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Attenuation of trichloroacetic acid-induced hepatocellular carcinoma by *Artemisia judaica* ethanolic extract in male rats

EL-Hassan M. Mokhamer^{1*}, Abdel-Aziz A. Zidan^{1,2}, Nourhan K. El.Ghayesh¹ and Karolin K. Abdel-Aziz¹

Abstract

Background: The liver cancer is one of the most frequent solid organ malignancies worldwide. Alternative medicine is deemed as one approach that may progress anticancer drugs efficacy and minimize their toxic effects. Similarly, this study was designed to establish the ethanolic extract from *Artemisia judaica* (AJ) plant and characterize extract that formed using gas chromatography–mass spectrometry analysis, then evaluate their anti-tumour activity alone or in combination with cyclophosphamide(CTX) against trichloroacetic acid (TCA)-induced hepatocellular carcinoma in rats.

Results: The obtained results revealed that a significant elevation in serum transaminases (ALT, AST), and alkaline phosphatase activities, as well as total serum bilirubin (T.Bil) levels, was recognized in TCA injected rats compared with the control group. In contrast, a significant decrease in liver antioxidant enzymes superoxide dismutase and catalase activities, as well as reduced glutathione level was observed in TCA treated rats compared with the control group. Furthermore, administration of AJ alone or in combination with CTX in rats presented a significant amelioration in all mentioned parameters and attenuated the increased malondialdehyde level in liver tissues compared to the TCA group.

Conclusions: It could be suggested that AJ hepatoprotective effect against chemical-induced hepatocarcinogenesis in combination with chemotherapy drug by reducing chemotherapy side effects confirmed by haematoxylin and eosin stain (H&E) observations, improvement of oxidative stress biomarkers, and liver enzymes.

Keywords: Hepatocellular carcinoma, Trichloroacetic acid, *Artemisia judaica* plant, Cyclophosphamide, Oxidative stress

Background

Hepatocellular carcinoma (HCC) is deemed one of the most challenging malignancies of multifactorial etiologies that shows increasing mortality indices (Gish, 2006). It has a poor prognosis, which is associated with its rapid progression, aggressive biological behaviour, and underlying chronic liver disease as cirrhosis (Mazzanti et al., 2016). HCC is characterized by

genetic variation accumulation, which causes the clonal selection of tumour cells having the performance of aggressive tumours (Marquardt et al., 2015). Moreover, mutagenic DNA adducts that arise from oxidative stress have the potential to serve as more direct and precise biomarkers to predict HCC risk and recurrence. Reactive oxygen species (ROS) resulted from oxidative stress are involved in the transcription and activation of a large series of cytokines and growth factors, which can contribute to further production of ROS. However, these free radicals can react with many classes of molecules or compounds such as proteins, membranes, DNA, and RNA which drives genomic damage and

*Correspondence: hassan.mokhamer67@gmail.com

¹ Zoology Department, Faculty of Science, Damanhour University, Damanhour, Egypt

Full list of author information is available at the end of the article

genetic instability to cause mutations which play a crucial role in incident diseases (Kurutas, 2015).

To understand the natural history and treatment of liver cancer, animal models have been used, as they serve as a critical bridge between laboratory-based discoveries and clinical trials to clarify the mechanism of hepatocarcinogenesis. Trichloroacetic acid (TCA) is a common laboratory chemical which used to induce HCC in rats by increasing inflammation and oxidative stress (Fouad et al., 2013). TCA induces tumours in laboratory animals through potential mechanisms such as DNA hypomethylation, peroxisome proliferation, oncogene activation, cell proliferation, and inhibition of intercellular communication (Harmon et al., 2011). Systemic treatment options for liver cancer are limited, and only a few are available. Cyclophosphamide (CTX) is widely applied in the clinical treatment of different types of cancers including lymphoma, breast cancer, HCC, and leukaemia (Zhu et al., 2015) although it produces many side effects such as severe cytotoxicity, hair loss, severe nausea, immunosuppression, and vomiting (Alyamkina et al., 2010). These side effects are the major limiting factor in clinical therapy. Therefore, in this study, metronomic (MET) chemotherapy was used due to its lower toxicity effect (Park et al., 2010).

Natural products have been considered as promising cancer chemo-preventive agents owing to advantages such as multi-target properties, easy availability, low toxicity, and reduced production cost (Bishayee & Sethi, 2016). Among them, *Artemisia judaica* (AJ) plant which grows widely in north and south Sinai, Egypt. It has been found that plants can generate a lot of secondary metabolites which occurs naturally, and may be important in pharmacology. These essential metabolites may include saponins, sterols, flavonoids, alkaloids, phenols, and tannins (Hashem et al., 2019). These metabolites enhance the plant to cure gastrointestinal disturbance, upset stomach, poor eyesight, cardiovascular disorders, abdominal disturbances, skin disorders, and weak immune systems as well as decrease the risk of atherosclerosis, cancer, and arthritis (Janačković et al., 2015).

