SUPPLEMENTARY MATERIAL

Comparative neuroprotective effects of native curcumin and its galactomannoside formulation in carbofuran-induced neurotoxicity model

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ABSTRACT

Toxicity of the pesticide carbofuran (CF), can be alleviated by curcumin, if not for its poor bioavailability. Hence, we investigated the effect of a bioavailable curcumin-galactomannan complex (CGM) on CF-induced neurotoxicity in rats in comparison to that of unformulated standard curcumin (CS). The CF (5mg/kg b.wt/day) treatment for 90 days produced chronicity model which were treated with either CS or CGM (100mg/kg b.wt and 250mg/kg b.wt/day) for another 30 days. Improvement in CF-induced behavioural was evident in endurance, motor co-ordination and pain response on both CS ($p<0.01$) and CGM ($p<0.001$) supplementation. Amelioration of CF-induced toxicity parameters, oxidative stress, and mitochondrial dysfunction on CS ($p<0.01$) and CGM ($p<0.001$) supplementation was further confirmed by histopathology of brain and liver tissues. But, CGM was more effective in mitigating CF toxicity, with results comparable to that of normal. Hence, CGM might be superior in toxicity management against CF.

Keywords: Carbofuran; neurotoxicity; curcumin; CGM; mitochondrial dysfunction

Experimental Section

Experimental design

Adult Sprague Dawley rats (male), procured from Veterinary College (Mannuthy, Kerala, India), weighing 170±20 g were used for the study. Animals were kept in an air-conditioned room at 22 ± 2^{o}C and relative humidity 60 ± 5% with 12 h light and dark cycle. After their acclimatization in ventilated cages at the animal house facility of M/s Amala Cancer Research Centre (Kerala, India), the experiments were carried out in strict accordance to the approved ethical norms by the Institutional Animal Ethics Committee (IAEC), recognized by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India (Registration No: 149/99/CPCSEA). Animals were provided with pellet diet and water *ad libitum*.

Carbofuran induced neurotoxicity studies were carried out as described by Binitha et al (2015a). Thirty six rats were divided in to six groups, (n=6), as described below.

Group I : Control (Untreated)
Group II : Carbofuran (CF) treated (5 mg/kg b.wt)
Group III : CF+ standard curcumin (CS) (100 mg/kg b.wt.)
Group IV : CF+ CS2 (250 mg/kg b.wt.)
Group V : CF+ CGM1 (100 mg/kg b.wt.)
Group VI : CF+ CGM2 (250 mg/kg b.wt.)

Standard curcumin (CS), CGM, and carbofuran dissolved in peanut oil were administrated by oral gavage at respective dosage as shown above. While carbofuran administration continued for 90 days to produce a chronic model, curcumin and CGM treatment was for 30 days. Carbofuran was dissolved in 0.5 mL peanut oil, CS was dispersed in 1% gum acacia solution, and CGM was dispersed in water for administration. All the animals were observed for clinical and behavioural changes. Body weight, food and water consumption were recorded for each group of animals on a weekly basis during the course of experiment.

Neurotoxicity study-Behavioural tests

Behavioural changes were monitored by a set a standardized behavioural tests, namely grip strength, rota-rod and pain threshold. All the tests were carried out 10 days before the sacrifice.

Rota-rod test

Rota-rod treadmill test is designed to evaluate the muscle strength and motor coordination (Jones and Roberts 1968). After initial acclimatization to the rotating rod experiment, the animals were placed on the rods at low rotation with a gradual acceleration to the final rotation speed. The duration the animal could stay from the start of the acceleration was recorded. The test was performed after 10 days of treatment and repeated for 5 consecutive days to obtain the average performance time in seconds

Grip strength test

In this test to assess the muscular strength, rodents were allowed to grip a horizontal griddle with forelimbs and hind limbs. The time taken for the animal to be fatigued and broke the grip was recorded as the endurance. Repetitions for 5 consecutive days gave the average endurance capacity in seconds (Meyer et al. 1979).

Measurement of pain-threshold

Haffner's tail clip method (1929) of mechanical stimulation was employed to measure pain threshold in rodents. An artery clip was applied to the root of tail of rat to induce pain and to elicit raised-tail response. The time between stimulation onset and response were measured by a stop watch. The procedure was repeated for 5 consecutive days and average pain threshold in seconds was taken for each animal.

