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Possible protective effects of Proanthocyanidin and N-acetylcystine on Tamoxifen induced hepatotoxicity in experimental rats

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Abstract

In the present work, an attempt has been made to study the possible protective role of Proanthocyanidin and N-acetylcystine againist Tamoxifen-induced Seventy female rats were divided into 7 groups and were administred with various doses of Proanthycynidin, N-Acetyl cystine and Tamoxifen. Hepatoprotection was prominant in the group treated with N-acetylcysteine than proanthocyanidin possibly due to its action as a GSH precursor resulting in increasing thiol pools, preventing oxidative stress and apoptosis. It is also concluded that the combination of N-Acetylcystine and Proanthocynidine is more effective as hepatoprotectant than N-Acetylcystine and Proanthocynidine alone may be due to the synergism between both drugs.

Keywords:
Tamoxifen,
Oxidative stress,
N-acetylcysteine,
Proanthocyanidin

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1.0 Introduction

Tamoxifen, a synthetic nonsteroidal antagonist of estrogen receptor (ER) is used in treatment of all stages of hormone dependent breast cancer in patients with early-stage breast cancer as well as those with metastatic breast cancer. It reduces level of estrogen by competition with estrogen for binding to its receptor in breast tissue [1]. Many cancer chemotherapeutic agents including Tamoxifen exert their anticancer properties by inducing apoptosis and oxidative stress through mechanisms that involve mitochondria and Nitric oxide (NO) [2]. Tamoxifen leads to oxidative liver damage and it has been elucidated to be a hepatocarcinogen in rodents, Many cases of Tamoxifen-induced hepatotoxicity have been reported including toxic hepatitis, massive hepatic steatosis, multifocal hepatic fatty infilteration, hepatic necrosis, hepatic cirrhosis and even hepatic cell carcinoma. Studies have suggested that oxidative stress and apoptosis underline in the pathogenesis of TAM – induced hepatotoxicity [3].
N-acetyl-L-cysteine or N-acetylcysteine (NAC), the acetylated variant of the amino acid L-cysteine, is an excellent source of sulfhydryl group (SH) and act as a precursor of reduced glutathione (GSH) promoting detoxification and acting directly as free radical scavenger which can interact directly with ROS and nitrogen species. NAC is used as a mucolytic agent in a variety of respiratory illnesses (e.g.chronic bronchitis); however it also appears to have beneficial effects in conditions characterized by decreased GSH or oxidative stress such as HIV infection, cancer, heart disease and cigarette smoking. NAC is also used in the management of acetaminophen poisoning caused by GSH depletion and glutathione reduction [4].
A new group of phytochemicals that has been attracting much attention from both the general public and health professionals are "proanthocyanidins". Oligomeric proanthocyanidins (OPCS) are poly-phenols and more specifically are polymers of flavenols. These are mainly found in various dietary sources such as grapes, cocoa and apples. Proanthocyanidins are famed for their potent antioxidant capacity and free radical scavenging properties. There is substantial evidence that proanthocyanidins intake from grapes or cocoa have anticarcinogenic and anti-inflammatory properties and increased antioxidant status among hypercholesterolemic, hyperlipidemic, hemodialysis patients and smokers [5].
The aim of this study was to further elucidate the role of oxidative stress and apoptotic cell death in Tamoxifen-induced liver injury. In addition, studying the possible protective effect of different antioxidants as N-acetylcysteine (NAC) and proanthocyanidin (PAC) on Tamoxifen-induced liver injury alone and in combination is also carried out.

2.0 Materials and methods

2.1 Chemicals

Tamoxifen citrate was obtained from medical union pharmaceuticals company, Cairo, Egypt. N-acetylcysteine (NAC) was obtained from Sedeco company, Cairo, Egypt, Proanthocyanidine (PAC) from Sigma company, Quesna, Tanta, Egypt.

2.2 Experimental animals

Female albino rats weighting 150-200 gm were obtained from the animal house of Tanta faculty of pharmacy. The rats were housed in cages (5 rats per cage) in a room temperature of 22±3Cº and exposed to dark/light cycle of the day. The rats were fed with bread and some green vegetables with free access to water . All procedures were conducted in respect of the acceptable human methods in the use of laboratory animals in medical research including the use of anesthesia and dissection procedures.

2.3 Study design

Fifty rats were divided into five groups, each group contain ten rats. Group-1 consisted of normal control rats injected interperitonialy by 0.1 saline for 6 successive days, Group-2 rats were interperitonialy injected with Tamoxifen 45mg/kg/day dissolved in 1 ml saline for 6

sucssive days [6]. Group-3 rats were treated orally with 150mg/kg NAC for 9 sucssive days [7] before TAM injection followed by 6 days with Tamoxifen injection.

