



The potential chemo preventive effect of ursolic acid isolated from *Paulownia tomentosa*, against N-diethylnitrosamine: initiated and promoted hepatocarcinogenesis

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ABSTRACT

The present study discusses the isolation of ursolic acid from the chloroform extract of *Paulownia tomentosa* (Thunb) Steud fruits and its cytotoxic effect has been assessed *in-vitro* was performed in different cells lines (A-549, MCF-7, HepG2) and *in-vivo* using N-diethylnitrosamine. The obtained results revealed that ursolic acid showed significant cytotoxic activity on MCF-7 and HepG2 cell lines in comparison to Doxorubicin as a reference drug. Moreover, we have assessed the inhibitory effects of *Paulownia tomentosa* fruit chloroform extract and the isolated ursolic acid on hepatocarcinogenesis was carried out for the first time using N-diethylnitrosamine, where the group treated with ursolic acid given orally after 8 weeks of cancer induction showed the most significant results in comparison to the chloroform extract. The effect of ursolic acid on intoxicated rats caused significant restoration of most of the normal hepatocytes architecture with regular dark nuclei and the group treated with *Paulownia tomentosa* fruits showed remarkable results with improvement in biochemical analysis.

1. Introduction

The liver is one of the most vital organs in the human body dealing with the metabolism and detoxification of external and internal chemical substances. Many toxins target the liver and cause hepatotoxic effects that can be observed through some biochemical parameters. As it is the main site of N-diethylnitrosamine (NDEA) metabolism, the production of ROS in liver may be responsible for its carcinogenic effects [1].

Many herbs have been proven to be effective as hepatoprotective agents; these hepatoprotective plants have the phytoconstituents such as iridoidglycosides, phenyl compounds, coumarins, essential oils, monoterpenoids, diterpenoids, triterpenoids, steroids, alkaloids and other nitrogenous compounds [2].

The chemical hepatocarcinogen NDEA is known to induce over expression of TGF- α (the growth factor transforming growth factor- α) which is closely involved in hepatocarcinogenesis and transformation in humans and animals. Furthermore, elevated expression of TGF- α in hepatic cirrhosis as TGF- α expression is higher in liver tissue of patients with chronic hepatic diseases compared with normal healthy subject

[3]. This phenomenon is thought to play an important role in the process of NDEA-induced hepatocarcinogenesis which is evidenced by changes in histopathological architecture and increased liver marker enzymes [4, 5].

Paulownia tomentosa (Thunb.) Steud. belongs to the family Scrophulariaceae, and it is commonly known as princess tree. It is a perennial tree native to China, Japan and the Far East but it has been adapted to the southern United States and it is even invasive now [6]. It has been used as an oriental medicine for the treatment of gonorrhoea, bruise and deodorant. A decoction of the leaves was used to wash foul ulcers and to promote the growth of hair and prevent greying [7].

Ursolic acid is a naturally occurring pentacyclic triterpene, found in many plants and has a chemo protective activity in human. It possesses many important biological activities, such as anti-inflammatory, hepatoprotective, antiulcer, hypolipidemic and anti-atherosclerotic [8]. Numerous reports discussed ursolic acid *in-vitro* activities against tumor cell lines [9]. Several suggest possible mechanisms of action for tumor inhibition; *i.e.* the proliferation of MCF-7 breast tumor cells by exerting an early cytostatic effect on the cell cycle at G1. The cytotoxic and cytostatic

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effects are likely to involve apoptosis (cell self-destruction). Also ursolic acid induced apoptosis in HepG2 human hepatoblastoma cells in a dose-dependent manner, with DNA fragmentation, enhanced release of cytochrome c, and activation of caspase-3 (mediator for apoptosis). Expression of p21WAF1 (tumor suppressor) was increased, indicating possible involvement in mediating cell-cycle arrest. Also ursolic acid was found to inhibit tumor invasion by inhibited expression of both MMP-2 and MMP-9 at micromole concentrations, a finding that is consistent with the observed ability of ursolic acid to inhibit MMP expression in fibrosarcoma cells, and also inhibits proliferation and colony formation of tumor and limit the ability of tumor to invade and metastasize [10]. Ursolic acid reduces the proliferation of many tumor cell lines and many possible mechanisms of action have been addressed. Studies in MCF-7 breast carcinoma cells showed an early G1 cytostatic effect for ursolic acid [11, 12]. Enhancement of intracellular Ca²⁺ signaling is thought to play a role in reducing proliferation [9]. Ursolic acid has been previously isolated from *Paulownia tomentosa* Leaves [13, 14]. The Essential oil of the flowers of *Paulownia tomentosa*. Growing in Egypt play a role as antimicrobial activity [15]. The methanolic extract was reported as antioxidant Hypoglycemic [16].

