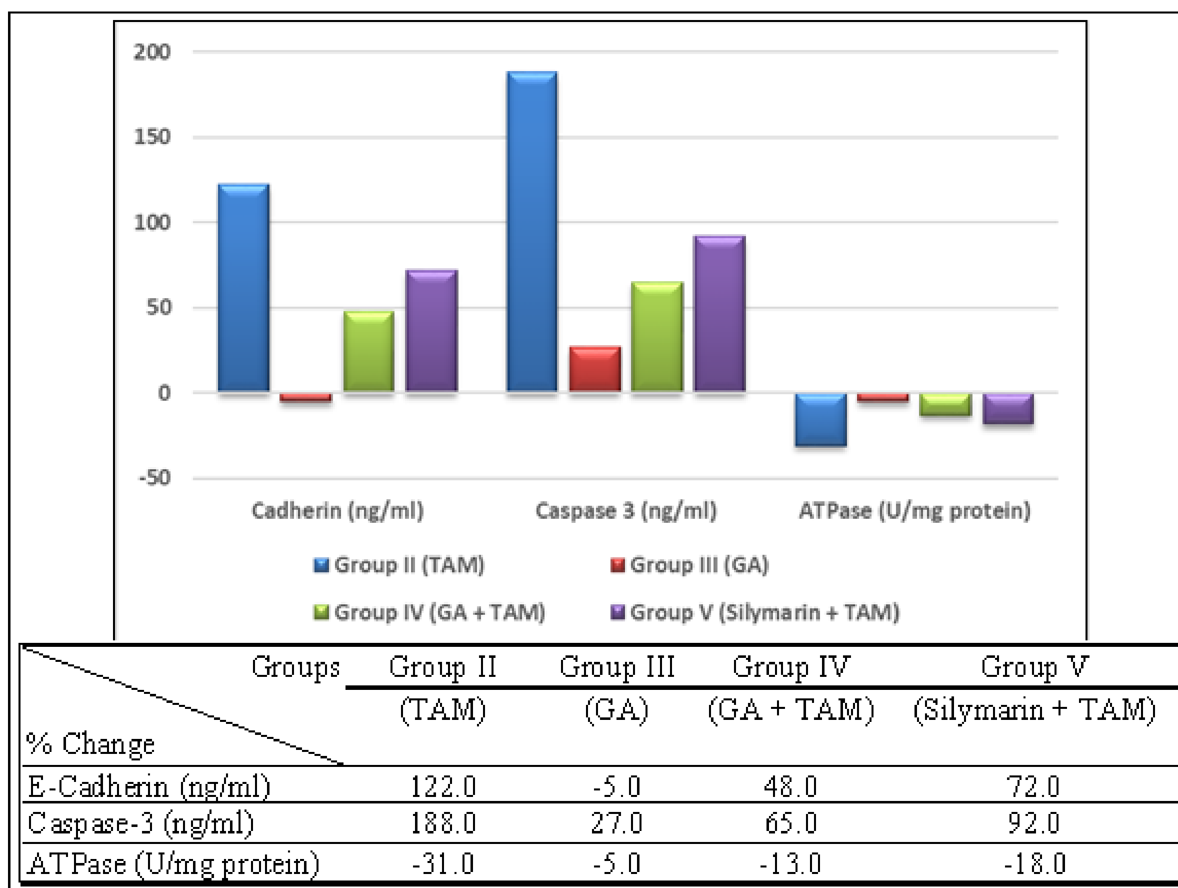
The liver samples of prophylactic group with GA showed few inflammatory cells infiltration in the portal area, as well as some fatty changes of the hepatocytes (Fig. 4G). Also, there was little focal hemorrhage in the parenchyma (Fig. 4H). On the other hand, inflammatory cells infiltration was detected in the periductal tissue surrounding the bile duct at the portal area associated with sever congestion in the portal vein were recorded in the prophylactic group of silymarin (Fig. 4I). Fatty change was detected in few hepatocytes associated with congestion in the sinusoids (Fig. 4J).

