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Hepatic Ameliorative Role of Amygdalin Against Phenolphthalein-Induced liver injury; involvement of Glial fibrillary acidic protein

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Abstract: Phenolphthalein (PTH) is an odorless and colorless chemical of laxative action and multiple side effects. It is primarily metabolized in the liver. Amygdalin (Vit B17) have antifibrotic, anti-inflammatory and antitumor activities. The histopathological and functional changes of PTH on the liver together with the postulated hepatoprotective effect of Amygdalin were investigated in this study. Glial fibrillary acidic protein (GFAP) was considered as a marker of liver pathological changes. Thirty adult male albino rats were divided into 3groups. Group I (control), group II (received PTH) and group III (received both PTH and Amygdalin). At day 21, liver functions were estimated and the liver tissue samples were examined by light and electron microscopes. The liver enzymes, total bilirubin, hydroxyproline, and serum GFAP were significantly increased in group II compared with the control and group III. The total proteins were significantly decreased in group II in comparison to the controls and highly significantly increased in group III compared to group II. Group II showed disturbed hepatic architecture with extensive congestion, degeneration with inflammatory cell infiltration and marked collagen deposition meanwhile group III showed preserved hepatic parenchyma and scanty inflammatory cells infiltration with decreased collagen. Group II had strong and diffuse immunoexpression and group III liver tissues were similar to the controls. The mean area percentage of GFAP immune staining showed a highly significant increased immunopositivity in group II when compared to the group I and a significant decrease in group III compared to group II. The ultrastructure of hepatic stellate cells of group II showed dilated rough endoplasmic reticulum and loss of lipid droplets while group III were similar to the controls. In conclusion, Amygdalin treatment had a hepatoprotective effect against PTH induced liver injury and GFAP level could be used as a predictor for HSCs activation.
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1. Introduction

Phenolphthalein (PTH) is a white water-insoluble powder that was initially used as an acid-base indicator (1). It is cheap fast and efficient laxative that was available in different pharmacological over-the-counter preparations (2). It is one of the stimulant laxatives that acts by increasing the peristalsis of the intestine through irritation of the mucosa or the intestinal smooth muscles (3). It has always been considered safe because adverse effects were noticed only with overdose(4). Skin is one the common organs reported to be affected by its adverse effects in the form of epidermal necrolysis and erythemamultiforme (5). Hypertension is also a reported side effect caused by the accompanying salt and water retention (6). PTH also induces apoptosis or necrosis in liver parenchymal cells (7). These adverse effects were mostly attributed to the high oxidative stress of PTH and its metabolites on the tissues (8).

Recently, carcinogenic effects of PTH gained more interest due to the widespread use and lack of adequate testing particularly in humans(3, 9). Carcinogenicity of PTH was thoroughly studied in animals' adrenal medulla, thymus gland, colon, lung, and ovaries (3, 10).

While many countries restricted the use of PTH, it is widely used as a component of pharmacological preparations in weight losing products, variety of ingestible commodities and as a laxative in some countries such as China. Moreover, PTH still has some scientific applications and industrial uses as an indicator of acids and alkalis and in determination of the depth of concrete carbonation (9).

Amygdalin (Vit B17) or the bitter apricot is a cyanogenic glycoside belongs to aromatic cyanogenic compounds that are widely distributed in plants like apricot and peach (11, 12). Amygdalin is reported to have multiple therapeutic effects as antifibrotic and anti-inflammatory (13, 14). Previous researches investigated the antitumor activity of Amygdalin against multiple types of carcinomas (15, 16). This effect gained increasing interests recently (11) and was attributed to its multiple anticancer actions as the ability to decompose the carcinogenic substances and the inhibition of cancer cell growth by blocking its nutrient source (17). Amygdalin has other favorable effects as it degrades into hydrocyanic acid which is an anticancerous and analgesic agent at the same time (18).

On the liver, Amygdalin was found to lower the levels of AST, ALT and to increase the content of hydroxyproline in CCl4 and D-galactosamine treated rats (19). The beneficial effects of amygdalin provoked its manufacturing into available anticancerous preparations. The use of amygdalin has been linked to improve symptoms and prolonged survival of patients with advanced cancer stages(11). Amygdalin by itself is non-toxic but it can be converted by some enzymes into poisonous substances, so it has to be used with caution (12).

