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The possible protective role of Graviola Leaf Extract against CCl4 hepatotoxicity in Male Albino Rats

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Abstract: The objective of the current study is to establish the protective effect of Graviola Leaf Extract (GLE) against Carbon tetrachloride (CCl_4) hepatotoxicity in male albino rats. Rats were divided randomly into four groups, each one contains 10 rats as follows: Group 1(control): rats received distilled water; group 2 (GLE): rats received GLE (200 mg / kg bw) through oral administration daily for one month; group 3 (CCl_4): rats received CCl_4 (1ml / kg bw) intraperitoneal administration as single dose at the last day of the experiment; group 4 (GLE+ CCl_4): rats received GLE and CCl_4 as group 2 and 3. The obtained results showed significant increase in hepatic enzymes (AST, ALT) and TB in serum and also showed significant increase in oxidative stress markers (MDA) as well as significant decrease in serum Alb and in antioxidant parameters (GSH, SOD, CAT) in CCl_4 treated group, it also causes significant increase in proinflammatory cytokines level (IL- 1β, TNF-α) and in apoptotic markers (Bax, caspase-3) and caused a significant decrease in anti-apoptotic protein (Bcl-2), as well as it caused DNA damage and significant increase in (Tail moment, Tail length, Tail DNA), Histology examination also showed hepatic tissue damage. On the other hand, GLE administration significantly improved the deviation resulting from CCl_4 in nearly all parameters. In conclusion GLE is able to reduce the hepatic damage caused by CCl_4 and GLE is recommended to be taken as a protective agent against hepatotoxicity

keywords: Graviola leaf extract, CCl_4, Hepatotoxicity, Antioxidant, Inflammatory marker

Abbreviations: GLE, graviola leaf extract; CCl_4, carbon tetrachloride; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TB, total bilirubin; MDA, malondialdehyde; Alb, albumin; GSH, reduced glutathione; SOD, Superoxide dismutase; CAT, catalase; IL- 1β, Interleukin 1 beta; TNF-α, Tumor necrosis factor alpha; Bax, Bcl-2 Associated X-protein; Bcl-2, B-cell lymphoma; DNA, Deoxyribonucleic acid.

1.Introduction

Liver is the largest and most metabolically complicated organ in the body. It has functions in detoxification of toxins and drugs in blood so it works as a filter, and processing ammonia into urea [1]. It plays an essential role in metabolism and has an important role in preserving and regulating the levels of glucose, lipid, bile production and plasma protein synthesis in the body [2].

Hepatotoxicity is the structural and functional damage of liver which are mainly caused by alcohol consumption, toxic chemicals, autoimmune disorders and many infections. Diseases mainly occur by inducing lipid peroxidation (LPO) and other hepatic injuries which caused by oxidative stress [3]. Most drugs and xenobiotics must pass through the liver which is the primary site for drug metabolism, it has large capacity for metabolic conversions, so it is highly vulnerable to detrimental pathological and physiological alterations described as liver or hepatic diseases [4].

Liver damage impairs the normal metabolism and excretion functions of the liver which in turn leads to inappropriate digestion of nutrients, inactivation of drugs, it also impairs

synthesis and secretion of several proteins and so many functions [5].

Carbon tetrachloride (CCl_4) is one of the widely known hepatotoxic agents which produces trichloromethyl free radical CCl_3^- which causes LPO by oxidizing the unsaturated fatty acids in phospholipid layers of cellular membranes and cause hepatic damages [6]. It is the most popular chemical compound to induce hepatotoxicity in rats. Hepatotoxicity caused by CCl_4 results in fatty degeneration, fibrosis and necrosis that leads to liver cirrhosis [7].

The incidence of hepatotoxicity has been rising in developing countries and it is life threatening condition which needs to be addressed with effective medication with less side effects [5]. Medicinal plants are known to have antioxidant, antiinflammatory and analgesic effect [8]. This is because of the phytochemical ingredients of these herbal medicines [9].