For spleen tissues of control group, there was no histopathological alteration and normal structure of the white and red pulps were recorded (Fig. 5A & 5B). Sever congestion was noticed in the red pulps with depletion in the lymphoid cells of the white pulps in spleen sections of TAM group (Fig. 5C & 5D). No histopathological alterations were recorded in GA spleen sections (Fig. 5E & 5F). There was mild congestion in the red pulps in spleen sections of prophylactic group treated with GA (Fig. 5G & 5H), while a depletion in the lymphoid cells at the central portion of the white pulps in the spleen tissue of prophylactic group treated with silymarin was observed(Fig. 5I & 5J).

Discussion

Liver is a vital organ that has astonishing metabolic and clearance functions to metabolize every drug or toxin introduced in the body. Regardless of the underlying cause, the prevalence of chronic liver disease is increasing globally. Despite its high regeneration capacity, prolonged hepatic damage may lead to severe inflammation and results in progressive fibrosis, cirrhosis, and eventually liver failure and hepatocellular carcinoma (Nallagangula *et al.*, 2018). It has been demonstrated that TAM is a potent hepatocarcinogen with both initiating and promoting activities to induce a formation of various types of neoplastic lesions in the livers. Previous studies documented that the hepatocarcinogenic activity of TAM in female rats is associated with its ability to induce both genotoxic and epigenetic abnormalities in the liver (Tryndyak *et al.*, 2006).

TAM and its N-desmethyltamoxifen derivative are metabolized in the liver by cytochrome P450-dependent monooxygenases, then subsequently esterified to reactive derivatives capable of interacting with DNA to cause the formation of deoxyguanosin-TAM and deoxyguanosin-N-desmethyltamoxifen DNA adducts (Kiyotani *et al.*, 2012). So, attempts have been made to enhance the performance of the drug and minimizing its adverse effects.



Groups / % Change	Group II (TAM)	Group III (GA)	Group IV (GA + TAM)	Group V (Silymarin + TAM)
E-Cadherin (ng/ml)	122.0	-5.0	48.0	72.0
Caspase-3 (ng/ml)	188.0	27.0	65.0	92.0
ATPase (U/mg protein)	-31.0	-5.0	-13.0	-18.0

Fig. 3. Percent change of the investigated cell death parameters in the studied groups.

The development of new molecules effective in treating or preventing hepatic damage remains a challenge in the field of drug development. Nowadays, medicinal plants are attracting attention due to their being low cost, highly safe, and their few side effects (Krishnappa *et al.*, 2014).

Recent studies have shown that GA possesses a wide range of biological activities such as anti-inflammatory, antioxidant and antitumor. GA also induced apoptosis in human cancer cell lines by regulation of mitochondria-dependent pathway, including Bcl-2, p53, and Bax (Perazzoli *et al.*, 2017).

Liver function tests

Hepatotoxicity is a significant complication of therapeutic drug use such in case of using anticancer drugs (Grigorian and O'Brien, 2014). The biochemical markers monitored in the liver are useful for assessment of tissue damage. The measurement of various enzymatic activities plays a significant role in disease investigation and diagnosis. The elevations of ALT and AST usually represent hepatocellular injury while elevations in ALP represent cholestasis. In the present study, TAM administration showed a highly significant increase in liver enzymes ALT, AST and ALP when compared to control which may be due to the leakage of these enzymes from the inflamed or damaged hepatocytes into the bloodstream (Suddek, 2014).