Chronic liver diseases are usually complicated by fibrosis which occurs primarily through activation of Hepatic stellate cells (HSCs) which are the known source of extracellular matrix collagen (20). HSCs undergo activation and trans-differentiation to myofibroblast-like cells and this is attributed secondarily to the effects of various cytokines, tissue mediators and growth factors (21). Liver carcinogenicity occurs predominantly in the setting of liver fibrosis, cirrhosis and inflammation as chronic liver diseases promote the development of transformed or premalignant hepatocytes (22). Finding precocious markers of activation of HSCs will be helpful in identifying early stages of hepatic fibrosis and may reduce fibrosis progression and prevents carcinogenicity (23, 24).

The glial fibrillary acidic protein (GFAP), is an intermediate filament protein that is commonly expressed by astrocytes in the central nervous system (25). It is expressed in vivo in the liver in a subpopulation of quiescent stellate cells. The percentage of GFAP-positive HSCs correlated with fibrosis progression (23) so, GFAP could represent a useful marker of early activation of HSCs in response to injury.

This study investigated the tissue and cellular changes induced by PTH and the postulated protective effect of Amygdalin. The level of GFAP was considered as a marker of liver tissue injury.

2. Materials and methods

Animals

Thirty adult male albino rats (200±20gm) were obtained from Mansoura University Animal house. The animals were kept under standard laboratory conditions assuring the animal welfare in steel wire cages in pairs with controlled room temperature (22 ± 5 C°) and humidity 40-70%. Throughout the study, the animals were fed with standard rodent diet ad libitum. All animal procedures were in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and the guidelines of Mansoura University IRB committee (Licence No. 145/2019).

The rats were randomly divided into 3 groups (10 rats each). Group I served as the control group. Group II received PTH alone and group III received both PTH and Amygdalin.

Drugs and administration

PTH (P. 105945 Millipore-Sigma, St Louis, MO, USA) supplied as crystalline powder dissolved in sterile distilled water and supplied to rats of group II and III as a single intravenous dose of 25 mg/kg through the tail vein (26).

Amygdalin (P. A6005, Millipore-Sigma). The solution was diluted with sterile distilled water. Group III animals received amygdalin 5 mg/kg intraperitoneally once a week for 3 weeks starting with the first dose of PTH (27).

Group I received a single injection of 0.5 ml of sterile distilled water through the tail vein.

Sampling and tissue preparation

Tail vein blood samples were obtained from all animals on day 21 of the initial drug administration (26). Blood samples were allowed to stand for 10mins at room temperature and then centrifuged at 1000rpm for 15mins on laboratory centrifuge (SM 800B, Surgifriend Medicals, England) and the supernatant (serum) was carefully removed with Pasteur pipette, and stored frozen at -10 Cº.

After blood samples collection, all rats were euthanized by intraperitoneal ketamine 50 mg/kg and xylazine 10 mg/kg. Liver samples were harvested from all animals, divided into 3 samples; the first one is fixed in 10% neutral buffered formalin for 24 hours and processed for the usual tissue handling for paraffin blocks preparation. Sections of 3-4 µm thickness were stained with hematoxylin and eosin (H & E), Masson's trichrome stains, and GFAP immune stain. The second sample was processed for electron microscopic examination and the last sample was used for biochemical assessment of hydroxyproline.

GFAP immune stain

For immunohistochemical studies, the paraffin sections were mounted on glass slides coated with 0.1% poly (L-lysine). The slides were deparaffinized,

incubated in 2.5% methanolic hydrogen peroxide (30 minutes), the endogenous biotin was blocked by the Biotin Blocking System (Dako, Milan, Italy) according to the supplied instructions then washed 3 times in phosphate-buffered saline.

Then the sections were further processed as described previously with diluted 1:40, mouse monoclonal anti-GFAP antibody (P. G3893, Millipore-Sigma) and counterstained with hematoxylin (23). Negative controls were performed with normal mouse antiserum instead of the primary antibody, which uniformly demonstrated no reaction. The hepatic cell population examined in the present study was represented by parenchymal HSCs that were distinguished from the other myofibroblasts of the liver by their specific position (perisinusoidally located) (28).

Tissue examination

The H & E, Masson's trichrome and anti-GFAP immunoreactive stained sections were examined under light microscope (Nikon Eclipse E600W, Japan) and the represented areas were photographed.

Image analysis

The degree of tissue fibrosis was estimated in Masson's trichrome-stained sections and the degree of GFAP immunopositivity was estimated by image analyzing system as previously described(29). Leica Qwin 500 image analyzer computer system was used. The percentage of the fibrosis area and the GFAP positive stained area over the whole observed field were assessed. Six slides of each stain per animal were used and 10different random fields were measured in each slide).

Electron microscopic examination

Small pieces of liver tissue were processed foe electron microscopic examination. The model of microscope used was JEOL JEM 1200 EXII Electron Microscope (Joel CX 100 transmission electron microscope operated at an accelerating voltage of 60 KV Ltd.) at Mansoura University, EM Unit (Egypt).