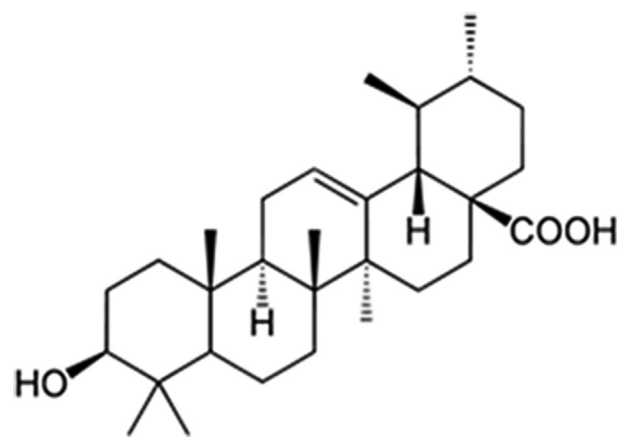


Fig. 1. Chemical structure of ursolic acid.

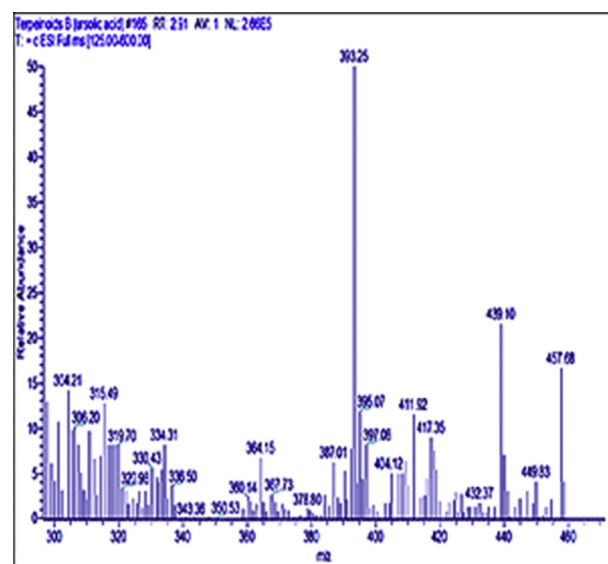


Fig. 2. Mass spectra of ursolic acid compound.

2. Material and methods

2.1. Plant material

Fresh mature fruits of *Paulownia tomentosa* were collected in September 2007 from the Agricultural Museum, Giza, Egypt. Identified by Mrs. Trease Labib consultant of plant taxonomy at the Ministry of Agriculture and former director of El-Orman botanical garden. A voucher specimen (No.06-06-03-16) was kept at the Herbarium of El-Orman Botanical Garden. The collected fruits were air dried, separately powdered and kept in tightly-closed containers.

2.1.1. Extraction and isolation

Successive extraction for *P. tomentosa* fruits (80g) using Soxhlet apparatus starting with petroleum ether till complete defatting followed by chloroform till exhaustion. The chloroform extract (11g) was purified using charcoal powder, filtered and the filtrate was collected, evaporated under vacuum till dryness and weighted, then kept to be used for isolation of the main active constituents and evaluation the *in vitro* and *in-vivo* anticancer effects. The chloroform extracts of *P. tomentosa* fruits, chromatographed using TLC alongside with ursolic acid for comparison, using solvent systems benzene-ethyl acetate (86:14 v/v) and benzene-methanol (9:1 v/v) as development solvent systems and the plate was sprayed with vanillin sulphuric reagent then heated at 110 °C for 10 min and the results showed the presence of ursolic acid the major compound in the chloroform extract of *Paulownia tomentosa* fruits which was isolated by preparative TLC then purified and crystallized from methanol.

2.1.2. Ursolic acid compound

White powder, m.p. = 283 - 285 °C, freely soluble in CHCl₃ and ether, partially soluble in alcohol.

¹H-NMR of Ursolic acid compound (Figs. 1 and 2): (CDCl₃, 400 MHz): δH 5.24 (1H, brs, H-12), 3.19 (1H, brs, H-3), 2.17 (1H, brs, H 18), 1.24 (3H, s, Me-23), 0.98 (3H, s, Me-24), 0.77 (3H, Me-25), 1.07 (3H, s, Me-26), 1.13 (3H, s, Me-27), 0.94 (3H, brs, Me-29), 0.92 (3H, brs, Me-30).