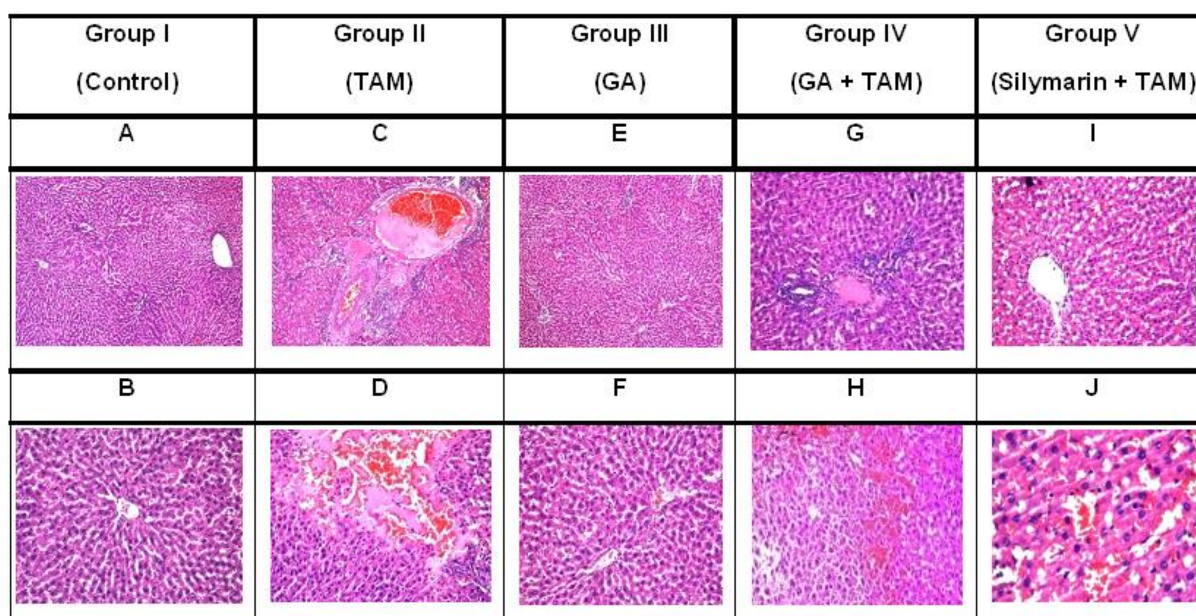


Fig. 4. Histological alterations of hematoxylin and eosin-stained liver tissue sections.
A (10X), B (40X); Control group. C (10X), D (40X); TAM group. E (10X), F (40X); GA group. G (40X), H (40X); Prophylactic group of GA + TAM. I (40X), J (80X); Prophylactic group of silymarin + TAM.

The present results also are in agreement with the recent study, which stated the possibility of abnormal liver function and risk of fatty liver development in patients on TAM therapy, where excessive accumulation of lipid in hepatocytes is the most common response of the liver to injury (Pan *et al.*, 2016). In this context, ALP is a marker enzyme, which is often employed to assess the integrity of plasma membrane. TAM administration in experimental animals of this study caused biochemical abnormality as seen by the elevation of ALP level. These results are on the same platform with the previous results that proved the alteration in hepatic function after TAM onset therapy, which characterized by observed elevations in ALP and GGT levels (Floren *et al.*, 1998). Also, some studies have referred to the occurrence of oxidative stress in hepatocytes as a result of TAM administration, accompanied by the formation of hydrogen peroxide (H_2O_2) and lipid degradation products, which may lead to the damage of hepatocellular cell membrane and consequently to the release of liver enzymes (Jatoba *et al.*, 2008). As stated by earlier studies, the increased ALP enzyme activity may be due to the increased functional activity of the tissues caused by the drug. Such increase in ALP activities can constitute threat to the life of cells that are dependent on a variety of phosphate esters for their vital process, since there may be indiscriminate hydrolysis of phosphate ester of the tissues (Yakubu *et al.*, 2006).

On the other hand, the hepatoprotective effects of GA against TAM-induced liver damage were examined in the present study. The results revealed the amelioration effect of GA in the prophylactic group, through normalization the levels of liver enzymes when compared to control group, with obvious decrement in the levels of all hepatic enzymes when compared to TAM group. These results are in the same line with recent studies (Karimi-Khouzani *et al.*, 2017) that showed significant ameliorations in abnormalities of fluoxetine-induced liver injury as represented by the improvement of hepatic enzymes after GA administration. The administration of GA as hepatoprotective agent showed more improvement in enzyme activities than that prophylactic group treated with the standard drug silymarin, therefore, GA assumes to be a promising hepatoprotective agent with particular importance to preserve liver function.
To further confirm the hepatoprotective effect of GA, the plasma total proteins content and albumin concentration were measured.

