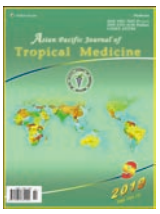

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# *Curcuma angustifolia* ameliorates carbon tetrachloride-induced hepatotoxicity in HepG2 cells and Swiss albino rats

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ABSTRACT

**Objective:** To determine the antioxidant and hepatoprotective potential of methanol extract of rhizome of *Curcuma angustifolia* (MECA) against carbon tetrachloride ($CCl_4$)-induced hepatic damage *in vitro* and *in vivo*.

**Methods:** DPPH, ABTS and reducing power assays were performed to estimate the antioxidant effect of MECA. *In vitro* cytotoxicity of MECA against HepG2 cells was evaluated, whereas serum biochemical parameters and levels of antioxidative enzymes were measured *in vivo* and *in vitro*. Additionally, histopathological studies were estimated in order to investigate the hepatoprotective efficacy of MECA. Furthermore, GC-MS analysis of the extract was performed to identify the chemical components.

**Results:** MECA exhibited strong antioxidant activity and attenuated $CCl_4$-induced decrease in the viability of HepG2 cells. Additionally, MECA significantly restored the ALT, AST, ALP, TP and albumin level in comparison with the $CCl_4$ group. After pre-treatment with MECA, effects of SOD, CAT and GSH were increased as well as lipid peroxidation amount decreased on $CCl_4$-induced hepatotoxicity in *in vitro* and *in vivo* model. Furthermore, histopathological observation confirmed that MECA reduced liver injury induced by $CCl_4$ in rats. GC-MS analysis confirmed the presence of bioactive constituents such as α -tocopherol (12.27%), phytol (7.61%), squalene (3.71%), β -sitosterol (2.19%), eugenol (2.59%), curcumenol (1.20%), β -elemene (1.00%) and eucalyptol (0.89%).

**Conclusions:** MECA contains antioxidant and hepatoprotective constituents such as α -tocopherol, phytol, squalene and eugenol and exerts hepatoprotective effect both *in vitro* and *in vivo*.

## 1. Introduction

Liver diseases remain a serious health problem worldwide because of the use of chemicals, alcohol, drugs and virus infiltration[1,2]. Chemicals like paracetamol, $CCl_4$, nitrosamines and polycyclic aromatic hydrocarbons could significantly induce hepatotoxicity[2]. These toxic chemicals induce the initiation of reactive oxygen species (ROS) which leads to severe hepatic damage[3,4]. Although ROS have many physiological functions in human metabolism, uncontrolled production of ROS leads to oxidative damage in cells

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resulting in impairment of protein functions and triggering cell death[5]. Traditional and alternative systems of medicine are being used from time immemorial in several countries including India, China and Japan to cure various diseases[6]. This has prompted the use of medicinal plants with therapeutic potential to treat various ailments. Medicinal plants rich in phenolic and flavonoid constituents are well known for their potential to scavenge free radicals and provide a significant value to the plants[7]. Synthetic drugs are utilized for curing several liver ailments but due to their side effects, scientists are paying attention towards the natural products for treatment of liver disorders as they are generally regarded as safe[8,9].

*Curcuma (C.) angustifolia* (Zingiberaceae), commonly known as East Indian Arrowroot, is widely grown throughout the Central, Southern and Eastern India[10]. Rhizome pulp, commonly known as Teekur kanda, is being used by the tribal people of Koraput and Phulbani districts of Odisha and Bastar district of Madhya Pradesh to cure hepatic disease like Jaundice[11]. The starch powder derived from the rhizome is highly nutritive and digestible and therefore it is suggested for weak children and infants[12]. Various parts of this species have been applied on the skin to relieve the pain of inflamed mucous membranes[13]. Rhizome pulp is also used for the treatment of headache and also gives cooling effects[14]. Starch of *C. angustifolia* used as Tugaksheeree is an essential ingredient in many Ayurvedic medicines[15]. Rhizomes part of *C. angustifolia* were mainly utilized by the tribal people of Madhya Pradesh and Chhattisgarh states of India for curing intestinal disorders, bone fracture, inflammation *etc*[16]. The plant *C. angustifolia* exhibits various pharmacological activities, including antioxidant antimicrobial, antifungal and antproliferative activities[10,17,18]. Experimental investigations of our previous study in essential oil of rhizomes of *C. angustifolia* identified the presence of bioactive compounds such as camphor, curzerenone, germacrone, 1,8-cineole, ar-curcumene, curzerene, curdione and xanthorrhizol[17].

In this study, an attempt has been made to evaluate the antioxidant as well as hepatoprotective activities of rhizome of *C. angustifolia* against $CCl_4$-induced hepatotoxicity in Human hepatoma cell lines (HepG2) and Swiss albino rats.

## 2. Material and methods

### 2.1. Plant sample and extract preparation

Rhizome samples were collected from R-Udaygiri, Gajapati district, Odisha (84º 12' E, 19º 9' N, 685 m above sea level) during December 2015. The plant was authenticated by Dr. P. C. Panda, Principal Scientist (Taxonomy and Conservation Division, RPRC, Bhubaneswar) and the voucher specimen (10191) was deposited in the Herbarium of Regional Plant Resource Centre, Bhubaneswar, Odisha, India. The cleaned and air-dried rhizome was made to coarse powder using mechanical grinder. For extract preparation, rhizome powder (1 kg) was extracted in methanol by soxhlet apparatus for 6 h and filtered. After filtration, concentration of extract was done using rotary evaporator to get dried extract and stored at 37 ℃ for further studies.

### 2.2. DPPH assay

The evaluation of free radical scavenging assay on DPPH radicals of the samples and reference standards (Ascorbic acid and BHT) were examined according to the reported protocol[17].

### 2.3. ABTS assay

The scavenging ability of test samples and reference standards (Ascorbic acid and BHT) were also assessed according to the reported protocol[19].

### 2.4. Reducing power ability

The reducing power capacity of the test samples and reference standards (Ascorbic acid and BHT) were assessed according to the reported protocol[17]. The absorbance was recorded at 700 nm using UV-Visible spectrophotometer (Thermo fisher evolution 220).

