# Possible Hepatoprotective Potential of *Cynara scolymus, Cupressus sempervirens* and *Eugenia jambolana* Against Paracetamol-Induced liver Injury: *In-vitro* and *In-vivo* Evidence

Zeinab Y. Ali [1,*], Hanan A. Atia [2] and Nancy H. Ibrahim [2]

1. Biochemistry Department, National Organization for Drug Control and Research (NODCAR), Giza 35521, Egypt.
2. Biochemistry Department, Faculty of Pharmacy (girls), Al-Azhar University, Cairo, Egypt.
zeinabyousef65@ymail.com, hananmoawad@gmail.com

**Abstract:** This study aimed to evaluate *in-vitro* antioxidant activities of hydroethanolic extract of leaves of *Cynara scolymus* L., *Cupressus sempervirens* L., and *Eugenia jambolana* Lam comparing with ascorbic acid, and their correlation with *in-vivo* hepatoprotective activity in rat model of paracetamol-induced hepatotoxicity, comparing with silymarin as reference agents. *In-vitro* study revealed that the tested extracts contain abundant amount of phenolic and flavonoids compounds attributed to their effective antioxidant potential in different models of assay that was decreased in the order of ascorbic acid ≥ *E. jambolana* > *C. sempervirens* > *C. scolymus*. *In-vivo* study, the pre-treatments with either extract (250 mg/kg/day, p.o) or silymarin (100 mg/kg/day, p.o.) for 4 weeks have good safety profile in normal rats and exhibited a marked hepatoprotection against single toxic dose of paracetamol (4 g. $kg^{-1}$ b.w, p.o.) as proved from 1. significant preserving the normal liver function parameters, 2. maintenance the hepatic redox status as evident from significant increase in antioxidant enzyme activities and reduced glutathione with inhibition of lipid peroxidation and protein oxidation. 3. decreasing nitric oxide and tumor necrosis factor alpha 4. membrane stabilizing effects as confirmed from significant increase in the hepatic $Na^+$-$K^+$-ATPase activity and decrease in lysosomal enzyme activities which were changed in the untreated paracetamol-intoxicated rats. These protection was decreased as silymarin ≥ *E. jambolana* > *C. sempervirens* > *C. scolymus*. In conclusion: *E. jambolana* may be applied as potential sources of natural antioxidant with hepatoprotective effect and may be recommended for early prophylaxis of patients with paracetamol-induced hepatic injure.

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**Key words**: phenolic; flavonoids; antioxidant; hepatoprotection; liver function; hepatic redox status; lipid peroxidation; protein oxidation; nitric oxide; tumor necrosis factor alpha; $Na^+$-$K^+$-ATPase; lysosomal enzymes

## 1. Introduction

Liver diseases remain to be serious health problems and the management of liver disease is still a challenge to the modern medicine. Liver plays an essential role in regulation of physiological processes, involved in several vital functions such as storage, secretion and metabolism. It also detoxifies a variety of drugs and xenobiotics and plays a central role in transforming, clearing the chemicals and is susceptible to the toxicity from these agents (Pal and Manoj, 2011).

Drug-induced liver injury is a potential complication of virtually every prescribed medication.. Paracetamol (N-acetyl-p-aminophenol; APAP), a highly popular analgesic and antipyretic drug, is quickly absorbed from the gastric intestinal tract and reaches peak serum levels in 1- 4 hours. It is safe at therapeutic doses; however the overdose following accidental ingestion or suicidal attempt causes a toxic response leading to the centrilobular necrosis in liver. APAP overdose, either alone, or in combination with other drugs, is account for 60% of cases of acute liver failure and leading to orthotopic liver transplant in the United States of America and United Kingdom (Sweetman, 2009; Mohit et al., 2011). The maximum recommended daily dose of APAP is 4 g in adults and 90 mg/kg in children. Toxicity is associated with a single acute APAP ingestion of 150 mg/kg or approximately 7-10 g in adults (FDA, 2009).

With therapeutic dosing, APAP is predominantly metabolized by conjugation with sulphate and glucuronic acid and normally, approximately 5% of the drug is oxidized by CYP450-dependent pathways to toxic highly reactive electrophilic metabolite, N-acetyl-p-benzoquinone imine (NAPQI), which is normally detoxified by reduced glutathione (GSH) and eliminated in the urine (Chen et al., 2009). Consequently, APAP has an excellent safety profile when a normal dose, however, high doses limit the ability of GSH to detoxify the over production of NAPQI result in the depletion of liver GSH. The excess NAPQI can covalently bind to cysteine groups (CYS) of critical hepatocyte cell proteins forming APAP-CYS adducts resulting in inactivation of these proteins (Davern et al., 2006). Also it raises the cytosolic calcium levels by inhibiting the Ca-ATPase activity in the plasma membrane and triggering the formation of reactive oxygen species

(ROS) (Das and Sarma, 2009). Moreover, these highly reactive molecules have toxic effects on membrane phospholipids, resulting in lipid peroxidation, oxidation of protein thiols, DNA fragmentation, cell lysis and cell death (Marotta et al., 2009; Jaeschkea and Bajt, 2010). Several studies proved that oxidative stress constitutes a major mechanism underlying the pathogenesis of paracetamol-induced liver damage (Singh et al., 2011).

The use of plants for their therapeutic value is a part of the human history in Egypt. Plant derived natural products have received considerable attention in recent years due to their diverse pharmacological properties (Govind and Sahni, 2011). Herbal drugs containing antiradical constituents are gaining importance in prevention and treatment of oxidative stress linked-diseases (Anand and Shrihari, 2011).