Table-1: Effect of NAC, PAC and TAM on serum enzymes ALT(GPT), AST(GOT)

Groups	ALT(U/ml)	AST(U/ml)
Normal	52.8 ± 9.07	126.4 ± 31.14
TAM	190.6 ± 22.57^{a*}	332.6 ± 59.63^{a*}
TAM+NAC	85.4±10.87^{a*b*c*d*}	212.8 ± 40.55^{a*b*d*}
TAM+PAC	112.6 ± 16.5a*b*c*	275.8 ± 21.65^{a*b*c*}
TAM+NAC+PAC	70.2 ± 1.9^{a*b*}	201 ± 10.7^{a*b*}

Values are expressed as mean ± SD. (n = 10). [a] level of significance as compared to normal control group, [b] level of significance as compared to Tamoxifen group, [c] level of significance as compared to (TAM+NAC+PAC) group and [d] level of significance as compared to (TAM+PAC) group. [*] significance at $p < 0.05$.

Figure-1: Effect of Tamoxifen treatment on serum level of ALT

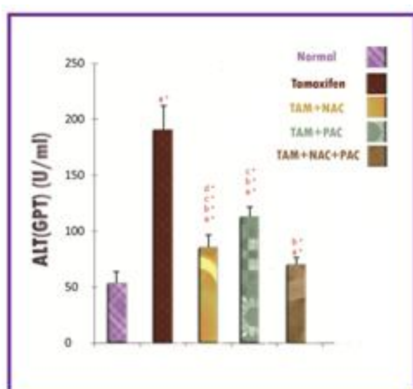
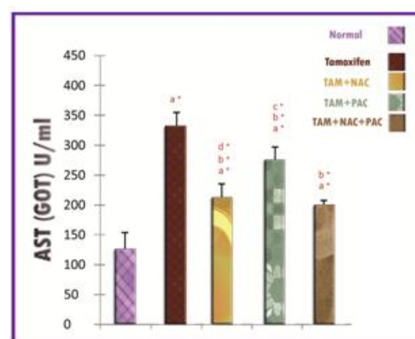


Figure-2: Effect of Tamoxifen treatment on serum level of AST



Group-4 rats were treated orally with 150mg/kg PAC for 9 sucssive days [8] before TAM injection followed by 6 days with Tamoxifen injection. Group-5 rats were treated orally with 150mg/kg NAC & 150mg/kg PAC for 9 successive days before TAM injection followed by 6 days with Tamoxifen injection. After the end of the decided periods of the studies, animals were anesthetized with diethyl ether, blood samples were collected from the carotid arteries using an anticoagulant heparin, centrifuged at 4000 rpm for 15 minutes at 4Cº, the plasma which is intended to be was used for assaying was stored on ice at -80Cº and the animals were sacrified and tissue samples from liver were taken to be fixed and processed .

2.4 Biochemical investigations

2.4.1. Measurement of ALT and AST

ALT and AST were measured spectrophotometrically at 546 nm and expressed as u/ml. The determination of ALT and AST activities based on measuring the concentration of pyruvate hydrazon or oxaloacetate hydrazon formed by the reaction of pyruvate or oxaloacetate with 2,4-dinitrophenylhydrazone [9].

2.4.2. Measurement of Thiobarbituric acid reactive substances (TBARS)

TBARS was measured spectrophotometrically using MDA kits. Thiobarbituric acid (TBA) react with malondialdehyde (MDA) in acidic medium at temperature of 95 C^0 for 30 min to form

thiobarbituric acid reactive product and the absorbance of the resultant pink product can be measured at 534 nm and expressed as nmol/ml [10].

Table-2: Effect of NAC, PAC and TAM on oxidative status (TBARS, GSH & NO)

Groups	TBARS (nmol/ml)	GSH (mmol/L)	NO (µmol/L)
Normal	3.46 ± 0.76	1.94 ±0.18	52.58 ± 3.23
TAM	10.5 ± 1.88[a*]	0.796 ± 0.11[a*]	78.1 ± 11.41[a*]
TAM+NAC	6.82 ± 1.08[a*b*c*d*]	1.552 ± 0.135[a*b*c*]	66.86 ± 12.94
TAM+PAC	5.5 ± 0.54[a*b*]	1.356 ± 0.16[a*b*c*]	63.196 ± 9.38[b*]
TAM+NAC+PAC	4.34 ± 1.006[b*]	1.746 ± 0.14[b*]	57.34 ± 6.64[b*]

Values are expressed as mean ± SD. (*n* = 10). [a] level of significance as compared to normal control group, [b] level of significance as compared to Tamoxifen group, [c] level of significance as compared to (TAM+NAC+PAC) group and [d] level of significance as compared to (TAM+PAC) group. [*] significance at $p < 0.05$.

Table-3: Effect of NAC, PAC and TAM on antioxidant enzymes (CAT, SOD & GPX)

Groups	CAT (U/L)	SOD (U/ml)	GPX (mu/ml)
Normal	317.34 ± 28.28	30.38 ±5.98	43.41 ±
TAM	241.42 ± 34.88[a*]	13.98 ± 2.64[a*]	11.76 ± 1.86
TAM+NAC	286.16 ± 40.66[b*]	18.94 ± 2.37[a*b*c*]	30.38 ± 3.06[a*b*c*d*]
TAM+PAC	262.36 ± 17.26[a*c*]	22.1 ± 2.6[a*b*c*]	24.18 ± 4.22[a*b*c*]
TAM+NAC+PAC	302.36 ± 21.21[b*]	26.84 ± 2.35[b*]	38.06 ± 2.34[a*b*]

Values are expressed as mean ± SD. (*n* = 10). [a] level of significance as compared to normal control group, [b] level of significance as compared to Tamoxifen group, [c] level of significance as compared to (TAM+NAC+PAC) group and [d] level of significance as compared to (TAM+PAC) group. [*] significance at $p < 0.05$.