EI/MS, m/z (rel.int): 456 (M⁺), 439.10 (23), 457.68 (18), 304.21 (15), 315.49 (13), 395.07 (12), 411.92 (12), 393.25 (100) [8].

2.1.3. Material for in-vitro cytotoxicity testing

The *in vitro* antitumor screening was performed adopting previously reported procedures. Cells were suspended in RPMI 1640 medium for HepG2, MCF7 and DMEM for A549, 1% antibiotic-antimycotic mixture (10,000 u/ml potassium penicillin, 10,000 mg/ml streptomycin sulfate and 25 mg/ml amphotericin B) and 1% L-glutamine at 37 °C, fewer than 5% CO₂ and 95% humidity. Cells were seeded at concentration of 1 ×

10⁴ cells/well in fresh complete growth medium in 96-well microliter plates for 24 h. Media was aspirated, fresh medium (without serum) was added and cells were incubated with different concentrations of sample to give a final concentration of (100, 50, 25, 12.5, 6.25, 3.125, 0.78 and 1.56 Ug/ml). 0.5% DMSO was used as negative control and 100 mg/ml of doxorubicin was used as positive control. MTT assay was used for assessment of cytotoxicity. After 48 h of incubation, medium was aspirated, 40 ml MTT salt (2.5 mg/ml) were added to each well and incubated for further 4 h. To stop the reaction and dissolving the formed crystals, 200 ml of 10% sodium dodecyl sulfate (SDS) in deionized water were added to each well and incubated overnight at 37 °C. The absorbance was then measured at 595 nm and a reference wave length of 620 nm. A statistical significance was tested between samples and negative control (cells with vehicle) using independent t-test by SPSS 11 program. The % cytotoxicity was calculated according to the formula: [1-(OD compound/OD negative control)] x100]. A probit analysis was carried for LC50 determination using SPSS 11 program.

2.2. Material for in-vivo hepatoprotective studies

2.2.1. Chemicals and biochemical kits

All chemicals used in the present study were of high analytical grade, products of Sigma (USA), Merck (Germany), BDH (England), diethyl

nitrosamine (Sigma Co.) was used for induction of hepatic carcinogens in rats. Kits used for the quantitative determination of different parameters were purchased from Bio-diagnostic (Egypt).

2.2.2. Animals

Male albino rats weighting 130–150g supplied from the animal house of National Research Centre (Dokki, Giza, Egypt) and were used for experimental investigation. Animals were kept for two weeks to accommodate on laboratory conditions; they were kept under constant environmental and nutritional conditions, given food and water all over the period of the experiment. Appropriate anesthetic and sacrifice procedures were followed ensuring that animals didn't suffer at any stage of the experiments. Anesthetic procedures complied and were approved according to the ethical committee of the National Research Centre in Egypt with registration No.13–016.

2.2.3. Experimental design

2.2.3.1. Induction of liver damage. Liver damage in rats was induced by intraperitoneal injection of (200 mg/kg body weight) with N-diethylnitrosamine once weekly for eight weeks. Forty male Wister albino rats, weighing 130–150 g will be used in this study. Animals will be divided into 5 groups each of eight rats.

- Group 1: Normal healthy control rats.
- Group 2: Orally administered with chloroform extract of fruits to determine if there were any side effects.
- Group 3: Given NDEA (200 mg/kg body weight) for 8 weeks.
- Group 4: Given NDEA (200 mg/kg body weight) along with chloroform extract of fruits that will be orally administered (500 mg/kg).
- Group 5 orally administered with isolated ursolic acid (500 mg/kg) after 8 weeks given NDEA.

After each experimental period of different groups NDEA, the sub-tongual vein was punctured in each animal in all groups, and blood samples were collected to be used for biochemical analysis. After blood collection, animals were sacrificed after decapitation, and then liver was rapidly removed, cut small sections then put in 10% formalin solution for histological and histopathological analysis, while the other sections were either used immediately or frozen at -80°C for other biochemical analyses. Histological and histopathological analyses were conducted according to Hirsch et al. [17] to evaluate structural alterations of the hepatic parenchymal cells.