Biochemical examination

Serum GFAP levels were determined by ELISA (Enzyme-Linked Immunosorbent Assay) using a GFAP ELISA Kit (NS830, Millipore, Temecula, CA) according to the manufacturer's instructions(30).

The serum concentrations of total protein, total bilirubin, and the activities of AST and ALT were measured using the specific diagnostic kits (Quimica Clinica Applicada, S. A. Spain). Total protein was determined by the Biuret method (31), bilirubin was estimated by the method described by Jendrassik and Grof (32). Alanine and aspartate aminotransferases were determined based on the colorimetric measurement of hydrazone formed with 2, 4 dinitrophenylhydrazine (33).

The tissue hydroxyproline was measured in the tissue sample homogenates as previously described by Fels (34).

Statistical analysis

Results are presented as the mean ± standard deviation. Comparison of the findings and their significance in normally distributed data was done by t-test. In abnormally distributed data Mann-Whitney U test was used. P values< 0.05 were regarded as statistically significant and P < 0.01 was regarded as highly significant.

3. Results

Light microscopy

The control liver sections stained with H & E showed the normal hepatic parenchyma with radiating cords of open face hepatocytes with intervening blood sinusoids. The hepatocytes had acidophilic cytoplasm; some of which are binucleated (Fig.1 A & B). The section from group II (PTH administrated rats) demonstrated prominent loss of the normal hepatic architecture with extensive congestion of the liver vasculature. The hepatocytes were separated by wide spaces and most of the cells demonstrated signs of degeneration (vacuolated cytoplasm and dark pyknotic nuclei). Variable degrees of inflammatory infiltrate were observed. (Fig.1 C & D). The liver sections from group III (Amygdalin treated rats) showed near normal relative preservation of the hepatic parenchyma except for scattered areas of hepatocytes degeneration and scanty inflammatory infiltrate (Fig.1 E & F).

PTH treated group showed a marked increase in collagen deposition around the portal triad. Group III showed a milder degree of collagen fibers deposition around portal triads (Fig. 2 A, B & C).

Morphometric analysis of the mean fibrosis area percentage showed a highly significant increased fibrosis in group II when compared to group I. Meanwhile, group III showed a significant reduction in fibrosis area percentage in comparison to group II (Tab.2 & Fig. 2 D).

GFAP immune staining of the liver sections of the control group showed positive staining of HSCs and the endothelium of the sinusoids. Group II had strong and diffuse immunoexpression mostly perisinusoidal. Group III liver tissues, were more or less similar to the controls (Fig. 3 A, B & C).

Morphometric analysis of the mean area percentage of GFAP immune staining showed a highly significant increased immunopositivity in group II when compared to the control group and a significant reduction in group III when compared with group II (Tab.1 & Fig. 3 D).

Electron microscopy

Electron microscopic examination of the HSCs of the control group showed typical cytoplasmic lipid

droplets, free ribosomes, and euchromatic nucleus. The PTH treated group (group II) HSCs cells showed dilated rough endoplasmic reticulum and loss of lipid droplets. On the other hand, the PTH-Amygdalin group (Group III) HSCs cells were more or less close to the control ones with small lipid droplets, scanty free ribosomes and a large intended euchromatic nucleus (Fig.4 A, B & C).