2.4.3. Measurement of reduced Glutathione (GSH)

The method based on the reduction of 5,5` dithiobis(2-nitrobenzoic acid) (DTNB) with glutathione reduced (GSH) to produce a yellow compound. The reduced chromagen directly proportional to GSH concentration and its absorbance can be measured at 405 nm and expressed as mmol/L [11].

2.4.4. Measurement of Nitric oxide (NO)

It determined according to the method of Montgomery and Dymock [12], which based on the fact that the final products of NO invivo are nitrite (NO^{2-}) and nitrate (NO^{3-}), thus one of the indicator of Nitric oxide production in biological fluids is the nitrite (NO2-). The measurement of NO depend on the using of Griess reagents which composed of sulphanilamide reagent and N-(1-naphthyl) ethylenediamine. Sulphanilamide reagent react with nitrite (NO^{2-}) in the presence of acid medium forming nitrous acid diazotize sulphanilamide product which coupled with N-(1-naphthyl) ethylenediamine. The resulting azo dye has a bright reddish purple color which can be measured spectrophotometrically at 540 nm and expressed as µmol /L.

2.4.5. Measurement of Catalase enzyme (CAT)

CAT activity was measured by using the method of as cited by Aebi and Fossati [13], it based on the fact that catalase enzyme catalyzes the break down of H2O2. In the presence of peroxidase (HRP), this reaction stopped and the remaining H2O2 react with 3,5-Dichloro-2-hydroxybenzene sulfonic acid (DHBS) and 4-aminophenazone (AAP) to form a chromophore

with a color intensity inversely proportional to the amount of catalase in the sample and measured spectrophotometrically at 510 nm and expressed as U/L.

2.4.6. Measurement of Superoxide dismutase enzyme (SOD)

SOD activity was measured by using the method of Nishikimi et al. [14]. This assay relies on the ability of the enzyme to inhibit the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye and % inhibition proportional with SOD activity. SOD activity measured spectrophotometrically at 560 nm and expressed as U/ml.

Figure-3: Effect of TAM administration on TBARS accompained with significant decrease in GSH

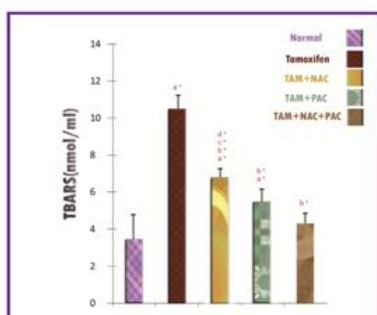


Figure-4: Effect of TAM administration on GSH

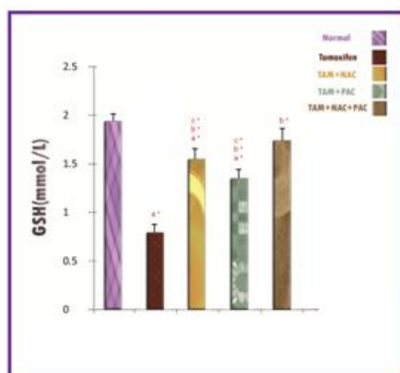


Figure-5: Effect of TAM exposure on NO level

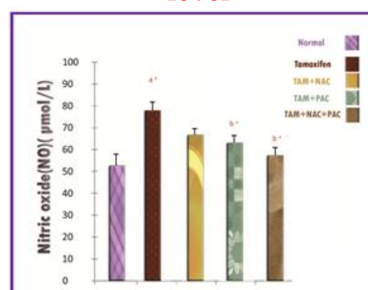
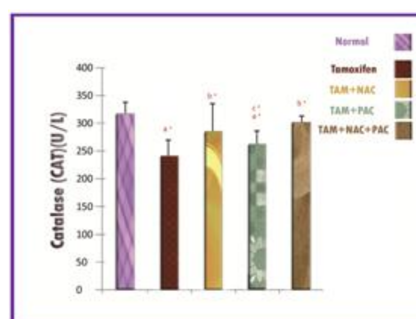


Figure-6: Effect of TAM exposure on Catalase



2.4.7. Measurement of Glutathione peroxidase enzyme (GPX)

GPX activity was measured spectrophotometrically according to the method of Paglia and Valentine 1967 [15]. This method is based on the oxidation of GSH by GPX in the presence of hydrogen peroxide H2O2 to produce GSSG. Then NADPH and GR were used for production of GSH and NADP. Enzyme activity was determined by measuring the disappearance on NADPH at 340 nm and was expressed as mu/ml.