2.2.4. Parameters in case of NDEA induction

2.2.4.1. Liver functions enzymes in serum. The Albumin level according to Dumas et al. [18], Alanine Aminotransferase Activity (ALT) and Aspartate Aminotransferase Activity (AST) according to Rietman and Frankle [19], Alkaline Phosphatase Activity (ALP) according to Babson et al. [20], and γ Glutamyl Transferase Activity (GGT) according to Szasz, [21].

3. Results

3.1. Determination of in-vitro cytotoxic activity

The isolated ursolic acid compound was tested for its *in-vitro* cytotoxic activity against (A-549), (MCF-7) and (HepG2) at concentration 100 $\mu\text{g}/\text{ml}$ using MTT assay [22]. The cytotoxic activity of the isolated compound gave 100% activity at 100 $\mu\text{g}/\text{ml}$ concentration; this led to secondary screening to calculate its IC_{50} value as in Table 1.

3.2. Determination of in-vivo cytotoxic activity

Experimental animals exposed to N-diethylnitrosamine NDEA,

Table 1

IC_{50} of cytotoxic active compound isolated from *P. tomentosa* (Thunb.) Steud. fruits extract against different cell lines.

Sample Name	$^{\text{a}}\text{IC}_{50}$ ($\mu\text{g}/1 \times 10^4$)		
	HEPG2	A-549	MCF-7
Compound 1	14.5	68.4	3.5
Doxorubicin	21.4	28.3	26.1

^a IC_{50} : compound concentration required to inhibit the cell viability by 50%.

showed that transaminase activities (AST, ALT & ALP) were increased in plasma by the release of these enzymes from hepatic parenchyma cells, indicating a considerable hepatocellular injury by 165.57%, 55.81% and 78.77% respectively as compared to the control group. Therapeutic treatment with *P. tomentosa*, chloroform extract and the isolated ursolic acid modulated the hepatic liver enzymes with 65.60%, 3.81% and 9.44% for chloroform extract treated group and with 7.53%, 112.62% and 0.32% for ursolic acid treated group respectively, with respect to control group. On the other hand control groups treated with either fruits extract or ursolic acid showing no significant change of the enzymes AST, ALT and ALP as revealed in Table 2, Fig. 3.

In case of albumin and total protein a significant reduction was recorded with NDEA given with different percentages 50.37% and 7.90% respectively, as compared to control group. After treated with fruits extract and ursolic acid, ameliorated these disturbances with different percentage of improvement, albumin record an elevation in the albumin level by 6.39% and 5.78% in groups treated with fruits and isolated ursolic acid respectively with comparison to control group, total protein results were close to the control group, although there was a slight elevation in case of ursolic acid treated group, according to GGT there was a significant increase in GGT liver enzymes by 71.8% as a result of NDEA induction with respect to control which ameliorated these disturbance by 20.377% and 38.61% respectively, as compared to the control group, no significant change in GGT was recorded in fruit extract normal group as compared to control. The significant decrease in GGT activity observed in rats treated with fruits extract and ursolic acid suggests a possible curative effect of *P. tomentosa* fruits extract in liver disturbance condition, which showed highly significant results near to control. For, hepatic glucose level, there was decrease by 42.83% in case of group given (NDEA), the treatment with fruits extract and ursolic acid ameliorated these decreases by 13.55% and 28.28% respectively compared to the control group as shown in Table 3, Fig. 4.

Table 4 & Fig. 5 showed the changes in rats liver weight, body weight and relative liver weight due to NDEA induction where there was an increase in liver weight by 3.2% as compared to control and decrease in the body weight by -4.79%, relative liver weight were increased slightly by 4.33% in NDEA group compared to control. Therapeutic treatment with fruits record 3.4%, 3.748% and 0.27% also according to ursolic acid record 10.8%, 16.65% and 6.233% with respect to control.