Methods

Chemicals and drugs

TCA and CTX were obtained from Sigma Chemical Company, USA (purity 99.0%). TCA was neutralized by 0.1 N NaOH (PH=7). Ethyl alcohol (96%), tween-80, diethyl ether, 10% neutral-buffered formalin, and the sodium hydroxide pellets were obtained from Al-Gomhoria Company for chemicals and medical supplies, Alexandria, Egypt.

Plant material collection and extraction

Fresh aerial parts of *Artemisia judaica* (AJ) plant were gathered from Arish-North Sinai, Egypt in 2019. The plant extract was prepared accordingly to the procedure described by Williamson et al. (1996) with slight modification. The plant material (2000 g) was air-dried at room temperature. The herb was ground to obtain a moderately coarse powder using mechanical mortar grinding and pass through a sieve to obtain the fine powder, the resultant dry powder soaked immediately in ethyl alcohol (96%) at room temperature (100 g/1L) for 9 days (in 3-day intervals), the organic phase filtered through filter paper, then the filtrate was concentrated under vacuum using the rotatory evaporator and percolated several times till exhaustion. The yield (a dark-green viscous residue) was chilled in the refrigerator at 5 °C until use. The tested solution was freshly prepared every day by dissolving 2 g of the viscous residue in 10 ml of 1% solution of tween-80 in distilled water. To identify the chemical constitution of the aerial parts from *Artemisia judaica* ethanolic extract gas chromatography-mass spectrometry (GC-MS) at the national institute for oceanography and fisheries, Alexandria, Egypt was applied. A literature preview was carried out to check the biological activities of different identified compounds.

Animals and management

One hundred healthy adult male Wister albino rats (age, 7–8 weeks), weighing 180–225 g, were obtained from the 'Egyptian Organization for Biological Products and Vaccines', Giza, Egypt, and housed in metallic boxes with controlled temperature (22–25 °C), photoperiod (12 h light: 12 h darkness). They were preserved under suitable commercial diet and tap water during the experiment. The Ethical Committee guidelines for animal care were used according to the medical research institute (appendix to guiding principles for biomedical research involving animals, 2011), Alexandria University, Egypt. After a two-week acclimatization period, animals were classified.

Experimental protocol

The rats were randomly allocated into five groups, each group including 20 rats. HCC was induced in the groups (2–5) with TCA as the following:

The first group served as control group (G1) was given only 1% solution of tween-80 (25 mg/kg/day, orally), for 6 weeks. The second group, the TCA group (G2) was orally administrated TCA (500 mg/kg/day) (Fouad et al., 2017), for 5 days and then 4 weeks later was given 1% solution of tween-80 (25 mg/kg/day, orally), for 6 weeks. The third group, the TCA + AJ group (G3) was orally treated with AJ (0.5 g/kg/ day)

(Nofal et al., 2009), for 6 weeks after being treated with the same dose of TCA as in G2. The fourth group, the TCA + CTX group (G4) was injected with CTX (20 mg/kg) (Park et al., 2010) intraperitoneally twice a week for 3 weeks and 1% solution of tween-80 (25 mg/kg/day, orally), for 6 weeks after being treated with the same dose of TCA as in G2. The fifth group, the TCA + AJ + CTX group (G5) was treated with the same doses of TCA, AJ, and CTX as described previously.

Blood and tissue sampling

At the end of the experiment, rats were sacrificed after being anaesthetized using diethyl ether and dissected. Blood samples were gathered by heart puncture and allowed to clot at room temperature. After coagulation and centrifugation at 5000 r.p.m for 5 min, the serum was separated and kept frozen at -80°C for biochemical analysis. Liver samples were immediately removed, weighed, and washed.

Histopathological investigation of liver tissue

Liver tissues from different groups were immersed in 10% buffered formalin, then embedded in paraffin wax at 56°C in a hot air oven and sectioned at 4 microns thickness. Finally, the obtained tissue sections were collected on glass slides stained by haematoxylin and eosin stain (H&E) for routine examination through the light microscope. A computer system with a camera for Olympus optical microscope was used for photographing hepatic histopathological changes at the medical technology centre, Medical Research Institute, Alexandria University, Egypt. The tissue architecture was captured under a microscope of $\times 400$.

Biochemical analysis

- Serum enzymatic activities of ALT, AST, and ALP, as well as the total serum bilirubin (T. BiL) levels, were measured according to commercially kits obtained from Spectrum Diagnostics Company, Cairo, Egypt.
- Liver tissue homogenate was used for evaluating CAT and SOD enzymatic activities besides GSH and MDA levels were estimated according to commercially kits obtained from the Bio-diagnostic Company (Dokki, Giza, Egypt). All biochemical parameters in serum and tissue were estimated by spectrophotometer ERMA (AE600N) In Food Safety and Nutrition Laboratory, Faculty of Veterinary Medicine, Damamhur University, Egypt.