*Cynara scolymus* L. (family of *Asteraceae*), *Cupressus sempervirens* L. (family of *Cupressaceae*), and *Eugenia jambolana* Lam. (family of *Myrtaceae*) traditionally used as an alternative medicine in Egypt. Ezz El-Din et al., (2010) reported that *C.scolymus* is rich in caffeoylquinic acid derivatives (cynarin and chlorogenic acid), flavonoids, volatile oils, phytosterols and tannins. Koriem, (2009) and Mazari et al., (2010) showed that *C. sempervirens* is rich in flavonoids (cupressuflavone, amenoflavone, rutin, quercitrin, quercetin, myricitrin) and phenolic compounds (anthocyanidin, catechines flavones, flavonols and isoflavones) tannins, catchol and essential oil. Moreover Magina et al., (2009) reported that *E. jambolana* is reach in flavonides, saponins and glycoside, volatile oils, gallic acid, tannins and flavonol glycosides. However, there are currently few reports concerning their hepatoprotective activity on the scientific evidence. The scientific evaluation of these plants may provide modern medicine with effective pharmaceuticals for the treatment of liver diseases. Therefore the current study was aimed to evaluate *in-vitro* antioxidant activities of hydroethanolic extracts of the leaves of aforementioned plants and their correlation with *in-vivo* hepatoprotective potential in experimental rat model of paracetamol-induced liver toxicity.

## 2. Materials and Methods

### 2.1. Chemicals

All chemicals were of analytical grade and purchased from Sigma-Aldrich Chemicals Co. (St Louis, MO, USA). Paracetamol (Abimol) was purchased from Glaxo Smithkline, Egypt.

### 2.2. Preparation of plant extracts

Fresh leaves of the three tested plants were collected, washed and air dried at room temperature for 3 weeks to constant weight. The dried leaves were later ground to powder and soaked in 70% ethanol separately for 48 hrs on an orbital shaker at room temperature. Extracts were filtered and the residue was re-extracted under the same conditions until extraction solvents became colorless. The combined filtrates were concentrated to dryness under reduced pressure at 40°C using a rotary evaporator and then the concentrated extract was lyophilized and kept at 4°C until used.

### 2.3. Animals

The study was conducted in female Swiss strain albino rats, weighing about 150 ± 20 g, obtained from the animal house of National Organization for Drug Control and Research (NODCAR), Animals were kept under standard laboratory conditions of light/dark cycle (12/12h.), temperature (25 ± 2°C) and fed on normal laboratory diet and water *ad libitium*. They were acclimatized for a week in the new environment before initiation of experiment.

### 2.4. Experimental design

#### 2.4.1. *In-Vitro* Study

*2.4.1.1. Total phenolic content:* The total phenolic content of the tested extracts were determined by Folin–Ciocalteau reagent in alkaline medium according to the method of Macdonald et al., (2001) with slight modifications. The results expressed as µg gallic acid equivalents (GAE) per mg dry extract (standard gallic acid curve equation: $y = 0.0063x$, $R^2 = 0.999$).

2.4.1.2. *Total flavonoid content*: The total flavonoid content was determined according to aluminum chloride colorimetric method as described by Zou et al., (2004). The result expressed as µg rutin equivalent (RE) per mg dry extract (standard rutin curve equation: $y = 0.0119x$, $R^2 = 0.9996$).

2.4.1.3. *Reducing power assay*: The reducing power of the tested extracts was determined by the method of Oyaizu (1986). Substances which have reduction potential react with potassium ferricyanide ($Fe^{3+}$) to form potassium ferrocyanide ($Fe^{2+}$), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm. A higher absorbance indicates a higher ferric reducing power.

2.4.1.4. *Antiradical screening activity:* These models of assay involve investigation of 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radical scavenging activity (Blois, 1958) and oxygen radical absorbance capacity (ORAC) which were covered the major radicals such as superoxide anion, $O^{2\bullet-}$ (Nishimiki et al., 1972), hydroxyl, $OH^{\bullet}$ (Jayasri et al., 2009), hydrogen peroxide, $H_2O_2$ (Ruch et al., 1989) and nitric oxide, NO (Govindarajan et al., 2003) that may be involved in the mechanism of paracetamol toxicity (Mandade, 2011). All tests were performed in triplicate and the graph was plotted with the average of the three determinations.

Antiradical activity expressed as $IC_{50}$ and compared with that of ascorbic acid as standards. $IC_{50}$ value was defined as the concentration of the sample in µg. $ml^{-1}$ required to scavenge 50% of free radicals (Boonchum et al., 2011). Therefore, the lower the $IC_{50}$ value, the higher is the antioxidant activity of the tested sample. The radical scavenged percent was calculated using the following formula:

$$\text{Radical scavenging}\,\% = \left(1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}}\right) X\, 100$$

### 2.4.2. Oral Acute Toxicity

Oral acute toxicity was conducted according to the method of Organization for Economic Co-operation and Development (OECD 1996). Five groups of six female albino rats each, weighing 150 ± 20 g b.w was used. Animals were kept fasting providing only water, after which each plant extract was orally administered by gastric tube in different gradual doses (1 to 5 $g.kg^{-1}$ b.w), and observed for any toxic symptoms and mortality for 72 hrs.

### 2.4.3. Effect on normal rats

A total of 40 albino Sprague Dawley rats were divided randomly into equal five groups (8 rats each). **Group 1** served as control. **Group 2-4** received once daily one of the hydroethanolic extracts of *C. scolymus, C. sempervirens and E. jambolana* at fixed daily dose of 1/20 $LD_{50}$ (250 $mg^{-1}$.kg b.wt, p.o), respectively. **Group 5** received a daily oral dose of silymarin (100 $mg.kg^{-1}$ b.w, p.o).