### 2.5. In vitro hepatoprotective activity

#### 2.5.1. Cell culture

HepG2 were acquired from NCCS, Pune, India and cultured in a humidified environment (5% carbon dioxide) at 37 ℃ along with Dulbecco's modified Eagle medium (DMEM) added with 10% fetal bovine serum (FBS) and antibiotics like penicillin (1U/mL) and streptomycin (100 μg/mL).

#### 2.5.2. In vitro cytotoxicity assay

The cytotoxicity of methanol extract of rhizome of *C. angustifolia* (MECA) was measured using MTT cell survival assay[20]. HepG2 cells ($1\times10^5$) were seeded in 96-well culture plate for 24 h in the presence of different concentrations (10, 20, 50, 100 and 200 μg/mL) of MECA. At the end of the incubation period, 20 μL of MTT reagent was added in each well. The plates were gently shaken and incubated for 4 h at 37 ℃. After 4 h, the supernatant was removed and 200 μL of DMSO was added and the plates were gently shaken. The absorbance (OD) was read at 570 nm by an ELISA plate reader (BMG Labtech, Ortenberg, Germany).

The percentage growth inhibition was calculated using the following formula:

Growth inhibition (%) = 100 - (Mean OD of treated group/Mean OD of control group)$\times$100

#### 2.5.3. $CCl_4$ induced toxicity in HepG2 cell line

To verify the *in vitro* hepatoprotective activity against $CCl_4$, HepG2 cells ($1\times10^5$ cells/well) were seeded into 96-well plates followed by incubation for 24 h. Normal control (Group Ⅰ) cells were incubated with DMEM in DMSO for the following 24 h. For $CCl_4$ (Group Ⅱ) treatment, cells were treated with DMEM with $CCl_4$ (1%, v/v) for the following 24 h. For silymarin (Group Ⅲ) treatment, HepG2 cells were stimulated with DMEM with silymarin at a concentration of 100 μg/mL and 1% (v/v) $CCl_4$ for the following 24 h. For MECA treatment (Group Ⅳ-Ⅴ), cells were incubated with DMEMA with MECA at 50 μg/mL, 100 μg/mL and 200 μg/mL in the plate

containing 1% (v/v) of $CCl_4$. Finally, the cytotoxicity assay was performed as described earlier. Further, AST, ALT, MDA and GSH level were measured following the manufacturer's instruction.

### 2.6. In vivo hepatoprotective activity

#### 2.6.1. Experimental animal model

Before starting the experiments, permission was taken from institutional animal ethical committee (IAEC) with approval letter number IAEC/SPS/SOA/23/2018 on dated 19th November 2018. The study was approved by University Animals Ethics Committee (Reg No: 1171/c/08/CPCSEA). Animal experimental procedures were performed according to CPCSEA guideline. Swiss albino rats (150-200 g) were acquired from the animal house of School of Pharmaceutical sciences, Siksha 'O' Anusandhan (Deemed to be University), Odisha, India for the experimental purposes. All the animals were kept in polycarbonate cages at 25 ℃-30 ℃ with a relative humidity of 45%-55% and they are exposed to 12 h day and 12 h night cycle. Before starting the experiment, acclimatization of the animals was processed in the laboratory conditions for 10 d and allowed to take standard pellet diets and water *ad libitum*.

#### 2.6.2. Acute toxicity study

The acute toxicity study of the test samples was performed according to the OECD guidelines 423. The test groups and control groups of the animals received from lower dose to maximum of 4 000 mg/kg dose of extract and distilled water along with Tween 80 orally, respectively. All the animals were observed critically up to 4 h followed by 72 h for acute toxicity sign. Animals were observed for a period up to 14 d for mortality caused by extracts[21].

#### 2.6.3. Experimental design

Animals were randomly assigned to five groups of 6 animals each. Group A (Control group) and Group B ($CCl_4$ intoxicated group) were given with vehicle (1% Tween-80 in normal saline) at a dose of 10 mL/kg b.w. orally once daily for 8 d. Group C (standard group) was given silymarin at a dose of 100 mg/kg b.w. orally once daily up to 8 d. Simultaneously, the protective effects of MECA at a concentration of 200 mg/kg b.w. and 400 mg/kg b.w. were tested in Group D and Group E, respectively, for 8 consecutive days. On the 8th d, rats in Group B-E were injected intraperitoneally with 1 mL/kg b.w. of $CCl_4$ in a ratio of 1:1 mixture in olive oil while Group A was given olive oil only[22].

#### 2.6.4. Determination of liver index

All the 12 h fasted animals were euthanized by cervical decapitation at the end of the experimental study period *i.e.* on the 9th day. The body weights of the animals were recorded before sacrificed whereas the liver weights of all the rats were recorded immediately after sacrifice.

Liver index (%) = liver weight/body weight × 100

#### 2.6.5. Measurement of serum biochemical parameters

On the 9th day, the blood was collected in sterile centrifuge tubes. Serum was separated and used to estimate the biochemical parameters like AST, ALP, ALT, TP and albumin, following the manufacturer's instructions of Ecoline diagnostic kits[23].

#### 2.6.6. Measurement of antioxidative enzyme activity in liver homogenates

Liver tissue of sacrificed rats were excised, gently washed in ice cold saline and homogenized in cold 10% PBS (50 mM, pH 7) using a Remi homogenizer followed by centrifugation. Then the supernatant was collected for the analysis of GSH, MDA, SOD and CAT following the manufacturer's instructions of Bio-Diagnostic commercial kits[24,25].

#### 2.6.7. Histopathological study

The liver tissue was dissected out and fixed in the 10% formalin, dehydrated in gradual ethanol (50%-100%), cleared in xylene and embedded in paraffin. Sections were cut using microtome into 5 μm thick and stained with hematoxylin and eosin dye for microscopic observation of histopathological changes in liver. Liver sections were evaluated according to the severity of the hepatic injury under the microscope (Nikon, Japan) and photographed[9].

### 2.7. GC-MS analysis

The chemical composition of MECA was carried out using Thermo Trace 1300 GC (Thermo-Fisher Scientific, Waltham, MA, USA) equipped with TSQ 8000 MS. Helium was used as a carrier gas. TG-5 MS was used as capillary column. The ion source and interface temperature was maintained at 230 ℃ and 280 ℃, respectively. The oven programming temperature was 60 ℃ for 2 min increased to 280 ℃ at 10 ℃/min and finally hold at 280 ℃ for 10 min.