Samples preparation for analysis

The animals were sacrificed by cervical dislocation under anesthesia and the brain was removed and rinsed with ice-cold isotonic saline. Brain samples were then homogenized with ice-cold 0.1 mmol/L phosphate buffer at pH 7.4 and the homogenates were centrifuged at 10,000×g for 15 min and the supernatant was used for the biochemical analysis.

Acetylcholinesterase (AChE) activity in brain was assayed. The tissue homogenates at 10% (w/v) were prepared in Tris-HCl buffer (0.1M, pH 7.0) and used for the assay. AChE activity was analyzed by butyrylthiocholine method, in which butyrylthiocholine was converted into thiocholine and butyrate by AChE, which further reacts with dithio-bis-nitrobenzoate to produce 2-nitromercapto-5-benzoate and is quantitated at 405 nm (Ellman et al. 1961).

Estimation of protein

Protein estimation was done by Lowry's method (Lowry et al. 1951) using bovine serum albumin (BSA) as standard.

Mitochondrial studies

Isolation of rat brain mitochondria

Rat brain mitochondria were isolated by the method of Susin (2000). The brain was homogenized in isolation buffer with ethylene glycol tetra acetic acid (EGTA) (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 1 mM EGTA, pH-7.2). The homogenates were centrifuged at 13,000×g for 5 min at 4-8°C. The pellet was re-suspended in the isolation buffer with EGTA and spun again at 13,000×g for 5 min. The resulting supernatant was transferred to new tubes and topped off with the isolation buffer containing EGTA and spun again at 13,000×g for 10 min. The pellet containing pure mitochondria was re-suspended in the isolation buffer without EGTA.

Assay of the enzymes in the mitochondrial fraction

Isocitrate dehydrogenase (ICDH) activity was determined from the rate of reduction of NAD+ in the presence of trisodium isocitrate at 340 nm and expressed as μmoles of NAD+ reduced/minute/mg protein using the extinction coefficient of NADH (6.3 mM-1) (Fatania et al. 1993). Succinate dehydrogenase (SDH) activity was determined from the rate of decrease in absorbance at 600 nm after treating the mitochondria with the reaction mixture containing sodium succinate in the presence of electron acceptor dichlorophenol indophenols (DCPIP) which is converted to its reduced form. The activity was calculated using the extinction coefficient of DCPIP (19.1 mM-1cm-1) and expressed as micromoles of DCPIP reduced/min/mg protein (Nulton-Persson and Szweda 2001). The activity of malate dehydrogenase (MDH) was determined from the rate of decrease in absorbance at 340 nm after treating the mitochondria with the reaction mixture containing oxaloacetate and NADH. The activity was expressed as μmoles of NADH oxidized/min/mg protein using the extinction coefficient of NADH 6.3 mM-1 cm-1 (Mehler et al. 1948). The activity of NADH dehydrogenase is expressed as n moles of NADH oxidized per minute per mg of protein (Minakami et al. 1962).

