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Cocos nucifera L. inflorescence extract: An effective hepatoprotective agent

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The flowering inflorescence of *Cocos nucifera*, a main constituent of several traditional drug formulations was investigated with a view to study the effect of the acetone extract of *C. nucifera* inflorescence (CnAE) on acetaminophen-induced hepatotoxicity. The total phenol and flavonoid contents of the extract are found to be 222.6 µg gallic acid equivalent/g and 120.8 µg quercetin equivalent/g, respectively. The LD_{50} value was >5000 mg/kg b.w. The antioxidant activity was assessed using three methods, namely, 2,2'- diphenyl-1-picryl hydrazyl assay, 2,2'-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) assay and ferric reducing antioxidant power assay and the IC_{50} values were found to be 65.72, 66.94 and 89.84 µg/mL, respectively. Effect of CnAE (100, 200 and 400 mg/kg b.w.) and silymarin (100 mg/kg b.w.) against acetaminophen-induced liver toxicity was evaluated in Wistar rats. The study showed that CnAE pre-treated groups remarkably prevented the increase in serum alanine amino transferase, aspartate amino transferase and alkaline phosphatase level and decrease in the level of liver superoxide dismutase, reduced glutathione, glutathione-*S*-transferase and glutathione peroxidise. The extract also suppressed the elevated level of malondialdehyde. The biochemical determinations supported the histopathological examination and blood parameter findings. The findings of our study indicated that the phenolic-rich CnAE could be an interesting alternative candidate against acetaminophen-induced hepato-toxicity and associated oxidative stress.

Keywords: *Cocos nucifera* inflorescence, Hepatoprotective effect, Phenolic content

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Cocos nucifera L. (fam. Arecaceae, Coconut tree), a monocotyledonous plant has been known as Kalpavriksha/Kalpataru/Kalpadruma in Sanskrit meaning wish-fulfilling divine tree in Indian mythology. *C. nucifera* is considered as the nature's greatest gift to man since every part of the tree is useful in one-way or other to mankind. The plant is native to Southeast Asia[1]. In Ayurveda, the inflorescence is used for the treatment of menorrhagia and back pain[2,3]. The fresh kernel is an ingredient of many Indian food preparations like puddings, sweets, curries, chutneys etc. *C. nucifera* has been the subject of investigation in several laboratories and a number of papers are available in the literature[4-10].

Liver diseases are one of the fatal diseases and over 10% of the world population are afflicted by liver diseases[11,12]. Available drugs are often limited in efficacy with serious adverse effects eventually causing hepatic damage and are generally expensive. About 50% of the cases of acute liver failure are caused by drug toxicity and is responsible for all forms of acute and chronic liver diseases[13]. Literature search shows that many of the hepatoprotective drugs in the market and under clinical investigations are of natural origin[14]. Therefore, we thought it desirable to study the effect of *C. nucifera* inflorescence against acetaminophen-induced liver toxicity.

Materials and methods

Drugs and chemicals

Chemicals, reagents and drug standards were obtained from Sigma-Aldrich, USA. The analytical kits for serum and blood parameters were purchased from Agappe Diagnostics Ltd., India.

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Collection of plant material and preparation of extract

The *Cocos nucifera* (Cn) inflorescence was obtained from southern Western Ghats region of Thiruvananthapuram district, Kerala, India. The fresh inflorescence (950 g) was extracted with 4 x 3000 mL acetone for 48 h and the extracts were filtered and pooled. The pooled extracts were concentrated by evaporation. The extract thus obtained (CnAE) was kept at 4°C until used.

Experimental animals

Wistar rats (male, 180-230 g) and Swiss albino mice (either sex, 25-30 g) were used in this study. Experiments were done according to OECD guidelines, after getting the approval of the Institute's Animal Ethics Committee (IAEC), Amala Cancer Research Centre, India (Reg. No.149/199/CPCSEA).

Qualitative analysis of phytoconstituents

Standard protocols were used for the preliminary phytochemical analysis of CnAE[15,16].