Statistical analysis

The results were expressed as Mean \pm SD and the statistical significance was evaluated by Mann–Whitney test using SPSS (version 20.0) program. Values were considered statistically significant when $p \leq 0.05$.

Results

Phytochemical Screening of *Artemisia judaica* extract

Artemisia judaica phytochemicals were resolved using GC-MS and the chromatogram is shown in Fig. 1. The Phyto-Compounds were found to be dominated by phenolic compounds, flavonoids, hydrocarbons, terpenes, and sterols listed in Table 1.

Effect of TCA, AJ extract, and CTX on body weights

The current study results revealed a significant decrease in body weights for the TCA group (G2) in comparison with the control group (G1) ($p \leq 0.05$) (Fig. 2). However, the mean body weights of the CTX treated rats alone

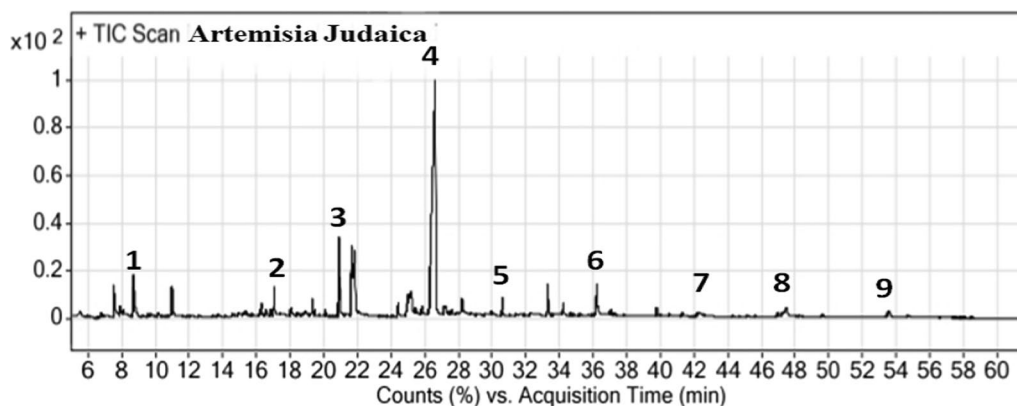
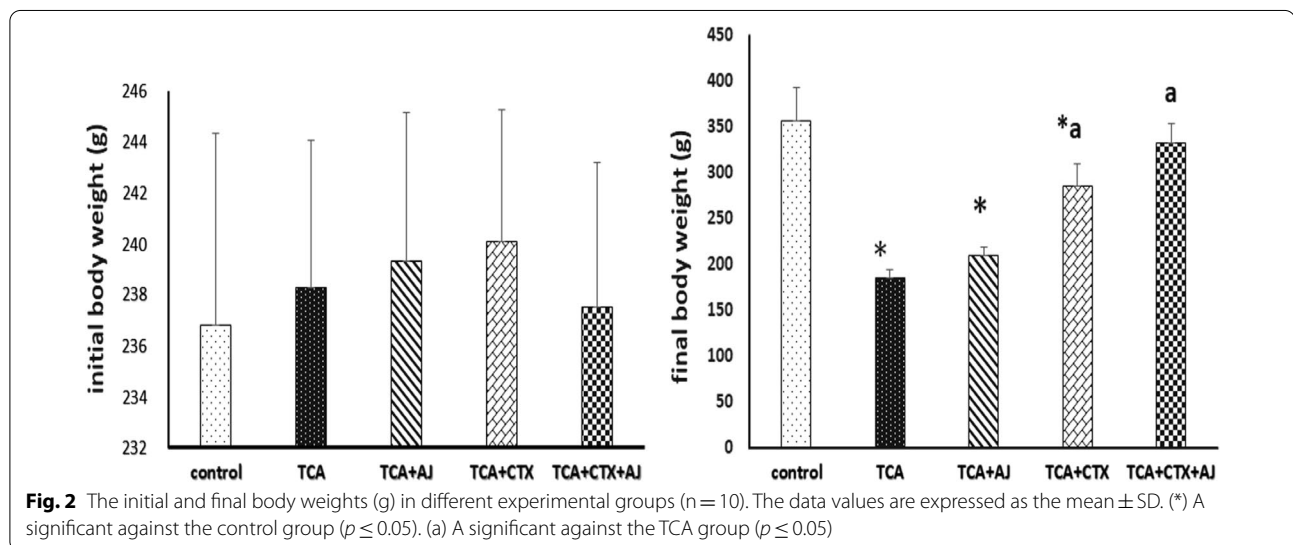


Fig. 1 GC-MS chromatogram of the AJ ethyl alcohol extract from Arish-North Sinai, Egypt showing the peaks of the identified compounds

