Antimutagenic Potential of *Cynara scolymus, Cupressus sempervirens* and *Eugenia jambolana* Against Paracetamol-Induced liver cytotoxicity.

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Abstract: Drug-induced liver injury is a potential complication of virtually every prescribed hot medication. Paracetamol (APAP) is one of the most commonly used drugs worldwide for its analgesic and antipyretic effect. Although it is considered to be safe and effective in the therapeutic range, the overdose following accidental ingestion or suicidal attempt causes a toxic response leading to the centrilobular necrosis in liver. Consequently, the present study was designed to evaluate antihepatotoxic and antimutagenic activities of hydroethanolic extract of ***Cynara scolymus* L., *Cupressus sempervirens* L., and *Eugenia jambolana* Lam** in experimental rat model of paracetamol-induced liver toxicity in rats, comparing with silymarin as reference agent. The results revealed that the pre-treatment with either hydroethanolic extract (250 mg/kg/day, p.o) or silymarin (50 mg/kg/day, p.o.) for 4 weeks has good safety profile in normal rats and exhibited a marked hepatoprotection against single toxic dose of paracetamol (4 $g.kg^{-1}$b.wt, p.o.) as proved from marked decline in the DNA fragmentations and inhibition in the percentage of chromosomal aberrations in bone marrow cells. 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. Further investigations are needed to isolation and characterisation of the active principles responsible for hepatoprotective activity.
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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).** Recently, it reported that lipid peroxidation and oxidative stress has been implicated in several drugs induced-hepatotoxicity (**Mohit *et al.*, 2011**).

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. Although it is safe at therapeutic doses, in overdose, APAP, whether accidental or deliberate, produces severe hepatotoxicity. 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)**. 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 (mostly CYP2E1) to toxic highly reactive electrophilic metabolite, N-acetyl-p-benzoquinone imine (NAPQI), which is normally detoxified by glutathione (GSH) and eliminated in the urine. **(Chen *et al.*, 2009)**, Consequently APAP has an excellent safety profile with 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)**. Natural antimutagens and ant carcinogens are able to inhibit or to reduce spontaneous DNA alteration. They react directly with mutagens or on the process of their activation. Antioxidants as flavonoids have been reported to scavenge free radicals thus prevent their interaction with cellular DNA **(Rajneesh et al., 2008)**.

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 and ellagic acid derivatives, tannins and flavonol glycosides**.** However, there are currently few reports concerning their hepatoprotective and antimutagenic activities 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 studies were aimed to evaluate antioxidant activities of hydroethanolic extract of leaves of aforementioned plants and their correlation with *in-vivo* antihepatotoxic and antimutagenic potential in experimental rat model of paracetamol-induced liver toxicity.

2. Materials and Methods

2.1. Chemical

All chemicals used in this study 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 colourless. The combined filtrates were concentrated to dryness under reduced pressure at 40°C using a rotary evaporator and then the concentrated extract was lyophilized.

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. Oral Acute Toxicity:

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

2.4.2. Effect on normal rats

A total of 40albino 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 dose of 250 $mg.kg^{-1}$ b.wt, p.o, respectively. **Group 5** received a daily oral dose of silymarin (50 $mg.kg^{-1}$ b.wt, p.o), respectively.

2.4.3. Protective effect against paracetamol toxicity

A total of 40 rats were randomly divided into five groups of eight rats each. **Group 6** received a single high dose of paracetamol (4 $g.kg^{-1}$ b.wt, p.o) after 28 days, served as positive control. **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 250 mg/Kg, p.o, respectively. **Group10** pre-treated once daily with silymarin (50 $mg.kg^{-1}$ b.wt, p.o). After 28 days of pre-treatments, rats

in **Groups 7-10** were administered with a single high dose of paracetamol (4 $g.kg^{-1}$ b.w).

2.5. Tissue sampling:

All animals were fasted overnight; all rats were sacrificed on the 29th day by decapitation. The livers were quickly excised, rinsed in cold saline, blotted and weighed. A part was used for DNA fragmentation assay.