Quantitative analysis of phytoconstituents

Estimation of total phenolic content

Folin-Ciocalteu method was used to find out the total phenolic content (TPC) of CnAE[17]. Each analysis was carried out in triplicate, values are expressed in mean ± SD and results are given in µg GAE (gallic acid equivalents)/g dry extract.

Estimation of total flavonoid content

Aluminum chloride colorimetric method was used to determine the total flavonoid content (TFC) of CnAE[18]. Each analysis was done in triplicate, values are expressed in mean ± SD and results are given in µg QE (quercetin equivalents)/g dry extract.

Acute toxicity study

The experiment was done in accordance with OECD guideline 423 (OECD, 2001). Mice were divided into 7 groups of 6 animals each. Prior to dosing with CnAE, mice were fasted overnight, but were allowed free access to water. Group I, normal (negative control) received distilled water and Groups II-VII were treated with 500, 1000, 2000, 3000, 4000 and 5000 mg/kg b.w. of CnAE (*po*). All the animals were observed continuously for first 3 h, then 1 h intermittently up to 24 h for behavioural changes. Post-treatment observation for toxic symptoms was continued, twice daily for 14 days.

Antioxidant activity assay

DPPH radical scavenging assay

The DPPH radical scavenging activity of CnAE was determined according to the method described by Aquino *et al.*[19]. Ascorbic acid was used as standard. The percentage of free radical scavenging activity of the sample was calculated according to the formula:

$$\% \text{ of DPPH radical scavenging} = [(A_0 - A_1) / A_0] \times 100$$

Where, A_0 = absorbance of the control and A_1 = absorbance of sample

ABTS radical scavenging assay

The ABTS radical scavenging activity of CnAE was performed by the previously described method[20].

FRAP assay

The FRAP assay was performed according to the method described by Benzie and Strain[21].

Acetaminophen-induced hepatotoxicity study

The hepatoprotective activity of CnAE was investigated according to the method described by Ajith *et al.*[22] with some modifications (Fig. 1). All the animals were euthanized after four hours of APAP treatment, blood and liver samples were collected for biochemical, haematological and histological studies.

Evaluation of serum enzyme status

The serum levels of aspartate aminotransferase (AST, EC 2.6.1.1) and alanine aminotransferase (ALT, EC 2.6.1.2) were measured by the method described by Reitman and Frankel[23], Alkaline phosphatase (ALP, EC 3.1.3.1) was measured by Kind and King method[24] using assay kits.

Evaluation of liver tissue antioxidant status

Liver samples collected were washed with ice-cold saline (0.89 %) and 10 % homogenate was prepared in PBS (0.05 M, pH 7) using a polytron homogenizer at 4 °C. The homogenate was centrifuged at 3000 rpm for 20 min to remove the cell debris. The supernatant obtained was used for the estimation of liver tissue antioxidant parameters such as superoxide dismutase (SOD, EC 1.15.1.1)[25], reduced glutathione (GSH)[26], glutathione peroxidase (GPx, EC 1.11.1.9)[27], glutathione S-transferase (GST, EC 2.5.1.18)[28] and malondialdehyde (MDA)[29].

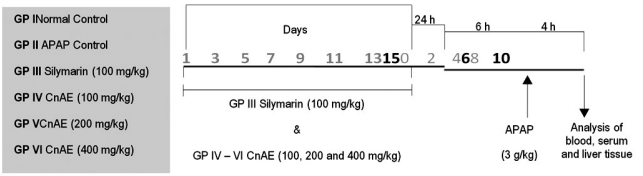


Fig. 1 — Experimental design and the interval for estimation of various parameters

Haematological study

Blood samples collected in EDTA coated vials were used to perform haematology analysis using an auto-haematology analyser (Mindray BC-2800Vet, China). The parameters tested are WBC, RBC and platelet counts.