Table 1 The major Phyto-compounds detected in AJ ethyl alcohol extract by GC–MS analysis from Arish-North Sinai, Egypt, and their biological activities

| Peak no | Detected compound | Biological activity |
|---------|---------------------------------------------------------|------------------------------------------------------------------------------------|
| 1 | Camphor | Antioxidant (Pandey & Singh, 2017) |
| 2 | Grandisol | Antibacterial and antimicrobial (Yang et al., 2015) |
| 3 | D-Limonene | Antioxidant and anticancer (Elansary et al., 2018) |
| 4 | 2,5-Octadecadiynoic acid, methyl ester | Anti-inflammatory (Hameed et al., 2016) |
| 5 | Cholestan-3-ol, 2-methylene-, (3 β ,5 α)- | Anti-inflammatory (Al-Rubaye et al., 2017) |
| 6 | n-Hexadecanoic acid | Anti-inflammatory and Antioxidant (Aparna et al., 2012) |
| 7 | α -Santonin | Anti-inflammatory, analgesic, and mild anticancer activities (Mabtin et al., 1988) |
| 8 | Heptacosane | Antimicrobial (Konovalova et al., 2013) |
| 9 | 17-Pentatriacontene | Anti-inflammatory and anticancer (Kumar et al., 2018) |



(G3) or in combination with AJ extract (G5) showed a significant increase in the body weights when compared to the TCA treated group (G2) ($p \leq 0.05$). No significant increase in the body weights was observed in the AJ extract treated group (G3) when compared to the TCA group (G2).

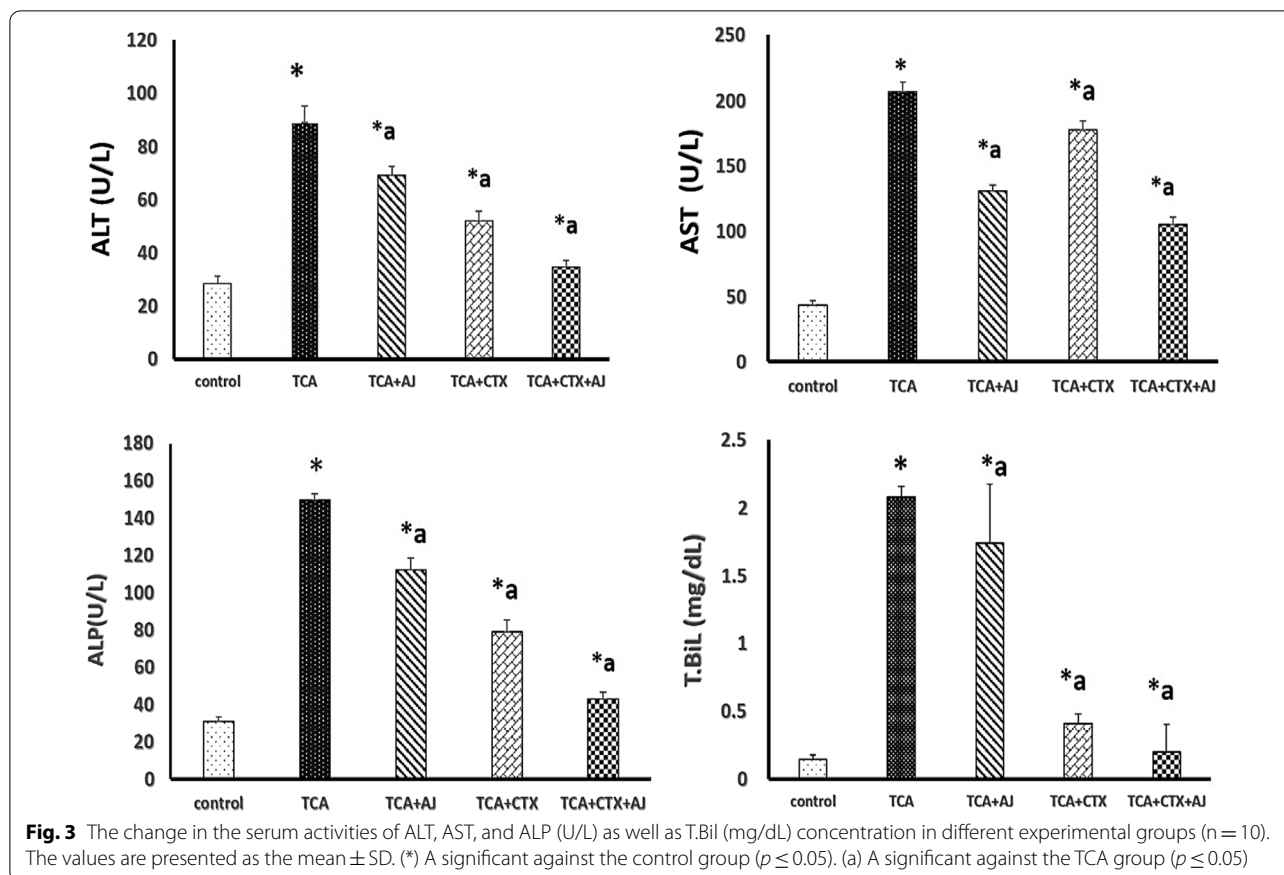
Effect of TCA, AJ extract, and CTX on liver enzymes and Total Serum Bilirubin