Antioxidants are compounds that act to protect the body from the damages that caused by free radicals because of its ability to scavenge free radicals in the human body. There are various antioxidants, such as vitamins, karotin, carotenoids, polyphenol and minerals [10].

Many natural and medicinal plants showed protective and curative effects against hepatotoxicity caused by CCl_4; this protective response was due to their antioxidant properties [11]. One of the medicinal plants which has many medicinal uses is graviola which belongs to the Annonaceae family that obtained much interest in the last decades due to its medicinal and protective properties [12].

Graviola is a plant that belongs to the Annonaceae family. It is also known as *Annona muricata*, soursop and guanabana, it is extend in tropical and subtropical regions of the world [13]. Graviola have many useful or phytochemical substances such as acetogenins, flavonoids, coumarins, phenolic compounds, alkaloids, steroids, terpenoids phytosterols, tannins and others [14]. Acetogenins (AGEs) are the main bioactive compounds of graviola and the Annonaceae family which has antioxidant and antiinflammatory effect [15].

Graviola leaf extract of (GLE) has antioxidant, antilipid peroxidation effect and is considered as hepatoprotective [16]. GLE has therapeutic properties against oxidative stress, inflammation, tumors, cancer and also has hepatoprotective effect against CCl_4 hepatotoxicity [17]. The hepatoprotecive occurs by decreasing LPO by scavenging the free radicals produced during CCl_4 metabolism due to its antiinflammatory and antioxidant properties [18].

1. Material and Methods

2.1. Materials

2.1.1. Chemicals

Carbon tetrachloride (CCl_4) was purchased from Sigma Chemical Company (St. Louis, MO, USA). CCl_4 was mixed (1:1) with olive oil and was administered intraperitoneally (1 ml/kg bw). GLE was purchased as capsules from Inkanatura World Peru Company (La Molina, Lima, Peru). The extract was dissolved in dist. H_2O to prepare 200 mg extract /kg bw, **(Product code: 7752494000280)**. All other chemicals were purchased from generic verified firms.

2.1.2. Animals

Forty white male albino rats (90-100)g used for this study. Rats were purchased from Egyptian Institute for Vaccine and Serological manufacture, Helwan, Egypt and were housed in the animal house of the Zoology Department, Faculty of Science, Mansoura University. Rats were placed in stainless steel cages containing wood-chip bedding, renewed daily. The rats maintained a 24 h cycle in a controlled temperature setting. one week before the start of the experiment, all rats were adapted acclimatized to the location. During the experiment time, the animals were provided with normal diet and water *ad libitum*.

2.2 Method

2.2.1 Animal grouping and mode of treatment

After one week of acclimatization period, Animals were divided randomly into four groups, each consisting of ten animals as follows: Control group: in this group, rats received distilled water. Graviola Leaf Extract (GLE) treated group: rats were given orally GLE daily for 4 weeks (200mg / kg bw). Carbon tetrachloride (CCl_4) treated group: CCl_4 (1 ml/kg bw) was administered

intraperitoneally as single dose. Graviola Leaf Extract and Carbon tetrachloride (GLE+ CCl_4) treated group: rats were given orally GLE daily for 4 weeks (200 mg / kg bw), followed by CCl_4 (1 ml/kg bw) was administered intraperitoneally as single dose.

2.2.2. Sample collection

After the experimental period (5 wks.), rats were sacrificed overnight (24 hours after CCl_4 treatment). Blood were collected in centrifuge tubes and left to clot then they centrifuged for 15 minutes at 4000 rpm. The sera of all samples were separated and put in labeled Eppendorf's tubes, and were frozen at -20^{o}C for different analyses of biochemical parameters.