2.5. Histopathological examination of liver sections

Liver sections from all rats were immediately fixed in 10% neutral buffered formaline, processed and paraffine embedded section with thickness of 3-5 µm were prepared at for routine heamatoxylin and eosin (H&E) staining and examined under light microscope (Olympus BX-50) to estimate liver damage.

2.6. Immunohistochemical staining (TUNEL staining) for determination of Apoptosis

TUNEL (TdT-mediated dUTP-biotin nick end labelin) staining relies on the ability of the enzyme terminal deoxynucleotidyl transferase (TdT) to incorporate labeled dUTP into free 3'-

hydroxyl termini generated by the fragmentation of genomic DNA into low molecular weight double-stranded DNA and high molecular weight single stranded DNA [16]. Ten high power (x400 magnification) fields were randomly chosen for each section. The neuclei of cells with clearly defined staining were counted ,and the mean number of neuclei in the 10 fields was estimated for each group .Each field was evaluated for the proportion of stained cells and staining intensity. The proportion of stained cells was assessed :- (±ve) = 1%- ≤5% apoptotic cells, (+) = ≤ 25% (mild), (++) = > 25 - < 50% (moderate), (+++) = ≥ 50% (severe).

Figure-7: : Effect of TAM exposure on SOD

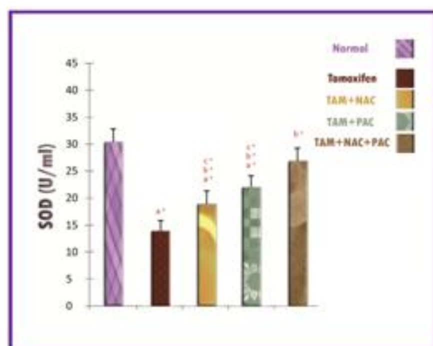


Figure-9: Liver sections from normal studied group

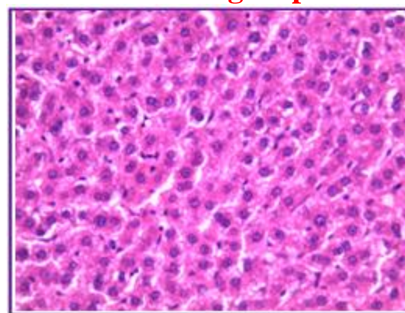


Figure-8: Effect of TAM exposure on GPX

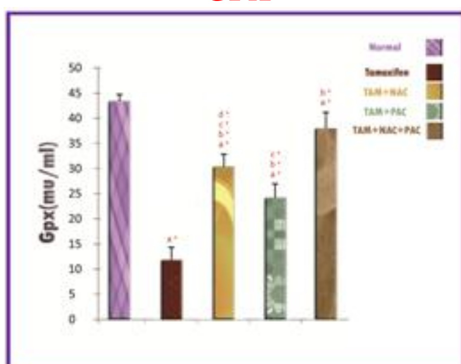
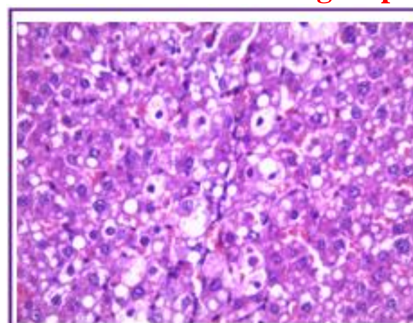


Figure-10: Liver sections from TAM+NAC treated group



2.7. Statistical analysis

Results are reported as mean ± SD of the mean. Statistical analysis of data was performed using one-way analysis of variance (ANOVA). The Tukey-Karmer post hoc test was applied to serve as significant among groups. The significance of results was ascertained at p<0.05. All statistical analysis were performed using GraphPad InStat 3 (GraphPad Software, Inc. La Jolla, CA, USA) software. Graphs were sketched using GraphPad prism version 4 software (GraphPad Software, Inc. La Jolla, CA, USA).

3.0 Results and discussion

3.1. Effect on serum enzymes (ALT, AST)

Results (Table-1, Figure-1and 2) showed that Tamoxifen treatment caused significant elevation in the serum level of ALT, AST (P<0.05) compared to normal control group. This elevation was significantly attenuated upon administration of NAC or PAC to TAM-treated rats, NAC exhibited more attenuation effect on serum enzymes on TAM-group compared to PAC. On the other hand, treatment of TAM-group with the combination NAC and PAC significantly reduced the ALT, AST level as compared with the corresponding TAM group. NAC&PAC combination

exhibited an observable attenuation effect on ALT, AST more than NAC alone or PAC alone on TAM group.