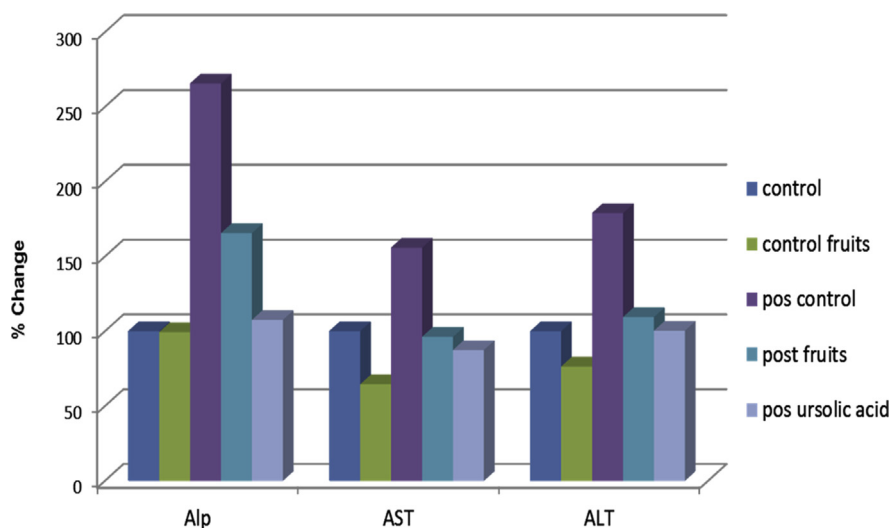
3.3. Histopathological findings

Histology examination of liver sections Fig. 6 of control animals (A) showed normal hepatocyte cell. H&E stain. (A*) normal hepatocyte cell Masson' trichome stains record normal collagen distribution. (B) NDEA group showed appearance of hepatocellular carcinoma with enlarged hyperchromatic nuclei and scattered mitosis after 8 weeks of NDEA administration H&E stain. (B*) cirrhosis cells and damage cell wall of hepatic cells shown through collagen increase distribution stained with Masson' trichome. (C) Revealed the effect of ursolic acid which orally given after 8 weeks of diethyl nitrosamine administration, restoration of most of the normal hepatocytes architecture with regular dark nuclei. (D) H&E stain showed significant regression of hepatic fibrosis in the cirrhotic rats treated with fruits extracts after 8 weeks of induction of cirrhosis and (D*) Masson' trichome stain showing initial improvement

Table 2Effect of the chloroform extract of *Paulownia tomentosa* (Thunb.) Steud. fruits & isolated ursolic acid on serum ALT, AST and ALP of rats.

Parameters	Control	Control Treated fruits	Diethyl nitrosamine	Diethyl nitrosamine Treatment fruits	Treated Ursolic acid	ANOVA P ≤
ALP	65.82 ± 0.85 ^c	65.37 ± 0.98 ^c	174.80 ± 4.26 ^a	109 ± 1.88 ^b	70.78 ± 1.27 ^c	0.0001
AST	51.49 ± 0.83 ^b	33.22 ± 0.54 ^d	80.23 ± 1.16 ^a	49.53 ± 0.98 ^{bc}	44.99 ± 0.67 ^{bc}	0.0001
ALT	28.49 ± 0.32 ^{cd}	21.75 ± 0.39 ^e	50.99 ± 1.2 ^a	31.18 ± 0.59 ^{bc}	28.58 ± 0.34 ^{cd}	0.0001

- Data are means ± SE of eight rats in each group.
- Data are expressed as U/ml for ALT, AST and ALP.
- Unshared letters between groups are significance value at $p \leq 0.05$.
- Statistical analysis is carried out using one way analysis of variance (ANOVA) by Co Stat Computer Program.

**Fig. 3.** The hepatoprotective activity of the chloroform fruits extract of *Paulownia tomentosa* (Thunb.) Steud. & isolated ursolic acid on serum ALT, AST and ALP of rats.**Table 3**Effect of the chloroform extract of *Paulownia tomentosa* (Thunb.) Steud. Fruits & isolated ursolic acid on serum albumin, total protein, GGT & hepatic glucose of rats.

Parameters	Control	Control Treated fruits	Diethyl nitrosamine	Diethyl nitrosamine Treatment fruits	Treated Ursolic acid	ANOVA P ≤
GGT	55.11 ± 0.97 ^d	42.66 ± 0.52 ^e	94.69 ± 1.77 ^a	62.95 ± 1.15 ^{cd}	76.39 ± 1.14 ^b	0.0001
Glucose	66.05 ± 0.62 ^a	53.42 ± 0.69 ^c	37.76 ± 0.69 ^e	57.10 ± 0.69 ^{bc}	47.37 ± 0.60 ^d	0.0001
T. Protein	17.7 ± 0.18 ^{ab}	18.44 ± 0.13 ^a	16.3 ± 0.1 ^c	17.46 ± 0.12 ^{ab}	18.45 ± 0.15 ^a	0.002
Albumin	5.34 ± 0.08 ^c	5.22 ± 0.08 ^c	2.65 ± 0.09 ^d	6.39 ± 0.09 ^{ab}	5.78 ± 0.1 ^{bc}	0.0001

- Data are means ± SE of eight rats in each group.
- Data are expressed as g/dl for serum protein and albumin as g/l
- Data are expressed as g/l hepatic glucose and U/mg protein for GGT
- Unshared letters between groups are significance value at $p \leq 0.05$.
- Statistical analysis is carried out using one way analysis of variance (ANOVA) by Co Stat Computer Program.

and initial regression of the fibrosis. The biochemical findings are supported by the histopathological observations, which revealed induction of hepatocellular damage and the enhancement of liver cells, through hepatic enzyme function Fig. 7.