### 2.4.4. Protective effect against paracetamol toxicity

A total of 40 rats were randomly divided into five groups of eight rats each. **Group 6** fed on normal diet for 28 days **Groups 7-9** pretreated once daily with one of the hydroethanolic extracts of *C. scolymus, C. sempervirens* and *E. jambolana* for 28 days at fixed dose of 1/20 $LD_{50}$ (250 $mg^{-1}$.kg b.wt, p.o). **Group 10** pretreated once daily with silymarin (100 $mg^{-1}$.kg b.w, p.o) (Premalakshmi and Thenmozhi, 2011) for the same period. Then groups 6-10 were administered with a single high dose of paracetamol (4 g. $kg^{-1}$ b.w, p.o) (Dixon et al., 1975), thus group 6 served as positive control.

### 2.5. Blood and tissue sampling

On the 29th day, all animals were fasted overnight, blood samples were withdrawn from the retro-orbital plexus vein. Sera were separated after centrifugation at 400 x g for 10 minutes, and stored frozen for assessment of liver function. All rats were sacrificed by decapitation. The livers were quickly excised, rinsed in cold saline, blotted and weighed. Parts of livers were freshly used for determination of GSH and preparation of lysosomal fraction. The remaining livers were stored at -80°C for further assessments.

### 2.6. Biochemical Analysis.

#### 2.6.1. Assessment of liver function

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) (Retimen and Frankel, 1957), alkaline phosphatase (ALP) (Belfield and Goldberg, 1971), total bilirubin (TB) (Tietz, 1995) and total protein (TP) (Lowry et al., 1951) were considered as markers of liver functional state.

#### 2.6.2. Assessment of hepatic oxidative stress biomarkers

Superoxide dismutase (SOD) (Marklund and Marklund, 1974); catalase (CAT) (Aebi, 1984), glutathione peroxidase (GPx) (Rotruck et al., (1973), glutathione S-transferase (GST) (Habig et al., 1974) and reduced glutathione (GSH) (Beutler et al., 1963) were estimated in liver tissues as indicators of oxidative stress. Tissue protein was determined also by the method described by Lowry et al., (1951). Malondialdehyde (MDA) a marker of lipid peroxidation was assayed using thiobarbituric acid reacting substance (TBARS) (Buege and Aus 1978) and protein carbonyl (PC) as a marker of protein oxidation (Levine et al., 1990) were assayed. Moreover nitric oxide (NO) was determined by the method of Wallace, (2004).

#### 2.6.3. Assessment of serum tumor necrosis factor alpha (TNF-α)

Serum TNF-α was determined using enzyme-linked immunosorbent assay (ELISA, RayBiotech, USA), according to the manufacture's instructions.

#### 2.6.4. Hepatic $Na^+/K^+$ATPase

Hepatic $Na^+/K^+$ATPase was determined by colorimetric assay according to Corcoram et al., (1987). ATP was hydrolyzed and the released Pi was measured by forming a complex with molybdate. Na,K-ATPase activity and calculated as the difference between ouabain-treated and untreated samples. Protein concentrations were determined by the method of Lowry et al., (1951).

#### 2.6.5. Assessment of the marker hepatic lysosomal enzymes

Hepatic lysosomal fraction was prepared by sucrose-density-gradient centrifugation in a refrigerating ultracentrifuge according to the method of Tanaka and Izuka, (1968). The extracellular activity of the marker lysosomal acid hydrolyases as acid phosphatase (ACP), N-acetyl-$\beta$-glucosaminidase ($\beta$-NAG), $\beta$-galactosidase ($\beta$-GAL) were measured according to the methods described by Van Hoof and Hers (1968) with slight modifications by Younan and Rosleff (1974). Protein in lysosomal fractions was measured by the method of Lowry et al., (1951).

**2.7. Statistical Analysis**

All results are presented as mean ± S.E. The statistical significance of the difference for biochemical results were analysed through one way analysis of variance (ANOVA). Comparisons of mean values of different groups were estimated by Turkey's Multiple Comparision Test (TMCT). $P \leq 0.05$ was considered significant.

**3. Results and Discussion**

***In-vitro* screening methods for antioxidant activity**

The medicinal values of the tested extracts lie in their active phytochemicals contents, which consists of a wide range of chemicals with different potency. The obtained result revealed that phenolic and flavonoids compounds were abundantly present in all of the three tested extracts. The highest concentrations of both phenolics and flavonoids were detected in *E. jambolana* (146.5 ± 5.63 µg GAE/mg extract and 49.7 ± 1.26 µg RE/mg extract) followed by *C. sempervirens* (86.3 ± 2.47µg GAE/mg extract and 26.4 ± 0.94 µg RE/mg extract) and *C. scolymus* (41.3 ± 1.59 µg GAE/mg extract and 11.9 ± 0.87 µg RE/mg extract), respectively. Plant phenolics included flavonoids composed of one or more aromatic rings with one or more hydroxyl groups. Their structure-activity studies have been established the importance of their planar structure; the number and position of their hydroxyl groups as well as the presence of the double bond in their various antioxidant activities (Atmani et al., 2009; Moein and Moein, 2010).

Reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Oyaizu, 1986). Figure (1) illustrates that all tested extracts exhibited a remarkable reducing potency to donate electron to stabilize the reactive free radicals and reduce the oxidized intermediates in concentration dependant manner as compared to the standard ascorbic acid. This reducing power decreased in the order of ascorbic acid > *E. jambolana* > *C.sempervirens* > *C. scolymus*.