2.6. DNA fragmentation assay

Liver tissues of the five animals/ group (200mg) were mechanically dissociated in hypotonic lysis buffer and centrifuged at 13.800 xg for 15 minutes. The supernatant containing small DNA fragments was separated immediately. Half of the supernatant was used for gel electrophoresis. While, the other half, as well as the pellet containing large pieces of DNA, were used for the colorimetric determination by Diphenylamine (DPA) assay (**Perandones *et al.*,. 1993**). The developed blue color was colorimetrically quantified spectrophotometrically at 578nm. Percentage of DNA fragmentation in each sample was expressed by the formula: % DNA fragmentation = (O.D Supernatant / O.D Supernatant + O.D Pellet) × 100. (O.D. optical denisty)

2.7. Chromosomal aberration In bone-marrow cells.

Bone-marrow metaphases prepared according to **Yosida and Amano (1965)** was used with some modifications. Rats were injected intraperitonealy with colchicine at a final concentration of 3 mg/kg b.w. 2hr before sacrificing. Bone-marrow cells from both femurs were collected .Slides prepared and stained with 7% Giemsa stain in phosphate buffer (pH6.8). A group of five rats were used for each treatment and 100 well-spread metaphases were analyzed for chromosomal aberrations: gaps, breaks, fragments, deletions and polyploidy metaphases were recorded in bone-marrow cell .

2.8. Statistical Analysis

All results are presented as mean ± S.E. The statistical significance of the difference for cytotoxic results were analysed through *students t-test*. $P< 0.05$-0.01 was considered significant.

3. Results and Discussion:

3.1. 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, 250 $mg.kg^{-1}$ b.w. day^{-1} of each extract was the dose selected for evaluation of hepatoprotective activity *in-vivo*.

3.2. Evaluation of the Antimutagenic Activity

As compared with the normal control group, the mean percentage of hepatic DNA fragmentation induced by tested extracts (**Fig.1**) is insignificant increased in normal rats. On the contrary, these extracts were able to inhibit DNA fragmentation induced 24hr after administration of a single toxic dose of APAP in the order of silymarin $\leq$ *E.jambolana* < *C.sempervirens* < *C.scolymus* by 56.91%, 54.68%, 46.44% and 39.89, respectively. (**Table1).** This protection attributed to inhibition of APAP reactive metabolite and ROS formed during the processes of microsomal enzymes activation which are capable of breaking DNA strands (**Kaur and Agarwal, 2007; Marotta *et al.*, 2009**). Our results were agreement with other *In vivo* studies; APAP induced chromosomal aberrations **(Kocisova et al., 1988)**, micronuclei **(Thomas, 1995)** and sister chromatid changes (SCE's) in bone-marrow cells of murines (**Giri, 1992, Farghaly, 2003)**.

Furthermore, **Figure (2)** shows that different levels of damage and sensitivity can be confirmed by agarose gel electrophoresis, shows the degree of DNA migration that correlated to the extent of DNA damage occuring in each sample. This study suggests that all tested extracts inhibit microsomal activation or directly protect DNA strands from the electrophilic metabolite of APAP. They may inhibit several metabolic intermediates and reactive oxygen species formed during the processes of microsomal enzymes activation which are capable of breaking DNA strand **Lee et al., 2000)**.

Table (2) shows the number and percentage of the structural and numerical aberrations induced in rat bone marrow cells after oral administration of APAP 24h and pre-treatment with different plant extracts and silymarin for 4 weeks**.** The number of metaphases with structural aberrations is insignificantly increased in normal rats treated with *C.scolymus*, *C.sempervirens* and *E.jambolana* as compared to control group, by 6.8%, 5.6%, 4.6% respectively and the control is 4.8% confirming their non-mutagenicity. Meanwhile, the number of metaphases with structural aberrations is significantly ($P < 0.01$) increased in APAP-intoxicated rats by 13.2% as compared to control group. Pre-treatment of different plant extracts were given prior to APAP treatment, decreased rates of clastogenic changes were observed in the order of *C.scolymus*, *C.sempervirens*, *E.jambolana* and silymarin by 7.6%, 7.4%, 5.8% and 5.8%, respectively. The pre-treatment with hydroethanolic extracts were capable to inhibit the cytogenetic damage in the form of gaps, breaks, acentric fragments and chromatid deletions observed after 24h sampling time with paracetamol. **Figure (3)** demonstrates several types of chromosomal aberrations in rat bone marrow cells induced after treatment with APAP. Chromosomal instability at a numerical and structural level is a hall mark of malignant tumours and

is particularly common in cancers of epithelial origin **(Klausner, 2002)**. This imply as a good marker of their antimutagenic and antineoplastic activity of the tested extracts.