2.2.3. Used kits

AST and **ALT** activities were measured in serum by using RAM diagnostic kit according to the colorimetric kit technique [19]. **Alb** was measured in serum using kit provided by Diamond diagnostics, Co., Cairo, Egypt, by the method of [20]. **TB** was estimated in serum using a kit provided by Diamond diagnostics, Co. Cairo, Egypt, it was estimated by the colorimetric kit technique [21]. The content of **MDA** and the activity of **GSH**, **CAT** and **SOD** in the homogenate were estimated according to the colorimetric method of [22], [23], [24], [25], respectively by using kits from Biodiagnostic, Co., Dokki, Giza, Egypt. **IL-1β** level was estimated by using a Rat ELISA kit purchased from Ray Biotech (Norcross, Georgia, USA), **TNF-α** was determined using a Rat ELISA kit provided by ALPCO (Salem, New Hampshire, USA), **Bax**, **Bcl-2** and **caspase-3** contents were determined using ELISA kits purchased from MyBioSource (San Diego, California, USA).

2.2.4. Statistical analyses

Using GraphPad Prism 5.0, data are analyzed. The findings of the experiment were presented as mean ± the standard error mean (SEM) (n= 10). Results was analyzed by a one-way analysis of variance (ANOVA) followed by multiple correlation testing by Newman–Keuls. Values deemed statistically significant P $\leq$ 0.05.

3. Results

Intraperitoneal administeration of CCl_4 caused marked increase in activities of serum liver enzymes (ALT and AST), also in TB and and caused a significant decrease in Alb content (table 1), and in antioxidant activities (SOD, GSH, CAT), and caused significant increase in MDA content (table 2) and proinflammatory cytokines (TNF-α, IL- 1β) and in apoptotic markers (Bax, caspase-3), as well as a significant decrease in anti-apoptotic

protein (Bcl-2) (table 3), it also caused DNA damage and marked increase in (Tail length, Tail DNA, Tail moment) (table 4) and in histology examination it showed hepatic tissue damage. On the other hand, GLE administration significantly improved the deviation resulting from CCl_4 in all parameters as shown in the following tables.

Table (1): Outcome on serum liver enzymes

Parameters	C	G	CCl_4	G+ CCl_4
ALT (U/L)	76.6 ±7.52	70.3 ±7.2	318^{a} ±21	128^{ab} ±8.95
AST (U/L)	77.47 ±7.34	75.7 ±7.2	321.2^{a} ±18.8	148.7^{ab} ±7.0
TB (mg/dl)	0.4 ±0.027	0.4 ±0.06	1.31^{a} ±0.12	0.427^{ab} ±0.1
Alb (mg/dl)	3.85 ±0.13	3.90 ±0.1	1.79^{a} ±0.25	3.75^{ab} ±0.16

Results for 10 rats in each group are expressed as means ± SE, a: significant as compared with control, b: significant as compared with CCl_4 group, at P$\leq$ 0.05. **C**: control group, **G**: Graviola extract, **CCl_4**: carbon tetrachloride.

Table (2): Hepatic antioxidant parameters

Parameters	C	G	CCl_4	G+ CCl_4
MDA (nmol/g)	835 ±16.75	832 ±15.9	1317 ^{a}±124.9	84^{1ab} ±14.37
SOD (U/g)	169.3 ±11.6	174.7 ±9.97	122.3^{a} ±9.5	167.9^{ab} ±9.75
GSH (mg/g)	4.55 ±0.1	4.56 ±0.1	1.57^{a} ±0.1	4.49^{ab} ±0.1
CAT (U/g)	202.9 ±15.3	206.4 ±15.1	127.9^{a} ±14.4	199.3^{ab} ±13.4

Results for 10 rats in each group are expressed as means ± SE, a: significant as compared with control, b: significant as compared with CCl_4 group, at P$\leq$ 0.05. **C**:control group, **G**: Graviola extract, **CCl_4**: carbon tetrachloride.