3.4. Statistical analysis

Data presented as mean ± standard error (SE), Statistical analysis is carried out using one way analysis of variance (ANOVA) Computer program by Co Stat Computer Program significant at $p \leq 0.05$.

4. Discussion

N-nitrosodiethylamine is one of the most important environmental carcinogen of this class, has been suggested to cause the generation of reactive oxygen species (ROS) resulting in oxidative stress and cellular injury [4]. Ursolic acid is a hydroxy penta cyclic triterpene, which has a chemoprotective activity in human and found in many plant, it is the

major component of some traditional medicinal herbs, It possesses many important biological activities, such as hepatoprotective, anti-inflammatory, antiulcer, hypolipidemic and ant atherosclerotic [8].

On the basis of physical, chromatographic and spectral data and also by comparison with published data [8], compound (1) was established and assigned as ursolic acid, which was identified in leaves, fruits and flowers using different chromatographic and spectroscopic techniques.

From the Table 1 and Fig. 2. The isolated ursolic acid showed the most promising and has the highest selectivity on MCF-7 with IC_{50} 3.5 μ g/ml even more than the reference drug 'Doxorubicin' with IC_{50} 26.1 μ g/ml. Moreover, ursolic acid showed strong cytotoxic activity against HepG2 cell line with IC_{50} 21.4 μ g/ml.

Elevated serum concentrations of this protein can be achieved in the adult by exposure to hepatocarcinogen agents. It has the high specificity for hepatocarcinoma. Its serum concentration confirms hepatocarcinoma and for the diagnosis of tumor response to therapy [5].

The significant observed improvement in all liver parameters, albumin, glucose and total protein in the treated rats with fruits ext. or ursolic

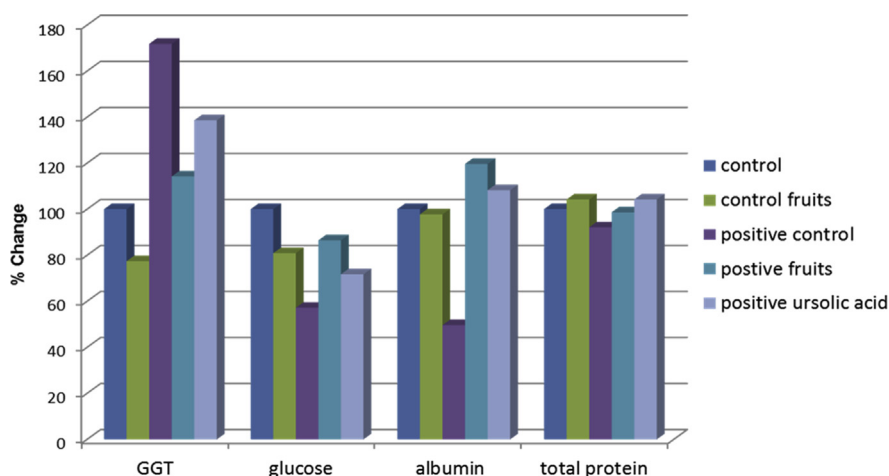


Fig. 4. The hepatoprotective activity of the chloroform fruits extract of *Paulownia tomentosa* (Thunb.) Steud. & isolated ursolic acid on GGT, glucose, albumin and total protein of rats.

Table 4
Effect of the chloroform extract of *Paulownia tomentosa* (Thunb.) Steud. fruits & isolated ursolic acid on liver weigh, body weight and liver ratio.

