

REPORT

Protective effect of *Cucurbita pepo* fruit peel against CCl₄ induced neurotoxicity in rat

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Abstract: *Cucurbita pepo* is a common vegetable used all over the world. In folk medicine it is used in gastroenteritis, hepatorenal and in brain anomalies. In the present study, protective effect of *Cucurbita pepo* fruit peel against CCl₄-induced neurotoxicity in rats was investigated. In this study, 36 Sprague-Dawley female rats (190±15 g) were randomly divided into 6 groups of 6 rats each. Group I was given 1 ml/kg bw (body weight) of corn oil intraperitoneally (i.p); Group II, III and IV were treated with 20% CCl₄ in corn oil (1ml/kg bw i.p.). However, animals of Group III and IV were also treated with CPME (methanol extract of *C. pepo* fruit peel) at 200 and 400mg/kg bw respectively. Animals of Group V and VI were administered only with CPME at 200 and 400mg/kg bw respectively. These treatments were administered 3 days a week for two weeks. Administration of CCl₄ cause acute neurotoxicity as depicted by significant depletion (P<0.05) in the activities of antioxidant enzymes; catalase, superoxide dismutase, peroxidase, glutathione reductase, glutathione-S-transferase, glutathione peroxidase, quinone reductase, while enhanced the γ -glutamyl transferase level in brain samples. CCl₄ intoxication decreased the reduced glutathione (GSH) level whereas markedly (P<0.05) enhanced lipid peroxidation in brain samples. Co-treatment of CPME significantly (P<0.05) protected the brain tissues against CCl₄ constituted injuries by restoring activities of antioxidant enzymes and ameliorated lipid peroxidation in a dose dependent fashion. These neuroprotective effects might be due to the presence of antioxidant constituents.

Keywords: *Cucurbita pepo*, CCl₄, oxidative stress, catalase, superoxide dismutase, reduced glutathione, lipid peroxidation.

INTRODUCTION

Carbon tetrachloride (CCl₄) intoxication as experimental model resembles oxidative stress caused by different pathophysiological conditions. Many studies have revealed that CCl₄ induced oxidative stress is significantly contributed in degeneration of tissues (Recknagel *et al.*, 1989; Sahreen *et al.*, 2014a). Tissue CCl₄-induced injuries have been manifested in different organs including brain, lungs and kidneys, however the major effect of CCl₄ intoxication was reported in liver (Khan *et al.*, 2012; Khan *et al.*, 2013; Sahreen *et al.*, 2014a). Still limited studies are available for brain tissue, however experimental studies have shown that like the hepatic tissue, brain tissue is also sensitive to CCl₄ (Jung and Henke, 1996).

Carbon tetrachloride metabolism by cytochrome p450 produces free reactive radicals more important are the trichloromethyl ($\bullet$ CCl₃) and trichloromethyl peroxy ($\bullet$ OOCCl₃) radicals. Trichloromethyl peroxy radicals remove hydrogen (H) atoms from unsaturated fatty acids producing lipid free radicals. Hydroperoxides of lipids have marked tendency to react with oxygen to generate more reactive radicals, which consequently initiated the

process of lipid proxidation. If radical scavengers do not neutralize these peroxy radicals, then these radicals continue to remove H atoms from lipid molecules and propagate the chain reaction of lipid peroxidation (Recknagel *et al.*, 1991) resulting in alteration of permeability of the membranous system with consequent loss of cellular Ca⁺² homeostasis and sequestration. This process enhances the level of degenerative enzymes and subsequently results in cellular damages (Halliwell and Gutteridge, 1999). As compared to trichloromethyl peroxy radical, trichloromethyl ($\bullet$ CCl₃) radical is more involved in covalent binding reactions (Slater, 1982). Trichloromethyl ($\bullet$ CCl₃) radical react covalently with sulfhydryl (SH) groups, such as protein thiols and GSH (reduced glutathione) which result in abnormal protein function. Reactive aldehydes, especially 4-hydroxynonenal are the fatty acids degradation products, which bind to proteins functional groups and hinder the important enzymes functions. CCl₄ have genotoxic potential due to ability of $\bullet$ CCl₃ to produce reactive species that covalently bind to DNA and induces chromosomal rearrangements (Diaz and Castro, 1981).

