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Some Studies on the Effect of *Annona muricata* and Cisplatin on Rats Suffering from Liver Cancer

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Abstract

This study aimed to evaluate the effect of Carbone tetrachloride (CCl_4) on inducing liver cancer beside the effect of Cisplatin and *Annona muricata* on the treatment of liver cancer in rats and to investigate the efficacy, safety and tolerability of *Annona muricata* leaf. A total of 56 healthy male-albino rats (3-4 months old), weighing between 180-200 g (Average body weight) were divided into two main groups. Group (1) contained 14 healthy rats left without any treatment (Negative control), then 42 healthy rats received IP injection with CCl_4 at dose of 1 ml/ kg/ twice every week for 8 weeks to develop Hepatocellular carcinoma (HCC), after the onset of liver cancer, rats were separated into three equal groups (14 in each) as follow: Group (2) included 14 rats suffered from liver cancer and left without any treatment (Positive control). Group (3) involved 14 rats suffered from liver cancer and received IP injection of Cisplatin at dose of 6 mg/kg/week for 4 weeks. Group (4) included 14 rats that had liver and received *Annona muricata* orally at dose of 300 mg/kg every day for 4 weeks. At the end of experiment blood samples were taken from all rats in all groups to preserve the serum for estimation of serum liver enzymes (Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and Alkaline phosphatase (ALP)), Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), B-cell lymphoma 2 (BCL2), Transforming growth factor beta (TGF-β) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB). Liver tissue was collected for gene expression determination of Caspase-3 and Caspase-9 levels, and Immunohistochemistry studies carried out to measure Silver-stained Nucleolar organizer regions (AgNORs) and alpha smooth muscle actin (α-SMA). In the current work, it has been found that; rats with HCC showed significant increase in ALT, AST, ALP, Nrf2, BCL2, TGF-β, NFκB, Caspase-3 and Caspase-9 levels in comparison with negative control rats. Rats with HCC treated with Cisplatin displayed non-significant decrease in serum AST, ALT, ALP and Nrf2 beside significant decrease in BCL2, TGF-β, NFκB, Caspase-3 and Caspase-9 in comparison with positive control rats. Rats with HCC treated with *Annona muricata* displayed significant decrease in AST, ALT, ALP, Nrf2, BCL2, TGF-β, NFκB, Caspase-3 and Caspase-9 in comparison with positive control rats. Photomicrograph of peroxidase stained rat liver of negative control group showing negative expression for AgNORs beside negative expression for α-SMA. Photomicrograph of peroxidase stained liver of rats with HCC showed positive severe expression for AgNORs beside moderate to severe positive expression for α-SMA. Photomicrograph of peroxidase stained liver of rats with HCC treated with Cisplatin showed positive mild to moderate expression neither for AgNORs beside mild to moderate positive expression for α-SMA. Photomicrograph of peroxidase stained liver of rats with HCC treated with *Annona muricata* showed positive mild expression for AgNORs beside negative to mild positive expression for α-SMA. In conclusion, the present study indicates that *Annona muricata* gives significant improvements in treatment of Albino rats with liver cancer, due to its antioxidant, anti-inflammatory and anticancer effects beside improvements of hepatorenal cellular carcinoma.

KEYWORDS
CCl_4, Cisplatin, *Annona muricata*, Liver cancer and Immunohistochemistry.

INTRODUCTION

Cancer starts when cells in the body begin to grow out of control (malignant growth) (Olude *et al.*, 2023). Hepatocellular carcinoma (HCC) is a primary liver cancer with high incidence rate, especially in regions with high prevalence of viral hepatitis infection. Autoimmune disorders, diabetes mellitus, obesity, alcohol consumption, and inflammation can also lead to initiation and development of HCC (Paskeh *et al.*, 2023). HCC is the most common primary liver cancer and the sixth tumor with higher incidence, ranking as the fourth deadliest neoplasm worldwide. Liver damage caused by different etiologic agents, mainly hepatitis C and B virus (HCV and HBV, respectively), contributes to HCC development through the stages of liver fibrosis and cirrhosis, which can take from years to decades. Its complex pathogenesis and molecular heterogeneity hinder HCC early diagnosis, making curative treatments impossible (Fondevila *et al.*, 2019). The treatment of cancer includes various traditional methodologies such as radiotherapy, chemotherapy, surgery, immunotherapy alone or in combination. However, efficacy of the methods was greatly reduced by their limitations, such as sensitivity of normal cells to irradiation, chemotherapeutic drug resistance, poor liver functional reserve, incomplete tumor resection, and development of intrinsic or acquired resistance (Kirtonia *et al.*, 2021). To overcome

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the disadvantages of the present methodologies, the discovery of novel anticancer agents with improved efficacy and minimal side effects continues. There has been an increasing global trend of traditional and complementary medicines used by people living with cancer.