Table (3): Anti-inflammatory and anti-apoptotic parameters

Parameters	C	G	CCl_4	G+CCl_4
IL-1β (pg/ml)	0.11 ±0.01	0.11 ±0.01	0.46^a ±0.06	0.15^b ±0.01
TNF-α (pg/ml)	0.13 ±0.01	0.1 ±0.02	0.48^a ±0.08	0.15^b ±0.01
Bcl-2 (ng/mg)	16.89 ±1.87	17.38 ±1.9	3.42^a± 0.66	13.63^b ±1.7
Bax (ng/mg)	5.02 ±0.40	4.83 ±0.37	16.64^a ±1.22	7.46^b ±0.61
caspase-3 (pg/ml)	4.97 ±0.60	4.10 ±0.47	16.99 ^{a}±0.90	7.46^b ±1.02

Results for 10 rats in each group are expressed as means ± SE, a: significant as compared with control, b: significant as compared with CCl_4 group, at P≤ 0.05. **C**: control group, **G**: Graviola extract, **CCl_4**: carbon tetrachloride.

Table (4): Commet assay parameters

Parameters	C	G	CCl_4	G+ CCl_4
Tail length (µm)	1.53± 0.06	1.41± 1.10	2.44^a± 0.09	1.97^{ab}± 8.95
Tail DNA (%)	1.49± 0.03	1.35± 0.03	2.22^a± 0.16	1.87^{ab}± 0.12
Tail moment (UNIT)	2.23± 0.03	1.87± 0.03	5.47 ^{a}±0.16	3.67^{ab}± 0.12

Results for 10 rats in each group are expressed as means ± SE, a: significant as compared with control, b: significant as compared with CCl_4 group, at P≤ 0.05. **C**: control group, **G**: Graviola extract, **CCl_4**: carbon tetrachloride.

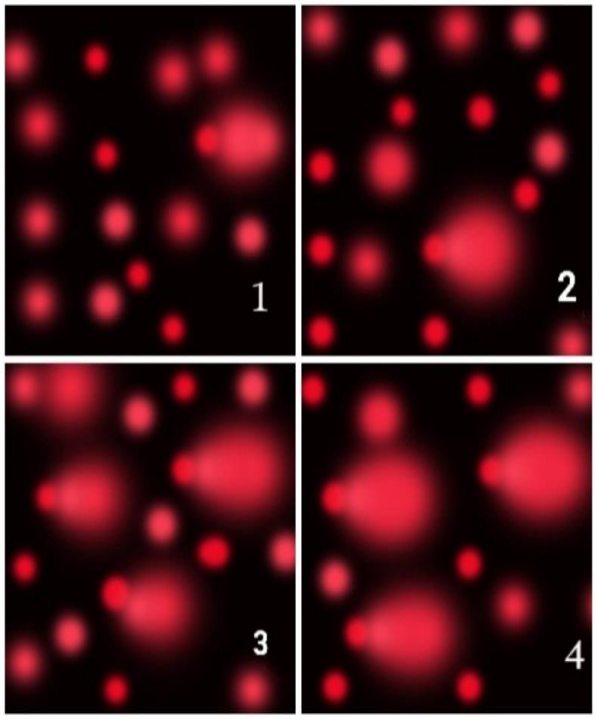

Figure (1): Representative photomicrographs of hepatic cells comet assay showing the effect of CCl_4 on DNA migration pattern in (A) control group (B) GLE treated group (C) CCl_4 treated group and (D) GLE and CCl_4 treated group.

Histopathological studies

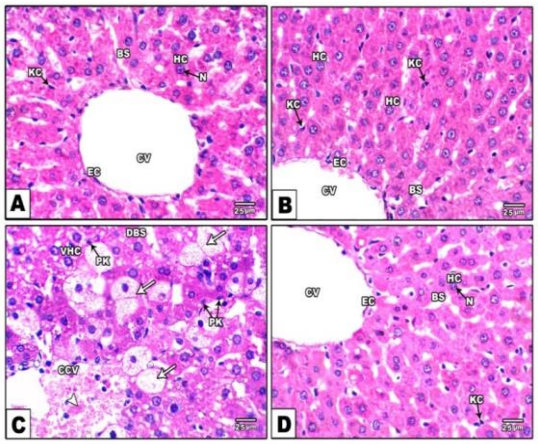

Figure (2): Liver sections of different treated groups at X400

Plate 1, Figure 2 (A-D): Photomicrographs of hematoxylin and eosin stained liver tissue sections **(Original magnification X400)**.