### 2.8. Statistical analysis

All the experimental data were expressed as mean±SD and statistical analysis was performed using AVONA followed by Tukey's multiple range test using Graphpad Prism software (version 6.0; GraphPad, Sandiego, CA, USA). Difference between groups at $P<0.05$ was considered statistically significant.

## 3. Results

### 3.1. In vitro antioxidant effect

Antioxidant activity of MECA was measured by DPPH assay, ABTS assay and reducing power assay. $IC_{50}$ values of the samples are used as an index to compare the free radical scavenging capacity of the sample. The lowest $IC_{50}$ values indicate a greater free radical scavenging capacity. Based on the calculated $IC_{50}$ values, the DPPH

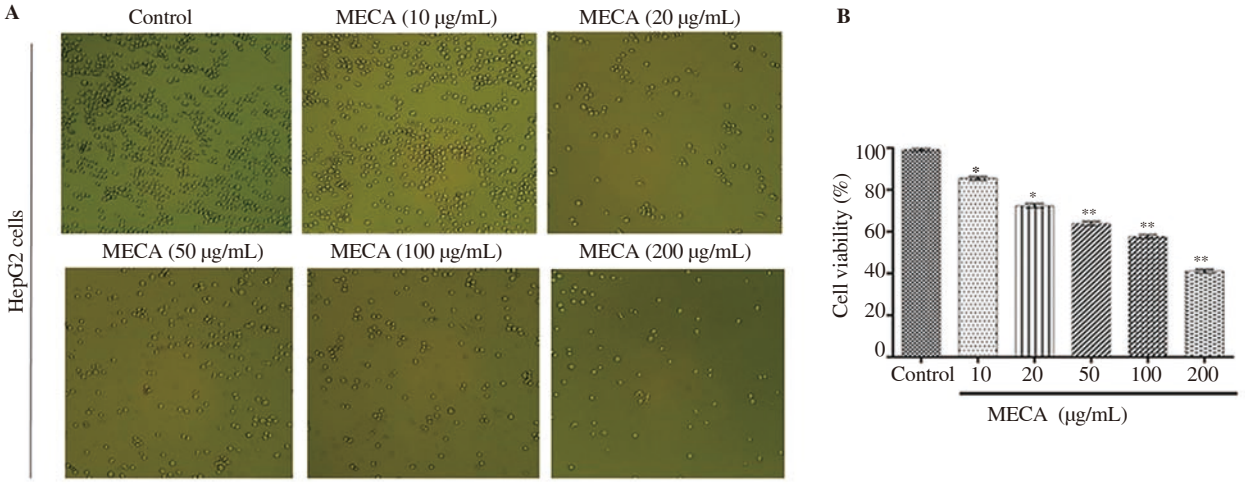


**Figure 1.** Effect of methanol extract of rhizome of *Curcuma angustifolia* (MECA)on proliferation of HepG2 cells as measured by MTT assay: (A) Cellular morphology of HepG2 cells after 24 h exposure to different concentrations of MECA (0-200 μg/mL) observed under phase contrast microscope; photograph was taken at 10×; (B) Percentage of cell viability of HepG2 cells after 24 h incubation with different concentrations of MECA (0-200 μg/mL). Data are represented as Mean±SD of three experiments $^{*}P<0.05$ and $^{**}P<0.01$ compared to control. MECA: methanol extract of rhizome of *Curcuma angustifolia*.

radical scavenging capacity of MECA, ascorbic acid and BHT were (206.31±0.75) μg/mL, (6.85±0.83) μg/mL and (19.68±0.54)μg/mL, respectively. Similarly, the ABTS radical scavenging effect followed the same trend with $IC_{50}$ values of the MECA, ascorbic acid and BHT being (92.43±0.34) μg/mL, (3.12±0.19) μg/mL and (16.17±0.81) μg/mL, respectively. The reducing ability of MECA and positive controls were in increasing order: ascorbic acid (5.02±0.51) μg/mL > BHT (7.48±0.79) μg/mL > MECA (114.56±0.26) μg/mL.

### 3.2. In vitro cytotoxicity assay

As shown in Figure 1, MTT assay demonstrated growth inhibition of HepG2 cells following treatment with MECA in a concentration dependent manner. No reduction in cell viability was observed in control (DMSO treated) group. Further, it was confirmed that MECA showed greater cytotoxicity towards HepG2 with very low $IC_{50}$ value of (149.31±1.24) μg/mL at 24 h (Figure 1B). The viability of HepG2 cells reduced from (99.95±1.27)% to (43.86±0.97)% with increasing concentration of MECA (0-200 μg/mL).

### 3.3. $CCl_4$ induced toxicity in HepG2 cell line

**Table 1.** Effect of methanol extract of rhizome of *Curcuma angustifolia* onthe viabilities of HepG2 cells treated with $CCl_4$.

| Groups | Cell viability (%) |
|---|---|
| Control | 99.95±1.27 |
| $CCl_4$ (1%, v/v) | $27.20±0.53^{**}$ |
| $CCl_4$+silymarin (100 μg/mL) | $59.32±1.72^{*\#\#}$ |
| $CCl_4$+MECA (50 μg/mL) | $38.65±0.95^{**\#}$ |
| $CCl_4$+MECA (100 μg/mL) | $47.13±0.84^{**\#\#}$ |
| $CCl_4$+MECA (200 μg/mL) | $61.43±1.51^{*\#\#}$ |

The values show the mean±SD ($n$=3). Statistically significant differences are represented by asterisks ($^{*}P<0.05$ and $^{**}P<0.01$ compared with the control group, $^{\#}P<0.05$ and $^{\#\#}P<0.01$ as compared with the $CCl_4$ treated group) as evaluated by ANOVA and Tukey's multiple range test. MECA: methanol extract of rhizome of *C. angustifolia*.

HepG2 cells were exposed to toxic chemical ($CCl_4$) and incubated with three different concentrations (50 μg/mL, 100 μg/mL and 200 μg/mL) of MECA and the effect on cell viability was assessed. As represented in Table 1, a significant decrease in cell viability was seen after treating with $CCl_4$ compared with control. The incubation of HepG2 cells exposed to $CCl_4$ and treated with MECA at 50 μg/mL, 100 μg/mL and 200 μg/mL increased the cell viability to (38.65±0.95)%, (47.13±0.84)% and (61.43±1.51)% compared to $CCl_4$, respectively. The standard drug silymarin (100 μg/mL) increased the cell viability to (59.32±1.72)% compared to that of $CCl_4$ treated group.