Cisplatin is a platinum-based chemotherapy agent used to treat various sarcomas, carcinomas, lymphomas, and germ cell tumors. Cisplatin is a potent anticancer agent, but therapeutic use is limited due to its nephrotoxic effects. Cisplatin is a platinum drug that has an anti-cancer effect (Ghosh, 2019). Cisplatin was initially discovered to prevent the growth of Escherichia coli and was further recognized for its anti-neoplastic and cytotoxic effects on cancer cells (Brown *et al*., 2019). Cisplatin has been widely used as a first-line platinum based-chemotherapy for lung cancer patients (Somruethai *et al*., 2021). It is one of the first-line anti-cancer drugs for the treatment of various malignant tumors in clinic. However, the therapeutic efficacy is compromised by the non-specific bio-distribution and insufficient tumor uptake that cause systemic toxicities (e.g., nephrotoxicity, neurotoxicity) and drug resistance (Rottenberg *et al*., 2021).

Natural products, especially those derived from plants, have been used to help mankind sustain its health since the dawn of medicine (Olude *et al*., 2023). The long history of employing natural products in ethnomedicine with low-prices and limited side effects, in contrast to expensive synthetic drugs with severe adverse side effects, was the main reason for the development of new pharmaceutical drugs from natural sources. *Annona muricata*, commonly known as soursop, graviola, guanabana, paw-paw and sirsak, is a member of the Annonaceae family comprising approximately 130 genera and 2300 species (Omoregie *et al*., 2020). *Annona muricata* leaf contained steroids, alkaloid, flavonoid, phenolic, and saponin. *Annona muricata* (Soursop) is one of the herbal plants that are widely used as anti-diabetic, anti-inflammatory, insecticidal, antimalarial, anticancer, antibacterial, and antioxidant (Hasmila and Soekamto, 2019). It also contains a phytochemical compound that correlated with the potential of the mechanism to reduce cancer cells. *Annona muricata* contains annonaceous acetogenins, annomuricin, annonacin, or curcumin; (David *et al*. 2021). It is known as graviola, soursop and guanabana, a herbal product used by people living with cancer. Anecdotal clinical evidence suggests that this herb has a potential anti-cancer activity. There are numerous pre-clinical studies detailing *Annona muricata*'s main bioactive constituents (Chan *et al*., 2023). Graviola (*Annona muricata*) has traditionally been used in many conditions including cancer, and both in vivo and in vitro studies have shown evidence of anti-cancer effects (Pham *et al*., 2019). *Annona muricata* has optimized the antitumor potential of cisplatin and could be utilized as a natural adjuvant to decrease Cisplatin's side effects (El-Khashab and Aniss, 2019). Ethanol extract of *A. muricata* leaves include flavonoids, alkaloids, cardiac glycosides, tannins, triterpenoids, saponins, and reducing sugars. Therefore, the compounds contained in *A. muricata* leaves have selective inhibitory properties on cancer cells that have a high proliferation rate (Hadisaputri *et al*., 2021). The objective of the current study was to elucidate the effects of Cisplatin and *Annona muricata* on liver cancer induced by CCl_4.

MATERIALS AND METHODS

Drugs

Cisplatin: Clear, colorless to pale yellow aqueous solution, sterile, available in vial of 50 ml solution that contains 50 mg of Cisplatin, was purchased from Sigma Chemical Co.

Annona muricata (Graviola): dry gray tablet of 500 mg, dietary supplement based on graviola, soluble in water, this Product was purchased from ORIGINI NATURALI Company.

Animals

A total of 56 healthy male-albino rats (3-4 months old), weighing between 180-200 g (average body weight) were purchased and taken to the animal house at the Department of Biochemistry Faculty of Science, Zagazig University, Egypt. Rats were housed in clean disinfected cages under standard conditions (room temperature with a 12 h light-dark cycle) and initially acclimatized with standard feed on barley, powder milk and clean water for 2 weeks before starting the experiments.