The mean serum enzymatic activities of ALT, AST, and ALP, as well as T.Bil concentration, showed a significant increase in the TCA group (G2) compared with the control group (G1) ($p \leq 0.05$). The mean serum enzymatic activities of ALT, AST, and ALP, as well as the T.Bil concentration, were significantly decreased in the AJ extract (G3) and the CTX (G4) treated groups when compared to the TCA group (G2) ($p \leq 0.05$). Also, there was a significant decrease in the liver enzymes levels and the T.Bil

concentration in the AJ extract and CTX treated group (G5) when compared to the TCA group (G2) (Fig. 3).

Effect of TCA, AJ extract, and CTX on liver oxidative stress biomarkers

The mean concentration level of GSH content, as well as the enzymatic activities of CAT and SOD, was significantly diminished in the TCA group (G2) compared with the control group (G1) ($p \leq 0.05$). Meanwhile, the mean concentration level of GSH content, as well as the enzymatic activities of CAT and total SOD, was significantly elevated in AJ extract (G3), CTX (G4), and AJ extract in combination with CTX (G5) treated groups when compared to the TCA-intoxicated group (G2) ($p \leq 0.05$). Also, there were insignificant changes in the GSH content as well as the enzymatic activities of CAT and SOD in the AJ extract and CTX treated group (G5) when compared to the control group (G1) (Fig. 4). The mean



concentration level of MDA was significantly elevated in the TCA group (G2) compared with the control group (G1) ($p \leq 0.05$). Conversely, the mean concentration level of the MDA was significantly decreased in the AJ extract and CTX treated groups (G3, G4, G5) when compared to the TCA treated group (G2) ($p \leq 0.05$). Also, there was no significant difference in the MDA concentration level in the AJ extract and CTX treated group (G5) when compared with the control group (G1) Fig. 4.

Histopathological changes

Normal liver architecture has been observed in the control group; histopathological changes, associated with TCA intoxication, as well as the AJ extract treatment (G3) are shown in Fig. 5. Also, liver architecture changes in CTX treated group (G4) and AJ extract combined with CTX treated group (G5) are shown in Fig. 6.

Discussion

Hepatocellular carcinoma (HCC) is one of the most fearful cancers worldwide with an incidence of over one million cases every year. It is induced by poisonous industrial chemicals, air pollutants, water contaminants, food additives, and other toxins (Kumar et al.,

2016). TCA is a chemical found in drinking water as part of a mixture of by-products resulting from water disinfection, which is absorbed rapidly, circulating to different organs, and causing severe organ damage (Aslani et al., 2019). TCA-induced severe toxic liver damage which is a well-characterized model for hepatocellular carcinoma has been performed (Fouad et al., 2017). Accordingly, deterrent measures are needed to find new slipways for HCC treatment.

Among the criteria used in estimating organ damage or injuries due to cancer development are the body weights. The present study revealed that there was a perceivable loss in body weight in TCA-intoxicated rats (G2) compared to the control rats consistent with Sweeney et al. (2009). This weight loss may be attributed to the cytotoxic effect of TCA which cause damage for liver tissue. On the other hand, the treatment of TCA-intoxicated rats with the AJ extract alone (G3) or in combination with CTX (G5) returned the body weight that could be attributed to the AJ content of flavonoids, essential oils, tannins, terpenes, and saponins which reduce the oxidative stress and protect against tissue damage. Also, the previous study of Park et al. (2010) elucidated the ameliorative impact of MET CTX