### 3.4. ALT, AST, MDA and GSH level in HepG2 cells treated with MECA

**Table 2.** Effects of methanol extract of rhizome of *Curcuma angustifolia* on biochemical parameters of the $CCl_4$-induced cytotoxicity in HepG2 cells.

| Groups | AST (U/L) | ALT (U/L) | MDA (nmol/mg) | GSH (nmol/mg) |
|---|---|---|---|---|
| Control | 49.64±1.11 | 11.54±0.58 | 3.76±0.10 | 21.16±0.02 |
| $CCl_4$ (1%, v/v) | $78.41±1.59^{**}$ | $22.54±0.59^{**}$ | $11.54±0.48^{**}$ | $8.57±0.07^{**}$ |
| $CCl_4$+silymarin (100 μg/mL) | $61.62±1.21^{*\#\#}$ | $16.63±0.72^{*\#}$ | $4.87±0.53^{\#\#}$ | $15.86±0.06^{*\#\#}$ |
| $CCl_4$+MECA (50 μg/mL) | $73.36±0.89^{**}$ | $19.43±0.61^{**\#}$ | $6.75±0.44^{*\#}$ | $11.76±0.03^{**\#}$ |
| $CCl_4$+MECA (100 μg/mL) | $65.19±1.00^{*\#}$ | $17.43±0.68^{*\#}$ | $5.54±0.03^{*\#\#}$ | $12.54±0.01^{*\#}$ |
| $CCl_4$+MECA (200 μg/mL) | $62.17±1.73^{*\#\#}$ | $16.54±0.86^{*\#}$ | $5.12±0.02^{*\#\#}$ | $13.87±0.03^{*\#\#}$ |

HepG2 cells were treated with silymarin (100 μg/mL) or MECA (50 μg/mL-200 μg/mL) and incubated in the presence of $CCl_4$ (1%, v/v). Control group was treated with vehicle and incubated with PBS instead of $CCl_4$. After incubation for 24 h, the supernatant was collected to biochemical analysis. The values show the mean±SD ($n$=3). Statistically significant differences are represented by asterisks ($^{*}P<0.05$ and $^{**}P<0.01$ as compared with the control group, $^{\#}P<0.05$ and $^{\#\#}P<0.01$ as compared with $CCl_4$ treated group) as evaluated by ANOVA and Tukey's multiple range test. MECA: methanol extract of rhizome of *C. angustifolia*.

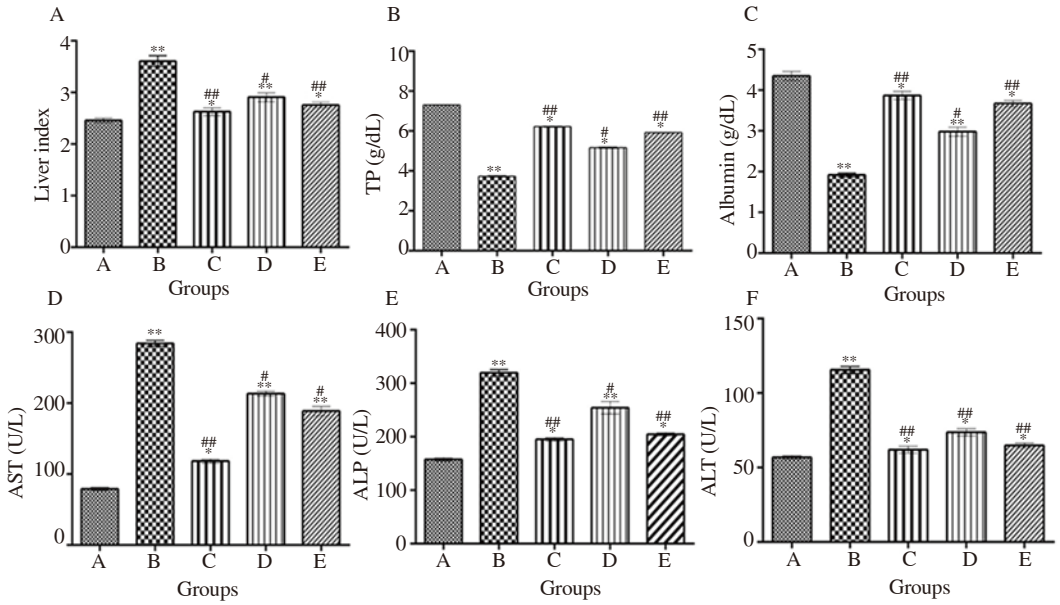


**Figure 2.** Effects of methanol extract of rhizome of *Curcuma angustifolia* (MECA) on (A) Liver index, (B) Total protein (TP), (C) Albumin, (D) AST, (E) ALP and (F) ALT serum levels after $CCl_4$-induced acute liver toxicity in rats. Group A: control; Group B: $CCl_4$; Group C: silymarin, 100 mg/kg; Group D and E: MECA at a concentrations of 200 mg/kg and 400 mg/kg, respectively. The values show the mean±SD ($n$=6). Statistically significant differences are represented by asterisks ($^{*}P$<0.05 and $^{**}P$<0.01 as compared with the control group, $^{\#}P$<0.05 and $^{\#\#}P$<0.01 as compared with the $CCl_4$ treated group).

The AST and ALT levels were assessed by the hepatoprotective activity of MECA. As shown in Table 2, exposure to $CCl_4$ increased the level of AST and ALT in the HepG2 cell culture medium by 57.96% and 95.32% compared to normal group respectively. Further pre-treatment of the cells at concentrations of 50 μg/mL, 100 μg/mL and 200 μg/mL of MECA significantly reduced the leakage of AST by 6.44%, 16.86% and 20.71% and ALT by 13.80%, 22.67% and 26.62%, respectively in comparison to $CCl_4$ treated group.