Table 1: Mean percentage of DNA fragmentation induced in rat liver cells 24h after orally treatment with paracetamol and pre-treatment with different plant extract for 4 week

Groups	DNA fragmentation	
	Mean%± S.E	Inhibition%
Normal control	2.98 ± 2.20	-
C.scolymus	4.31 ± 1.36^{ns}	-
C.sempervirens	3.07 ± 1.03^{ns}	-
E.jambolana	3.00 ± 1.36^{ns}	-
Silymarin	2.54 ± 1.32^{ns}	-
APAP	12.51 ± 1.13$^{###}$	-
C.scolymus + APAP	7.52±1.29**	39.89
C.sempervirens + APAP	6.70 ±1.20***	46.44
E.jambolana + APAP	5.67 ± 0.35***	54.68
Silymarin + APAP	5.39 ± 0.46***	56.91

Each value represents the mean of 5 rats ± S.E.
ns: non significant, significant at $^{###}P$ <0.001 vs control, Significant at $^{**}P$ <0.01; $^{***}P$ <0.001 vs APAP.

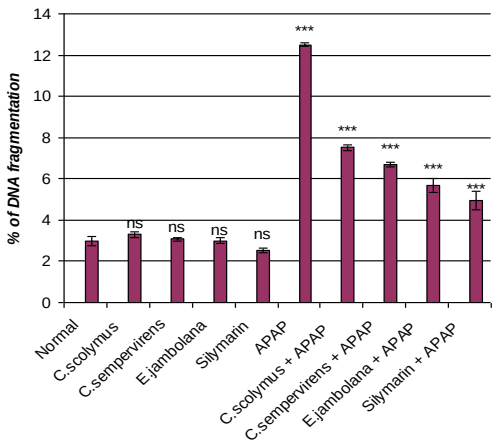

Figure 1. DNA fragmentation of rat liver cells by Diphenylamine (DPA) assay

The genotoxic effect of APAP was earlier evaluated by **Brunborg et al., (1995)** who showed that it was blocked DNA replication by inhibiting deoxyribonucleotide (dNTP) synthesis and may also by interfering with DNA repair. **Farghaly,.(2003)** confirm the mutagenic effect of paracetamol in somatic and germ cells of mice**.** High level of APAP, reactive metabolites (NAPQI) and ROS exhibit a cascade of oxidative damage resulting in oxidative stress which in turn induces deleterious actions including chromosomal aberration and DNA fragmentation which

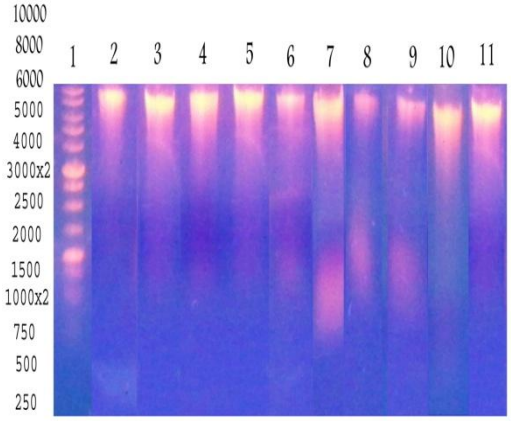

Figure 2. DNA fragmentation of rat liver cells by agaros gel electrophoresis.
Lane 1: 1kb DNA ladder.
Lane 2: DNA of normal control.
Lane 3-6: DNA of *C.scolymus, C.sempervirens, E.jambolana* and silymarin, treated rats, respectively.
Lane 7: DNA of APAP-intoxicated rats.
Lane 8-11: DNA of *C.scolymus* +APAP, *C.sempervirens* + APAP, *E.jambolana* + APAP and silymarin + APAP treated rats, respectively.

propagates hepatocellular injury and centrilobular liver necrosis **(Jaeschkea and Bajt, 2010)**. This study suggested that anti-genotoxic activity of the tested extracts may be attributed to the presence of important quantities of flavonoids **(Park et al., 2004)**. By using Comet assay quercetin and kaempferol reduced the DNA damage induced in sperm and lymphocytes by four oestrogenic compounds and by H_2O_2 **(Cemeli et al., 2009)**. **Patel et al., (2010)** demonstrated that, the phytochemical extract of silymarin significantly reduced doxorubicin (anticancer drug) hepatotoxicity and associated apoptotic and necrotic cell death. **Miccadei et al., (2008)** reported that artichoke (*C. scolymus)* extract reduced cell viability and had an apoptotic activity on a human liver cancer cell line (Hep G2 cells) after 24h of treatment in a dose-dependent manner. *Eugenia jambolana* leaves are found to reduce radiation-induced DNA damage in cultured human peripheral blood lymphocytes **(Migliato, 2005).** In this context, our study reveals that *E.jambolana* is one of the best performing extracts in terms of both antigenotoxic and antimutagenic ability to neutralize free radicals and prevent cellular DNA damage. Also *Cupressus sempervirens* extract was tested in *vitro* for effects on cytokines in human monocytes and for potential cytotoxic/pro-apoptotic