Experimental design

The experimental rats were divided into two main groups. Group (1) contained 14 healthy rats that were left without any treatment (Negative control), then 42 healthy rats received IP injection with CCl_4 at dose of 1 ml/ kg/ twice every week for 8 weeks to develop Hepatocellular carcinoma (HCC), after the onset of liver cancer, rats were separated into three equal groups (14 in each) as follow: Group (2) included 14 rats suffered from liver cancer and left without any treatment (Positive control). Group (3) involved 14 rats suffered from liver cancer and received IP injection of Cisplatin at dose of 6 mg/kg/week for 4 weeks (Jambhulkar *et al*., 2014). Group (4) included 14 rats that had liver and received *Annona muricata* orally at dose of 300 mg/kg every day for 4 weeks (Evy *et al*., 2016).

Ethical approval

The Zagazig University Animal Ethics Committee accepted this work with the approval number ZU-IACUC/1/F/37/2019.

Samples collection

Blood samples were taken from all rats in all groups at the end of the experiment from the orbital plexus by using a heparinized micro-hematocrit tube and placed in a clean, dry centrifuge tube, left to clot at 25˚C before being centrifuged at 3000 rpm/20 min to preserve the serum for further analysis. The obtained clear serum was transferred to clean and dry sterilized plastic eppendorf tubes with a good stopper and kept at -20ºC until determination of biochemical analysis, serum liver enzymes; Aspartate aminotra aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), B-cell lymphoma 2 (BCL2), Transforming growth factor beta (TGF-β), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB).After that the rats were euthanized via halothane anesthesia; the livers were harvested and divided into two-part:

The 1st part: Collected liver tissue from each group were frozen at -20˚C for further Caspase-3 and Caspase-9 expression levels were studied employing the qRT-PCR method.

The 2nd part: Collected liver tissue from each group were fixed in 10% buffer formalin solution, processed with paraffin embedding technique and stained with hematoxylin and eosin for immunohistochemistry study, liver sections on positively charged coated slides were used for IHC technique using Abs marking the target elements. Slides used with the antibody α-SMA (eBioscience, Tokyo, Japan) (Mikami *et al*., 2006) for estimation of hepatocellular carcinoma for evaluation of cancer-Associated fibroblasts

markers in hepatocellular carcinomas and other slides used with antibody AgNORs (Dako, Denmark) (Bukhari *et al*., 2007) which correlate with the proliferative activity of neoplasms generally and carcinomas specially.

Statistical analysis

Data were expressed as the mean ± standard deviation (SD). Differences between groups were determined by one–way analysis of variance (ANOVA). Post hoc testing was performed for inter group comparisons using the least Significant Differences (Duncan) test, and p value < 0.05 was considered significant according (Tambane and Dunlop, 2000).

RESULTS

In the current work, it has been found that rats with HCC showed significant increase in ALT, AST, ALP, Nrf2, BCL2, TGF-β, NFκB, Caspase-3 and Caspase-9 levels in comparison with negative control rats. Rats with HCC treated with Cisplatin displayed non-significant decrease in serum AST, ALT, ALP and Nrf2 beside significant decrease in BCL2, TGF-β, NFκB, Caspase-3 and Caspase-9 in comparison with positive control rats. Rats with HCC treated with *Annona muricata* displayed significant decrease in AST, ALT, ALP, Nrf2, BCL2, TGF-β, NFκB, Caspase-3 and Caspase-9 in comparison with positive control rats. Photomicrograph of peroxidase stained liver of rats of negative control group showing negative expression for Silver-stained Nucleolar organizer regions (AgNORs) (Fig. 1A).

Photomicrograph of peroxidase stained liver of rats with HCC of positive control group showing positive severe expression for AgNORs (Fig. 1B). Photomicrograph of peroxidase stained liver of rats with HCC treated with Cisplatin showing positive mild to moderate expression for AgNORs (Fig. 1C).