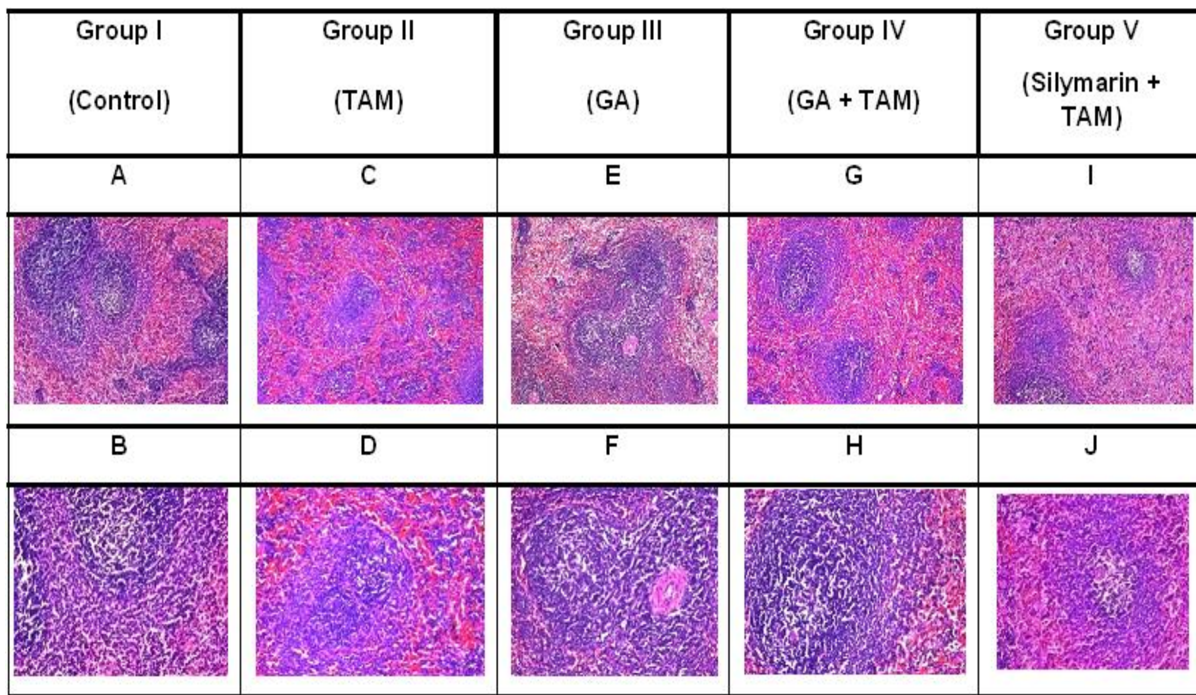


Fig. 5. Histological alterations of hematoxylin and eosin-stained spleen tissue sections.
A (10X), B (40X); Control group. C (10X), D (40X); TAM group. E (10X), F (40X); GA group. G (10X), H (40X); Prophylactic group of GA + TAM. I (10X), J (40X); Prophylactic group of silymarin + TAM.

The current results showed no significant changes in the protein and albumin content in GA group when compared to control group which suggest the hepatoprotective effect of GA against the toxic action promoted by TAM. Meanwhile, results showed no significant changes in total protein content with sharp decrease in albumin level in TAM-intoxicated group. Decreased plasma albumin levels in TAM group indicate liver failure when there is a widespread distortion of the normal hepatic architecture. These changes may be due to the capacity of TAM to induce oxidative stress (Abdel-Moneim *et al.*, 2015).

Cell death markers

E-cadherin

E-cadherin and other members of the cadherin family play a crucial role in establishing and maintaining the integrity of epithelial tissues. The majority of human cancers (80–90 %) are of epithelial origin (Sewpaul *et al.*, 2009). Loss of E-cadherin expression in epithelial cells leads to decreased adhesion and enhanced migration/invasion at the epithelial-to-mesenchymal transition (EMT) during late-stage tumorigenesis which characterized by invasive tumor growth and metastasis.

Moreover, studies in a transgenic mouse model of carcinogenesis have demonstrated that the loss of E-cadherin is a rate-limiting step in the transition from benign tumors to malignant tumors and the subsequent formation of metastases (Repetto *et al.*, 2014).

Soluble E-cadherin (sE-cadherin) is an 80 kDa peptide degradation product of the 120 kDa E-cadherin molecule which is generated by a calcium ion dependent proteolytic process. Matrix metalloproteinase, trypsin, kallikrein 7, and plasmin are examples of molecules that are capable of performing this proteolytic process (Symowicz *et al.*, 2007). The maintenance of epithelial membranes in healthy individuals involves a continuous turnover of E-cadherin where low levels of sE-cadherin are found in serum (Katayama *et al.*, 1994).