Figure-11: Liver sections from TAM+PAC treated group

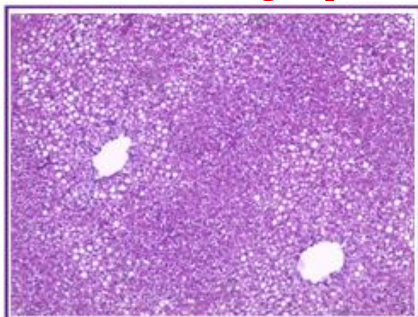


Figure-13: Mild diffuse vacular changes with mild dilated engorged central vien and blood sinusoid in TAM+NAC+PAC group

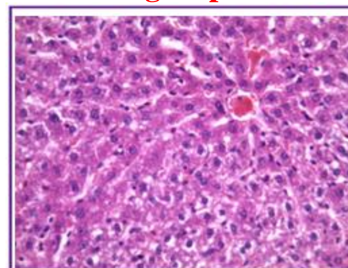


Figure-12: Liver sections from TAM+NAC+PAC treated group

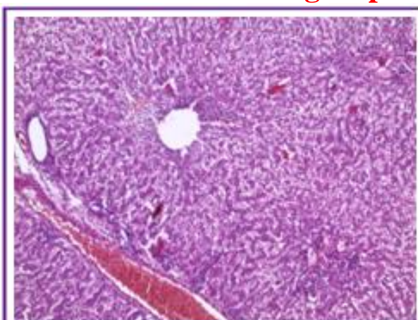
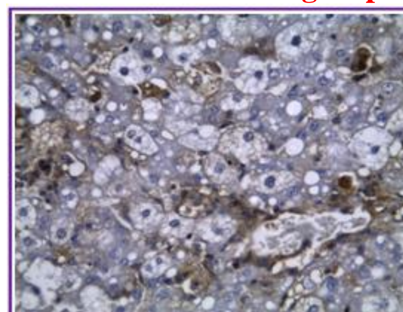


Figure-14: Apoptotic figures of liver cells observed in TAM group



3.2. Effect on oxidative status (TBARS,GSH,NO)

3.2.1. Effect on TBARS and reduced glutathione GSH

Table-2, Figure-3 and 4 showed that TAM administration produced significant increase in TBARS accompained with significant decrease in GSH ($P < 0.05$) compared to control group. Administration of NAC or PAC to TAM- group induced significant decrease in TBARS with significant increase in reduced glutathione GSH ($P < 0.05$). While, administration of NAC and PAC together showed significant reduction in TBARS accompanied with significant elevation compared to administration of NAC alone or PAC alone to TAM-group as combination restored TBARS and GSH almost near normal level.

3.2.2. Effect on Nitric oxide NO

Results (Table-2 and Figure-5) of study influence of Proanthocyanidin and N-acetylcystine on Nitric oxide free radical showed that TAM exposure resulted in a significant increase in NO level ($p<0.05$) as compared with control group. Administration of PAC to TAM-intoxicated rats exhibited significant reduction in NO level ($p <0.05$) compared to TAM-group while NAC administration reduce NO level but with less ability to reduce it significantly compared to TAM-treated group. On the other hand, combination between NAC and PAC significantly decrease NO level ($p < 0.05$) as compared to TAM-group and manage to restore NO level near normal level. Combination exerted more ameliorated effect on NO level than NAC or PAC administration to TAM-treated rats.

3.3. Effect on antioxidant enzymes (CAT, SOD, GPX)

Data from Table-3, Figure-6-8 showed that TAM administration produced significant decline in the level of all antioxidant enzymes (CAT, SOD, GPX) ($p<0.05$) compared to normal control

group. NAC supplementation to TAM-intoxicated rats showed significant elevation in the levels of CAT, SOD and GPX ($p<0.05$) compared to TAM-treated group. While, PAC administration to TAM-group showed significant elevation in SOD and GPX levels but with less ability to increase CAT level significantly. Effect of NAC treatment was more than PAC treatment to TAM-group in CAT & GPX activity while with less extent in SOD level. On the other hand (NAC & PAC) combination exhibited more amelioration effect on antioxidant enzymes than NAC or PAC on TAM-group, as combination significantly elevated CAT, SOD, GPX ($p<0.05$) compared to TAM-group and manage to restore CAT, SOD near normal level while GPX still significantly high.

3.4. Histopathological examination

The protective effect of NAC or PAC or NAC and PAC combination against TAM-induced hepatotoxicity were further confirmed by histopathological examination. Liver sections from normal studied group sections showed normal liver architectiure, parenchymal cells, portal tracts and vascular spaces (Figure-9). TAM group animals showed diffuse vacular degeneration of hepatocytes with multiple apoptotic figure and extensive congested blood sinosoids (Figure-10). TAM and NAC group animals showed central diffuse zonal vacular degeneration of hepatocyte with dilated central viens in liver sections (Figure-11). TAM and PAC group showed congested central viens with central focal zonal necrosis and infiltration by mononuclear cells associated with dilated sinusoids and vacular degeneration of hepatocytes (Figure-12). TAM+NAC+PAC group; animals showed mild diffuse vacular changes with mild dilated engorged central vien and blood sinusoid (Figure-13).