Histopathological study

Liver samples were fixed in 10% formalin and embedded in paraffin. Sections (5 µm) were prepared from each liver sample using rotary microtome and are stained with hematoxilin-eosin (H&E). All the slides stained were examined for pathological findings of liver toxicity using a microscope (Labomed LX 400, USA).

Liquid chromatography-high resolution mass spectrometry analysis

Liquid chromatography-high resolution mass spectrometry (LC-MS, 1290 Infinity UHPLC System, 1260 infinity Nano HPLC with Chipcube, 6550 iFunnel Q-TOFs) analysis was carried out for the identification of chemical constituents in CnAE[30]. The mass spectra obtained were processed using Agilent MassHunter Q-TOF B.05.01 (B5125.1) software.

Statistical analysis

All data were represented as mean ± SD. Linear regression analysis was carried out for standards to calculate total phenolic and flavonoid contents, and the IC_{50} value was analysed using non-linear regression using Microsoft Excel 2007 (Microsoft Corporation, USA). In animal studies, significant difference between the mean values were statistically analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's t test using In Stat 3 software (GraphPad Software, Inc., USA). $p<0.05$ was considered to be statistically significant.

Results

Evaluation of phytoconstituents

Preliminary phytochemical screening of CnAE showed the presence of phenolics, coumarins, steroids, terpenoids, saponins and carbohydrates. The total phenolic and flavonoid content were found to be 222.61 µg GAE/g dry extract and 120.83 µg QE/g dry extract, respectively.

Acute toxicity study

No mortality was recorded after 14 days of treatment (Table 1). Therefore the LD_{50} value is >5000 mg/kg b.w. All the animals in the treated group did not show any significant decrease in body weight, food and water consumption for all the 14 days, when compared with the zero day values. Also there is no behavioural change after the drug administration. The cage side observations of animals are presented in Table 2.

DPPH radical scavenging assay

It was observed that CnAE dose-dependently increased the DPPH radical scavenging activity (Fig. 2). Higher concentration of CnAE (100 µg/mL) showed 72.50% of DPPH radical scavenging activity. The IC_{50} value of CnAE was found to be 65.72 µg/mL and of reference standard ascorbic acid was 7.63 µg/mL.

ABTS radical scavenging assay

The result showed that CnAE demonstrated a dose-dependent ABTS radical scavenging activity (Fig. 3).

Table 1 — Mortality latency and toxic symptoms of animals administered with different concentrations of CnAE in acute toxicity study

Treatment	Dose (mg/kg b.w.)	Sex	D/T	Mortality latency (h)	Toxic symptoms
Control	-	M	0/3	-	None
		F	0/3	-	None
CnAE	500	M	0/3	-	None
		F	0/3	-	None
CnAE	1000	M	0/3	-	None
		F	0/3	-	None
CnAE	2000	M	0/3	-	None
		F	0/3	-	None
CnAE	3000	M	0/3	-	None
		F	0/3	-	None
CnAE	4000	M	0/3	-	None
		F	0/3	-	None
CnAE	5000	M	0/3	-	None
		F	0/3	-	None

D/T = Dead/Treated mice

Table 2 — Cage side observations of animals administered with different concentrations of CnAE in acute toxicity study

Parameters observed	Cage side observations (500 – 5000 mg/kg b.w)
Condition of the fur	Normal
Skin	Normal
Subcutaneous swellings	Nil
Abdominal distension	Nil
Eyes – dullness	Nil
Eyes – opacities	Nil
Pupil diameter	Normal
Ptosis	Nil
Colour and consistency of the faeces	Normal
Wetness or soiling of the perineum	Nil
Condition of teeth	Normal
Breathing abnormalities	Nil
Gait	Normal