Parameters	Control	Control treated Fruits	NDEA	NDEA Treated fruits	Treated Ursolic acid	ANOVA P ≤
Liver weigh	5.00 ± 0.06 ^{ab}	4.31 ± 0.02 ^c	5.16 ± 0.11 ^{ab}	5.17 ± 0.05 ^{ab}	5.54 ± 0.14 ^a	0.021
Body weigh	136.33 ± 1.5 ^{bc}	141.9 ± 0.69 ^b	129.8 ± 0.94 ^{cd}	141.4 ± 1.10 ^{bc}	159.0 ± 1.87 ^a	0.0001
Liver ratio	3.69 ± 0.04 ^{ab}	3.04 ± 0.012 ^c	3.85 ± 0.12 ^{ab}	3.68 ± 0.058 ^{ab}	3.46 ± 0.057 ^{bc}	0.004

- Data are means ± SE of eight rats in each group.
- Data are expressed as grams.
- Unshared letters between groups are significance value at p ≤ 0.05.
- Statistical analysis is carried out using one way analysis of variance (ANOVA) by Co Stat Computer Program.

acid, suggests a possible protective effect of ursolic acid in liver disturbance condition which showed highly significant results as compared to control. Lower level of total protein showing the reduction in biosynthesis of protein due to destructions and dissociation of polyribosomes on endoplasmic reticulum caused by toxicity of NDEA [23].

Liver mass recovery accelerates by using ursolic acid, it stimulates

hepatocyte proliferation after partial hepatectomy, and this may be associated with the stimulation of C/EBPβ expression [24]. NDEA has been shown to be metabolized to its active ethyl radical, which can interact with DNA causing mutation and subsequent oncogenesis [25]. Ursolic acid has been shown to inhibit DNA replication [26].

The present study has assessed the inhibitory effects of *Paulownia tomentosa* (Thunb.) Steud. Flowers, fruits and isolated ursolic acid on hepatocarcinogenesis for the first time using diethyl nitrosamine induced rats' hepatocarcinogenesis model. Transaminase activities AST, ALT, ALP, GGT, were increased remarkably in plasma by the release of these enzymes from hepatic parenchymal cells, which were indicating a considerable hepatocellular injury while albumin, total protein showing reduction against NDEA treatment in agreement [4].

There was significant decrease of AST, ALT, GGT and ALP activities observed in rats treated with fruits and ursolic acid suggested a possible protective effect of *P. tomentosa* especially with ursolic acid in liver disturbance condition also Treatment with fruits extract ameliorated the disturbances in all total protein, albumin, glucose with different percentage of improvement. These biochemical findings were further confirmed by histopathological studies. On histopathological sections of the liver cell, fruits and ursolic acid treatment revealed that it had anti-tumor activity, evidenced from the absence of cellular necrosis and inflammatory infiltrates in the liver section of rats. Ursolic acid is able to restore the biochemical and histological changes caused by the

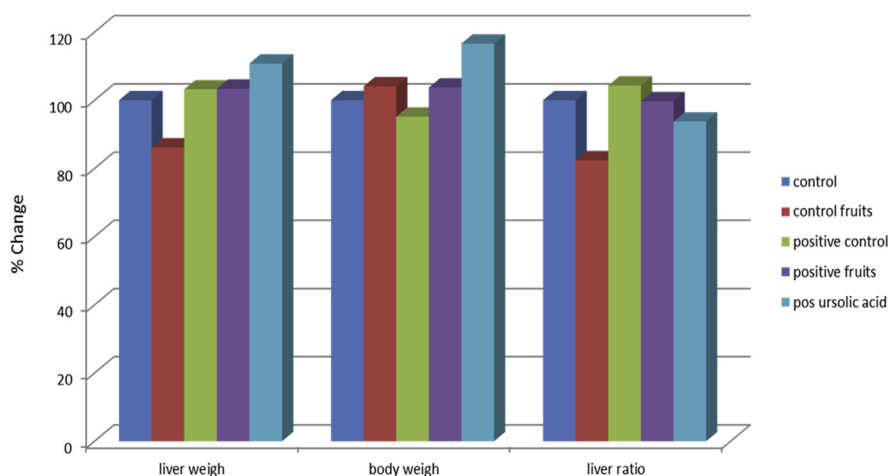


Fig. 5. The hepatoprotective activity of the chloroform fruits extract of *Paulownia tomentosa* (Thunb.) Steud. & isolated ursolic acid on liver weigh, body weight and liver ratio.