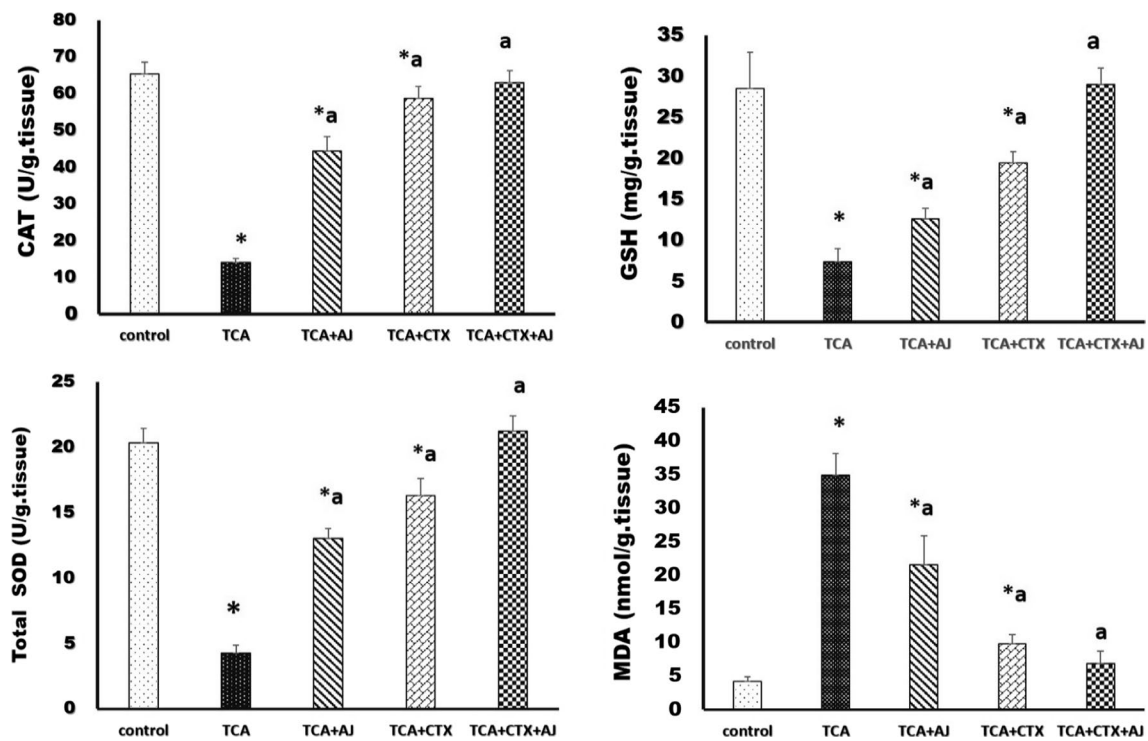


Fig. 4 The change in CAT and SOD activities (U/g. tissue) besides GSH (mg/g. tissue) and MDA (nmol/g. tissue) concentration in liver tissues for different experimental groups. The data are expressed as the mean \pm SD. (*) A significant against the control group ($p \leq 0.05$). (a) A significant against the TCA group ($p \leq 0.05$)

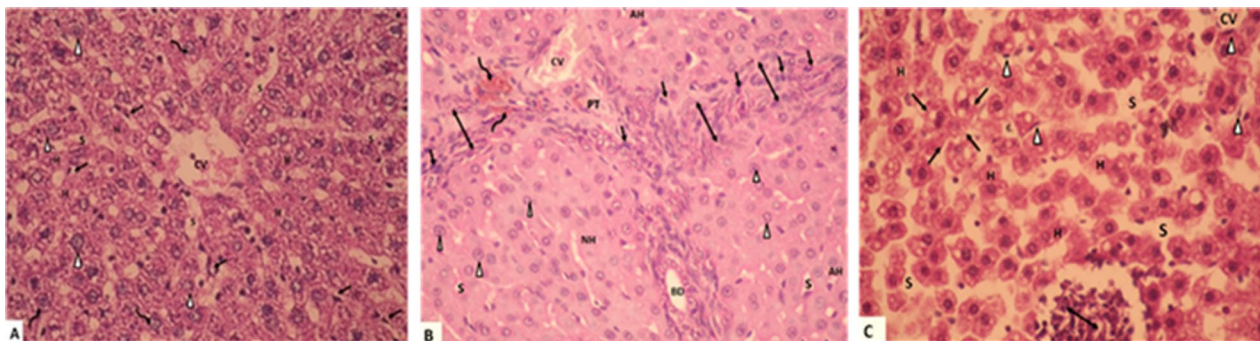


Fig. 5 The histological examination of paraffin sections of the liver viewed under a light microscope in (G1), (G2), and (G3), respectively (H&E \times 400). **A** Normal liver tissue revealing polyhedral hepatocytes (H) with Kupffer cells (arrow), normal central vein (CV), normal blood sinusoids (S), and rounded vesicular nuclei (head arrow). **B** TCA treated liver tissue revealing Thick fibrous tissue septa (two head arrow) dilated bile duct (BD) and dilated central vein (CV). **C** AJ treated liver tissue revealing area of aggregated lymphocytes (two head arrow), presence of few Kupffer cells (head arrow) and the hepatocytes (H) were markedly regulated to the normal architecture and directed to blebs cytoplasm (arrows)

treatment (G4) on body weight loss in experimentally induced HCC.

Under chemical-induced liver damage, aspartate and alanine aminotransferases enter the circulatory system due to alteration of hepatocyte membrane permeability (Saad et al., 2014). In the agreement with previous studies (Fouad et al., 2013), the data of the present study

confirmed the elevation in serum activity levels of AST and ALT in TCA-intoxicated rats (G2) compared to the control group (G1). This can find a good corroboration in the recent study of TCA to induce hepatic oxidative stress where Ni et al. (1996) elucidated that TCA bio-transformation (with the help of cytochrome P450) usually converted to dichloroacetic acid via a dichloroacetic