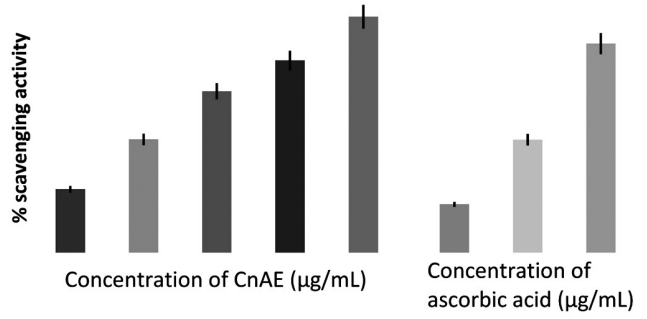


Fig. 2 — DPPH radical scavenging activity of CnAE at different concentrations

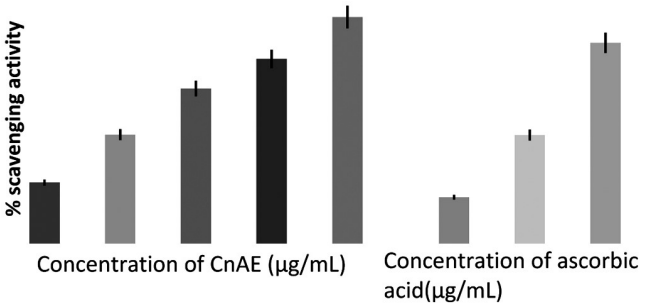


Fig. 3 — ABTS radical scavenging activity of CnAE at different concentrations

At the higher concentration (100 µg/mL), CnAE scavenged 69.96% of ABTS radicals. The IC_{50} value of CnAE was found to be 66.94 µg/mL and of reference standard ascorbic acid was 9.32 µg/mL.

FRAP assay

CnAE showed ferric ion reducing activity in a dose-dependent manner (Fig. 4). At 100 µg/mL concentration, CnAE showed 53.75% of ferric reducing antioxidant power. The IC_{50} value of CnAE is found to be 89.84 µg/mL and of reference standard ascorbic acid was found to be 11.06 µg/mL.

Acetaminophen-induced hepatotoxicity

Oral administration of acetaminophen (3 g/kg bw) caused significant hepatic damage as evidenced by the altered serum biochemical parameters, liver antioxidant status, haematological parameters and histopathology.

Serum enzyme activity

Acetaminophen administration significantly augmented the serum AST, ALT and ALP levels (p<0.001), when compared with the normal control group (Table 3). However, pre-treatment with CnAE at doses of 100, 200 and 400 mg/kg b.w. decreased the serum AST, ALT and ALP levels to varying extents in the APAP-treated rats. CnAE (400 mg/kg b.w.) significantly suppressed the elevated levels of AST, ALT and ALP in serum (p<0.05).

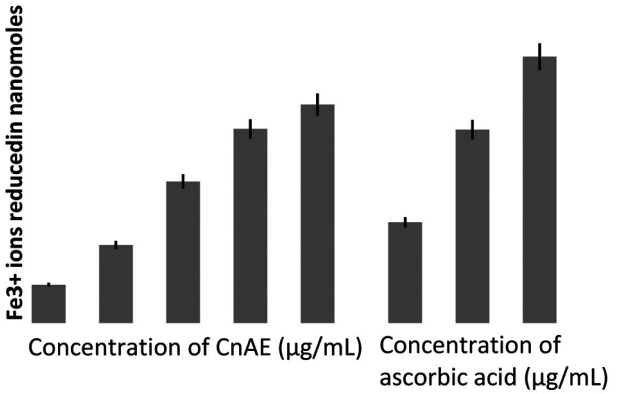


Fig. 4 — Ferric reducing antioxidant power of CnAE at different concentrations

Liver antioxidant status

The study demonstrated that APAP administration to the rats affected the liver antioxidant status, as evidenced by changes in SOD, GSH, GPx and GST activities and MDA level, when compared to the normal control group (Table 4). The APAP-induced decrement in SOD, GSH, GPx and GST activities was dose-dependently attenuated in CnAE pre-treated groups. The middle and high doses of CnAE (200 and 400 mg/kg b.w.) significantly augmented the SOD, GSH, GPx and GST activities (p<0.001). The GST activity of high dose of CnAE (400 mg/kg bw) treated group is comparable with the silymarin treated group.