Photomicrograph of peroxidase stained liver of rats with HCC treated with *Annona muricata* showing positive mild expression for AgNORs (Fig. 1D). Photomicrograph of peroxidase stained liver of rats of negative control group showing negative expres-

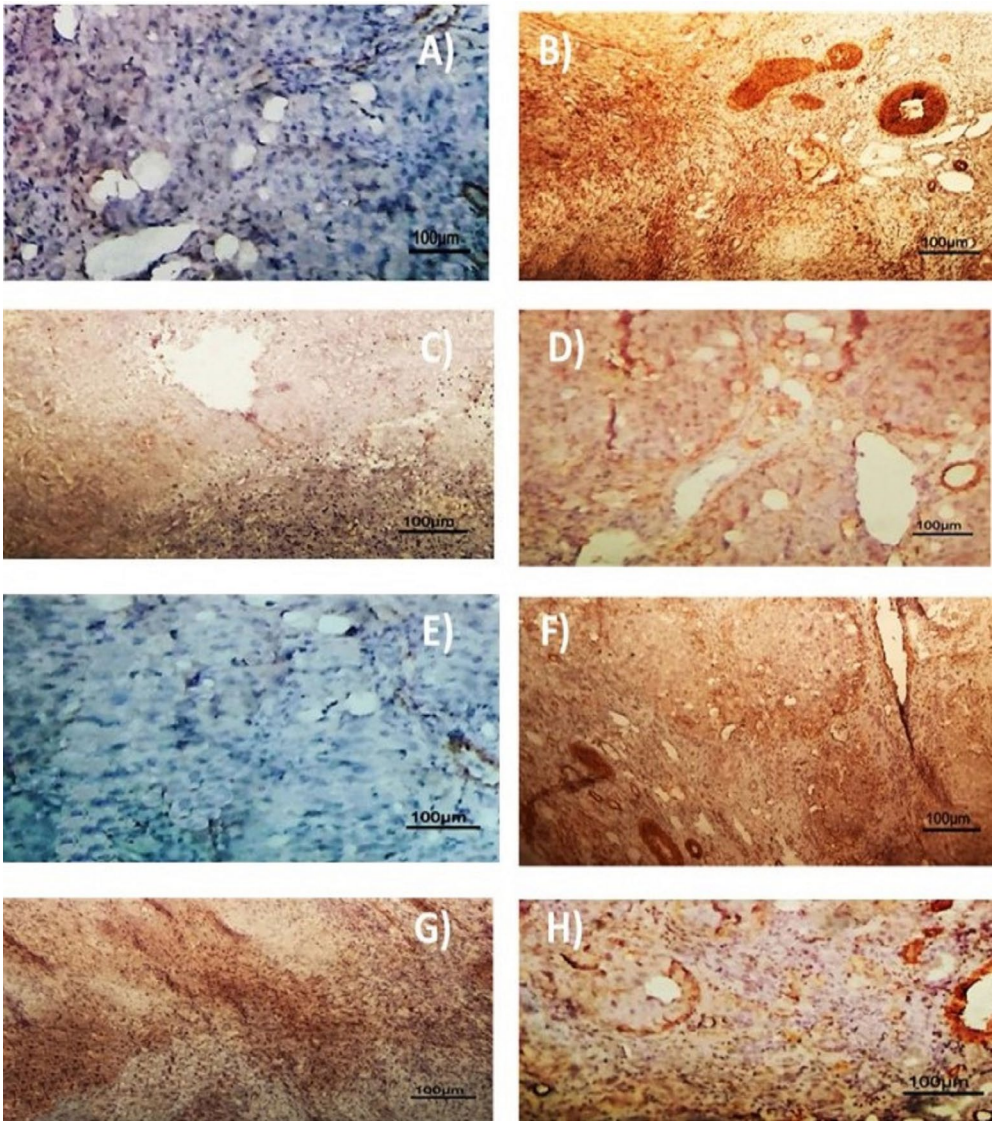

Fig 1. A) Photomicrograph of peroxidase stained rat liver of negative control group showing negative expression for AgNORs (scale bar = 100 µm). B) Photomicrograph of peroxidase stained rat liver of positive control group received CCl_4 showing positive severe expression for AgNORs (scale bar = 100 µm). C) Photomicrograph of peroxidase stained liver of rat suffering from liver cancer treated with Cisplatin showing positive mild to moderate expression for AgNORs (scale bar = 100 µm). D) Photomicrograph of peroxidase stained liver of rat suffering from liver cancer treated with *Annona muricata* showing positive mild expression for AgNORs (scale bar = 100 µm). E) Photomicrograph of peroxidase stained rat liver of negative control group showing negative expression for α-SMA (scale bar = 100 µm). F) Photomicrograph of peroxidase stained rat liver of positive control group received CCl_4 showing moderate to severe positive expression for α-SMA (scale bar = 100 µm). G) Photomicrograph of peroxidase stained liver of rat suffering from liver cancer treated with Cisplatin showing mild to moderate positive expression for α-SMA (scale bar = 100 µm). H) Photomicrograph of peroxidase stained liver of rat suffering from liver cancer treated with *Annona muricata* showing negative to mild positive expression for α-SMA (scale bar = 100 µm)

sion for alpha smooth muscle actin (α-SMA) (Fig. 1E). Photomicrograph of peroxidase stained liver of rats with HCC of positive control group showing moderate to severe positive expression for α-SMA (Fig. 1F). Photomicrograph of peroxidase stained liver of rats with HCC treated with Cisplatin showing mild to moderate positive expression for α-SMA (Fig. 1G).

Photomicrograph of peroxidase stained liver of rats with HCC treated with *Annona muricata* showing negative to mild positive expression for α-SMA (Fig. 1H).

DISCUSSION

In the current work, it has been found that rats with HCC showed significant elevation in serum ALT, AST, and ALP activities in comparison with the negative control rats. The same results were reported by Hany *et al.* (2019) and Maaly *et al.* (2020) who found that CCl_4 induces liver damage, accompanied by a significant increase in liver enzymes. The previous finding fit in with those previously reported by Haytham *et al.