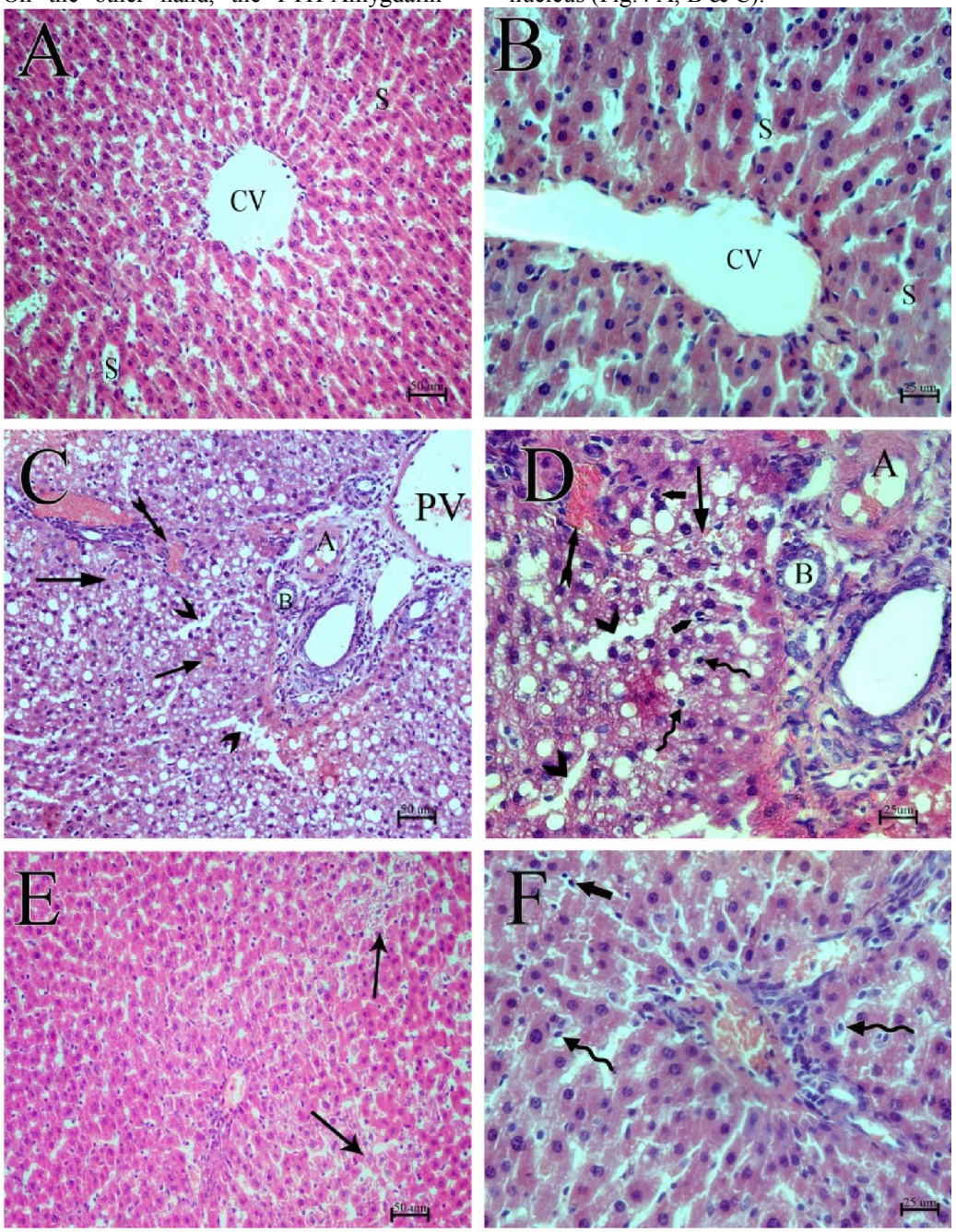

Fig. 1: Histopathological specimens of the liver tissue stained with H & E; A & B the control group (Group I) showing the normal liver architecture with normal central veins (CV) and blood sinusoids (S). The radiating cords of hepatocytes had open face hepatocytes with acidophillic cytoplasm C & D: the PTH treated group (group II) showing congestion of the portal vein radicles (PV) and hepatic sinusoids (tailed arrows). The bile canaliculi (B) and hepatic arterioles (A) appeared normal. Most of the hepatocytes had vaccolated cytoplasm and pyknotic nuclei (curved arrows). Areas of widened separation between the hepatocytes (arrow heads) extensive interstitial hemorrhage (arrows). Inflammatory cell infiltration can be seen intervening between the hepatocytes (short arrows). E & F: PTH-Amg group (group III) apparently normal hepatocyes (arrows). With higher magnification, few hepatocytes with pyknotic nuclei and vacculated cytoplasm (curved arrows) and scanty inflammatory cells infeltartion (short arrows) could be seen.