APAP-administration drastically increased the liver MDA level (p<0.001) and pre-treatment with CnAE suppressed the level of MDA in a dose-dependent manner. At higher dose of 400 mg/kg bw the level of MDA significantly decreased (p<0.01). Treatment with drug standard, silymarin also manifested a significant decrease in MDA level as compared to toxin control.

Haematology

Acetaminophen-induced hepatic toxicity caused an increase in RBC and WBC counts and a decrease in platelet count (Table 5). Oral administration of different concentrations (100, 200 and 400 mg/kg b.w.) of CnAE every day for fifteen days prior to the APAP-treatment resulted in attenuated levels of all the haematological parameters tested, in a dose dependent manner. CnAE at a dose of 400 mg/kg bw significantly (p<0.001) augmented the platelet count and declined the RBC and WBC counts

Table 3 — Effect of CnAE on liver function enzyme activities of experimental rats

Experimental groups	AST(IU/L)	ALT(IU/L)	ALP(IU/L)
Group I (Normal control)	124.21±3.83	6.29±0.60	135.63±3.34
Group II (APAP control)	203.64±9.19***	22.09±2.43***	218.55±11.58***
Group III (Silymarin 100 mg/kg)	138.06±7.84*	8.85±0.66^{ns}	126.26±3.44^{ns}
Group IV (CnAE 100 mg/kg)	180.47±2.79***	15.63±1.27***	180.47±2.95**
Group V (CnAE 200 mg/kg)	166.82±1.86***	14.36±0.99***	176.13±5.33**
Group VI (CnAE 400 mg/kg)	149.08±4.01***	10.19±1.24*	163.46±4.38*

Values are mean ± SD, n=6; (∗∗∗) p<0.001, (∗∗) p<0.01 and (∗) p<0.05 compared with normal control.

Table 4 — Effect of CnAE on liver antioxidant status of experimental rats

Experimental groups	GSH (U/mg protein)	SOD (U/mg protein)	GPx (U/mg protein)	GST (U/mg protein)	MDA (mmol/mg protein)
Group I (Normal control)	53.46±5.23	6.73± 0.54	85.71±2.72	94.17±3.765	4.35±0.59
Group II (APAP control)	10.51±0.76***	1.71±0.36***	41.75±2.99***	44.13±2.83***	13.40±0.84***
Group III (Silymarin 100 mg/kg)	44.04±4.47*	5.51±0.38**	78.13±2.56**	81.06±1.62***	5.63±0.38^{ns}
Group IV (CnAE 100 mg/kg)	23.84±3.66***	4.26±0.29***	49.60±1.37***	49.56±3.09***	10.15±0.53***
Group V (CnAE 200 mg/kg)	30.19±1.47***	4.98±0.17***	56.04±2.50***	65.16±2.88***	8.75±0.62***
Group VI (CnAE 400 mg/kg)	35.22±1.25***	5.25±0.17***	74.39 ±1.92***	76.76±1.51***	6.35±0.50**

Values are mean ± SD, n=6; (∗∗∗) p<0.001, (∗∗) p<0.01 and (∗) p<0.05 compared with normal control.

Table 5 — Effect of CnAE on haematological parameters of experimental rats

Experimental groups	RBC ($\times10^6$/μL)	WBC ($\times10^3$/μL)	Platelet ($\times10^3$/μL)
Group I (Normal control)	8.23±0.85	13.94±1.18	1053.33±107.30
Group II (APAP control)	16.50±1.62***	32.90±2.56***	247.83±40.85***
Group III (Silymarin100 mg/kg)	10.33±0.68**	19.22±0.70***	634.67±50.24***
Group IV (CnAE 100 mg/kg)	14.99±0.84***	30.38±3.51***	288.67±37.43***
Group V (CnAE 200 mg/kg)	13.31±0.60***	26.59±1.33***	335.67±40.13***
Group VI (CnAE 400 mg/kg)	11.36±0.80***	20.63±1.08***	580.00±28.56***

Values are mean ± SD, n=6; (∗∗∗) p<0.001, (∗∗) p<0.01 and (∗) p<0.05 compared with normal control

(p<0.001). The restorative effect of high dose of CnAE (400 mg/kg bw) is comparable to the silymarin treated group.