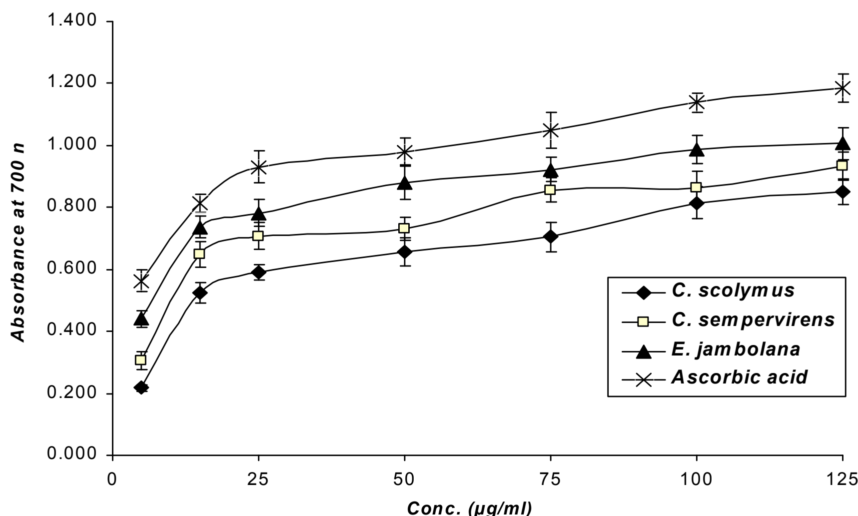


Figure 1. Reducing power of tested extracts in comparable to ascorbic acid

Based on the results in Figure (2), *E. jambolana* seams to be the most effective extract in the scavenging of different radicals at low $IC_{50}$ values after ascorbic acid followed by *C. sempervirens* and *C. scolymus,* respectively. It was effective extract in the scavenging of DPPH$^{\bullet}$, OH$^{\bullet}$, $H_2O_2$ and NO$^{\bullet}$ radicals at the lowest $IC_{50}$ values of 14.1 ± 1.13, 160.3 ± 10.0, 13.2 ± 1.10 and 83.1 ± 5.33 µg/ml, respectively. The antiradical potency of *E. jambolana* against different reactive molecules decrease in the order: $H_2O_2 \geq$ DPPH > $O^{2\bullet-}$ > NO$^{\bullet}$ > OH$^{\bullet}$. While, *C. sempervirens* leaves extract was the most effective in the scavenging of $O^{2\bullet-}$ at $IC_{50}$ value of 33.5 ± 1.51 µg/ml. Therefore, the present *in-vitro* study proved that all tested extracts exhibited effective and concentration dependent antioxidant activity. This activity was more pronounced in the hydroethanolic leaves extract of *E. jambolana*, which contains the highest amount of phenolic compounds. Thus our results confirmed that there is strong correlation between the antioxidant activity and total phenolic content, which are in agreement with studies of Boonchum et al., (2011).

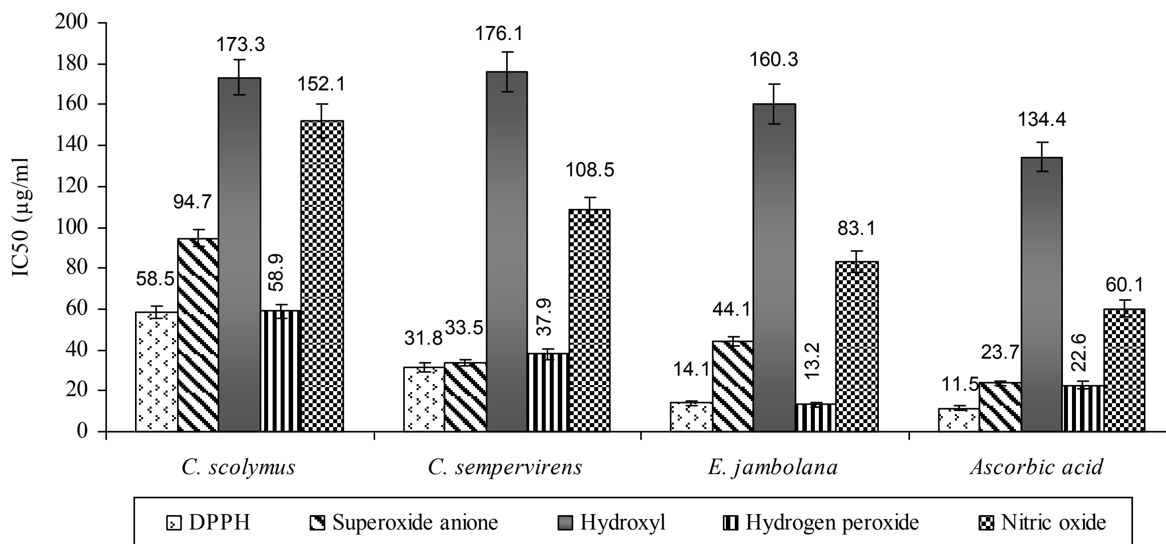


Figure 2. Comparison of the total antioxidant activity of tested extracts against ascorbic acid

**Oral acute toxicity test**

The oral acute toxicity test for the three tested extracts showed no lethality or signs of toxicity up to a dose level of 5 g. $kg^{-1}$ b.w and were considered as safe. Therefore, one-twentieth of the maximum dose (250 mg. $kg^{-1}$ b.w. $day^{-1}$) of each extract was selected for evaluation of hepatoprotective activity *in-vivo*.

**Effect on liver function**

The pretreatment of the normal rats with the hydroethanolic extracts of *C. scolymus, C. sempervirens* and *E. jambolana* (250 mg.$Kg^{-1}$ b.w/day) or silymarin (100 mg.$Kg^{-1}$ b.w/day) for 4 weeks has no significant change in all liver function parameters confirming their safety profile (the data not shown). While, the depicted data in Table (1) support and substantiate that the oral administration of a single toxic dose of paracetamol (APAP, 4 g/kg) induced a significant increase ($P<0.001$) in serum levels of the ALT, AST, ALP and TB by 80.3%, 62.2%, 81.1% and 125.4%, respectively and a significant decrease ($P<0.001$) in serum TP by 25.1% as compared to the normal control group. Meanwhile, as compared with the untreated paracetamol-intoxicated group, the pre-treatment with the three tested extracts or silymarin for 4 weeks was significantly ($P<0.001$) able to maintain the aforementioned parameters near healthy levels after treatment with the single toxic dose of APAP (4 g.$kg^{-1}$, p.o.). These beneficial effects were decreased in the order of silymarin $\geq$ *E. jambolana* > *C. sempervirens* > *C. scolymus.*