3.5. Immunohistochemical results

Immunohistochemical tunnel staining of sections from different groups confirmed histopathological and chemical analytical results as increased apoptotic figures were proportional to the degree of damaged hepatocytes. Immunohistochemical staining of liver sections for tunnel in Tamoxifen-treated animals showed sever (+++) numerous apoptotic figures ($\geq 50\%$) of liver cells were positive in all liver sections of this group (Figure-14). There was a decreased number of apoptotic figures in both (TAM+NAC) group & (TAM+PAC) where TAM-group treated with PAC showed moderate apoptosis (++) (> 25-<50%) (Figure-15) while TAM-group treated with NAC showed moderate to mild apoptosis (+) $\leq 25\%$ (Figure-16). On the other hand,TAM-group receving both proanthocyanidins (PAC) and N-acetylcysteine (NAC) together showed mild (+) to (±) apoptotic cells [1%- $\leq 5\%$] (Figure-17).

4.0 Discussion

The present study was carried out in order to investigate protective role of NAC and PAC alone or in combination on TAM-induced hepatotoxicity. The current study showed that Tamoxifen treatment caused significant increase in serum level of ALT and AST. This elevation could be attributed to hepatic structural damage as these enzymes are normally localized in the cytoplasm and are released into circulation after cellular damage has occured, through adversely interaction of Tamoxifen active metabolites, 4-hydroxy Tamoxifen and α-hydroxy Tamoxifen, with lipid bilayers of model and native membrane, an effect which may contribute to the cytostatic effects of Tamoxifen [17]. Also, Tamoxifen is known to increase hepatic lipid peroxidation as assessed by the high level of thiobarbituric acid reactive species (TBARS) which explains the observed leakage of cellular ALT,AST into the circulation which suggest the hepatocellular damage [18].
In addition, this study showed that Tamoxifen treatment caused a significant depletion in the reduced glutathione (GSH) accompanied with significant elevation of TBARS level; a product of lipid peroxidation, compared to normal control group. This could be explained on the basis of Tamoxifen impaired mitochondrial functions which resulted in mitochondrial oxidative damage, ROS generation, ATP depletion, enhanced LPO (lipid peroxidation), depleted hepatic GSH and elevated GSSG [19]. Also, Tamoxifen is recorded to cause reduction in glutathione reductase

"GR" enzyme activity which reduce oxidized glutathione GSSG to reduced glutathione GSH and hence there is a depletion in GSH level and accumulation or elevation in GSSG level [20], leading to reduction of glutathione peroxidase (GPX) activity as glutathione peroxidase utilizes GSH for H_2O_2 detoxification into water and organic peroxides (R-o-o-H) and this would eventually result in H_2O_2 accumulation which in turn leads to exhaustation of antioxidant superoxide dismutase (SOD) and catalase (CAT) enzymes [21] and that was confirmed by our results as there was significant depletion in the antioxidant enzymes (CAT,SOD,GPX).

Figure-15: Apoptotic figures of liver cells observed in TAM+PAC group

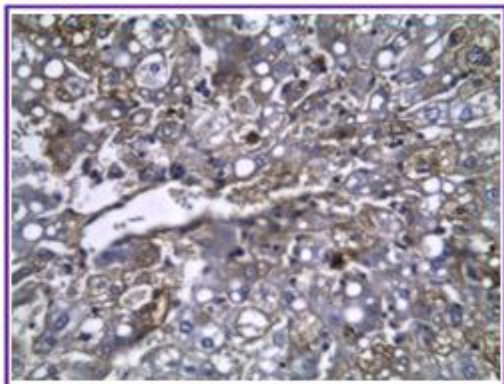


Figure-16: Apoptotic figures of liver cells observed in TAM+NAC group

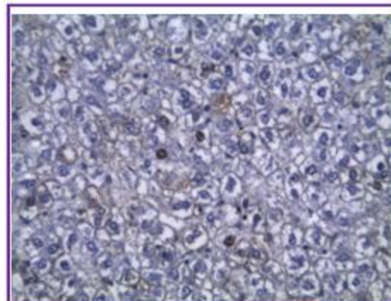
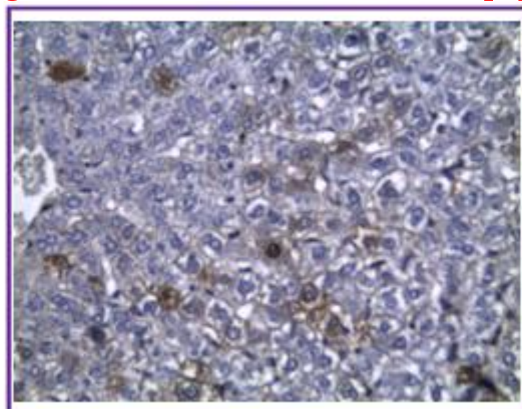


Figure-17: TAM group receiving both proanthocyanidins (PAC) and N-acetylcysteine (NAC) together showed mild to moderate apoptotic cells



Regarding the influence on Nitric oxide, it was found that Tamoxifen treatment caused a significant elevation in Nitric oxide (NO) level compared to normal control group [22]. Tamoxifen increases the intra-mitochondrial ionized Ca^{+2} concentration which stimulates mitochondrial Nitric oxide synthase (mtNOS) in the mitochondrial liver. Mitochondrial Nitric oxide synthase (mtNOS) stimulation increased Nitric oxide (NO) production which react with superoxide anion O_2^- to produce peroxynitrite (ONOO-). peroxynitrite increases lipid peroxidation which leads to oxidative stress and Cytochrome c release from the mitochondria. Cytochrome c is one of the key events during most forms of apoptosis including that induced by Tamoxifen [23] which could explain the abnormalities biochemical results and histopathological examination encountered in this study.