Histopathology

Liver sections of normal control group showed normal cellular architecture and the toxin control showed fatty infiltration and necrosis together with massive neutrophilic and lymphocytic infiltration. However, in CnAE pre-treated groups these changes were reduced to moderate to low (Fig. 5).

LC-MS analysis

Liquid chromatography-mass spectrometry analysis of CnAE showed the presence of 92 compounds. Of these, some of the compounds tentatively identified are chlorogenic acid, apiin, emodin 8-glucoside, petunidin, dihydromyricetin and swietenine (Fig. 6 & Table 6).

Discussion

The present study revealed the anti-hepatotoxic effect of the acetone extract of *C. nucifera* flowering inflorescence. Quantitative analysis revealed that *C. nucifera* inflorescence is rich in polyphenols (222.6 μg GAE/g dry extract) and flavonoids (120.8 μg QE/g dry extract). In DPPH assay, the phenolic rich CnAE reduces the DPPH radical to the corresponding hydrazine by releasing hydrogen ions. The highest percentage of DPPH radical scavenging activity of CnAE was found to be 72.50% at 100 μg/mL. In ABTS assay, the blue green colour of ABTS radicals is reduced by the quenching effect of CnAE as similar to the DPPH assay[31]. CnAE at a dose of 100 μg/mL showed the highest percentage (69.96%) ABTS radical scavenging activity. The CnAE also showed significant ferric reducing power by donating hydrogen ions. The highest percentage of ferric reducing antioxidant capacity of CnAE was 53.75% at 100 μg/mL. Ascorbic acid was used as the reference standard.

The elevated level of intracellular enzymes like AST and ALT are the indication of oxidative membrane damage and instability in hepatocytes[32].