Since herbs have wide contribution in many areas of human life such as nutrition, flavoring, cosmetics, beverages and medicine. During recent decades medicinal plants were studied extensively for their antioxidant

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potential (Bokhari *et al.*, 2013; Sahreen *et al.*, 2014b). The intake of fresh vegetables, fruit and other dietary natural products having antioxidants possess marked role to mitigate cancer and cardiovascular anomalies (Willcox *et al.*, 2004). The risk of mortality from these diseases lowers down by the higher intake of plant foods. About 60% anti-infective and antitumoral agents have natural origins, which are available commercially (Cragg *et al.*, 1997).

Cucurbitacins are highly diverse oxygenated triterpenoid molecules possessed by different genera of Cucurbitaceae family including *Cucurbita pepo* (Tannin-Spitz, 2007; Haritunians *et al.*, 2008; Wakimoto *et al.*, 2008). It is reported that cucurbitacins have excellent antioxidant potential and this potential is due to their free radical scavenging ability like singlet oxygen, hydroxyl radical and superoxide anions. Moreover various others reports proved that cucurbitacins have excellent potent antioxidant, anti-inflammatory and anti-proliferative activities. The most important thing is that these compounds were found safe for body cells without having any toxic effect.

Cucurbita pepo is a gourd like squash of Cucurbitaceae family. Many active components are present in it. The pharmacological investigations of *Cucurbita pepo* indicated the presence of active compounds such as hexane cucurbitane glycoside, syringic acid, cucurbitane type triterpenes, alkaloids, tannin and vitamin A, E, and C (Wang, 2007). Sugars, fats and fibers are also present. Minerals such as iron, boron, cobalt, zinc, magnesium, potassium and calcium are present. Both D-chiro-inositol (a molecule having insulin like activity), and antioxidants are thought to be responsible for protective effects of pumpkin (Oloyede *et al.*, 2014). Anti mutagenic and anti cancer effects of *C. pepo* have also been established (Parasad *et al.*, 2011). Ingestion of dried fruit of *C. pepo* lowered the cholesterol level and reduced the hepatic damage (Silveria *et al.*, 1996) and ulcer (Sarkar and Guha, 2008). Polyunsaturated fatty acids are the most prominent and integral constituents of brain tissues. It has been investigated that CCl₄ induces injuries in various tissues through lipid peroxidation (Khan *et al.*, 2013). *Cucurbita pepo* is used as brain tonic and other disorders in local system of medicine (Gossell-Williams *et al.*, 2011; Adnaik, 2012). Plant based products are mostly endowed with antioxidants to prevent the oxidative stress caused by ROS. This relationship has led to considerable interest in determining the free radical scavenging capacity of extracts. In this study protective effects of *Cucurbita pepo* fruit peel were investigated against neuronal oxidative toxicity induced with CCl₄ in rats.

MATERIALS AND METHODS

Extract preparation

At maturity fruits of *Cucurbita pepo* were collected in August 2011 from the local market of Rawalpindi, 1968

Pakistan and were peeled off. Peels were then shade dried for four weeks. 2kg of dried peels were ground and extracted with 4 liters of 95% methanol for 3 days and filtered (Khan and Zehra, 2013). Filtrate was evaporated on rotary evaporator to obtain methanol extract (CPME) and kept at 4°C for additional *in vivo* investigations.

Animals and experimental design

Healthy Sprague-Dawley female rats (36 rats) of weight 190±15g were maintained at room temperature (25±3°C) in ordinary cages. Rats were divided into 6 groups (06). Animals of Group I were administered with 1ml/kg bw (body weight) of corn oil intraperitoneally 3 days a week for two weeks. Group II, III and IV were given 20% CCl₄ in corn oil (1ml/kg bw) intraperitoneally 3 days a week for two weeks. Rats of Group III and IV were also co-treated with *C. pepo* peel extract (CPME) at 200 and 400 mg/kg bw intraperitoneally 3 days a week for two weeks. Rats of Group V and VI were given CPME only at 200 and 400 mg/kg bw intraperitoneally 3 days a week for two weeks. Authentication of the protocol for the present study was granted by Ethical Committee of Quaid-i-Azam University Islamabad for laboratory animal feed and care. All experiments were conducted at the primate facility at Quaid-i-Azam University, Islamabad.