The intracellular level of MDA and GSH were also measured. In comparison to control group, $CCl_4$ treated cells resulted remarkable increase in MDA and decrease in GSH level (Table 2). Pre-treatment with 50 μg/mL, 100 μg/mL and 200 μg/mL of MECA remarkably decrease the intracellular content of MDA by 41.51%, 51.99% and 55.63% and increased the GSH level by 37.22%, 46.32% and 61.84%, respectively compared to $CCl_4$ treated group.

### 3.5. Acute toxicity study

Methanol extract of rhizome of *C. angustifolia* did not showed any mortality and acute signs of toxicity such as convulsions, change in skin, fur, lethargy, salivation, diarrhea, sleeping and other toxicity signs up to 72 h even when the extract concentration was increased to 4 000 mg/kg b.w. The observation of acute toxicity showed that the treated animals were safe and devoid of any toxicity effects and dose was determined according to the 1/20th and 1/10th of maximum treated dose in all the experiments.

### 3.6. Liver index and serum biochemical markers in Swiss albino rats

Effects of MECA on liver index of rats were evaluated. Liver index was decreased to 2.91 and 2.76 after administration of MECA at 200 mg/kg (Group D) and 400 mg/kg (Group E), respectively, in comparison with $CCl_4$ treated group (3.61) (Figure 2A). Furthermore, it was found that MECA at 200 mg/kg (Group D) and 400 mg/kg (Group E) showed remarkable improvement of total protein by 38.61% and 58.71% and albumin by 55.20% and 91.15%, respectively compared to $CCl_4$ treated group (Group B) (Figure 2B and C). Additionally, pretreatment of MECA at concentrations of 200 mg/kg (Group D) and 400 mg/kg (Group E) remarkably decreased the AST level by 24.93% and 33.57%, ALT by 36.23% and 43.89% and ALP by 20.44% and 35.95%, respectively compared to $CCl_4$ treated group (Figure 2D-F).

### 3.7. Liver antioxidant enzymes

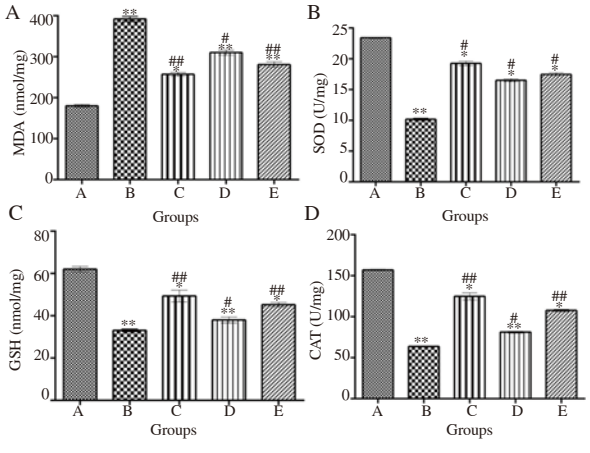


**Figure 3.** Effects of *Curcuma angustifolia* (MECA) on (A) MDA, (B) SOD, (C) GSH, (D) CAT after $CCl_4$-induced acute liver toxicity in rats. Group A: control; Group B: $CCl_4$; Group C: silymarin, 100 mg/kg; Group D and E: MECA at concentration of 200 mg/kg b.w. and 400 mg/kg b.w., respectively. The values show the mean±SD ($n$=6). Statistically significant differences are represented by asterisks ($^{*}P$<0.05 and $^{**}P$<0.01 as compared with the control group, $^{\#}P$<0.05 and $^{\#\#}P$<0.01 as compared with the $CCl_4$ treated group) as evaluated by ANOVA and Tukey's multiple range test.

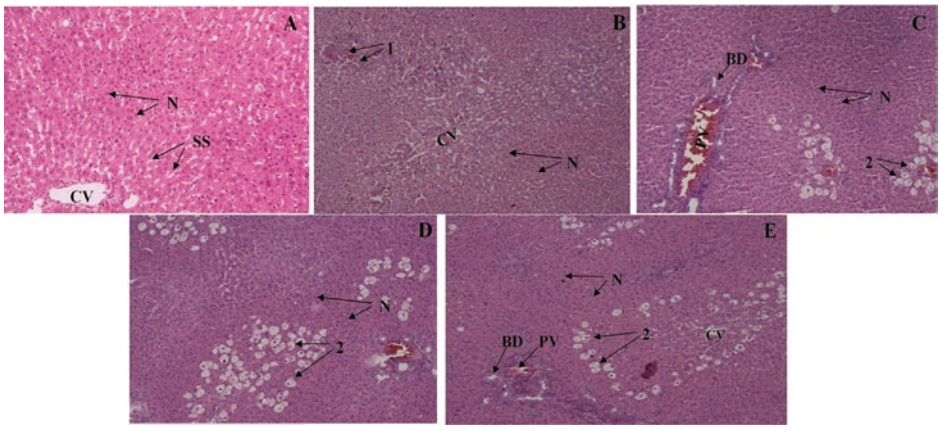


**Figure 4.** Histopathology of the liver. (A) The control group shows normal histological appearance with the presence of the central vein (CV), nucleus (N), and sinusoidal space (SS); (B) The $CCl_4$-treated group exhibits severe damage with dilated sinusoidal and infiltration of inflammatory cells (1); (C) The group administrered with silymarin showed rounded nucleus with little hydropic degeneration (2); (D and E) Groups administered with methanol extract of rhizome of *Curcuma angustifolia* at a concentration of 200 mg/kg b.w. and 400 mg/kg b.w. show the presence of the central vein, rounded nucleus, bile duct (BD), portal vein (PV) and scant hydropic degeneration. (Magnification 10×).

Pre-treatment of MECA at 200 mg/kg (Group D) and 400 mg/kg (Group E) alleviated the MDA levels by 20.89% and 28.34%, respectively compared with $CCl_4$ treated group (Figure 3A). Furthermore, it was observed that pretreatment of MECA (200 mg/ mL and 400 mg/mL) caused remarkable increase in the content of SOD by 62.28% and 71.61% , GSH by 14.96% and 36.75% and CAT by 27.44% and 66.93%, respectively (Figure 3B-D).