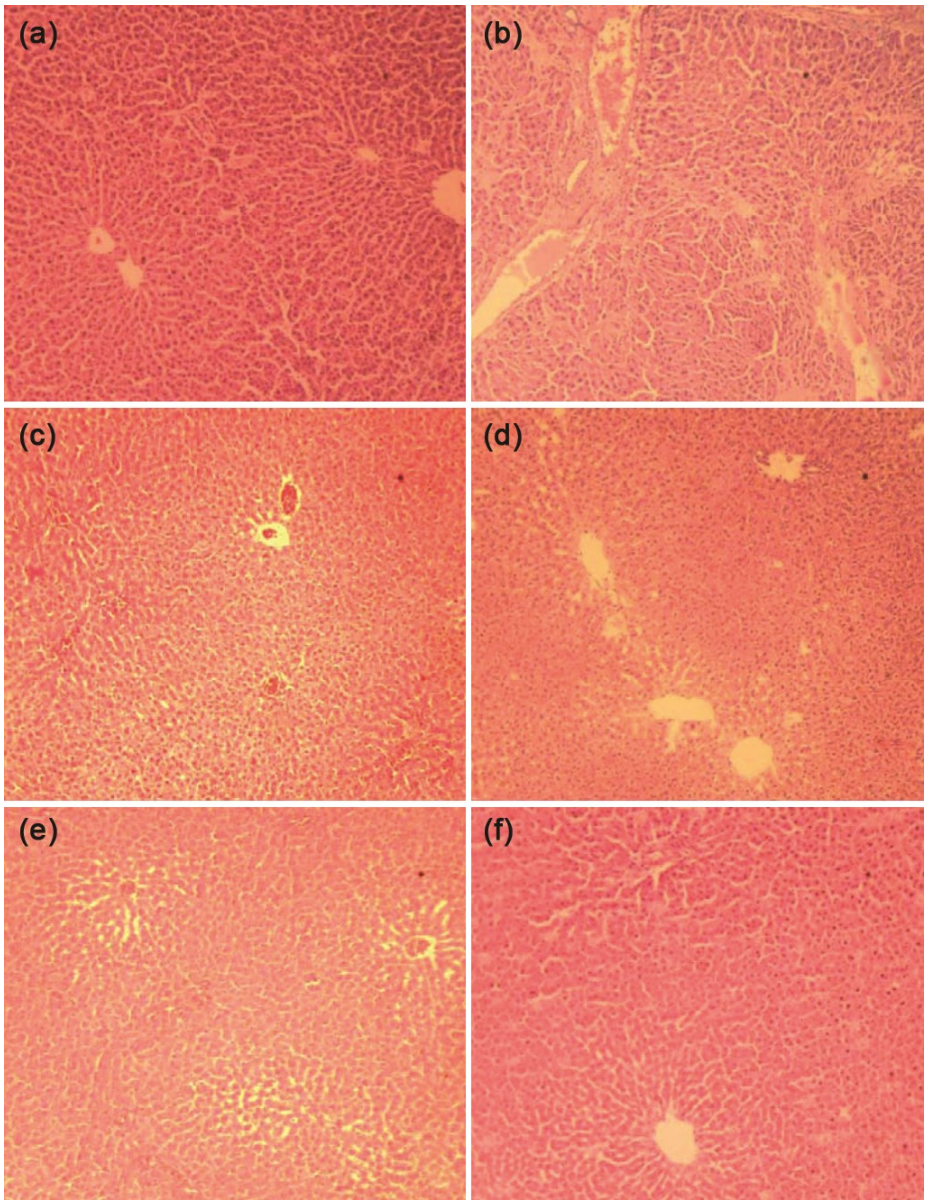


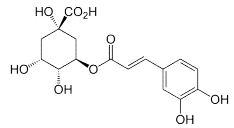
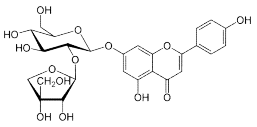
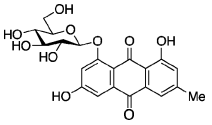
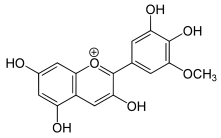
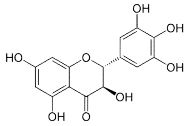
Fig. 5 — Photomicrographs of liver sections stained with H & E: (A) liver from a rat in normal control group pre-treated with distilled water; (B) liver from APAP-control group rat pre-treated with distilled water; (C) liver from a rat pre-treated with silymarin (100 mg/kg) followed by APAP treatment; (D) liver from a rat in the group pre-treated with CnAE (100 mg/kg) followed by APAP treatment; (E) liver from a rat in the group pre-treated with CnAE (200 mg/kg) followed by APAP treatment; (F) liver from a rat in the group pre-treated with CnAE (400 mg/kg) followed by APAP treatment.

In the present study, pre-treatment with CnAE demonstrated a significant decline in serum concentrations of AST, ALT and ALP. All the concentrations of CnAE reduced the serum enzyme levels as compared to the levels in APAP-treated group, demonstrating dose dependent membrane stabilizing activity of CnAE. CnAE at higher concentration (400 mg/kg bw) exhibited greater reduction in AST, ALT and ALP levels (p<0.001) and is comparable with silymarin.

Malondialdehyde is an indicator of tissue damage due to lipid peroxidation and is involving a series of chain reactions[33,34]. In our study, APAP alone administered group showed a drastic increase in MDA levels as compared to the normal group (p<0.001). However, pre-treatment with CnAE markedly prevented the increase in MDA formation.

Reactive oxygen species induced oxidative stress combined with weakened cellular antioxidant system underlies the disruption of cellular homeostasis[35].