Body weights of rats in each group were recorded before and at the end of experiment. After sacrifice under anesthesia brains were taken and washed with saline solution, weighted and processed for various biochemical studies.

Biochemical studies of brain samples

Whole brain was homogenized with 10 times (w/v) ice cold phosphate buffer (100 mM, pH 7.4). Supernatant was taken after centrifugation at 12000×g for 30 min at 4°C for various biochemical studies.

Assay for total protein

Quantity of protein in brain sample was estimated with standard biuret method. For this assay standard AMP diagnostic kit (Krenngasse 12, 8010 Graz, Austria) was used.

Assay for antioxidant parameters

Activity of catalase (CAT) and peroxidase (POD) was determined by using hydrogen peroxide and guaiacol as the substrates respectively (Chance and Maehly, 1955). Superoxide dismutase activity (SOD) was assessed by using phenazine methosulphate (Kakkar *et al.*, 1984). Glutathione-S-transferase activity (GST) was estimated according to Habig *et al.* (1974) whereas the method reported by Carlberg and Mannervik (1975) was used to estimate the glutathione reductase activity (GSR). To estimate the glutathione peroxidase assay (GSH-Px) in brain samples method of Mohanddas *et al.* (1984) was followed. Activity of quinone reductase (QR) was determined according to Benson *et al.* (1980). Reduced

glutathione contents (GSH) in the brain homogenate were determined by following the method of Jollow *et al.* (1974). However, estimation of lipid peroxidation (TBARS) was carried out according to the method of Wright *et al.* (1981).

STATISTICAL ANALYSIS

Means $\pm$ standard deviation (SD) were determined for each group. Treatment effects for different groups were assessed by computer software SPSS 13.0 through one way analysis of variance. Significance level among treatments was determined by *post hoc* LSD at 0.05% and 0.01% probability level.

RESULTS

Effect of CPME on body and brain weight of rat

The protective potential of CPME against the general toxicity of CCl₄ in rat is given in table 1. In this experiment body weight of the rats was significantly ($P<0.01$) decreased whereas absolute brain and relative brain weight was increased with CCl₄ treatment against the untreated rats. The protective capacity of CPME on the CCl₄ induced toxicity was evident as it increased ($P<0.05$) the body weight, while decreased the absolute brain and relative brain weight in a dose dependent manner. Co-administration of CPME at 400mg/kg bw showed the highest protective potential for the body weight, absolute brain and relative brain weight in

relation to CCl₄ treated animals. In comparison to untreated animals administration of CPME alone at both 200 and 400mg/kg bw did not alter ($P>0.05$) the above parameters.

Effect of CPME on CAT, POD and SOD in brain

The antioxidant potential of CPME was determined by estimating different radical scavenging assays such as the activity level of CAT, POD and SOD in brain of rat (table 2). The level of antioxidant enzymes; CAT, POD and SOD and total protein content was decreased by the toxicity of CCl₄ in rat's brain with respect to untreated animals. The functional integrity of these enzymes mutually protect the body by scavenging of free radical damages and constitute the first line defense to maintain a balance in cellular activities. The extract used in our study (CPME) showed the potential to counteract the formation of free radicals due to CCl₄ toxicity in brain thereby elevated the activity level of CAT, POD and SOD and restore the total protein concentration. The results obtained from this study show that administration of CPME at 200 and 400mg/kg bw did not influence the activity level of CAT, POD and SOD and the content of total protein ($P>0.05$) in brain samples with respect to the control group.

Effect of CPME on GSR, GST, GSH-Px and QR in brain

Antioxidants present in the extract (CPME) under study

Table 1: Effect of CPME on % increase in body weight, absolute brain weight and relative brain weight of rat

Treatments	% increase in body weight	Absolute brain weight (g)	Relative brain weight (% of body weight)
Control	18.6 $\pm$ 0.730**	1.43 $\pm$ 0.062**	0.703 $\pm$ 0.033**
CCl ₄ (1 ml/kg bw)	12.6 $\pm$ 0.417 ⁺⁺	1.60 $\pm$ 0.037 ⁺⁺	0.877 $\pm$ 0.054 ⁺⁺
CPME (200 mg/kg bw) + CCl ₄	17.1 $\pm$ 0.212 ⁺ *	1.55 $\pm$ 0.043 ⁺	0.773 $\pm$ 0.029*
CPME (400 mg/kg bw) + CCl ₄	17.8 $\pm$ 0.119**	1.53 $\pm$ 0.056**	0.758 $\pm$ 0.018*
CPME (200 mg/kg bw)	17.2 $\pm$ 2.24**	1.38 $\pm$ 0.047**	0.715 $\pm$ 0.024**
CPME (400 mg/kg bw)	18.4 $\pm$ 3.18**	1.40 $\pm$ 0.052**	0.705 $\pm$ 0.036**