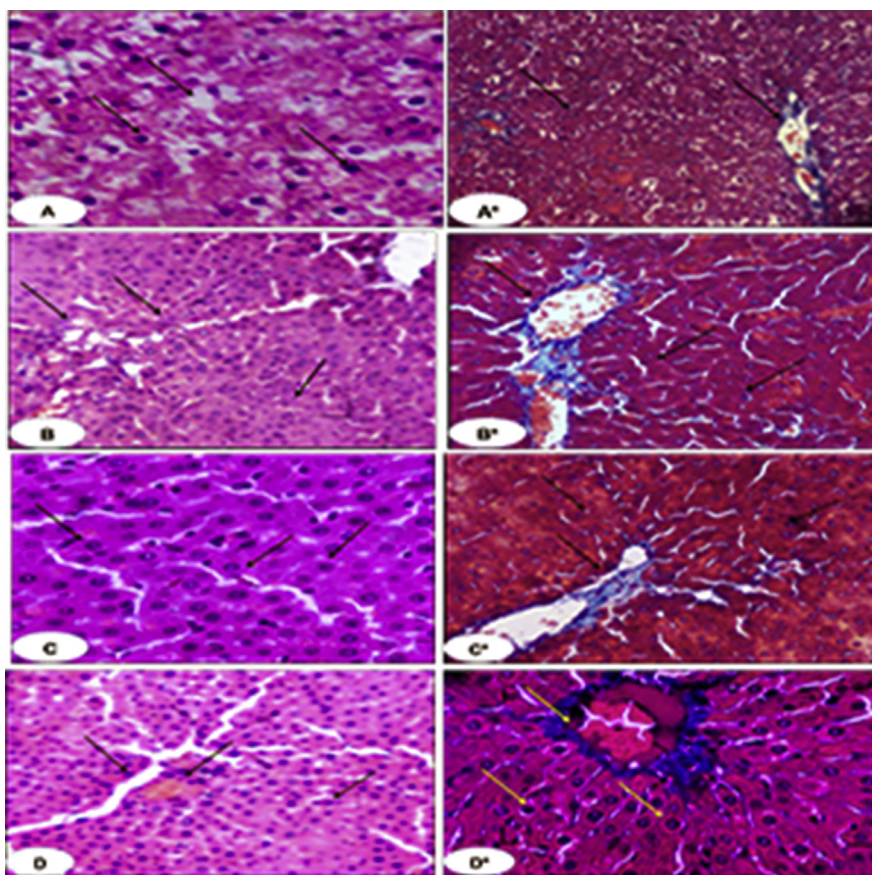


Fig. 6. Liver sections of control animals [H&E] stain (A), (A*) normal hepatocyte cell Masson' trichome stains. (B) DEN group [H&E] stain. (B*) collagen increase distribution stained with Masson trichome. (C) Revealed the effect of ursolic acid [H&E] stain (C)*the same section stained with Masson's trichome. (D) H&E stain cirrhotic rats treated with fruits extracts (D*) Masson' trichome stain showing initial improvement.

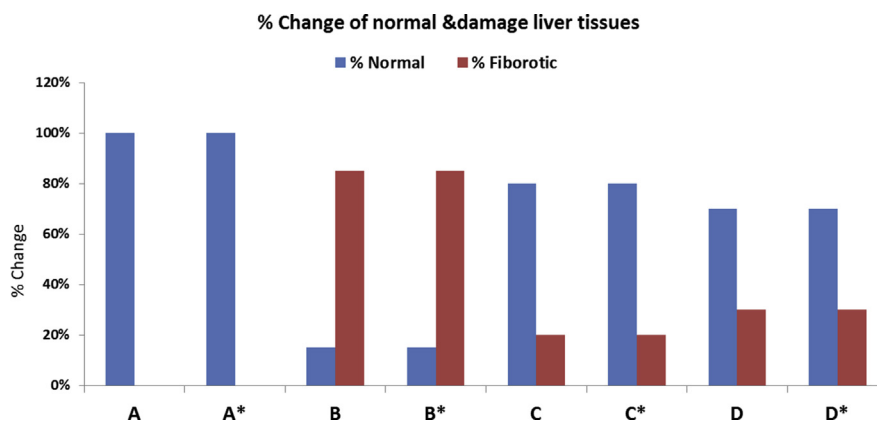


Fig. 7. A quantitative analysis of Fig. 6.

development of hepatocellular carcinoma.

5. Conclusion

The present study has assessed the inhibitory effects of *Paulownia tomentosa*, fruits and the isolated ursolic acid on hepatocarcinogenesis is for the first time using a NDEA induced rats hepatocarcinogenesis model. Ursolic acid plays a role in accelerating liver proliferation, recovering liver function, and protecting the integrity of hepatocytes against liver damage.

Declarations

Author contribution statement

Sanaa Ali, Nabaweya Ibrahim, Magdy Mohammed, Seham El-Hawary, Esraa Refaat: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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