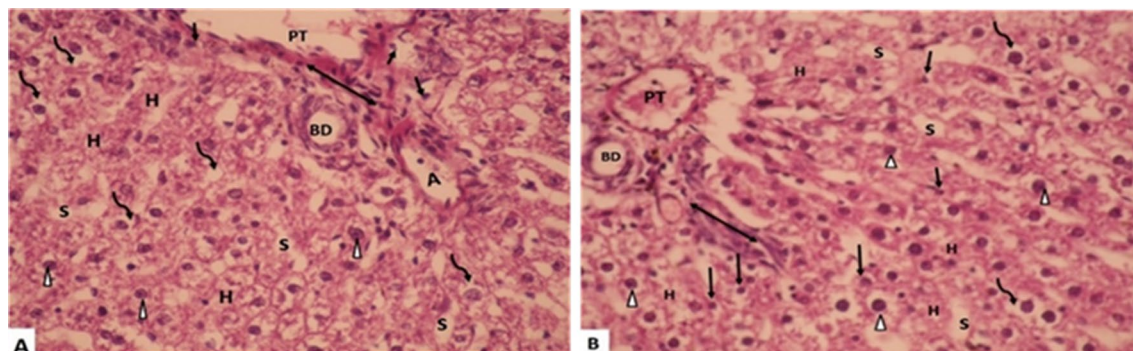


Fig. 6 The histological examination of paraffin sections of the liver detected under a light microscope in (G4) and (G5), respectively (H&E \times 400). **A** CTX treated liver tissues revealing a decrease in fibrosis (two head arrow) around the portal tract (PT), reduction of infiltrating lymphocytes (arrow) and hepatocytes (H) with hyperchromatic nuclei (head arrows). **B** CTX and AJ extract treated liver tissues revealing some hepatocytes (H) with less vacuolated cytoplasm (waved arrow) and hyperchromatic nuclei (arrowheads), some fibrous cells (two head arrow) are also shown and many pyknotic hepatocytes (arrows)

acid-free radical causes oxidative DNA damage and produces lipid peroxidation. Meanwhile, Fig. 3 represented the ameliorative effects of the AJ extract treatment alone (G3) or in combination with a chemotherapy drug (G5). Moreover, the AJ extract treatment against TCA-intoxicated rats (G3) had shown more ameliorative effect on AST enzymatic activity than that of CTX treatment when used alone (G4); these may be due to the cardiotoxicity impact of CTX (Abdallah et al., 2019) which is indicated by the elevated AST enzymatic activity in the CTX group (G4) as it is a mitochondrial enzyme that is released from heart, liver, and skeletal muscles.

On the other hand, the noticeable elevation of ALP enzymatic activity as a marker for TCA-intoxicated rats might be due to a bile duct obstruction, hence the failure of enzyme excretion, and thus its elevation in the blood (Wiwanitkit, 2001). Alleviating the activity levels of ALP ectoenzyme in post-treatment with the AJ extract alone (G3) or in combination with the CTX drug (G5) might be assigned to the decrease in mechanical obstruction in the bile duct. The hepatoprotective effect of AJ (reported in the current study) is consistent with the previous study of Ahmed et al. (2017) who elucidated the hepatoprotective effect of the AJ extract against doxorubicin-induced hepatic damage. Also, the previous study of Mohamed et al. (2019) elucidated the ameliorative effect of CTX treatment (G4) on ALP enzymatic activity level.

The total serum bilirubin (T.Bil) level is a sensitive biomarker for hepatocyte damage. The decreased biliary secretion of conjugated bilirubin rather than increased bilirubin load due to haemolysis leads to increased serum bilirubin. In hepatic tumours, haemolysis (plus deranged liver function) leads to hyperbilirubinemia. In the present investigation, TCA-intoxicated rats showed a significant elevation in the levels of total serum bilirubin compared

to the control group which may be due to the hepatocellular or obstructive jaundice in HCC (Abdel-Hamid et al., 2011). After treatment with the AJ extract either independently (G3) or in combination with the CTX drug (G5), a reduction in T.Bil level was observed due to therapeutic and hepatoprotective efficacy of alkaloids and flavonoids in the AJ extract. These findings are in agreement with Janghel et al. (2019) who reported the protective effect of alkaloids and flavonoids from plants on jaundice levels.

The histopathological examination in the present study highlighted and confirmed the biochemical results since it is evidence that the liver tissues after TCA intoxication were showing disturbed liver architecture with thick fibrous tissue septa and infiltration of lymphocytes as well as dilation and congestion of hepatocyte sinusoids with large rounded vesiculated nucleus Fig. 5B. The present findings concurred with many other studies which reported that necrotic hepatocytes resulted from TCA intoxication with marked elevations in serum aminotransferase levels and jaundice. On the other hand, administration of CTX to TCA-intoxicated rats showed scattered macro- and micro-vacuolated hepatocytes.