APAP is one of the most common pharmaceuticals associated with both intentional and unintentional poisoning. Recent studies provide evidence that high doses of APAP limit the ability of GSH to detoxify the over production of NAPQI result in the depletion of liver GSH pool (Singh et al., 2011). In the current study, a marked elevation in serum levels of ALT, AST and ALP which are normally located in the cytosol indicated the cellular leakage and loss of functional integrity of the cell membrane, and used as an index of liver damage and as a complicating case of APAP intoxification (Parmar et al., 2010; Mandade, 2011). Consequently, the maintenance of these serum enzymes near the normal value in plant extract pretreated-groups is an indication of stabilization of plasma membrane thereby preserving the structural integrity of cell as well as repairing and regeneration of hepatic tissue damage that caused by a high dose of APAP.

The hyperbilirubinemia that observed in the untreated APAP-intoxicated group may be attributed to excessive heme destruction and blockage of biliary tract. As a result of blockage of the biliary tract there is a mass inhibition of the conjugation reaction and release of unconjugated bilirubin from damaged hepatocytes. Meanwhile, the reduction in T.P may be attributed to the damage produced and localized in the endoplasmic reticulum leading to decrease in protein synthesis (Kanchana and Sadiq, 2010).

Pretreated with the three extracts or silymarin for 4 weeks was able to prevent the increase in the serum level of TB suggesting that they offered liver protection. While, significant reduction ($P<0.001$) in serum TP in untreated-APAP intoxicated group may be attributed to the initial damage produced and localized in the endoplasmic reticulum leading to its functional failure with a decrease in protein synthesis (Takate et al., 2010). Meanwhile, the marked elevation ($P<0.001$) in the TP in pretreated groups can be suggesting the stabilization of endoplasmic reticulum leading to protein synthesis and enhanced hepatocyte regeneration.

Table 1. Protective effect of different pretreatments on liver function parameters in APAP-intoxicated rats

| Groups | ALT (U/ml) | AST(U/ml) | ALP (IU/L) | TB (mg/dl) | TP (g/dl) |
|---|---|---|---|---|---|
| **Control** | 21.8 ± 0.56 | 52.8 ± 0.94 | 322.1 ± 8.82 | 0.80 ± 0.03 | 6.02 ± 0.18 |
| **APAP** | 39.3 ±0.80$^{\#\#\#}$ | 85.6 ± 2.44$^{\#\#\#}$ | 583.3 ± 13.7$^{\#\#\#}$ | 1.80 ± 0.06$^{\#\#\#}$ | 4.51 ± 0.13$^{\#\#\#}$ |
| ***C. scolymus* + APAP** | 28.5 ± 1.30$^{***}$ | 63.6 ± 1.86$^{***}$ | 412.0 ± 17.0$^{***}$ | 1.23 ± 0.03$^{***}$ | 5.11 ± 0.13$^{***}$ |
| ***C. sempervirens* + APAP** | 27.2 ± 0.99$^{***}$ | 58.1 ± 2.55$^{***}$ | 365.0 ± 12.4$^{***}$ | 1.05 ± 0.03$^{***}$ | 5.35 ± 0.09$^{***}$ |
| ***E. jambolana* + APAP** | 25.1 ± 0.90$^{***}$ | 56.9 ± 1.75$^{***}$ | 366.1 ± 13.0$^{***}$ | 0.98 ± 0.02$^{***}$ | 5.51 ± 0.13$^{***}$ |
| **Silymarin+ APAP** | 25.9 ± 0.64$^{***}$ | 55.4 ± 0.82$^{***}$ | 335.4 ± 11.8$^{***}$ | 0.95 ± 0.02$^{***}$ | 5.78 ± 0.15$^{***}$ |

Each value represents the mean of 8 rats ± S.E. Significant at $^{\#\#\#}P$ <0.001 vs control.
Significant at $^{***}P$ <0.001 vs APAP.

**Effect on the hepatic antioxidant capacity**

In the current study, the results confirmed that the hepatic antioxidant defense system was insignificantly increased in the pretreated-groups for 4 weeks indicated their safety profile (the data not shown). However, data depicted in Table (2) demonstrate that a single toxic dose of APAP was associated with significant (P<0.001) impairment of the hepatic antioxidant defense system as confirmed from decrease in hepatic SOD, CAT, GPx and GST activities and GSH by 30%, 20.1%, 26.1%, 17.5% and 30.1%, respectively as compared to the normal control group. While, the pre-administration of tested extracts (250 mg.Kg$^{-1}$ b.w) or silymarin (100 mg.Kg$^{-1}$ b.w) for 4 weeks exhibited a significant (P<0.001) increase in hepatic antioxidant capacity. This improvement was in the order of silymarin ≥ *E. jambolana* > *C. sempervirens* > *C. scolymus*.

Table 2. Protective effect of different pretreatments on the hepatic oxidative stress markers in APAP-intoxicated rats

