

The Beneficial Effect of Cape Gooseberry Juice on Carbon Tetrachloride-Induced Neuronal Damage

Ebtesam M. Al-Olayan¹, Manal F. El-Khadragy^{1,2}, Sawsan A. Omer³, Mohamed Tarek M Shata⁴, Rami B. Kassab² and Ahmed E. Abdel Moneim^{*,2}

¹Chair Vaccines Research of Infectious Diseases, Faculty of Science, King Saud University, Riyadh, Kingdom of Saudi Arabia; ²Department of Zoology and Entomology, Faculty of Science, Helwan University, Cairo 11795, Egypt; ³Department of Zoology, Faculty of Science, King Saud University, Riyadh 11451, Kingdom of Saudi Arabia; ⁴Department of Internal Medicine, Division of Digestive Diseases, University of Cincinnati College of Medicine, Cincinnati, OH, USA



Abstract: *Objective:* Cape gooseberry (*Physalis peruviana* L.) belongs to the Solanaceae family. *Physalis* has many medicinal properties however, the beneficial effect of *physalis* in protecting against neurotoxins has not yet been evaluated. This experimental study investigated the protective effect of *physalis* juice against the oxidative damage induced by carbon tetrachloride (CCl₄) in the rat brain.

Methods: The degrees of protection by *physalis* in brain tissues were evaluated by determining the brain levels of lipid peroxidation, nitric oxide, glutathione content and antioxidant enzyme activities (superoxide dismutase, catalase, glutathione-S-transferase, glutathione peroxidase and glutathione reductase), after CCl₄ induction in the presence or absence of *physalis*. Adult male albino Wistar rats were divided into 4 groups, Group I served as the control group, Group II was intraperitoneally treated with 2 ml CCl₄/kg bwt for 12 weeks, Group III was supplemented with *physalis* juice *via* the drinking water for 12 weeks, Group IV was supplemented with *physalis* juice and was intraperitoneally injected weekly with CCl₄.

Results: Treatment with CCl₄ was significantly associated with a disturbance in the oxidative status in the brain tissues; this was marked by a significant ($p < 0.05$) elevation in the lipid peroxidation and nitric oxide levels with a concomitant reduction in glutathione content compared to the control, along with a remarkable reduction in antioxidant enzymes. The administration of *physalis* along with CCl₄ juice significantly ($p < 0.05$) alleviated the changes in enzymatic antioxidant activity when compared to the CCl₄ treated group. Furthermore, *physalis* juice supplementation inhibited apoptosis, as indicated by the increase of Bcl-2 immunoreactivity in brain tissue.

Conclusion: Our results suggest that *physalis* juice could be effective in preventing neurotoxicity and the neuroprotective effect of *physalis* might be mediated *via* antioxidant and anti-apoptosis activities.

Keywords: *Physalis peruviana* L., carbon tetrachloride, oxidative stress, antioxidant, brain.

INTRODUCTION

The imbalance between the generation of reactive oxygen species (ROS) and the antioxidant defense capacity of the body causes oxidative stress. ROS are implicated in the causation and progress of several diseases, including cancer, Alzheimer's disease, Parkinson's disease, diabetes and liver cirrhosis [1].

Carbon tetrachloride (CCl₄) is a colorless and non-inflammable volatile liquid with a distinct odor. Inhalation of its vapor depresses central nervous system activity and causes hepatic and renal failure by exerting a destructive and poisonous effect on the cells [2]. CCl₄ by itself does not have cytotoxic effects but its metabolic products, such as generated trichloromethyl free radical, which can react with oxygen to produce the trichloromethyl peroxy radical, is responsible for its toxicity and the production of covalent

binds to macromolecules to form protein, lipid and nucleic acid adducts. The toxicity of CCl₄ is a multifactorial process involving lipid peroxidation, inflammatory cytokines production, loss of calcium homeostasis and nucleic acid hypomethylation [3]. Documented evidence suggests that CCl₄ has been commonly used as hepatotoxin in experimental hepatopathy [4], and a single exposure to it rapidly leads to severe hepatic necrosis and steatosis. However, the oxidative damage induced by CCl₄ is a well-established animal model for neuronal damage [5, 6].