### 3.8. Histopathological studies

From the histopathological studies, the liver architecture of control group (Group A) revealed normal hepatocytes with complete absence of centrolobular necrosis. Cords of hepatocytes can be seen radiating from central vein towards portal triads (Figure 4A). In contrast, $CCl_4$ intoxicated group (Group B) revealed the dearrangement of liver parenchyma, sinusoidal congestion and infiltration of inflammatory cells (Figure 4B). Silymarin treated group (Group C) showed hepatocytes with mild acute portal triaditis with some hydropic degeneration (Figure 4C). No evidence of granuloma or necrosis was observed. In contrast, $CCl_4$-intoxicated rats pretreated with MECA at a concentration of 200 mg/kg (Group D) illustrated hydropic degeneration and no evidence of granuloma or malignancy were noticed (Figure 4D). However, pretreatment of MECA at a concentration of 400 mg/kg b.w. (Group E) reduced the damage caused by $CCl_4$. No necrosis and chronic inflammation were observed (Figure 4E).

### 3.9. GC-MS analysis of MECA

The chemical composition of MECA was carried out by GC-MS (Table 3). A total of 28 compounds representing 92.15% of total peak area were identified in MECA. Compound identification was performed by comparing their mass spectra database of NIST/EPA/NIH (NIST 08) mass spectral library. GC-MS analysis of MECA showed the presence of various secondary metabolites like terpenes, fatty acids, esters, phytosterols, phenols and vitamin. α -tocopherol was the main component (12.27%) followed by stigmasterol (10.58%), phytol (7.61%), α -linolenic acid (7.24%), linoleic acid (5.42%) and curdione (4.76%).

**Table 3.** Chemical constituents identified from the methanol extract of rhizome of *Curcuma angustifolia*.

| Sl. No | RT | Compound name | Molecular formula | Molecular weight (g/mol) | Peak area (%) |
|---|---|---|---|---|---|
| 1 | 4.44 | Butanoic acid | $C_4H_8O_2$ | 88.11 | 2.79 |
| 2 | 4.64 | 2-hexenal | $C_6H_{10}O$ | 98.14 | 1.11 |
| 3 | 7.26 | Eucalyptol | $C_{10}H_{18}O$ | 154.25 | 0.89 |
| 4 | 9.84 | Camphor | $C_{10}H_{16}O$ | 152.23 | 1.47 |
| 5 | 14.65 | Carvacrol | $C_{10}H_{14}O$ | 150.22 | 0.60 |
| 6 | 15.54 | Eugenol | $C_{10}H_{12}O_2$ | 164.20 | 2.59 |
| 7 | 15.70 | β -elemene | $C_{15}H_{24}$ | 204.36 | 1.00 |
| 8 | 15.99 | β -Elemenone | $C_{15}H_{22}O$ | 218.34 | 0.53 |
| 9 | 16.15 | tau-Cadinol | $C_{15}H_{26}O$ | 222.37 | 0.89 |
| 10 | 16.31 | β -eudesmol | $C_{15}H_{26}O$ | 222.37 | 1.82 |
| 11 | 16.74 | Germacrone | $C_{15}H_{22}O$ | 218.34 | 1.18 |
| 12 | 17.41 | Curcumenol | $C_{15}H_{22}O_2$ | 234.34 | 1.20 |
| 13 | 17.50 | Xanthorrhizol | $C_{15}H_{22}O$ | 218.34 | 1.19 |
| 14 | 17.65 | Curdione | $C_{15}H_{24}O_2$ | 236.36 | 4.76 |
| 15 | 18.00 | Farnesyl acetate | $C_{17}H_{28}O_2$ | 264.41 | 1.66 |
| 16 | 18.43 | trans ferulic acid | $C_{10}H_{10}O_4$ | 194.18 | 1.27 |
| 17 | 19.03 | Palmitic acid, methyl ester | $C_{17}H_{34}O_2$ | 270.45 | 3.15 |
| 18 | 19.63 | Farnesyl acetone | $C_{18}H_{30}O$ | 262.44 | 5.04 |
| 19 | 20.23 | Palmitoleic acid | $C_{16}H_{30}O_2$ | 254.41 | 2.17 |
| 20 | 20.69 | γ -Linolenic acid, methyl ester | $C_{19}H_{32}O_2$ | 292.46 | 4.42 |
| 21 | 20.89 | Phytol | $C_{20}H_{40}O$ | 296.53 | 7.61 |
| 22 | 21.13 | Linoleic acid | $C_{18}H_{32}O_2$ | 280.45 | 5.42 |
| 23 | 21.67 | α -Linolenic acid | $C_{18}H_{30}O_2$ | 278.43 | 7.24 |
| 24 | 24.08 | Squalene | $C_{30}H_{50}$ | 410.72 | 3.71 |
| 25 | 24.98 | α -tocopherol | $C_{29}H_{50}O_2$ | 430.71 | 12.27 |
| 26 | 25.62 | Campesterol | $C_{28}H_{48}O$ | 400.68 | 3.40 |
| 27 | 26.61 | Stigmasterol | $C_{29}H_{48}O$ | 412.69 | 10.58 |
| 28 | 27.22 | β -sitosterol | $C_{29}H_{50}O$ | 414.71 | 2.19 |

## 4. Discussion

The free radical scavenging ability towards the DPPH radical is an effective indicator of antioxidant[26]. Previous study in the ethanolic extract of *C. angustifolia* has revealed 58.35% of DPPH radicals scavenging activity[27]. Similarly, Thapa and Basistha[28] have reported in *C. angustifolia* cold and hot methanol extract to have $IC_{50}$ value of 357.4 μg/μL and 341.5 μg/μL respectively. Reducing power acts as a significant reflection of antioxidant properties. In this assay, the samples could reduce ferric ion to its ferrous form. The quantity of ferrous ion could be identified by evaluating the development of Perl's Prussian blue at wave length of 700 nm. The effect of reducing power indicates that electron donating capacity of MECA thereby neutralizing free radicals by forming stable products. Carbon tetrachloride is readily metabolized through hepatic metabolism to form trichloromethyl radicals. These trichloromethyl radicals after reacting with ROS to form trichloromethyl peroxide radicals ($CCl_3O$). Trichloromethyl peroxide radicals along with hydrogen peroxide, superoxide and hydroxyl radicals cause severe liver damage[20]. Nowadays, $CCl_4$ is being widely used to induce hepatotoxicity through *in vitro* and *in vivo* models[7]. For primary level of screening, nowadays, *in vitro* cytotoxicity as well as hepatoprotective activities of medicinal plant extracts has gained much more importance[29]. HepG2 cells are considered as an alternative model to human hepatocytes for studying the liver function, metabolism and *in vitro* toxicity, as they possess biochemical and morphological characteristics similar to that of the normal hepatocytes[30-32]. Treatment of MECA at various doses restored the altered serum biochemical parameters, MDA and GSH levels.