* (2019). CCl_4 induced hepatic injury is a very classic model widely used for hepatoprotective drug screening. The acute hepatotoxicity of CCl_4 lies in its biotransformation to trichloromethyl free radical (CCl_3) or trichloroperoxyl radical (CCl_3O_2-) produced by the mixed-function cytochrome P450 (CYP) oxygenase system of the endoplasmic reticulum, which causes oxidative stress and membrane damage. These changes were accompanied by increase of the activites of AST, ALT and ALP. The liver is a target organ for the CCl_4 toxicity due to its detoxifying function in protecting the body. CCl_4 is a well-known hepatotoxic industrial solvent so it is used in diverse experimental models. Keeping with this line Hany *et al.* (2019) observed that CCl_4 resulted in increases in AST, ALT and ALP activities. Liver fibrosis was induced by CCl_4 in adult male albino rats in a study done by Haytham *et al.* (2019).

Rats with HCC treated with Cisplatin displayed non-significant decreases in serum AST, ALT and ALP sctivities compared with the positive control rats. Study was carried out by Zhang *et al.* (2021) showed that interventional ultrasound injection of Cisplatin in the treatment of HCC has a definite effect. It can effectively relieve liver damage, by decreasing AST, ALT and ALP activties, reduce adverse reactions and improve serum tumor marker levels. Cisplatin can bind to DNA and cause cross-reaction and damage to DNA functions and further inhibits cancer cells from mitosis; Injecting Cisplatin into the tumor can cause cell protein degeneration, coagulation, necrosis, fixation and dehydration of the lesion tissue. The destruction of local endothelial cells in the lesion can cause thromboembolism, thereby blocking the blood supply to the tumor, leading to cell death. Our findings coordinate with Maheshwari *et al.* (2019) who mentioned that Cisplatin-treated rats with hepatorenal damage, showed increases in AST, ALT and ALP activities in comparison with normal rats.

Rats with HCC treated with *Annona muricata* displayed significant decreases in AST, ALT and ALP activities in comparison with positive control rats.

Our results agree with Naglaa and Kasem (2019) who stated that *Annona muricata* leaves clarified modulatory role against the cellular damage produced by carboplatin by reduction of serum AST, ALT and ALP activities. *Annona muricata* fruit and Turmeric combination treated group showed decreases in serum AST, ALT, ALP activities, degeneration of hepatocytes and their epithelial cells appeared normal with few normal vacuoles, which indicated that mixture of both can be used as a natural source of antioxidant (AL-Mashhadani *et al.*, 2023). Our results agreed with Ojowu *et al.* (2020) who mentioned that extracts of *Annona muricata* leaves induced a protective effect against CCl_4 toxicity and improved hepatorenal function by lowering CCl_4-elevated serum enzyme markers like AST, ALT and ALP. The ethanol extract of *Annona muricata* L. leaf can inhibit liver tissue damages in hepatitis model rats, possibly due to the presence of acetogenins as an antioxidant agent (Kuswinarti *et al.*, 2021). Our result revealed that rats with HCC showed significant increase in Nrf2 and BCL2 in comparison with the negative control rats.