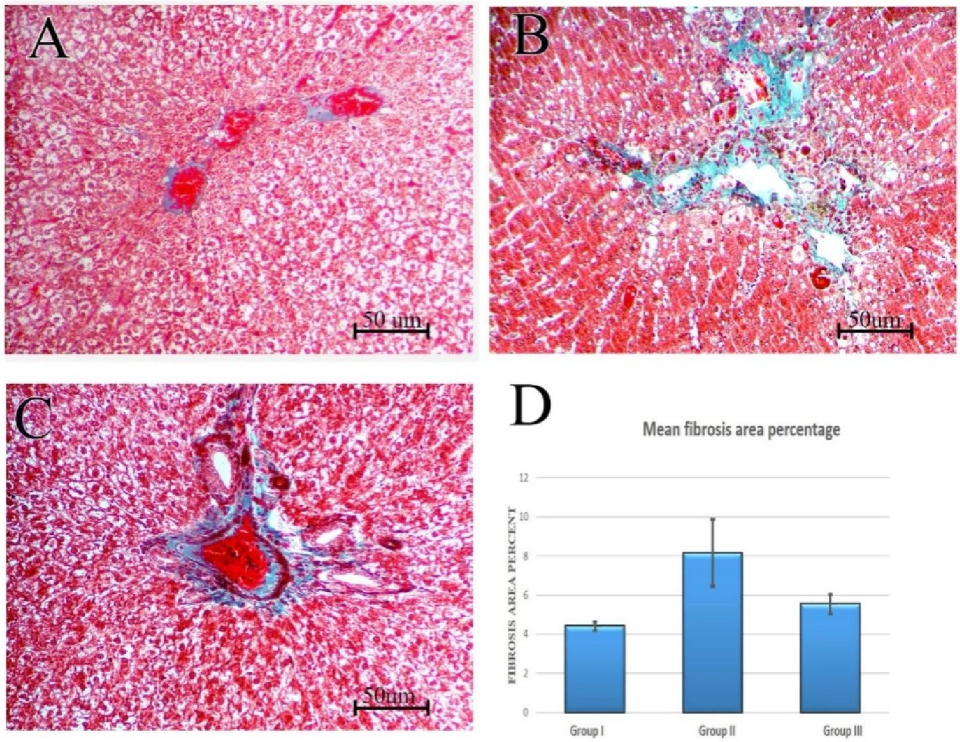

Fig. 2: Masson trichrome stain, A: the control group showing blue stained collagen fibers around the portal triade. B: PTH treated group, showing mrked deposition of blue-stained collagen fibers in the portal triade and between the distorted liver architecture. C: The PTH-Amygdalin treated group had less marked collagen fibers deposition. D: Diagram of the mean fibrosis area percentage in the 3 animal groups.

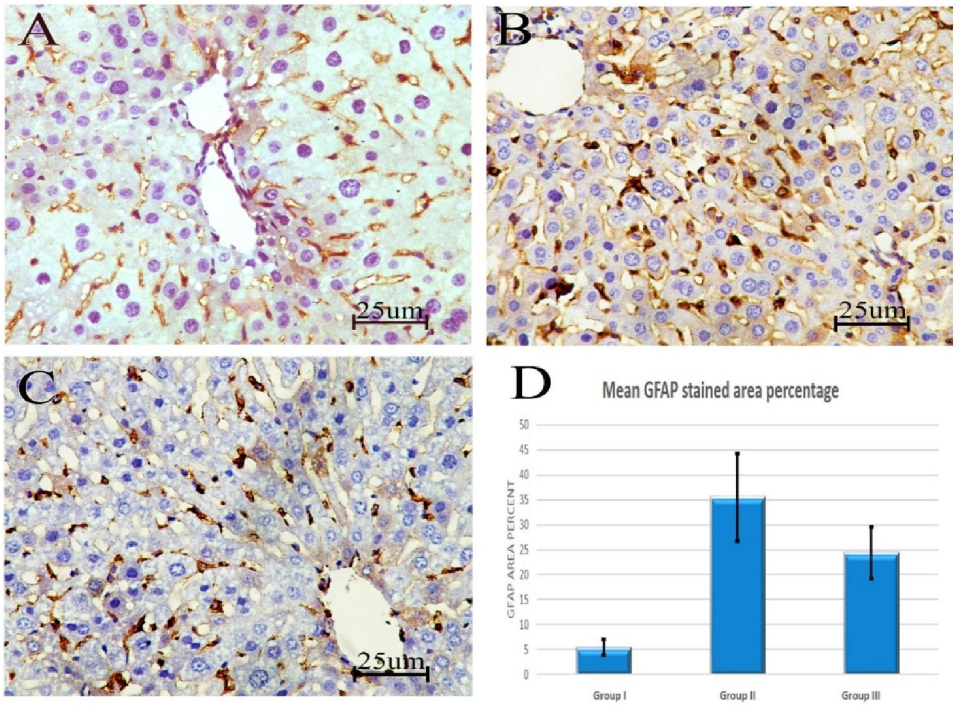

Fig. 3: GFAP immune staining of the liver section of the control group (A) showing positive staining of the stellate cell and the endothelium of the sinusoids. The endothelium of the central veins is negativly stained. B: the PTH group showing strong and diffuse immunoexpression mostly perisinusoidal. C: The PTH-Amg. group showed less marked dye expression. D: Diagram of the mean GFAP stained area percentage of the 3 animal groups

Table 1 Quantitative measurements of the mean area percent fiberosis in masson trichrome stained section and the Serum Glial fibrillary acidic protein (GFAP) positive staining.