Oxidative stress, dysfunction of cytochrome P450, inflammation, and mitochondrial dysfunction are considered as the main mechanisms that explain liver damage (Zhang et al., 2018). Flavonoids showed a promising ameliorative impact on liver damage induced by toxins (Li et al., 2018). The herein results depicted a significant decline in the hepatic enzymatic activities of AST, ALT, and ALP (G3); this may be due to the antioxidant properties of flavonoids in the AJ extract which reduce ROS production, regulating the expression of hepatic liver biosynthesis and metabolism genes. The anti-inflammatory activity of AJ extract may be the another mechanism by which it ameliorated the disturbed liver architecture in

the current investigation. The anti-inflammatory effect of AJ was previously reported to relieve inflammatory reactions in various studies, such as rheumatoid arthritis, encephalomyelitis, hepatitis, and colitis (Pandey & Kumar, 2016). Moreover, flavonoids isolated from *Artemisia judaica* showed a protective effect on experimentally hepatic damage through decreasing the level of phosphorylated c-JNK, extracellular signal-regulated kinase (ERK), and P38 mitogen-activated protein kinases (p38MAPK). It diminished the increases of nuclear factor kappa (NF- κ B) and c-Jun nuclear translocation while enhancing the nuclear level of nuclear factor erythroid-2-related factor 2 (Nrf2), indicating its role in enhancing antioxidative defence system and reducing inflammation (Khan et al., 2019).

Lipid peroxides estimated as MDA are applied as a remarkable index of oxidative stress to estimate the oxidative damage in patients with liver cancer (Lorente et al., 2016). The oxidative toxic effect of TCA can be noticed in (G2) Fig. 4 as the elevation of MDA together with diminished GSH level as a non-enzymatic antioxidant, CAT and SOD activities, as major antioxidant enzymes critically needed for the scavenging of MDA as a marker of oxidative stress compared to the control group. The present study depicted a significant decline in MDA level indicating the antiperoxidative effect of the AJ extract alone (G3) or in combination with CTX (G5), which was manifested by a marked increase in GSH level as well as the enzymatic activities of CAT and SOD. It was suggested that the phenolic content may explain the antioxidant impact of AJ (Hashem et al., 2019), that having hydroxyl groups linked to the aromatic ring can act as hydrogen donors to scavenging of free radicals (Valentao et al., 2003). Moreover, the phenolic compounds can reduce the metal ions. So, the phenolic content of the *Artemisia judaica* might hold the key for its antioxidant properties. It has been proven that somatic cell mutations might be caused by the direct effect of ROS which are considered as cancer promoters and oncogenes (Reczek & Chandel, 2017). Moreover, most chemotherapeutic drugs can induce malignant cell death through their abilities to produce ROS (Wang & Yi, 2008). These explain the different roles of ROS in different stages of tumour development and death (Fu et al., 2014).

The histopathological investigation in the present study highlighted and depicted the restoration of hepatic cells features, with scattered apoptotic cells in few areas and minimal congested sinusoids, mild infiltration of the hepatocytes with the minimal portal tract and central vein dilation (Figs. 5, 6). Moreover, group: 5 (Fig. 6B) showed a decrease in vacuolated hepatocytes with the ameliorative impact of liver cells figure. These findings reflected and gave a good interpretation

of the sharp decrease in the liver enzymatic activities in sera and the ameliorative impact of liver antioxidant biomarkers with a decline in the chemotherapy side effects.

Conclusions

Although in early stages of HCC, surgery is the main effective and curative treatment option. The previous studies demonstrate that *Artemisia judaica* extract is a natural immune supporter, antioxidant, anti-inflammatory, and has a mild anticancer impact. So, we could suggest its ameliorative effect against chemical-induced hepatocarcinogenesis in combination with chemotherapy drug by the improvement of oxidative stress biomarkers, and liver enzymes as well as may reduce the chemotherapy side effects. This result provides scientific validation for the use of *Artemisia judaica* plant in traditional medicine.

Abbreviations

ALT: Alanine aminotransferase; AJ: *Artemisia judaica*; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; CAT: Catalase; CTX: Cyclophosphamide; DNA: Deoxyribonucleic acid; GC-MS: Gas chromatography-mass spectrometry; GSH: Reduced glutathione; HCC: Hepatocellular carcinoma; ROS: Reactive oxygen species; RNA: Ribonucleic acid; SOD: Superoxide dismutase; TCA: Trichloroacetic acid; T.BiL: Total bilirubin; MDA: Malondialdehyde.

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Authors' contributions

KKA and HMM designed the research idea, overall work, and wrote the manuscript. AAZ helped with the experimental protocols. HMM and NKE worked on calculations and statistical analysis. HMM revised the drafted manuscript and made necessary corrections. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The Ethical Committee guidelines in this study for animal care were used according to the medical research institute (appendix to guiding principles for biomedical research involving animals, 2011), Alexandria University, Egypt.

Consent for publication

Not applicable.

Competing of interests

The authors declare that they have no competing interest.

Author details

¹Zoology Department, Faculty of Science, Damanhour University, Damanhour, Egypt. ²Center of Excellence in Cancer Research, Tanta University, Global Educational Hospital, Tanta, Egypt.

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