Cytochrome C is one of the key events during the most characteristic moroghological features of apoptosis as:-cytoskeletal changes, nuclear membrane breakage, cell bleeding and formation of apoptotic bodies which are then phagocytosed by macrophages [24]. Shao et al [25] in studying Tamoxifen as a treatment for breast cancer reported that, Tamoxifen increased the expression of inducible Nitric oxide synthase (iNOS) and Nitric oxide (NO) production by myoepithelial cells of breast tissue leading to apoptosis and this antiproliferative effect exerted by Nitric oxide (NO) may contribute to TAM-induced growth suppression of breast cancer. And so, the occurrence of apoptosis in the current work was predicted from the significant elevation of Nitric oxide "NO" levels in this work which was supported by the immune-histochemical staining for tunnel showed that high or sever apoptosis (+++) ($\geq$ 50% of liver cells were apoptotic in Tamoxifen treated group).
According to our study, administration of NAC to TAM-treated rats significantly reduced ALT,AST compared to TAM group. This effect could be attributed to NAC, as a precursor of reduced glutathione (GSH), tends to prevent liver damage, decreased lipid peroxidation, suppresses the leakage of enzymes through cellular membranes, preserves the integrity of the plasma membranes and hence restores GPT (ALT) and GOT(AST) enzymes levels [26].
This study showed that treatment of TAM-group with NAC induced significant elevation in the reduced glutathione GSH and antioxidant enzymes CAT, SOD, GPX level accompanied with significant reduction in the TBARS level. The lowered level of TBARS (MDA) and hydroperoxides caused by oral administration of NAC could be related to its structure. NAC contain sulfhydryl (SH) group which is responsible for a great deal of the metabolic activity and antioxidant capacity of NAC, SH group scavenge reactive oxygen species such as superoxide radical O_2^-, hydrogen peroxide H_2O_2 and hydroxyl radical OH^- resulting in reduction and prevention of lipid peroxidation, while the acetyl substituted amino group makes the NAC molecule more stable against the oxidation [27]. As a source of sulfhydryl (SH) group, NAC can stimulate GSH biosynthesis by enhancement of glutathione-S-transferase activity and promotes the uptake of cystine from the culture medium for cellular GSH biosynthesis [28]. In addition to stimulation of GSH biosynthesis, NAC stimulate cytosolic enzyme activites involved in NADP reduction and enzyme activites involved in glutathione reduction as "glutathione reductase" which involved in the reduction of oxidized glutathione GSSG into nonenzymatic antioxidant reduced glutathione "GSH" and thus correct the reduction in GSH concentration resulting in a significant preservation in the membrane fluidity and the activites of CAT, mitochondrial SOD and the different forms of glutathione peroxidase "GPx" [29].
Oral treatment of Tamoxifen-treated rats with NAC showed insignificant decrease in the NO level compared to TAM-intoxicated group. This could be explained by the report recording that NAC attenuated the formation of Nitric oxide and peroxynitrite (ONOO-) through the reduction in inducible Nitric oxide synthase "iNOS" over expression and this related to the inhibitory effect of NAC on the activation of nuclear factor-KB "NF-kB" which induced Nitric oxide synthase "iNOS" over expression [30]. In addition, NAC scavenges and inactivates superoxide anions (O_2^-) that would prevent also (ONOO-) formation which may resulted in a decreased in ROS generation and oxidative damage occured after TAM administration and at the same time inhibited Cytochrome C release and so apoptosis pathway induction , and that could be one of the mechanisms of apoptosis suppression by NAC.
Also, the protective role of NAC aganist TAM-induced cytotoxicty and apoptosis could be explained by its ability to stimulate synthesis of GSH and so increased cellular thiol pools as depletion of GSH has been correlated with apoptosis, GSH depletion caused Cytochrome C release even in the absence of apoptosis suggesting that Cytochrome C release could be the consequence of a redox disequilibrium arising from increased mitochondrial generation of ROS associated with apoptosis [31]. NAC which exhibited antiapoptotic property may be useful