BCL-2 is a family of proteins plays a crucial role in regulating apoptosis. There is abundant evidence that the activation of Nrf2 is able to suppress carcinogenesis, especially in its early stage. Nrf2 maintains the cellular redox homeostasis and exerts anti-inflammatory functions and further anticancer activities, hence supports cell survival (Wu *et al.*, 2019). Same results were reported by Yang *et al.* (2019) who recorded that CCl_4-agitated apoptosis was intensely reduced through increasing BCL-2 (anti-apoptotic factor) signaling pathways and up-regulated Nrf2 expression in the CCl_4-intoxicated rat's liver. Similar observation was recorded by Chun *et al.* (2019) who stated that the expressions of Nrf2 and BCL-2 protein in hepatic tissue were elevated in rats received tested dose of CCl_4.

Rats with HCC received Cisplatin revealed non-significant decrease in Nrf2 beside significant decrease in BCL2 in comparison with positive control rats. These results agreed with Basel *et al.* (2020) who reported that Cisplatin induced decrease in BCL-2

Table 1. The result of serum liver enzymes (AST, ALT and ALP), Nrf2, BCL2, TGF-β and NFκB and liver tissue gene expression Caspase-3 and Caspase-9 from rats.

Groups	Liver enzymes (U/l)			Nrf2 ng/mL	BCL2 pg/mL	TGF-β pg/mL	NFκB ng/mL	Caspase	
	AST	ALT	ALP					3	9
Group 1	46.83±1.52b	33.81±1.87b	83.54±1.34b	0.70±0.09c	84.52±2.89d	23.5±1.89d	1.20±0.25d	1.28±0.21b	1.42±0.19b
Group 2	74.59±1.62a	60.21±1.34a	110.33±1.92a	2.60±0.17a	157.83±4.55a	73.7±1.96a	3.4±0.43a	4.24±0.74a	4.45±0.48a
Group 3	72.21±2.39a	58.81±1.87a	106.42±1.68a	2.20±0.20a	144.50±5.68b	53.5±1.76b	2.5±0.13b	1.64±0.29b	1.91±0.74b
Group 4	49.24±2.44b	36.59±1.55b	86.42±1.44b	1.10±0.21b	112.23±4.69c	32.5±1.98c	1.50±0.21c	1.50±0.29b	1.61±0.35b

Group 1: negative control; Group 2: positive control; Group 3: treated with Cisplatin; Group 4: treated with *Annona muricata*.
Values within the same column followed with different letters (a, b, c, d) are significantly different at <0.05.

Table 2. Microscopical assessment of malignancy in different treated groups.

Groups	Grade	Area of malignancy (/1.3*10^6 µm)	Area of malignancy Average (%)	Grades of malignancy (range)
Group 1		0	0	0
Group 2		0.7902±0.064	60.82	II –IV
Group 3		0.4842±0.540	37.25	II – III
Group 4		0.2976±0.480	22.9	II

Group 1: negative control; Group 2: positive control; Group 3: treated with Cisplatin; Group 4: treated with *Annona muricata*.
Grades of malignancy: 0: No malignancy; Grade II: 25-50%, moderately differentiated; Grade III: 50-75%, poorly differentiated; Grade IV: 75-100%, anaplastic.

and down-regulation of Nrf2 in CCl_4-induced rat's cancer. Cisplatin decreased liver BCL-2 gene in rats suffering from liver cancer (Omayma *et al*., 2019). Cisplatin induced apoptosis and oxidative stress and modulating Nrf2 signaling pathway, the activated Nrf2-ARE signaling pathway can induce the transcription of protective genes, such as HO^{-1}, glutathione-S-transferase and NQO1, to resist oxidative stress damage caused by various stimulating factors (Zhenjian *et al*., 2021).