Fig. (A): Photomicrographs of hematoxylin and eosin stained liver tissue section of control rat, showing normal appearance of central vein (**CV**) with normal endothelial cells (**EC**), hepatocyte (**HC**) with nuclei which located centrally (**N**), blood sinusoid (**BS**), and Kupffer cells (**KC**) **(Original magnification X400)**.

Fig. (B): Photomicrographs of hematoxylin and eosin stained liver tissue section of GLE treated rat displaying normal appearance of blood sinusoids (**BS**), central vein (**CV**) with normal endothelial cells (**EC**), hepatocyte (**HC**), and Kupffer cells (**KC**) **(Original magnification X400).**

Fig. (C): Photomicrographs of hematoxylin and eosin stained liver tissue section of CCl_4 rat showing severe damage of hepatic tissue represented by congested central vein (**CCV**) with hemorrhage (**arrow head**), diffused microvesicular steatosis with a "foamy" cytoplasmic appearance(**arrow**), vacuolated hepatocytes (**VHC**), Pyknosis (**PK**), and dilated blood sinusoids (**DBS**). **(Original magnification X400).**

Fig. (D): Section in liver of GLE & CCl_4 treated rat displaying typically normal central vein (**CV**) with normal endothelial cells (**EC**), blood sinusoid (**BS**), hepatocytes (**HC**) with nuclei which located centrally (**N**), and normal

distribution of Kupffer cells (**KC**) **(Original magnification X400),** bar= 40 µm.

4.Discussion

The present study showed that CCl_4 resulted in significant disruption in all studied hepatotoxicity parameters. On the other hand, oral administration of GLE resulted in marked improvement in most tested parameters. CCl_4 caused marked increase in the activity of liver enzymes such as AST and ALT which are recommended for cellular leakage and defect in activities of hepatic cell membrane, these enzymes are mainly used as biomarkers for hepatocellular injury, the obtained results go parallel with that of [26]. CCl_4 also caused marked increase in total bilirubin level, the possible explanation is that CCl_4 induced the generation of ROS that has the possibility to elicit tissue damage [27], the present results go parallel with that of [3]. On contrary it caused marked decrease in the Alb level, this may be due to the dissociation and disruption of polyribosomes on rough endoplasmic reticulum [6]. In this investigation, a significant decrease in CAT and SOD activities and GSH content were observed in CCl_4 treated animals. These results revealed the role of CCl_4 in oxidative stress in the liver, the results go parallel with that of [28]. One probable mechanism for the decreased activities of CAT and SOD beside GSH content, it may resulted by the oxidative stress which caused by the active metabolite trichloromethyl radicals [27]. GSH plays an important role in antioxidant mechanisms, so it participates to keep the normal structure and function of cells it may be due to its redox reactions also it plays an essential role in the detoxification of free radical that produced by CCl_4 metabolism [29]. The decrease in GSH content may be due to oxidative stress which causes utilization of GSH by antioxidant enzymes [30]. In this study, a significant increase in the level of MDA in CCl_4 treated animals was observed, MDA is a biomarker of tissue damage as it is a LPO secondary product [31], so the increase in the level of MDA is considered as an indicator for LPO of cell membrane and the defect in antioxidant system to defense against free radicals produced by CCl_4 [27], the present results go parallel with [32]. On the other hand, pretreatment with GLE significantly reduced the elevated activity of liver enzymes toward normal levels, referring to hepatic tissue repair and restoring the integrity of plasma membrane, the obtained results go parallel with the study of [33]. GLE reduced the level of total bilirubin, the possible reason may be due to the inhibitory effects of GLE on cytochrome P450 or promotion of its glucuronidation [34]. The hepatoprotective role of GLE may be due to its content of flavonoids, saponins, alkaloids, tannins, ascorbic acid and also its content of glucosides which can be turn into glucuronic acid and conjugate with bilirubin for excretion [35], the obtained results go parallel with [17]. Pretreatment with GLE also caused a marked increase in Alb level than its level in CCl_4 treated group, this is a reflection of the hepatoprotective effect of GLE [36], these results go parallel with that of [34], who demonstrated the same results. GLE pretreated animals showed a marked increase in liver GSH, CAT and SOD when compared to animals administrated CCl_4 alone. these results are in agreement with several studies [37]. The possible explanation is that GLE significantly reduced LPO and maintained the integrity of the plasma membrane so it prevent the liver damage, reduced rate of LPO arising from increased levels of GSH, CAT and SOD due to the antioxidant activities of the GLE [38]. Pretreatment with GLE significantly decreased MDA level, the probable mechanism is that GLE reduced LPO and caused an increase in the antioxidant activities, this can be due to the fact that GLE has been shown to possess antioxidant properties because of its content of acetogenins that has an important role in free radical scavenging [39], the obtained results go parallel with that of [40]. In this study, CCl_4 caused a marked increase in TNF-α and IL-1β levels in CCl_4 treated animals, the possible mechanism is that CCl_4 and its metabolites can activate inflammatory cells in the liver, then it cause a release of pro-inflammatory cytokines such as IL-1β and TNF-α from Kupffer cells and stimulate other to produce excessive IL-1β and TNF-α, causing cell membrane damage and stimulate the inflammatory response [41], the results are in the same line with [42]. Pretreatment with GLE caused significant decrease in IL-1β and TNF-α, this occurred due to its anti-inflammatory effect of GLE through the suppression of inflammatory mediators