Mean $\pm$ SD (n=6 number)

*, ** indicate significance from the CCl₄ group at $P<0.05$ and $P<0.01$ probability level

⁺, ⁺⁺ indicate significance from the control group at $P<0.05$ and $P<0.01$ probability level

Table 2: Effect of methanol extract of *Cucurbita pepo* peels on CAT, POD and SOD of rat's brain

Treatment	Total protein (μ g/mg tissue)	CAT (U/mg protein)	POD (U/mg protein)	SOD (U/mg protein)
Control	3.09 $\pm$ 0.58**	4.75 $\pm$ 0.57**	22.70 $\pm$ 2.08**	8.93 $\pm$ 1.53**
CCl ₄ (1 ml/kg bw)	1.44 $\pm$ 0.45 ⁺⁺	2.60 $\pm$ 0.36 ⁺⁺	10.83 $\pm$ 2.04 ⁺⁺	4.17 $\pm$ 0.77 ⁺⁺
CPME (200 mg/kg bw) + CCl ₄	2.52 $\pm$ 0.17*	3.90 $\pm$ 0.63 ⁺ **	18.70 $\pm$ 1.41 ⁺ **	8.03 $\pm$ 1.17**
CPME (400 mg/kg bw) + CCl ₄	2.86 $\pm$ 0.26**	4.15 $\pm$ 0.39**	20.88 $\pm$ 1.96**	8.26 $\pm$ 0.66**
CPME (200 mg/kg bw)	2.97 $\pm$ 0.17**	4.68 $\pm$ 0.27**	21.82 $\pm$ 1.62**	8.82 $\pm$ 1.24**
CPME (400 mg/kg bw)	2.94 $\pm$ 0.21**	4.60 $\pm$ 0.50**	22.32 $\pm$ 2.23**	9.18 $\pm$ 1.59**

Mean $\pm$ SD (n=6 number)

*, ** indicate significance from the CCl₄ group at $P<0.05$ and $P<0.01$ probability level

⁺, ⁺⁺ indicate significance from the control group at $P<0.05$ and $P<0.01$ probability level

Table 3: Effect of methanol extract of *Cucurbita pepo* peels on brain GSR, GST, GSH-Px, QR and γ -GT of rat

Treatment	GSR (nM/min/mg protein)	GST (nM/min/mg protein)	GSH-Px (nM/min/mg protein)	QR (nM/min/mg protein)	γ -GT (U/L)
Control	85.11 $\pm$ 7.57**	206.9 $\pm$ 13.1**	52.94 $\pm$ 6.54**	131.2 $\pm$ 9.70**	5.0 $\pm$ 0.63**
CCl ₄ (1 ml/kg bw)	33.84 $\pm$ 3.05 ⁺⁺	120.7 $\pm$ 6.89 ⁺⁺	23.12 $\pm$ 5.33 ⁺⁺	68.29 $\pm$ 4.88 ⁺⁺	10.4 $\pm$ 1.06 ⁺⁺
CPME (200 mg/kg bw) + CCl ₄	67.02 $\pm$ 2.30 ⁺ *	166.6 $\pm$ 28.4 ⁺ *	33.77 $\pm$ 9.94 ⁺ *	98.7 $\pm$ 11.5 ⁺ *	7.78 $\pm$ 1.74 ⁺ *
CPME (400 mg/kg bw) + CCl ₄	72.7 $\pm$ 10.1 ⁺ *	189.2 $\pm$ 13.1**	45.34 $\pm$ 7.25*	111.4 $\pm$ 8.36**	6.11 $\pm$ 0.94 ⁺⁺ *
CPME (200 mg/kg bw)	83.2 $\pm$ 9.22*	205.2 $\pm$ 23.2**	51.57 $\pm$ 4.32**	128.2 $\pm$ 16.8**	4.92 $\pm$ 0.62*
CPME (400 mg/kg bw)	80.8 $\pm$ 11.2*	208.4 $\pm$ 29.4**	54.25 $\pm$ 4.78**	132.0 $\pm$ 17.6**	4.81 $\pm$ 0.74*