	Group I	Group II	Group III
Mean area percentage of fibrosis	4.42±0.23	8.16±1.73** p1<0.001	5.55±0.49* p2<0.05
Mean area percentage of GFAP positive staining	5.39±1.57	35.47±8.80** p1<0.001	24.35±5.19* p2<0.05

Data are the mean ± SD (n = 10).
P1: statistical significance between group II versus the control group.
P2: statistical significance between group III versus the group II.
p>0.05 not significant (ns); p<0.05 *; p<0.01 **

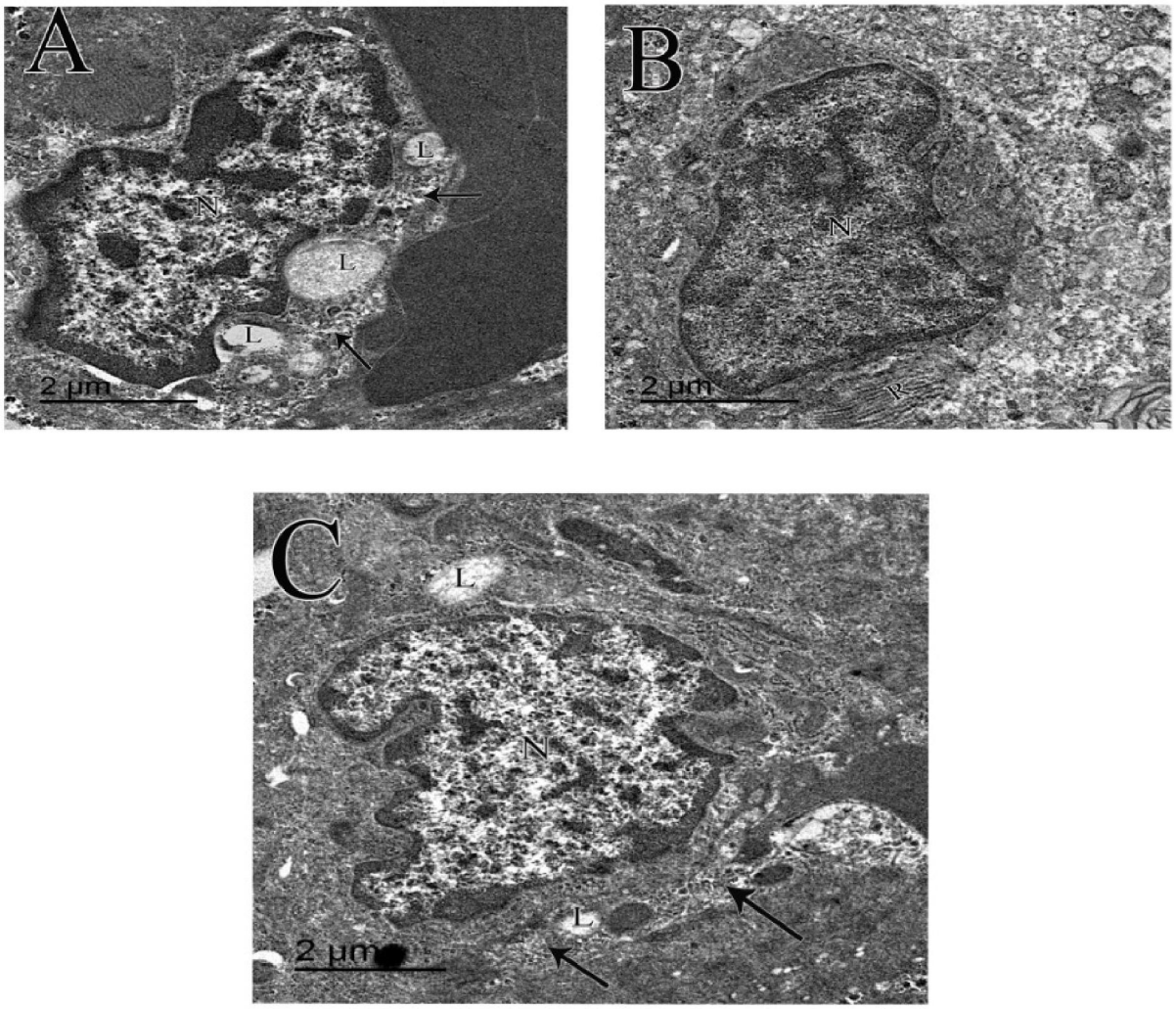

Fig.4: Electron micrgraph of HSCs of A: the control group showing a control HSCs cell with abundant lipi droplets (L), free ribosomes (arrows) and intended euchromatic nucleus (N). B: the liver parenchyma of PTH treated group showing HSCs with rounded euchromatic nucleus (N), dilated rough endoplasmic reticulum (R) and clear cytoplsamdevoied of the lipid droplets. C: the parenchyma of PTH-Amg group showing the HSCs with small lipid droplets (L), free ribosomes (arrows) and a large intended euchromatic nucleus (N).