| Groups | SOD (u/mg protein) | CAT (u/mg protein) | GPx (u/mg protein) | GST (u/mg protein) | GSH (mg/g tissue) |
|---|---|---|---|---|---|
| **Control** | 3.72 ± 0.07 | 64.2 ± 1.84 | 5.52 ± 0.14 | 5.07 ± 0.10 | 1.49 ± 0.04 |
| **APAP** | 2.61 ± 0.09$^{\#\#\#}$ | 51.4 ± 2.39$^{\#\#\#}$ | 4.08 ± 0.08$^{\#\#\#}$ | 4.19 ± 0.08$^{\#\#\#}$ | 1.04 ± 0.05$^{\#\#\#}$ |
| ***C. scolymus* + APAP** | 3.78 ± 0.12$^{***}$ | 66.4 ± 1.23$^{***}$ | 5.72 ± 0.11$^{***}$ | 4.94 ± 0.09$^{**}$ | 1.39 ± 0.06$^{***}$ |
| ***C. sempervirens* + APAP** | 3.83 ± 0.13$^{***}$ | 71.7 ± 0.93$^{***}$ | 5.75 ± 0.12$^{***}$ | 5.55 ± 0.11$^{***}$ | 1.45 ± 0.04$^{***}$ |
| ***E. jambolana* + APAP** | 4.07 ± 0.17$^{***}$ | 73.8 ± 1.41$^{***}$ | 5.99 ± 0.18$^{***}$ | 5.74 ± 0.19$^{***}$ | 1.58 ± 0.06$^{***}$ |
| **Silymarin + APAP** | 3.92 ± 0.11$^{***}$ | 69.1 ± 1.46$^{***}$ | 5.89 ± 0.16$^{***}$ | 5.84 ± 0.14$^{***}$ | 1.64 ± 0.05$^{***}$ |

Each value represents the mean of 8 rats ± S.E. Significant at $^{\#\#\#}P$ <0.001 vs control. Significant at $^{**}P$ <0.01; $^{***}P$<0.001 vs APAP. $U_{SOD}$ - enzyme concentration required to inhibit 50% autoxidation of pyrogallol in min., $U_{CAT}$ - enzyme concentration required to decompose 1 m moles of hydrogen peroxide in min., $U_{GPX}$ – m moles of glutathione utilized/min. $U_{GST}$- m moles of glutathione-chlorodinitrobenzene conjugate formed in min.

Our study suggested that the marked depletion in the level of antioxidant enzymes activities observed after ingestion of a single toxic dose of APAP is a clear manifestation of inactivation or failure of the antioxidant enzymes to scavenge the excessive formation of free radicals resulting in accumulation of free radicals and redox imbalance which in turn propagates the oxidative stress and liver injury. Consistence with our results, it was reported that APAP overdose may cause hepatotoxicity by mechanisms triggering the formation of reactive oxygen species (ROS), such as superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($HO^\bullet$), reactive nitrogen species (RNS), such as nitric oxide and

peroxynitrite (ONOO•). The high level of ROS exhibited a cascade of oxidative damage resulting in oxidative stress which in turn induces various deleterious actions including activation of lipid peroxidation, oxidation of protein thiols especially in the mitochondria, inflammatory response and DNA fragmentation that propagate hepatocellular injury and centrilobular liver necrosis. Therefore, oxidative stress constitutes a major mechanism underlying the pathogenesis of APAP-induced liver damage (FDA, 2009; Jaeschkea and Bajt, 2010).

Glutathione plays a role in the removal of free radical species through GPx and GST activities and maintenance of membrane protein thiols (Mandade, 2011). The present study suggested that the decreased intracellular pool of GSH after ingestion of large dose of APAP results in inadequate detoxification mechanism and failure of antioxidant defense mechanisms to prevent the formation of excessive accumulation of highly toxic metabolites (NAPQI) and free radicals. In accordance with our results, Parmar et al., (2010) and Mandade, (2011) illustrated that the GSH depletion in hepatic mitochondria is considered the most important mechanism in the paracetamol induced hepatotoxicity.

Consequently, the natural supplements with potential for serving as a source or enhancing synthesis of GSH and activate the antioxidant defense system are particularly important in diminishing the cumulative oxidative damages and counteract APAP toxicity. In the current study, APAP-intoxicated groups that pretreated with either tested leaves extracts or silymarin for 4 weeks were able to prevent the decrease in the hepatic antioxidant enzymes activities and GSH; therefore they prevent the accumulation of excessive free radicals and protect the liver from APAP intoxication. Among all the studied plants, *E. jambolana* has greatest ability to reduced oxidative stress by increasing hepatic GSH pool and preventing the oxidative stress state.

**Effect on some hepatic oxidative stress markers**

In accordance with the previous studies (Marotta et al., 2009, Mandade, 2011), our results in Table (3) illustrated that as compared with normal group, treatment with single high dose of APAP (4 g. $kg^{-1}$, p.o.) was significantly (P<0.001) enhanced the hepatic lipid peroxidation (MDA), protein oxidative modification, NO and circulated proinflammatory cytokines as serum tumor necrosis factor–alpha (TNF-α), associated with decrease in the $Na^+/K^+$ATPase activity (Mandade, 2011) in the untreated APAP-intoxicated rats. However, the pre-administration of tested extracts or silymarin for 4 weeks exhibited a significant improvement in the above mentioned parameters. The maximum hepatoprotection was observed in silymarin-preteated group, while among the three studied plants, *E. jambolana* exhibited good hepatoprotective potential followed by *C. sempervirens* against single toxic dose of APAP as compared to untreated APAP-intoxicated rats.

***Effect on Lipid peroxidation:*** Lipid peroxidation frequently occurs in our body, especially in cell membrane phospholipids. The high level of poly-unsaturated fatty acid and methylene groups in their double bonds make them sensitive to oxidation by free radicals that enhanced the free radical chain reaction and oxidative degeneration of cell membranes. Malondialdehyde (MDA), a major end product and marker of lipid peroxidation, can bind covalently to DNA and proteins and alter their functions (Boonchum et al., 2011).

In the current study, the increased level of MDA that induced by APAP suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanism (Mandade, 2011). While, a massive decrease in hepatic lipid peroxidation in plant extract-pretreated groups indicated that the tested plants possess antioxidative properties (Parmar et al., 2010). It is evident that the antioxidant property of the tested extracts prevented the formation of an oxygen radical species and thus able to inhibit or delay the oxidation process by blocking the initiation or propagation of oxidizing chain reactions and thus inhibited the lipid peroxidation. Therefore, the protective activity of the tested extracts may be attributed to their antioxidant potential.