Cape gooseberry (*Physalis peruviana* L.) belongs to the Solanaceae family, which is distributed throughout the tropical and subtropical regions of the world and presents herbal characteristics and perennial habits [4]. *Physalis* has various biological functions, due to its rich polyphenol ingredients, provitamin A, vitamin B-complex, vitamin C and many minerals [7]. Polyphenol ingredients in *physalis* have also been reported to have a variety of biological activities, including antioxidant, anticancer, and anti-inflammatory activities, the ability to fortify the optic nerve, and sedative, analgesic, antiseptic, antimicrobial and

*Address correspondence to this author at the Department of Zoology and Entomology, Helwan University, Cairo, Egypt; Tel: (+2) 01153322079; E-mail: aest1977@hotmail.com

antispasmodic activities. Antidiabetic properties have also been reported [4, 8]

Recently Al-Olayan *et al.* [4] reported that the juice of physalis attenuated CCl₄-induced hepatotoxicity. However, the potency of physalis to protect against CCl₄-induced neurotoxicity has not yet been examined. Therefore, the aim of this study was to investigate the possible protective effect of physalis juice against CCl₄-induced neurotoxicity. We hypothesized that this juice would protect against CCl₄-induced adverse effects in the brain due to its intrinsic biochemical and antioxidant properties which result in improved the antioxidant defense mechanisms in the brain.

MATERIALS AND METHODS

Chemicals and Experimental Animals

Carbon tetrachloride (CCl₄; CAS Number 56-23-5) and Tris-HCl were purchased from Sigma (St. Louis, MO, USA). Thiobarbituric acid and trichloroacetic acid were purchased from Merck. All other chemicals and reagents used in this study were of analytical grade. Double-distilled water was used as the solvent.

Adult male Wistar albino rats weighing 200–250 g (8-10 weeks) were obtained from the Holding Company for Biological Products and Vaccines (VACSERA, Cairo, Egypt). After an acclimatization period of one week, the animals were divided into four groups (7 rats per group) and housed in wire bottomed cages in a room under standard conditions of illumination with a 12 hr light-dark cycle at 25±2°C. The rats were provided water and a balanced diet *ad libitum*. All experimental procedures involving animals were conducted in accordance with the guidelines of the National Program for Science and Technology of the Faculty of Science, King Saud University. The study protocol was approved (No. 30/7/1435) by Ethical Committee of King Saud University, Riyadh, with collaboration with the College of Science and Zoology Department (Helwan University).

Plant Material

The fresh fruits of physalis were collected from the market of East Cairo, Egypt during February-March 2012. The plant material was authenticated in the Botany Department, Faculty of Science, Helwan University, Cairo-Egypt by a plant taxonomist. The plant material was identified based on its taxonomic characters and by direct comparison with the herbarium specimens available at the herbarium of the Botany Department.

Physalis Juice Preparation and Stability

The fresh fruits of physalis (10 kg) were separated from their calyxes and homogenized. The pulp was filtered off, the filtrate was clear and yellow in color, and the filtrate was immediately diluted with distilled water at 1:5 ratio (V/V) and stored at 4 °C for no longer than 2 months. Physalis juice stability was assessed by measuring the initial total phenolic and flavonoid contents and evaluating the alterations after 2 and 3 days of exposure to the same conditions as the juice supplied to the animals. According to our previous study, flavonoid content in physalis juice was 89.4 µg/mg quercetin

equivalents of flavonoids/ml juice. The total polyphenolic content was 121.3 µg/ mg gallic acid equivalents of polyphenols/ml juice [4].

Determination of the Neuroprotective Effects of Physalis Juice on Brain Injury Induced by CCl₄ in Rats

To study the protective effects of physalis juice on CCl₄ mediated neurotoxicity, the rats were randomly allocated into four groups of seven rats per group. Group I served as the control and was intraperitoneally (i.p.) treated each week with saline (300 µl per rat). Group II (CCl₄) was injected i.p. weekly with 2 ml CCl₄/kg bwt for 12 weeks as described by Sohn *et al.* [9]. Group III received juice (20%) supplied in dark water bottles and renewed every 2-3 days for 12 weeks. Group IV received physalis juice as in group III and was also injected intraperitoneally with 2 ml CCl₄/kg bwt for 12 weeks.

Twenty-four hours after CCl₄ intoxication, the animals were killed under mild ether anesthesia. The brains of the rats were carefully removed and washed twice in ice-cold 50 mM Tris-HCl, pH 7.4. Then, each brain was weighed and immediately homogenized to yield a 10 % (w/v) homogenate in ice-cold medium that contained 50 mM Tris-HCl, pH 7.4. The homogenates were centrifuged at 3000 rpm for 10 minutes at 4°C. The supernatants were used for the various biochemical determinations.

Oxidative Stress Measurements

Lipid peroxidation (LPO) in the brain was determined using 1 ml of 10% trichloroacetic acid and 1 ml of 0.67% thiobarbituric acid in a boiling water bath for 30 min. Thiobarbituric acid reactive substances were determined by the absorbance at 535 nm and expressed as the amount of malondialdehyde (MDA) formed [10]. The nitric oxide (NO) level was determined by the optimized acid reduction method in an acidic medium and in the presence of nitrite. The formed nitrous acid diazotise sulphanilamide was coupled with N-(1-naphthyl) ethylenediamine. The resulting azo dye had a bright reddish-purple color that could be measured at 540 nm [11]. In addition, the neuronal glutathione (GSH) was determined by the reduction of Elman's reagent (5,5'-dithiobis (2-nitrobenzoic acid)) with GSH to produce a yellow compound. The reduced chromogen was directly proportional to the GSH concentration, and its absorbance could be measured at 405 nm [12].