Further, *in vivo* hepatoprotective effect was carried out in *in vivo* model to additionally support the hepatoprotective nature of the MECA. The intra peritoneal administration of $CCl_4$ provoked the hepatic damage in rats which was showed by the elevated level of liver index in comparison to control group. However, pretreatment of MECA significantly elevated the liver index towards normal. Increase in the liver weight may also be associated with an inflammatory process induced by the hepatic toxicity on rat model[33]. A reduction in the protein levels is usually seen in hepatotoxic conditions due to the difficulty in the biosynthesis of protein[34]. Pretreatment of MECA significantly normalized the level of protein, similar finding was made by other researcher in *Tragia involucrata* root extracts against $CCl_4$-induced hepatotoxicity in rats[35]. Similarly, administration of $CCl_4$ significantly decreased the level of serum albumin, which indicated an alteration of albumin synthesis in the liver associated with hepatic intoxication and impairment of liver functions[24]. Besides, pretreatment of MECA significantly increased in the level of albumin, suggests the stabilization of endoplasmic reticulum leading to protein synthesis. An increased release of serum AST, ALT and ALP levels indicated structural disintegrity of the liver[36]. Similar observations of increased AST, ALT and ALP levels were seen in $CCl_4$-induced hepatotoxicity[1,2,4]. A significant increase in lipid peroxidation was seen in the $CCl_4$-intoxicated animals as compared to the normal group, which was evident by elevated MDA levels. An increase in MDA levels results in formation of excessive free radicals thus leading to tissue damage[37]. It has been documented that SOD, CAT and GSH are considered as the first line of defense against damage caused by oxidative stress[35]. Elevated levels of GSH protect the cells against potential oxidative damage[38]. SOD and CAT are crucial enzymes in the enzymatic antioxidant defence mechanism that catalyzes the detoxification reactions of toxic oxygen metabolites[39]. Our results are in agreement with the study of another researcher who also reported the increase in level of SOD, GSH and CAT in chickpea extract (CA250 and CA500) as compared to $CCl_4$ treated group[24]. The treatment of MECA reduced the destruction caused by $CCl_4$ by reducing the nuclear degeneration and restoration of fatty acid changes and lobular architecture of liver. Thus, histopathological studies of liver cross-sections confirm the hepatoprotective effect of MECA at both doses but 400 mg/kg b.w. showed better protective effect against $CCl_4$ induced hepatic injury as compared to 200 mg/kg b.w.

α-tocopherol is known to lower the increased level of liver enzymes, thereby, ameliorating the oxidative liver damage[40]. β-sitosterol, a steroid was reported to improve the antioxidant potential in 1,2-Dimethylhydrazine-induced colon cancer in rats[41]. Squalene, extracted from the aerial parts of *Albizia procera*, displayed protective effect against paracetamol induced liver injury *in vivo*[42]. Eugenol has strong antioxidant activity and showed protective effect against DNA damage[43]. β-elemene has the ability to reduce the pathological progression of $CCl_4$-induced hepatic fibrosis by preventing hepatic stellate cells activation in rats[44]. Curcumenol, exhibited protective effect against D-GalN-induced cytotoxicity and suppressed LPS (lipopolysaccharide)-induced nitric oxide (NO) production in mouse macrophage[45].

In conclusion, our findings revealed that administration of MECA showed a strong hepatoprotective effect based on the measurement of serum biochemical parameters and antioxidant enzymes in HepG2 cells and rats. Findings were further supported by the histopathological examinations which showed normal hepatic architecture with few pathological changes in MECA treated with $CCl_4$ intoxicated rats. *C. angustifolia* might be used in the field of pharmaceutical products for the prevention and treatment of liver injury. Further, details of molecular mechanisms underlying the hepatoprotective effect are needed to validate MECA as a potent hepatoprotective agent.

## Conflict of interest statement

The authors declare that there is no conflict of interest.

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**Supplemental Materials**

*Severe dengue case definition*

Virologically-confirmed dengue cases were classified as severe if they fulfilled the World Health Organization 1999 definition of DHF grade I, II, III, or IV, *i.e.*,:

Clinical Manifestations

a) Fever: acute onset, high and continuous, lasting 2-7 days.

b) Any of the following hemorrhagic manifestations (including at least a positive tourniquet test): petechiae, purpura, ecchymosis, epistaxis, gum bleeding, and hematemesis and/or melena.

Laboratory findings

a) Thrombocytopenia (platelet count=100 000/$mm^3$ or less).

b) Plasma leakage as shown by hemoconcentration (hematocrit increased by 20% or more) or pleural effusion (seen on chest X-ray) and/or hypoalbuminemia.

The first two clinical criteria, plus thrombocytopenia and signs of plasma leakage are sufficient to establish a clinical diagnosis of DHF. Pleural effusion (seen on chest X-ray) and/or hypoalbuminemia provide supporting evidence of plasma leakage.

DHF was graded as follows:

Grade I: Fever accompanied by non-specific constitutional symptoms; the only hemorrhagic manifestation is a positive tourniquet test.

Grade II: Spontaneous bleeding in addition to the manifestations of Grade I patients, usually in the form of skin and/or other hemorrhages.

Grade III: Circulatory failure manifested by rapid and weak pulse, narrowing of pulse pressure (20 mmHg or less) or hypotension, with the presence of cold clammy skin and restlessness.

Grade IV: Profound shock with undetectable blood pressure and pulse.

The additional signs and symptoms were also taken into account by the IDMC when defining severe dengue:

1) Thrombocytopenia: platelet count ≤ 50 000/$mm^3$

2) Any hemorrhage requiring blood transfusion

3) Objective evidence of capillary permeability documented by one or several of the following:

a) Increase in hematocrit by ≥ 20% compared to normal for age, or [(Maximum hematocrit-minimum hematocrit)/min]*100% ≥ 20%

b) Pleural or abdominal (ascites) effusion (diagnosed either by clinical signs or radiography or other imaging method)

c) Hypoproteinemia

4) Signs of circulatory failure manifested by:

a) Narrow pulse pressure < 20 mmHg, or hypotension for age (as defined by systolic pressure < 80 mmHg in children < five years and systolic pressure < 90 mm Hg in children ≥ five years), and

b) Rapid and weak pulse, and

c) Signs of poor capillary perfusion (cold and clammy extremities, delayed capillary refill).