***Effect on Protein Oxidation:*** Protein oxidation is defined as the covalent modification of a protein induced either directly by reactive oxygen species (ROS) or indirectly by reaction with secondary by-products of oxidative stress (Gill and Tuteja, 2010). Oxidative modifications of protein alter their biological properties, leading to their fragmentation, increased cross linking, aggregation and enzyme dysfunction (Gracanin et al., 2009). The protein carbonyl derivatives (PC) are chemically stable and result from oxidative cleavage of the protein backbone, direct oxidation of amino acids such as lysine, arginine, histidine, proline, glutamic acid and threonine, or by binding of aldehydes produced from lipid peroxidation (Baslé et al., 2010). Therefore, the assay of carbonyl proteins (PC) provides a convenient technique for detecting and quantifies the oxidative modifications of protein. In the present study, only groups that pretreated with *C. sempervirens* and *E. jambolana* were able to inhibit the oxidative modification of liver proteins. The beneficial effects of tested extracts can be attributed to the antioxidant activity of the bioactive constituents of the tested leaves extracts.

***Effect on Nitric Oxide:*** Nitric oxide (NO•) is a gaseous free radical, an important mediator of both

physiological and pathophysiological processes. It is produced by endothelial nitric oxide synthase (NOS), enzymes that catalyze the conversion of L-arginine into citrulline and $NO^{\bullet}$ in the presence of NADPH and $O_2$ (Wallace, 2004). $NO^{\bullet}$ has a short half-life in vivo of a few seconds or less. Therefore, the levels of the more stable NO metabolites, nitrite ($NO_2^-$) and nitrate ($NO_3^-$), have been used in the indirect measurement of NO in biological fluids (Li et al., 2004).

Nitric oxide has recently been recognized as an important mediator in paracetamol-induced hepatotoxicity (Nagi et al., 2010). In the current study, a single toxic dose of APAP enhance the production of high levels of nitric oxide (nitrate plus nitrite) within the liver, by multiple cell types (Kupffer cells, hepatocytes, and endothelial cells). Peroxynitrite, a reaction product of nitric oxide with superoxide, is now considered, in part, responsible for the paracetamol-induced hepatotoxicity (Wallace, 2004). It is normally detoxified by GSH, but when GSH is depleted by the toxic metabolite of paracetamol (NAPQI), peroxynitrite exhibits a cascade of oxidative damage resulting in oxidative stress which in turn induces deleterious actions ending with hepatocellular injury and centrilobular liver necrosis. Meanwhile, the improvement in the groups received the plant extracts, is attributed to the presence of the flavonoides as a free radical scavenger, that scavenge the excess NO and peroxynitrite, it may also suppress the NOS (Nagi et al., 2010).

***Effect on $Na^+/K^+$ATPase:*** The sodium-potassium adenosine triphosphatase ($Na^+$-$K^+$-ATPase) is an integral membrane enzyme found in all cells and is responsible for the pumping function that transports $Na^+$ and $K^+$ against their concentration gradients across plasma membranes on expense of the energy liberated from hydrolysis adenosine triphosphate (ATP). Its activity has been proposed to be used as a potential indicator for membrane structure and function. The prolonged inhibition of this pump lead to several alterations as gradual $Na^+$ accumulation, cell swelling and death (Bogdanova et al., 2005).

In the current study, the reduction in liver $Na^+$-$K^+$-ATPase activity in the APAP-intoxicated rats indicating hepatocellular damage. The hepatocyte membrane appears to be the critical locus of oxidative alterations that responsible for membrane damage in APAP-induced hepatotoxicity in rats. Under oxidative stress, the covalent binding of free radicals to hepatocellular proteins leads to their oxidative modifications that alter their biological properties and enhance the oxidative damage of membrane ATPase protein. In addition, enhanced membrane lipid peroxidation by free radicals leads to change in membrane fluidity, which in turn alters the membrane-bound enzymes as ATPase activities (Mandade, 2011). This observation is well correlated with significant increase in hepatic MDA and PC in APAP-intoxicated rats. However, the pretreatments with tested plant extracts provide protection against APAP- induced membrane damage. Thus they are considered as useful agents for the membrane-stabilization and normalization of APAP-induced impaired membrane function that decrease in the order of silymarin ≥ *E. jambolana* > *C. sempervirens* > *C. scolymus*.

Table 3. Protective effect of different pretreatments on some of oxidative stress markers in APAP– intoxicated rats

| Groups | MDA (nmol/g tissue) | Protein carbonyl (µmol/mg protein) | Nitric oxide (µg/g tissue) | $Na^+/K^+$ATPase (nmol $P_i$/hr/ mg protein) | Serum TNF-α (Pg/ml) |
|---|---|---|---|---|---|
| **Control** | 0.48 ± 0.02 | 1.46 ± 0.05 | 77.8 ± 1.19 | 0.63 ± 0.02 | 32.8 ± 1.58 |
| **APAP** | 0.67 ± 0.04$^{###}$ | 2.47 ± 0.04$^{###}$ | 95.7 ± 2.13$^{###}$ | 0.42 ± 0.02$^{###}$ | 47.3 ± 1.96$^{###}$ |
| ***C.* scolymus + APAP** | 0.56 ± 0.23$^{*}$ | 2.30 ± 0.03$^{ns}$ | 86.3 ± 2.76$^{*}$ | 0.52 ± 0.01$^{*}$ | 43.3 ± 2.19$^{ns}$ |
| ***C. sempervirens* + APAP** | 0.59 ± 0.02$^{*}$ | 2.18 ± 0.04$^{**}$ | 81.1 ± 1.67$^{***}$ | 0.54 ± 0.02$^{**}$ | 39.4 ± 0.88$^{*}$ |
| ***E. jambolana* + APAP** | 0.51 ± 0.01$^{***}$ | 1.87 ± 0.06$^{***}$ | 79.5 ± 1.36$^{***}$ | 0.56 ± 0.03$^{**}$ | 38.3 ± 1.42$^{**}$ |
| **Silymarine + APAP** | 0.48 ± 0.01$^{***}$ | 1.79 ± 0.07$^{***}$ | 74.3 ± 1.81$^{***}$ | 0.59 ± 0.03$^{***}$ | 36.3 ± 1.43$^{***}$ |

Each value represents the mean of 8 rats ± S.E. ns: non significant, Significant at $^{###}$P <0.001 vs control, Significant at *P <0.05; **P <0.01; ***P <0.001 vs APAP

***Effect on serum tumer necrosis factor-alpha (TNF-α):*** Other factors contributing to the APAP hepatotoxicity include proinflammatory cytokines mediators, such as tumor necrosis factor-α (TNF-α) (Mohit et al., 2011).