prevent apoptosis induced by chemotherapy [32] and this could be confirmed immunohistologically by tunnel test that showed mild apoptosis (+) [≤ 25% apoptotic cells].
In the contrary of our results, Pechanova et al [33] documented that NAC treatment increased endothelial Nitric oxide synthase "eNO" activity by increasing eNOS expression on both mRNA and protein levels resulting in increased NO production which leads to blood pressure reduction as super oxide anion O_2^- increased in the hypertension and accelerated the inactivation of NO by peroxynitrite formation which leading to hypertension suggested that NAC has or possess antihypertensive properties [33].
On the other hand, administration of PAC to TAM-intoxicated rats significantly decreased ALT, AST serum levels compared to TAM group. This could be attributed to the fact that PAC decreased the lipid peroxidation induced by Tamoxifen treatment, these effects may be due to its potential act as a hydrogen-donating free radical scavenger and singlet oxygen quencher activity [34].
Our results showed that administration of TAM treated rats with PAC revealed a significant reduction in TBARS level accompanied with a significant elevation in the level of reduced glutathione GSH, SOD and GPX while there was insignificant increase in CAT levels compared to TAM-treated group. Proanthocyanidins is reported to possess antioxidant through the following mechanisms;(i) free radical scavenging property and (ii) metal chelating activity [35]. Proanthocyanidins scavenges a wide range of free radicals including the most active hydroxyl radicals which initiated lipid peroxidation, hence it may be assumed that it decreased the concentration of lipid free radicals and so terminated the initiation and propagation of lipid peroxidation, prevented the "GSH" depletion on free radical scavenging and induced the antioxidant enzymes activities in the liver as CAT, SOD and GPX enzymes [36] .
Regarding Nitric oxide, treatment of TAM-group with PAC significantly reduced the Nitric oxide level compared to TAM-treated group. Proanthocyanidins has a potent inhibitory action on Nitric oxide production presumably through the inhibition of Ca^{+2} dependent Nitric oxide synthase by the inhibition of excessive Ca^{+2} influx due to its metal chelating activity and inhibition of Ca^{+2} release from intracellular stores [37]. In addition, Lyu and park [38] reported that PAC and other flavanoids repress NO production in macrophages and human peripheral blood mononuclear cells. PAC inhibited Nitric oxide production through prevention of Nitric oxide synthase stimulation by Ca^{+2} and thus peroxynitrite formation decreased resulting in inhibition of lipid peroxidation, Cytochrome C release and apoptotic pathway intiation by Tamoxifen treatment.
On the other hand, proanthocyanidins or grape seed proanthocyanidins could induce apoptosis through stimulation of the tumor suppressor protein P53 by increasing levels of P53 and its activated/phosphorylated form upon PAC treatment indicates that the induction of apoptosis by PAC is mediated through this protein [39]. Previous studies have shown that PAC inhibit the overexpression of Bcl-2 protein which inhibits apoptosis by inhibiting the release of Cytochrome C and apoptosis inducing factor (AIF) from the mitochondria to the cytoplasm, suggesteing that proanthocyanidine (PAC) could be a potential cancer chemopreventive agent against various types of cancers [40]. This indicated immunohistochemically by tunnel test showing moderate apoptosis (++) [>25-<50% apoptotic cells]. Hence, it could be said that NAC is better than PAC at the level of routine histopathological and immmunohistopathological examination of liver sections.
Results from biochemical results indicated that N-acetyl cysteine caused a highly hepatoprotection more than proanthocyanidine in all the measured parameters except for TBARS, Nitric oxide"NO" and SOD enzyme, where PAC was found to cause more increase in TBERS, NO and SOD enzyme and that could be attributed to the free radical scavenging property and metal chelating activity of proanthocyanidin. While, the combination treatment of Tamoxifen intoxicated rats with NAC+PAC together expectedly caused highly hepatoprotective

effect against TAM-induced liver injury more than TAM group treated with NAC alone or TAM group treated with PAC alone and that indicated by the clinical serological parameters causing significant attenuation in ALT,AST serum level but still significantly high compared to normal control group.
In addition, the combination caused significant reduction in TBARS and Nitric oxide with significant elevation in GSH level and managed to restore TBARS, NO and GSH near normal level. The serum level of antioxidant enzymes (CAT, SOD & GPX) were significantly elevated in TAM+NAC+PAC group where CAT and SOD restored towards normal level while level of GPX still significantly high compared to normal control group suggesting a synergism effect between NAC and PAC. This biochemical results further indicated and supported by the histopathological examination of liver sections and immunohistochemical stainig for tunnel test showing (±) [1%-≤ 5%] of apoptotic cells.

4.0 Conclusion

In conclusion, the results of the present study indicate that the antioxidant potential of N-acetylcysteine is better than proanthocyanidin as a hepatoprotective against hepatotoxicity induced by Tamoxifen due to its action as a GSH precursor resulting in increasing thiol pools, preventing oxidative stress and apoptosis. It can also be concluded from the present study that the combination of NAC and PAC is more valuable than NAC or PAC alone due to the synergism between both drugs.

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Abbreviations

(TAM) Tamoxifen, (NAC) N-acetylcysteine, (PAC) proanthocyanidin, (ALT) alanine aminotransferase, (AST) aspartate aminotransferase, (TBARS) thiobarbituric acid reactive substances, (NO) Nitric oxide, (GSH) reduced glutathione, (CAT) catalase, (SOD) superoxide dismutase , (GPX) glutathione peroxidase, (ER) estrogen receptor, (DCIS) ductal carcinoma in situ, (SH) sulfhydryl group,(ROS) reactive oxygen species, (OPCS) oligomeric proanthocyanidins, (MDA) Malondialdehyde, (TUNEL) TdT-mediated dUTP-biotin nick end labelin, (TdT) terminal deoxynucleotidyl transferase, (PAP) peroxidase antiperoxidase.

Conflict of interest:

All authors declare any conflict of interest

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