Biochemical Analysis

The liver enzymes (ALT and AST), Serum total bilirubin, serum hydroxyproline, and serum GFAP were highly significantly increased in group II (PTH treated group) when compared with the control (Table 2). Meanwhile, these measurements were highly significantly decreased in group III (PTH and Amygdalin treated group) when compared to group II. On the other hand, the total proteins were highly significantly decreased in group II in comparison to the controls and highly significantly increased in group III compared to group II.

Table 2 Effects of Amg. on PTH-induced hepatotoxicity. Total proteins, Total Bilirubin, Serum alanineaminotransferase (ALT), aspartate aminotransferase (AST), Serum Glial fibrillary acidic protein (GFAP) and serum hydroxyproline.

	Group I	**Group II**	**Group III**
ALT u/L	16.54±0.52	27.78±0.28** p1<0.001	26.75±0.35** p2<0.001
AST u/L	25.21±0.77	39.30±0.53** p1<0.001	36.58±0.46** p2<0.001
Total Protein (g/L)	13.97±0.12	11.54±0.44** p1<0.001	13.57±0.39** p2<0.001
Total Bilirubin (µmol/L)	8.0±0.03	20.23±0.06** p1<0.001	8.69±0.68** p2<0.001
Hydroxyproline (µg/ml)	1.20±0.06	1.98±0.02** p1<0.001	1.22±0.03** p2<0.05
Serum GFAP (ng/ml)	3.63±0.34	9.11±0.60** p1<0.001	3.82±0.28** p2<0.001

Data are the mean ± SD (n = 10).
P1: statistical significance between group II versus the control group.
P2: statistical significance between group III versus the group II.
p>0.05 not significant (ns); p<0.05 *; p<0.001 **

4. Discussion

The odorless and colorless properties of the PTH potentiate the excessive exposure to the drug(9). Most researchers investigated PTH carcinogenicity being the evident side effect. In animals, it was proved that PTH side effects are dose-dependent and noticeable only in higher and extended doses than that used in human (9, 35). PTH side effects other than carcinogenicity are still in need of more investigations. Studying the PTH- induced histopathological changes on the liver will help to overcome the adverse effects of this drug aiming to achieve more benefits of such cheap and effective laxative.

PTH is primarily metabolized in the liver by conjugation with glucuronic acid with formation of a small amount of sulfation (36, 37). The formed PTH glucuronide is excreted in bile and is liable to deglucuronation by the colon flora. 10-30% of the drug is eliminated by the kidney in either free or conjugated forms (38, 39). PTH was reported to have the ability to generate free radicals (8) besides its clastogenic activities(40).

After oral administration, it is understood that the intestine will be the first targeted organ and followed by the liver. However, PTH was detected in the liver even before it reaches the intestine (41). Most of the previous studies about the effects of PTH on the liver were limited to changes in the liver weight and/or function (42, 43). Nonetheless, our study showed extensive histopathological changes in the livers of the PTH group. The hepatocytes showed cellular degenerative changes in the form of vacuolated cytoplasm and pyknotic nuclei with extensive interstitial hemorrhage and inflammatory cell infiltration. No definitive signs of carcinogenicity were detected. These changes were accompanied by increased serum bilirubin, ALT and AST and decreased total protein.

Contrary of our finding, some studies reported no evidence of PTH effect on the hepatocytes DNA, apoptosis or necrosis (7). These adverse effects were only evident on high doses and they were attributed to the high oxidative stress of PTH and its metabolites on the tissues(8).

Models of precancerous liver lesions reported various degrees of histopathological changes as the multiple proliferative foci of hepatocytes, nuclear atypia, fibrosis and vacuolation (44). In our study, PTH-induced severe histopathological changes but in our opinion, they were not significant enough to prove

the carcinogenic effects probably due to the smaller PTH dose or the short time of its exposure in experiment. Although, exposure to PTH has been reported to cause tumors at several different organs in mice and rats, the data available from epidemiological studies is inadequate to evaluate the actual relationship between human cancer and exposure specifically. Additionally, no evidence is available to suggest that mechanisms by which PTH causes tumors in experimental animals would also apply in humans (9).