action as well as for their influence on the cell cycle of cancer cell line **(Bremner et al., 2009)**.

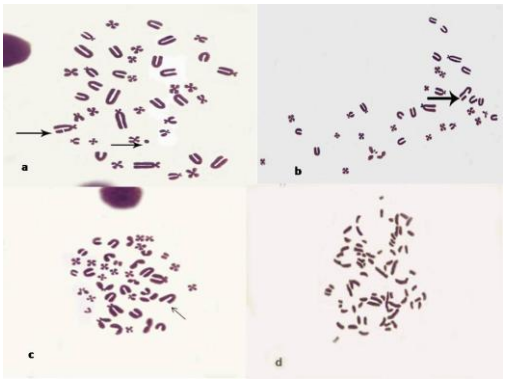

Figure 3. Metaphases with chromosomal aberrations (a) chromatid gap and fragment, (b) break, (c) deletion and (d) tetraploidy in rat bone-marrow cells after oral treatment with 4 g

The present study proved that the oxidative stress is a major mechanism in the development of paracetamol-induced hepatotoxicity and provide strong evidences that *C. scolymus, C. sempervirens* and *E. jambolana* hydroethanolic extracts posses antihepatotoxic and antimutagenic effects upon hepatic 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.

In conclusion, *C.scolymus*, *C.sempervirens E. jambolana* can be considering as a potential source of natural antioxidant with hepatoprotective activity. Further detailed investigations on these plants are needed in order to identify and isolate the hepatoprotective components in the 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.

Table 2: Number and percentage of chromosomal aberration in rat bone marrow cells after oral administration of APAP at 4 gm/kg b.wt. for 24 hr and pretreatment with different plant extracts and silymarin for 4 weeks.

Groups	No. of abnorml metaph.	Metaphases with Aberration		No. and (%) of metaphases with				
		Including gap Mean% ± S.E	Excluding gap Mean% ± S.E	Chromatid Gap	Break and /or fragment	Deletion	More than one aberration	polyploidy
Control	24	4.8 ± 0.20	2.4 ± 0.24	12 (2.4)	6 (1.2)	4 (0.8)	-	2 (0.4)
C.scolymus	34	6.8 ± 0.37	4.4 ± 0.24	12 (2.4)	16 (3.2)	6 (1.2)	-	-
C.sempervirens	28	5.6 ± 0.74	3.6 ± 0.74	10 (2.0)	12 (2.4)	4 (0.8)	-	2 (0.4)
E.jambolana	23	4.6 ± 0.24	3. 0 ± 0.45	8 (1.6)	6 (1.2)	3 (0.9)	-	6 (1.2)
Silymarin	23	4.6 ± 0.24	2.8 ± 0.20	9 (1.8)	7 (1.4)	5 (1.0)	-	2 (0.4)
APAP	66	13.2 ± 0.58**	10.0 ± 0.90**	16 (3.2)	29 (5.8)	5 (1.0)	8 (1.6)	8 (1.6)
C.scolymus + APAP	38	7.6 ± 0.67*	3.8 ± 0.58	18 (3.6)	12 (2.4)	5 (1.0)	2 (0.4)	1 (0.2)
C.sempervirens + APAP	37	7.4 ± 0.93*	4.8 ± 0.86	13 (2.6)	10 (2.0)	8 (1.6)	5 (1.0)	1 (0.2)
E.jambolana + APAP	29	5.8 ± 0.37	3.6 ± 0.40	11 (2.2)	10 (2.0)	3 (0.6)	3 (0.6)	2 (0.4)
Silymarin + APAP	25	5.0 ±0.58	2.8 ± 0.40	11 (2.2)	9 (1.8)	3 (0.9)	-	2 (0.4)

*significant at p > 0.05.
** significant at p > 0.01.

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