Mean $\pm$ SD (n=6 number)*, ** indicate significance from the CCl₄ group at P<0.05 and P<0.01 probability level⁺, ⁺⁺ indicate significance from the control group at P<0.05 and P<0.01 probability level**Table 4:** Effect of CPME on brain GSH and TBARS of rat

Treatment	GSH (μ M/g tissue)	TBARS (nM/min/mg protein)
Control	5.03 $\pm$ 0.41**	4.13 $\pm$ 0.80**
CCl ₄ (1 ml/kg bw)	2.05 $\pm$ 0.34 ⁺⁺	9.14 $\pm$ 1.16 ⁺⁺
CPME (200 mg/kg bw) + CCl ₄	4.05 $\pm$ 0.69*	5.99 $\pm$ 0.46 ⁺⁺ *
CPME (400 mg/kg bw) + CCl ₄	4.26 $\pm$ 0.64**	4.86 $\pm$ 1.11**
CPME (200 mg/kg bw)	5.26 $\pm$ 0.42**	4.28 $\pm$ 0.35**
CPME (400 mg/kg bw)	4.96 $\pm$ 0.74**	4.13 $\pm$ 0.67**

Mean $\pm$ SD (n=6 number)*, ** indicate significance from the CCl₄ group at P<0.05 and P<0.01 probability level⁺, ⁺⁺ indicate significance from the control group at P<0.05 and P<0.01 probability level

have the potential to stop the chain reactions and to alleviate the burden of oxidative stress induced with CCl₄ in rat's brain. Activity level of phase II antioxidant enzymes such as GSR, GST, GSH-Px and QR was increased whereas activity of γ -GT was decreased (P<0.01) in brain samples by CPME co-administration, which were adversely affected by the CCl₄ intoxication to rats (table 3). The effect of CPME was dose- dependent with peak effect produced at 400mg/kg bw to scavenge the CCl₄ induced oxidative stress. In this study, the effect of CPME at different doses (200 and 400mg/kg bw) alone was not significantly different (P>0.05) from the control group.

Effect of CPME on TBARS and GSH in brain

Intraperitoneal injection of CCl₄ to rats caused the induction of oxidative injuries in brain with an increase in lipid peroxidation (concentration of TBARS) while a decrease in the concentration of reduced glutathione (GSH). Co-administration of CPME produced significant reduction in lipid peroxidation while enhance GSH level in rat's brain (P<0.01). This effect was dose-dependent with peak effect produced at the highest dose of 400 mg/kg bw (table 4). However, CPME at doses of 200, 400 mg/kg bw alone did not affect the lipid peroxidation and concentration of GSH as comparable with control rats.

DISCUSSION

Enhanced lipid peroxidation and alteration in the antioxidant status are significantly related with the

oxidative stress induced disorders. Free radicals most likely are involved in lipid peroxidation of the membranous system. Brain tissues are considered to be more vulnerable to lipid peroxidation to reactive oxygen species of CCl₄ on account of its aerobic metabolic activity and greater accumulation of polyunsaturated fatty acids in the neuronal membranes (Del Maestro and McDonald, 1987; Adams *et al.*, 1991). CCl₄ after administration is concentrated in different organs. Liver and brain tissues rapidly intake and metabolize the CCl₄ in to highly injurious reactive oxygen species such as trichloromethyl ($\bullet$ CCl₃) and trichloromethyl peroxy ($\bullet$ OCCl₃) radical (Sanzgiri *et al.*, 1997; Weber *et al.*, 2003). Human body is protected from free radical damages by a cooperative scavenging mechanism of antioxidant enzymes and dietary nutrients. Enzymic antioxidants include; SOD, POD, CAT and GSH-Px. Their coordinated system is essential for free radical detoxification. SOD converts superoxide radical which is highly reactive to H₂O₂ while GSH-Px and CAT decompose H₂O₂ and protect the body while minimizing the potential damages of hydroxyl (OH) radicals. According to Szymonik-lesiuk *et al.* (2003) CCl₄ intoxication results in changes in expression level and consequently decreases the levels of CAT and SOD in liver tissue. Our present investigation corroborates with the above findings and reflected that CCl₄ intoxication lead to significant reduction in phase I enzymes such as SOD, POD and CAT in brain tissue. Two-week administration of CPME significantly restored the levels of SOD, POD and CAT towards normal group. These

results are in agreement with earlier findings (Khan and Younus, 2011) where various doses of *Digera muricata* extract enhanced the levels of SOD, POD and CAT against the CCl₄ intoxication (Khan *et al.*, 2009).