The PTH-induced histopathological changes were alleviated in Amygdalin treated group. These findings were accompanied by improvement of the liver cell functions. This effect can be explained by the fact that Amygdalin is reported to an efficient antioxidant (45) and its ability to decompose carcinogenic substances in the body (46). Moreover, Amygdalin decomposes during digestion into pharmacologically active metabolites as hydrocyanic acid which is an antitumor, and benzaldehyde, which is an analgesic (18).

Hepatic fibrosis is the pathological healing process resulting in abnormal proliferation and accumulation of connective tissue in the liver (47). It occurs as a normal tissue response to injury in which the liver heals by scar formation which unfortunately disturbs the liver parenchyma (48). The key step of liver fibrosis is the conversion of the HSCs from quiescent cells into proliferative, contractile and fibrogenic myofibroblasts-like cells (24).

In our study, Masson trichrome stain revealed a marked increase in collagen bundles deposition around portal triads in group II while, group III showed a milder degree of collagen fibers deposition around portal tract with congested vasculature which is further substantiated by the quantitative assessment of the mean area percentage of fibrosis and the changes in the level of tissue hydroxyproline (increased in group II and decreased in group III).

PTH was reported to induce fibrosis in the liver parenchyma secondary to the induced severe liver tissue injury and inflammatory cell infiltration (49). The process of fibrosis starts with activation of the HSCs by the produced mediators and growth factors produced from inflammatory cells (50). In response, fibrogenic myofibroblasts like cells synthesize collagen, glycoproteins, and proteoglycans with subsequent deposition in the extracellular matrix of the liver. This process is also aggravated by the accompanying impaired collagenolysis (51).

Amygdalin treated group, on the other hand, showed a significant decreased fibrosis in both histopathological examination and quantitative measurements. This action of Amygdalin can be explained by its protective effect on the liver tissue which prevented subsequent inflammatory cell infiltration and avoided over activation of HSCs.

As a marker of HSCs activation, our study used GFAP as a specific antibody for detection of the activation of the HSCs. A significant increase in the serum levels of GFAP in group II was reported secondary to PTH treatment. A reversal of this finding was reported after Amygdalin pretreatment in group III. This finding was also supported by microscopic examination of GFAP immune staining and the quantitative measurement of the mean area of immunopositivity.

GFAP is the main intermediate filament protein in mature astrocytes and the up-regulation in its expression pattern is characteristic of the astrocyte activity (30). However, the exact function of GFAP remains obscure. It has been suggested that GFAP helps astrocytes to maintain mechanical strength and their shape (52). GFAP expression is not limited to the central nervous system (30). An overall increase of GFAP expression was reported in a variety of diseases away from the nervous system such as in muscles (53) or liver (24). Overexpression of GFAP is probably caused by activation of the mitogen-activated protein kinase termed Erk2 (extracellular signal-regulated protein kinase) (54).

Early activation of the HSCs was monitored by different markers such as alpha-smooth muscle actin, however, GFAP was found to be superior to alpha-smooth muscle actin due to its high specificity as an early predictor of liver cirrhosis (55). GFAP expression was reported to increase in acute liver injury and a downward expression in chronic liver injuries (24). We reported an increase in GFAP expression after PTH administration in group II as the short duration of the study (21 days) did not allow for chronicity.

Upon activation, HSCs acquire myofibroblast phenotype with increased levels of GFAP expression (56). In our study, livers of PTH treated animals showed concomitant increased serum GFAP and GFAP immunopositivity with the engagement of HSCs in the development of liver fibrosis (57). Other human and animal studies reported the involvement of GFAP expression in vascular remodeling in the hepatic damaged tissue, in which neovascularization significantly increases during the development of liver fibrosis and carcinogenicity (58, 59).

This finding was supported by the evidenced ultrastructural changes in the HSCs. A significant diminution of the lipid droplets of the HSCs and proliferation of the rough endoplasmic reticulum are signs of changing nature of the cells (60). The decreased number of fat droplets with the progression of the disease suggested the transitional stage of the

cells in its way to change myofibroblast phenotype (61).

Our study showed that Amygdalin administration protected against the PTH-induced histopathological changes, which is evident by decreased tissue fibrosis and improved liver function parameters. These effects were owed to the antioxidant property of Amygdalin. GFAP levels reflected the degree of change in the liver pathology. Other promising properties of Amygdalin such as anticarcinogenic and analgesic effects favor its use to counteract the side effects of PTH.

Conclusion

Amygdalin treatment demonstrated an evident hepatoprotective effect against PTH induced liver injury and fibrosis and GFAP level measurement can be a good predictor for early detection of HSCs transformation.

Author contributions

Both YME and HAM prepared the animals and chemicals, performed the experiments, examined the slides and prepared the figures, interpreted the results and wrote the manuscript. They also read, reviewed and approved the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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