These mediators are generated in large quantities in the hepatic response to injury by hepatic nonparenchymal cells including macrophages, Kupffer cells and hepatic stellate cells. In APAP-intoxication, the release of cell contents from necrotic cells leads to an innate immune response with excessive production of proinflammatory mediators such as TNF-α (Jaeschkea and Bajt, 2010). The antioxidants are capable of reducing hepatic inflammation thus slowing or even preventing progression to fibrosis (Rasool et al., 2010). In accordance to previous studies, our results demonstrated that APAP increase serum TNF-α. Furthermore, the pretreatment with the tested extracts were significantly reduced the elevated TNF- level in APAP- treated rats. This improvement is a definite indication of their direct antioxidant potential and was in the order of silymarin $\geq$ *E. jambolana* > *C. sempervirens* > *C. scolymus*.

**Effect on the hepatic lysosomal enzyme activities in normal rats:**

Lysosomes, intracellular organelles, contain about 50 different degradative enzymes. They have a central role in cellular homeostasis as sites for digestion of foreign materials and for degradation of intracellular components undergoing autolytic processing (Abdel-Hamid and Morsy, 2010). The loss of integrity of the lysosomal membrane and subsequent discharge of enzymes into the blood stream is a characteristic feature of hepatic diseases (Premalatha and Sachdanandam, 2000). Estimation of these enzymes level has been considered as a useful quantitative marker to describe the extent of the hepatocellular damage.

The current study proved that the intracellular release of lysosomal enzymes precedes cellular death by initiating the cellular injury process ultimately causing tissue necrosis (Premalatha and Sachdanandam, 2000). The results in Table (4) indicate that APAP caused significant increase in the extracellular hepatic lysosomal enzyme activities (ACP, *β*-NAG and *β*-GAL) in APAP-intoxicated rats as compared with control confirming its labilizing effect. However, pretreatment with silymarin, *E. jambolana, C. sempervirens and C. scolymus* antagonize such effect by reduce the release of these enzymes. This beneficial effect may be due to the potential stabilizing effect of their bioactive constituents. *E. jambolana* exhibited the most pronounced stabilizing effect.

Table 4. Protective effect of different pretreatments on the hepatic lysosomal enzyme activities

| ***Groups*** | ***ACP*** | ***B-NAG*** | ***B-GAL*** |
|---|---|---|---|
| | *(n moles phenol liberated /min/mg protein)* | | |
| **Control** | 156.0 ± 7.15 | 39.8 ± 1.28 | 44.7 ± 1.84 |
| **APAP** | 233.3 ± 7.35$^{###}$ | 57.7 ± 2.28$^{###}$ | 64.7 ± 2.40$^{###}$ |
| ***C. scolymus* + APAP** | 184.3 ± 3.60$^{***}$ | 52.2 ± 3.62$^{ns}$ | 60.5 ± 2.84 $^{ns}$ |
| ***C. sempervirens* + APAP** | 167.8 ± 5.15$^{***}$ | 49.4 ± 2.07 $^{ns}$ | 56.8 ± 2.53 $^{ns}$ |
| ***E. jambolana* + APAP** | 161.1 ± 7.07$^{***}$ | 47.2 ± 1.47$^{*}$ | 49.5 ± 2.07$^{***}$ |
| **Silymarin + APAP** | 158.8 ± 8.09$^{***}$ | 42.6 ± 1.53$^{***}$ | 46.1 ± 1.70$^{***}$ |

Each value represents the mean of 8 rats ± S.E. ns: non significant,
Significant at $^{###}P$ <0.001 vs control. Significant at $^{*}P$ <0.05; $^{***}P$ <0.001 vs APAP.

The present study proved that the oxidative stress is a major mechanism in the development of APAP-induced hepatotoxicity and provide strong evidences that *C. scolymus, C. sempervirens* and *E. jambolana* hydro-ethanolic extracts posses hepatoprotective effect upon injury induced by single high toxic dose of paracetamol that is most probably mediated through antioxidant potential of their bioactive constituents. This antioxidant property is attributed to the presence of phenols, flavanoids and other phytochemicals in each crude extract. Thus our study provides a scientific base for the medicinal uses of these plants and validates their folkloric use in oxidative stress linked-diseases. These plants may offer new alternatives to the limited therapeutic options that exist at present in the treatment of liver diseases or their symptoms, and they should be considered for future studies.

## 4. Conclusions

*E. jambolana* can be considering as a potential source of natural antioxidant with hepatoprotective activity. Further detailed investigations are needed in order to identify and isolate the hepatoprotective components in this extract and to justify its use in polyherbal formulations prescribed in the treatment of

liver disorders. Finally, the education of the public and medical profession is needed to increase awareness of the potential toxic effects of paracetamol overdose.

**Correspondence to:**
Zeinab Y. Ali (Ph.D)
National Organization for Drug Control and Research (NODCAR), Giza 35521, Egypt
Telephone: +2-02-35850005,
Fax: +2-02-35855582
e.mails: zeinabyousef65@ymail.com

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