[43], these results go parallel with that of [44]. Apoptosis is a form of programmed cell death in which Bax, Bcl-2 and caspase-3 are important control factors, in the obtained results CCl_4 caused a marked increase in Bax and caspase-3 levels in CCl_4 treated animals than the levels in untreated control animals, while reducing the anti-apoptotic Bcl-2 expression which inhibits proapoptosis protein Bax and prevent apoptosis process, the possible mechanism is that CCl_4 liver metabolism cause a release of large amounts of ROS causing inflammation, apoptosis and liver necrosis [45], this was in the same line with [46]. On the contrary, pretreatment with GLE caused significant decrease in Bax and caspase-3 expressions and significant increase in Bcl-2 level when compared with CCl_4 treated group, which inhibits DNA fragmentation because of the antioxidant effect and reduce the mitochondrial permeability and the apoptosis of hepatocytes [47]. DNA damage is considered as apoptosis indicator; thus, comet assay was used to elucidate CCl_4 effect on DNA by examining the tail length, tail moment and tail DNA in the liver. CCl_4 administration showed a marked increase in all comet parameters compared with the control group. These findings are in a harmony with the previous results of [48]. On contrary, pretreatment with GLE showed a marked decrease in DNA tail, tail length and tail moment in the liver cells compared with CCl_4 treated group. This might be due to that GLE has an important role in DNA damage inhibiting due to the antioxidant components of GLE which have an effective role in DNA protect against ROS and oxidative damages [49], this is in the same line with [50].

Histopathological examination of liver supported the obtained biochemical results. The hepatic tissue of CCl_4 treated animals revealed inflammatory cell infiltration, hepatocytes degenerative changes and extensive damage in form of necrosis. At the same time, it also caused damage to the parenchyma of the liver and hepatic architecture. Most of the hepatocytes were vacuolated and appeared to have large cytoplasmic vacuoles. These results are in accordance with that of [51], the possible explanation is due to the free radicals which produced from CCl_4 metabolites causing LPO [52]. On the other hand, pretreatment with GLE exhibited hepatic protection represented by restoration the normal hepatocytes architecture and being arranged in the form of cords, in a polyhedral shape and radiate in peripheral cords with minimized hepatic parenchymal distress, lower inflammatory response and absence of necrosis. These results confirmed the results of biochemical studies [53], this was in the same line with [47].

5. Conclusion

The obtained results of this study, conclude that GLE has potential hepatoprotective effects against CCl_4 hepatotoxicity, which we propose to be because of the antioxidant and anti-inflammatory effects which inhibit LPO and prevent cellular damage.

6.References

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