Rats with HCC received *Annona muricata* revealed significant decrease in Nrf2 and BCL2 in comparison with positive control group of rats. In another in vitro experiment, Olas (2023) studied the tested extract (200 mg/kg b.w.) also demonstrated antioxidant potential in CCl_4 treated rats and reported that both the aqueous and methanolic extracts from graviola leaves have antioxidant properties and can protect DNA against H_2O_2 damage. The antioxidant properties of the used graviola extract appear to be associated with the reduction of other oxidative stress-related factors, including Nrf2. Graviola reduced the expression of BCL2 (Mustafa *et al*., 2020). Results by Shmeas (2020) showed that *A. muricata* extract down-regulated anti-apoptotic gene Bcl2 (Borjac, 2022). All target genes of Bcl-2, which were up-regulated in colorectal tissues of 1, 2-dimethylhydrazine-injected groups, were significantly minimized upon the administration of *A. muricata* extract. Our results revealed that rats with HCC showed significant increases in TGF-β and NFκB in comparison with the negative control rats.

The obtained data are in accordance with those obtained by Maaly *et al*. (2020) who reported that CCl_4 induced increase in hepatic tissue oxidative stress parameters and TGF-β. Although its expression is tightly regulated in normal tissue, overexpression of TGF-β has been identified in multiple tumor types, including pancreatic adenocarcinoma (Ahmed *et al*., 2019). CCl_4 induced hepatic damage and increase TGF-β (Jeaburua and Oriakhib, 2021). Intraperitoneal administration of CCl_4 enhanced NF-κB expression and facilitated phosphorylation of signaling factors in mitogen-activated protein kinase (Ko *et al*., 2020).

Rats with HCC treated with Cisplatin showed significant decrease in TGF-β and NFκB in comparison with the positive control group of rats. Our data was reinforced by Omayma *et al*. (2019) who reported that Cisplatin induced significant decreases in TGF-β and NFκB concentrations. Our results are supported by Yan and Wei (2020) who stated that Cisplatin can ameliorate the systemic toxicity caused by CCl_4 via down-regulation of the ROS/NF-κB signaling pathway in the rat organs. Cisplatin induced non-significant increase in NFκB (Mohamed *et al*., 2020). Rats with HCC treated with *Annona muricata* showed significant decrease in TGF-β and NFκB in comparison with the positive control group.

In vivo research on rats gavaged precisely with *A. muricata* presented a substantial reduction in the liver index and hepatocyte propagation, with much lower cell injury, by down-regulating TGF-β1 in liver parenchymal tissue (Al-Medhtiy *et al*., 2022). Another study stated that natural products derived from *A. muricata* could be used to develop anti-metastasis therapeutics by the induction of EMT-related transcription factors; it has been proven that TGF-β/Smads pathway is the most potent EMT inducer. It may be possible to utilize an extract of the leaves of *A. muricata* to inhibit the activity of TGF-β and hence control subtype 3 EMT (Ilango *et al*., 2022). Our result is in harmony with Wu *et al*. (2021) who recorded that the ethanol extract of leaves of *Annona muricata* L. was able to reduce viability and trigger apoptosis of the liver cancer cells through ER stress pathway. The extract inhibits the cancer cell migration and invasion through suppressing NF-κB activation, suggesting that NF-κB is involved in apoptotic signal transduction.

In our result, rats with HCC showed significant increase in Caspase-3 and Caspase-9 levels in comparison with the negative control rats. This is in parallel with Yang, *et al*. (2019) who revealed that CCl_4 induced hepatocellular apoptotic changes with increase Caspase-3. In vivo study by Peng *et al*. (2023), revealed that CCl_4 causes liver fibrosis and elevates Caspase-3 and Caspase-9 levels. Our findings demonstrated that rats with HCC treated with Cisplatin induced significant decrease in activity of Caspase-3 and Caspase-9 in comparison with positive control group. These recorded results are supported by Omayma *et al*. (2019) who stated that Cisplatin induced decrease in Caspase-3 activity in comparison with normal rats. The obtained findings are supported by Basel *et al*. (2020) who stated that Cisplatin, an important antineoplastic agent used to control several types of cancers, decreased gene expression of apoptotic markers Caspase-3 and Caspase-9 activity in male reproductive toxicity. Our findings is supported by Fatemeh *et al*. (2021) who reported that Cisplatin decreased oxidative stress and immune reactivity of Caspase-3 in liver tissue in comparison with rats that suffered from liver cancer.