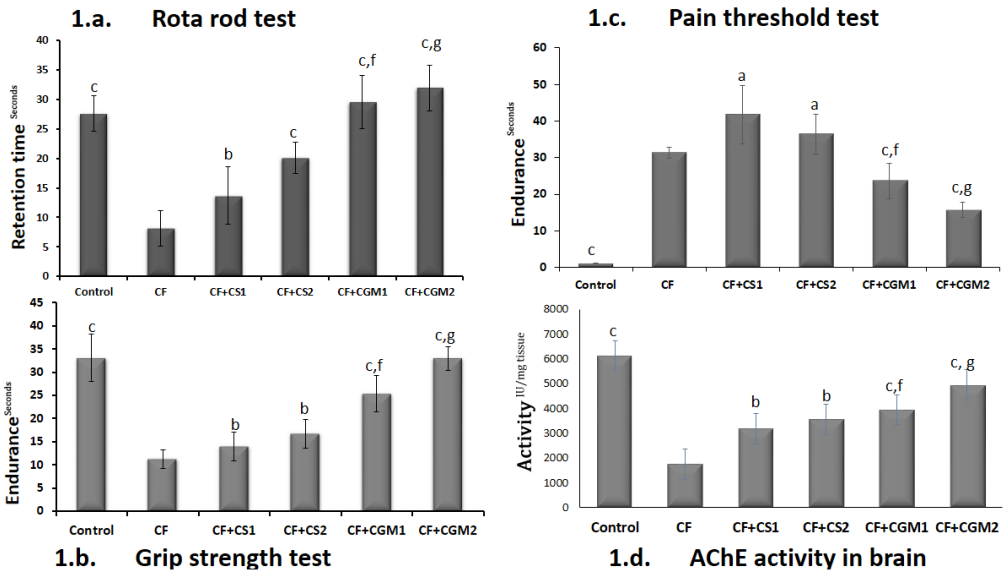
Statistical analysis

The values are expressed as mean ± SD. The statistical significance was compared between control and experimental groups by one way analysis of variance (ANOVA) followed by appropriate post hoc test (Tukey multiple comparison test) using GraphPad InStat software (version 3.05). Data of treated animals were

compared with untreated animals. A value of p< 0.05 was considered statistically significant.

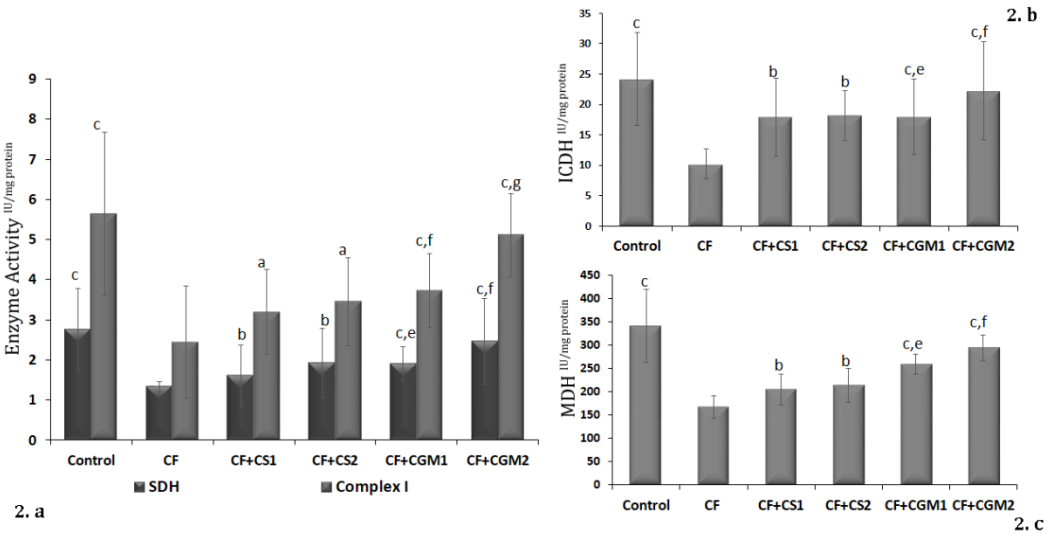
Results and discussion

Figure S1: Behavioural and biochemical assessment of neurotoxicity



Retention on rota-rod, grip strength and pain threshold recorded in seconds. AChE activity given in IU/mg tissue. Significant differences in p values are given as:
a- *p<0.05;* b- *p<0.01;* c- *p<0.001;* d- *p>0.05 -in comparison to CF;*
e- *p<0.05;* f- *p<0.01;* g- *p<0.001;* h- *p>0.05 -for CGM in comparison to CS.*

Figure S2: Activity of brain mitochondrial enzymes



Activities of the enzymes given as IU/mg protein in mitochondrial fraction.
Significant differences in
p values are given as:
a- *p<0.05;* b- *p<0.01;* c- *p<0.001;* d- *p>0.05 -in comparison to CF;*
e- *p<0.05;* f- *p<0.01;* g- *p<0.001;* h- *p>0.05 -for CGM in comparison to CS.*
IU for SDH is micromoles of DCPIP reduced/min; IU for ICDH is μmoles of NAD+ reduced/minute; IU for MDH is μmoles of NADH oxidized/min

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