The current study revealed that TAM administration caused an increase in the E-cadherin level either alone or combined with GA or silymarin in the prophylactic groups. These increased levels may be due to the cleavage of membrane-bound E-cadherin by certain proteases (Sundfeldt *et al.*, 2001; Soyama *et al.*, 2008).

Further, the treatment with GA showed normal pattern of E-cadherin when compared to control group with significant ameliorations in abnormalities of TAM-induced liver injury as represented by the recovery of sE-cadherin status. This result suggests the role of GA in attenuation of tumorigenicity which may be due to down regulation of epithelial-mesenchymal transition (EMT)-related genes, such as E-cadherin, N-cadherin and vimentin and hence decrease the sE-cadherin levels (Jin *et al.*, 2017). On the other hand, another study (Guimaraes *et al.*, 2016) showed that GA can upregulate E-cadherin expression in oral squamous cell carcinoma which significantly reduces cell proliferation, invasion, and migration by increasing transmembrane E-cadherin, suggesting that GA might be a potential anticancer compound.

Caspase-3

Caspases are a family of protease enzymes that have proteolytic activity and are able to cleave proteins at aspartic acid residues. Once caspases are initially activated, there seems to be an irreversible obligation towards cell death. Caspase-3 is a general mediator of physiological and stress-induced apoptosis. It acts as an effector caspase in the end phase of apoptosis due to its ability to cleave a variety of substrates, including a molecule involved in DNA repair, DNA fragmentation factor, protein kinase C, and others. The cleavage of these substrates correlates with the morphological and biochemical changes seen in apoptotic cells (Susin *et al.*, 2000).

Although many studies suggested the antiproliferative effect of TAM through inducing the apoptosis in many different types of cell line such as MCF-7 human breast cancer cells (Perry *et al.*, 1995) and HeLa cell (Grenman *et al.*, 1988), but unfortunately many adverse effect on the normal hepatocytes were detected through determination of some cell death markers. The present work indorsed the apoptotic effect of TAM. This was observed by increasing the serum level of caspase-3 in all TAM groups, which emphasized the potential apoptotic effect of TAM as anticancer drug.

Likewise, recent studies (Han *et al.*, 2017) proved the capability of TAM to suppress breast cancer by inducing the apoptotic events relying on caspase-3/TRAIL activation. Also, TAM, a drug used in treating many types of cancer such as breast cancer and recently for brain tumors could induce apoptosis via caspase-3 activation in human glioblastoma cell lines (Zartman *et al.*, 2004).

The ameliorative effect of GA as anti-apoptotic agent was noticed through the decreasing level of caspase-3 in prophylactic group of TAM-induced hepatotoxicity. These results are in harmony with other studies (Liu *et al.*, 2016)which suggested that GA could suppress alveolar epithelial cell apoptosis in acute lung injury via inhibiting the STAT1-caspase-3/p21 associated pathway. The same findings were reported by another study (Kim *et al.*, 2012) that proved the efficacy of GA to counteract the ototoxicity by suppressing the activation of caspase-3/NF-kappaB and preventing the destruction of hair cell arrays in the organ of Corti. Another study (Mard *et al.*, 2015) documented the gastroprotective effect of GA on ischemia-reperfusion injury by lowering the caspase-3 as seen by decreasing the total area of acute gastric mucosal lesions. On the contrary, another studies (Sun *et al.*, 2016) documented the efficiency of GA to induce apoptosis to inhibit the growth of HepG2 and SMMC-7721 HCC cells which suggests the use of GA as a potential agent to be a novel compound for the treatment of HCC. Additionally, different concentrations of dodecyl gallate could induce apoptosis by up regulating the caspase-dependent apoptotic pathway and inhibiting the expression of anti-apoptotic Bcl-2 in different manners (Cheng *et al.*, 2016). The similar results were reported by earlier studies (Lo *et al.*, 2011)which indicated that GA could induce apoptosis in A375.S2 human melanoma cells, affect morphological changes, decrease the percentage of viable cells and induce apoptosis in a dose- and time-dependent manner.

Na^+/K^+ ATPase activity

Over and above, apoptosis requires energy, because it is a highly regulated process involving a number of ATP-dependent steps.