Rats with HCC treated with *Annona muricata* induced significant decreases in activities of Caspase-3 and Caspase-9 in comparison with positive control group. In a study by Elmas *et al*. (2022), *Annona muricata* administration caused decrease in caspase 9 level, which correlates with the increased levels of total oxidant status, also it was observed that caspase 3 levels were somewhat lower. These findings are supported by Mustafa *et al*. (2020) who stated that *Annona muricata* induced reduction in Caspase-3 and Caspase-9 levels in comparison with diseased rats. The anticancer effects of the *Annona muricata* could be attributed to induction of oxidative damage and apoptosis on the cancer cells (Kariyil *et al*., 2021).

Photomicrograph of peroxidase stained liver of rats with HCC showed positive severe expression neither for AgNORs beside moderate to severe positive expression for α-SMA. Another mechanism suggests that CCl_4 breaks DNA strands and affect AgNORs and they scatter with unusual size and shape and leads to genotoxicity (Memon *et al*., 2019). The study by Srivastava *et al*. (2019) showed a correlation of high mean AgNORs counts with HPV positivity and persistence of squamous intraepithelial lesions of the cervix. These results indicate that image cytometry may be a valuable method for quantitating DNA and AgNORs, and that provides valuable information that may have prognostic significance for patients with breast cancer (Hamed *et al*., 2020). The number of AgNORs/nuclei expressions in lung cancer-bearing mice was substantially increased when compared to normal control animals (Chandrashekar *et al*., 2022). Same observations were reported by Akouavi *et al*. (2021) who found that liver fibrosis induced byCCl_4 was consistent with the amount of deposition of collagen and the expression of α-SMA. These fibrogenic cells express α-SMA and produce extracellular matrix (ECM), with disorganization and loss of function of hepatic parenchyma (Haytham *et al*., 2019). The α-SMA expression and the number of collagen increased in a CCL_4-induced liver fibrosis animal model (Hermansyah *et al*., 2021). The obtained data are in accordance with those obtained by Maaly *et al*. (2020) who reported that hepatic injury in the CCl_4 group was manifested by elevation of hepatic fibrosis and immunostaining expression of α-SMA. In vivo study, CCl_4-induced hepatic fibrosis in rats stimulated the activation of hepatic stellate cells, as indicated by significantly increased α-SMA (Wang *et al*., 2021).

Photomicrograph of peroxidase stained liver of rats with HCC treated with Cisplatin showed positive mild to moderate expression for AgNORs beside mild to moderate positive expression for α-SMA. In the same direction, Xingbo *et al*. (2019) stated that Cisplatin induced expression for AgNORs and α-SMA. Cisplatin induces significant reduction of the amount of AgNORs spots was substantially augmented in the nucleus of lung cancer mice (Chandrashekar *et al*., 2022). Remarkably, treatment with Cisplatin triggered significantly higher expression immunohistochemical detection of α-SMA in the hepatic portal area (Gad El-Hak *et al*., 2022). In rats with kidney fibroblasts, administration of Cisplatin, upregulate α-SMA suggesting that administration of Cisplatin induces kidney fibroblast-to-myofibroblast transformation (Yu *et al*., 2022).

Photomicrograph of peroxidase stained liver of rats with HCC treated with *Annona muricata* showed positive mild expression for AgNORs beside negative to mild positive expression for

α-SMA. The same result was obtained by Subin *et al.* (2019) who mentioned that *Annona muricata* act as antioxidant, anti-inflammatory and anticancer, so AgNORs and α-SMA remain within normal levels. The extracts of *A. muricata* effectively prevent liver cirrhosis by the marked down-regulation of α-SMA expression. The defensive result of *A. muricata* against hepatotoxicity could be due to its capacity to avoid hepatocyte propagation, decrease oxidative stress and lipid peroxidation, and its antioxidant and free radical scavenger properties. *A. muricata* resisted hepatocyte fibrosis by down-regulating α-SMA expression (Al-Medhtiy *et al.*, 2022).

CONCLUSION

The present study documented the beneficial effects of *Annona muricata* in the treatment of Albino rats with liver cancer, possibly via its antioxidant, anti-inflammatory effects.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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