5) Visceral manifestations such as:

a) Neurological symptoms (convulsions or change in level of consciousness)

b) Hepatic failure or elevation of hepatic enzyme (> 5-fold normal level)

c) Metabolic (hypoglycemia) or electrolyte (hyponatremia, hypocalcaemia) disturbances or volume overload (acute pulmonary edema or congestive heart failure)

d) Other visceral manifestations such as cardiomyopathy, acute renal failure, acute respiratory failure, cholecystitis

**Table S1.** Incidence of hospitalized VCD during the entire six-year follow-up period, each phase of the trial for each serotype.

| Serotype | CYD-TDV group | | Control group | | Relative risk (95% *CI*) |
|---|---|---|---|---|---|
| | Cases | Annual incidence rate (95% *CI*) | Cases | Annual incidence rate (95% *CI*) | |
| Active phase (Y1–Y2) | | | | | |
| Any | 32/2 621 | 0.6 (0.4–0.8) | 30/1 312 | 1.1 (0.7–1.6) | 0.53 (0.31–0.91) |
| 1 | 8/2 621 | 0.1 (0.1–0.3) | 9/1 312 | 0.3 (0.2–0.6) | 0.45 (0.15–1.30) |
| 2 | 20/2 621 | 0.4 (0.2–0.6) | 15/1 312 | 0.5 (0.3–0.9) | 0.67 (0.32–1.40) |
| 3 | 1/2 621 | <0.1 (0.0–0.1) | 3/1 312 | 0.1 (0.0–0.3) | 0.17 (0.00–2.08) |
| 4 | 0/2 621 | 0.0 (0.0–0.1) | 2/1 312 | <0.1 (0.0–0.3) | 0.00 (0.00–2.66) |
| Unserotyped* | 3/2 621 | <0.1 (0.0–0.2) | 1/1 312 | <0.1 (0.0–0.2) | 1.50 (0.12–78.81) |
| Hospital phase (Y3–Y6) | | | | | |
| Any | 85/2 098 | 1.0 (0.8–1.3) | 46/1 058 | 1.1 (0.8–1.5) | 0.93 (0.64–1.36) |
| 1 | 21/2 098 | 0.3 (0.2–0.4) | 11/1 058 | 0.3 (0.1–0.5) | 0.96 (0.44–2.21) |
| 2 | 29/2 098 | 0.4 (0.2–0.5) | 11/1 058 | 0.3 (0.1–0.5) | 1.33 (0.64–2.95) |
| 3 | 21/2 098 | 0.3 (0.2–0.4) | 10/1 058 | 0.2 (0.1–0.4) | 1.60 (0.48–2.52) |
| 4 | 15/2 098 | 0.2 (0.1–0.3) | 12/1 058 | 0.3 (0.2–0.5) | 0.63 (0.28–1.47) |
| Unserotyped* | 0/2 098 | 0.0 (0.0–0.0) | 3/1 058 | <0.1 (0.0–0.2) | 0.00 (0.00–1.22) |
| Entire study (Y1–Y6) | | | | | |
| Any | 117/2 272 | 0.9 (0.7–1.0) | 76/1 142 | 1.1 (0.9–1.4) | 0.77 (0.57–1.05) |
| 1 | 29/2 272 | 0.2 (0.1–0.3) | 20/1 142 | 0.3 (0.2–0.4) | 0.73 (0.40–1.36) |
| 2 | 49/2 272 | 0.4 (0.3–0.5) | 26/1 142 | 0.4 (0.2–0.6) | 0.95 (0.58–1.59) |
| 3 | 22/2 272 | 0.2 (0.1–0.2) | 13/1 142 | 0.2 (0.1–0.3) | 0.85 (0.41–1.84) |
| 4 | 15/2 272 | 0.1 (0.1–0.2) | 14/1 142 | 0.2 (0.1–0.3) | 0.54 (0.24–1.20) |
| Unserotyped* | 3/2 272 | <0.1 (0.0–0.1) | 4/1 142 | <0.1 (0.0–0.1) | 0.38 (0.06–2.23) |

* RT-PCR was unable to establish dengue serotype in virologically confirmed cases.

**Table S2.** Incidence of hospitalized VCD during the study for any serotype, in children aged 4–5 years at enrolment into the initial study.

| | CYD-TDV group | | Control group | | |
|---|---|---|---|---|---|
| Time period | Cases | Annual incidence rate (95% *CI*) | Cases | Annual incidence rate (95% *CI*) | Relative risk (95% *CI*) |
| Year 1 | 1/482 | 0.2 (0.0–1.2) | 2/230 | 0.9 (0.1–3.1) | 0.24 (0.00–4.58) |
| Year 2 | 3/465 | 0.6 (0.1–1.7) | 1/219 | 0.4 (0.0–2.3) | 1.41 (0.11–74.17) |
| Active phase | 4/474 | 0.4 (0.1–1.0) | 3/225 | 0.6 (0.1–1.9) | 0.63 (0.11–4.32) |
| Year 3 | 5/393 | 1.4 (0.5–3.2) | 1/192 | 0.6 (0.0–3.1) | 2.44 (0.27–115.54) |
| Year 4 | 5/393 | 1.3 (0.4–2.9) | 3/192 | 1.6 (0.3–4.5) | 0.81 (0.16–5.24) |
| Year 5 | 4/387 | 1.0 (0.3–2.6) | 0/192 | 0.0 (0.0–1.9) | NC |
| Year 6 | 12/370 | 3.2 (1.7–5.6) | 5/184 | 2.7 (0.9–6.2) | 1.19 (0.39–4.32) |
| Hospital phase | 26/386 | 1.7 (1.1–2.5) | 9/190 | 1.2 (0.6–2.2) | 1.42 (0.65–3.45) |
| Entire study | 30/415 | 1.2 (0.8–1.7) | 12/202 | 1.0 (0.5–1.7) | 1.21 (0.60–2.60) |

NC, not calculable.