Glutathione system includes different phase two metabolizing enzymes e.g. GSH-Px, GST and GSR. These enzymes metabolize different toxins and also regulate genomic and biochemical changes (Mc Gregor and Lang, 1996). Reduced glutathione is an important thiol protein, which participates in different defense processes against oxidative stress. It eliminates superoxide anion and H₂O₂ from the body. Low level of GSH in living organisms is responsible for tissue injury and various anomalies related to oxidative stress (Limon-Pacheco *et al.*, 2007). Reduced glutathione scavenge diverse free radicals effectively. In systems of oxidative stress GSH is oxidized to GSSG by GSH-Px activity while GSR activity converted back GSSG to GSH (Meister and Anderson, 1983). Glutathione peroxidase is found in the cell membrane. It is thought that GSH is usually dependent on GSH-Px for its protective system against oxidative breakdown of lipids, which occur by GSH-Px mediated reduction of endogenous H₂O₂ (Sing and Pathak, 1990; Khan and Siddique, 2012). According to Adams *et al.* (1991) GSH-Px more efficiently detoxify H₂O₂ than CAT and SOD in brain tissues. Moreover, GSH-Px in combination with GSH terminates lipid peroxidation chain reaction by amelioration of H₂O₂ and many other hydroperoxides (Sun *et al.*, 1988; Mak *et al.*, 1996). Results reported in this study suggested that free radicals generated by metabolism of CCl₄ cause significant reduction in protein concentration as well as the catalytic potential of phase II enzymes e.g. GSH-Px, GSR, GST in brain samples. Protective potential of the CPME is evident in this study as its administration along with CCl₄ ameliorated the oxidative stress by increasing the activities of GSR, GSH-Px, GST and QR and protein content whereas reduces the activity of γ -GT against the CCl₄ treated group similar to other findings (Farombi *et al.*, 2002; Khan and Zehra, 2013).

Reactive oxygen species formed by CCl₄ intoxication can covalently bind to macromolecules and in cellular membranes can promote lipid peroxidation. Peroxidation of polyunsaturated fatty acids generates malonaldehydes (Khan and Younus, 2011; Saeed *et al.*, 2012). Superoxide dismutase eliminates peroxide anion radicals and inhibits the propagation of fatty degeneration (Escobar *et al.*, 1996). In this study CCl₄ administration significantly increased the TBARS while decreased the GSH level in brain tissues. Increase in TBARS levels indicated CCl₄ induced oxidative damages in brain tissues, similar to other reports (Clemedson *et al.*, 1990; Krasteva *et al.*, 2007; Park *et al.*, 2008). In present investigation, the TBARS level in the brain tissues moved towards normal group after treatment with CPME. The elimination of

CCl₄ induced neuronal toxicity with the extract may involve restoration of antioxidant enzymes (Srivastava and Shivanandappa, 2010; Sahreen *et al.*, 2013).

Our study also revealed the marked changes on body and organ weight induced by the administration of CCl₄ in rat. Intraperitoneal administration of CCl₄ significantly decreased the body weight of rat, which was suggested due to degeneration and necrosis of body tissues (Noyan *et al.*, 2006). Findings of this study are similar to the above reports where CCl₄ intoxication reduced the body weight conversely to the increase in absolute brain and relative brain weight. Decrease in body weight while increase in absolute and relative brain weight was significantly restored to normal control group by treatment with CPME. According to Khan and Ahmed, (2009) and Lin *et al.* (2008) significant loss in body and gain in brain weight due to CCl₄ was restored significantly by *Digera muricata* and *Solanum nigrum*, respectively.

CONCLUSION

Methanol extract of *Cucurbita pepo* fruit peel showed significant neuroprotective activity against CCl₄ induced alterations, which might be due the presence of its bioactive compounds. Further research work for isolation and purification of bioactive constituents is needed.

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