Moreover, depletion of cellular ATP was found to cause switching of the form of cell death, from apoptotic cell death to necrotic cell death (Oropesa-Avila *et al.*, 2013). Na^+/K^+ ATPase is an integral membrane-bound enzyme found in plasma membrane of all animal cells that maintains the intracellular Na^+ and K^+ concentrations (Baker Bechmann *et al.*, 2016). The activity of this enzyme is necessary for the regulation of the cellular ionic homeostasis and maintaining the electrochemical gradient required for ion channel function and secondary active transport. Although, these ion concentrations are maintained by membrane bound ATPase enzymes, but these enzymes are inactivated under oxidative stress leading to alterations in lipid composition. Additional functions for Na^+/K^+ ATPase in the cell have been proposed, as a signal transducer triggers several cell-signaling pathways and transcription activator affecting cell proliferation, cell motility and apoptosis where the oxidative damage of membrane ATPase is crucial for cell death (Wang and Yu, 2005). Recent study (Gonçalves-de-Albuquerque *et al.*, 2017) suggested the utility of enzyme as a target for cancer treatment where binding of chemotherapeutic agents to the enzyme resulting in the proliferation, differentiation and promotion of autophagy or apoptosis. These effects vary depending on the cell type.

Many studies reported that there are a large number of chemotherapeutic agents are regulated by Na^+/K^+ ATPase. In the present experiment, results of Na^+/K^+ ATPase activity demonstrated that TAM can lead to a significant depression in the activity of liver Na^+/K^+ ATPase. This may lead to general deficit in cell membrane transport function, and may be explained as an indicator for the alterations in membrane function in patients on TAM therapy which in turn results in decreased hepatocellular function. These findings agree with other studies which stated that TAM is an inhibitor of Na^+/K^+ ATPase in case of tumor and normal cells (Repke and Matthes, 1994).
During apoptosis, there is strong evidence indicating an early increase in intracellular Na^+ followed by a decrease in both intracellular Na^+ and K^+, suggesting a regulatory role for these cations during different stages of apoptosis.

Previous studies have shown that Na^+/K^+ ATPase is involved in controlling of Na^+ and K^+ homeostasis during apoptosis and some of anti-apoptotic molecules can influence these ionic fluxes (Lefranc and Kiss, 2008). Other study showed that the alterations in hepatic Na^+/K^+ ATPase activities substantially impaired the defense mechanisms against oxidative stress (Lopez-Lopez *et al.*, 2011). In addition, treatment with GA could restore the enzyme activities and consequently maintain the normal ion homeostasis. This was consistent with a previous study (Yang *et al.*, 2015) which stated that GA administration was significantly maintained the antioxidant status and ATPase activity in *Wistar* rats.
On the other hand, the activities of Na^+/K^+ ATPase in liver were significantly improved in prophylactic groups when compared to TAM-intoxicated group. The previous findings documented that the activities of Na^+/K^+ ATPase in liver were significantly increased after administration of GA. This might be due to the membrane stabilizing activity of GA, owing to their antioxidant potential. This potential can reverse the membrane alterations by inducing the chemo preventive and antioxidant enzyme system. Those play an important role in the cellular stress response, during which a majority of oxidative toxicants are removed from the cells (Latief *et al.*, 2016). Eventually, GA supplementation had a beneficial effect that associated with a normalization of membranes by partial restoration of Na^+/K^+ ATPase activity. This finding suggest its role to enhance Na^+/K^+ ATPase activity by improving the antioxidant capacity of the cell to protect all membrane lipids against oxidative degeneration (Verma *et al.*, 2008).

Conclusion

In conclusion, there is an urgent need to discover new compounds that may be combined with TAM to improve its efficacy and overcome toxicity. Treatment with GA along with TAM can ameliorate TAM-induced changes. GA can be used as a promising adjuvant chemo preventive agent for the protection of liver and supports its antioxidant potential during TAM therapy. Also, GA can trigger apoptosis through the modulation of caspase-3, through its potential anti-apoptotic effect in reducing the cellular toxicity of the anticancer drug TAM.

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Limitations

Further *in vitro* and *in vivo* studies are recommended with induction of breast cancer to assess the positive effects of GA.

Disclosure

The authors report no conflicts of interest in this work.

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