Table 6 — Compounds tentatively identified from HR LC-MS analysis of CnAE

Sl No.	Compound name	Retention time	Molar mass	Molecular formula	Molecular structure
1	Chlorogenic acid	5.392	354.0916	$C_{16}H_{18}O_9$	
2	Apiin	5.959	564.1426	$C_{26}H_{28}O_{14}$	
3	Emodin 8-glucoside	6.509	432.1017	$C_{21}H_{20}O_{10}$	
4	Petunidin	6.537	317.0631	$C_{16}H_{13}O_7$	
5	Dihydromyricetin	8.487	320.0491	$C_{15}H_{12}O_8$	

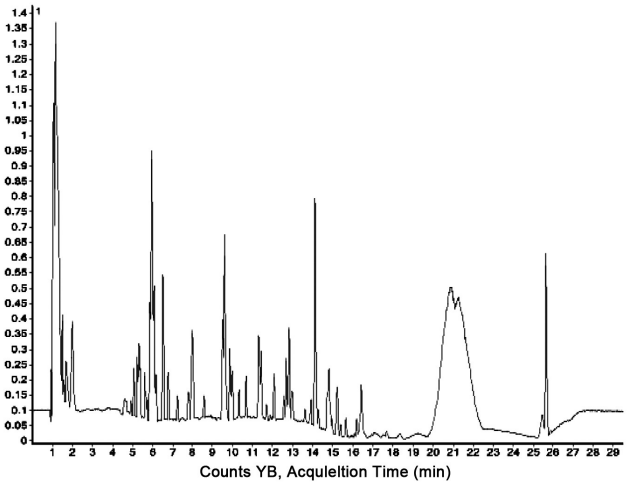


Fig. 6 — HR LC-MS chromatogram of CnAE

Several studies have shown that depletion of this intracellular antioxidant defence systems are the major mechanism in the development of APAP-induced oxidative stress[36,37]. Our results showed that APAP-treatment significantly decreased the liver SOD, GSH, GPx and GST levels (p<0.001), which is in agreement with earlier reports[38,39]. However, it was noticed that the rats pre-treated with CnAE (200 and 400 mg/kg b.w.) significantly raised the antioxidant status towards normal against the APAP-induced oxidative stress. These results suggested that CnAE might have a liver protective effect by decreasing APAP-induced oxidative stress.

It is found that the histopathological results are in good concurrence with biochemical estimations. The normal control group showed cells with a well preserved cytoplasm, normal lobular architecture and well defined nucleus. The higher concentration of CnAE (400 mg/kg bw) showed more prominent signs of recovery than the other concentrations tested. Earlier reports on similar studies showed a marked and widespread inflammation and infiltration in the APAP-control group[40,41]. The silymarin treated group also significantly reversed APAP-induced liver injury. In line with diagnostic significance, haematological parameters namely RBC, WBC and platelet counts were also monitored. It was found that APAP-treatment significantly increased the RBC and WBC counts and reduced the platelet count. Pre-treatment with CnAE significantly ameliorated the APAP-induced effect.

Several studies have shown antioxidant and hepatoprotective activities of different classes of polyphenols[38,42]. Polyphenols interact with the cells mainly through receptors or enzymes involved in signal transduction[43-45]. Interestingly, compounds tentatively identified by LC-MS analysis are phenolic/flavonoid class of compounds. A thorough literature search revealed that all these identified compounds

(chlorogenic acid, apiin, emodin 8-glucoside, petunidin and dihydromyricetin) showed significant antioxidant and hepatoprotective activities[46-52]. This might be a reason for the antioxidant and hepatoprotective effect of CnAE. Collectively, our data strongly exposed the clinical potential of the polyphenol-enriched fraction from *C. nucifera* inflorescence as a nutraceutical/ functional food ingredient for the prevention of toxin-induced hepatic injury.

Conclusion

In conclusion, our findings clearly demonstrated the hepatoprotective effect of phenolic-rich acetone extract of *Cocos nucifera* inflorescence. The detailed chemical and pharmacological investigations can be helpful in finding the desired bioactive molecules and their mechanism of action.

Conflict of interest

The authors declare that we have no conflict of interest.

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