Enzymatic Antioxidant Status

The activities of neuronal antioxidant enzymes, such as superoxide dismutase (SOD), were assayed by measuring the ability of the enzyme to inhibit the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye. Brain catalase (CAT) was assayed by adding 50 µl of brain homogenates to 30 mM H₂O₂ in 50 mM of potassium phosphate buffer (pH 7.8), and the consumption of H₂O₂ was measured at 340 nm for 120 s at 20 s intervals. Catalase activity was expressed in units per g tissue (U/g tissue). Glutathione reductase (GR) was assayed indirectly by GR catalysis of the reduction of glutathione in the presence of

NADPH, which is oxidized to NADPH^+ . The decrease in absorbance at 340 nm was measured. Glutathione-S-transferase (GST) was determined by measuring the conjugation of 1-chloro-2,4-dinitrobenzene with reduced glutathione. The conjugation is accompanied by an increase in absorbance at 340 nm. The rate of increase is directly proportional to the GST activity in the sample [13]. Finally, glutathione peroxidase (GPx) activity in the brain homogenates was measured using the methods of Paglia and Valentine [14]. The assay is an indirect measure of the activity of GPx. Oxidized glutathione, produced upon reduction of organic peroxide by GPx, is recycled to its reduced state by the enzyme glutathione reductase. The oxidation of NADPH to NADP^+ is accompanied by a decrease in absorbance at 340 nm.

Immunohistochemical Analyses of Bcl-2

For immunohistochemistry, brains were fixed in 10% neutral formalin for 24 hours and paraffin blocks were obtained. Then, brain sections (4 μm) were deparaffinized and boiled in Declere (Cell Marque, Hot Springs, AR, USA) to unmask the antigen sites; the endogenous activity of peroxidase was quenched with 0.03% H_2O_2 in absolute methanol. Brain sections were incubated overnight at 4 °C with a 1:200 dilution of rabbit anti-Bcl-2 antibody in phosphate buffered saline (PBS). Following removal of the primary antibody and repetitive rinsing with PBS, slides were incubated with a 1:5000 dilution of biotinylated anti-IgG secondary antibody. Bound antibody was detected with avidin biotinylated peroxidase complex ABC-kit Vectastain and diaminobenzidine substrate. After appropriate washing in PBS, slides were counterstained with hematoxylin. All sections were incubated under the same conditions with the same concentration of antibody and at the same time; therefore, the immunostaining was comparable among the different experimental groups.

Statistical Analysis

Results were expressed as the mean \pm standard error of mean (SEM). Data for multiple variable comparisons were analyzed by one-way analysis of variance (ANOVA). For the comparison of significance between groups, Duncan's test was used as a *post hoc* test according to the Statistical Package for the Social Sciences (SPSS version 17.0).

RESULTS

To examine the effect of CCl_4 on oxidative stress markers, lipid peroxidation and nitric oxide levels in the brain homogenates of rats treated with CCl_4 were measured (Figs. 1, 2). LPO measured in terms of MDA levels was significantly ($p < 0.05$) enhanced in the brain tissue. However, the supplementation of physalis juice to rats induced a significant decline in the content of MDA ($p < 0.05$) compared to the control values (343.8 ± 10.61 and 674.6 ± 12.88 nmol/g, respectively). Moreover, physalis juice was found to have the ability to decrease the increase in the content of NO in the brain homogenates induced by CCl_4 when supplemented concurrently with CCl_4 compared to the CCl_4 group (125.6 ± 5.67 and 162.5 ± 3.65 $\mu\text{mol/g}$, respectively).

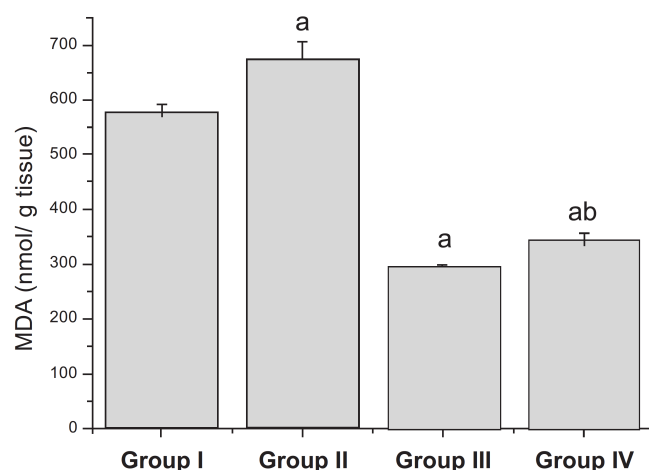


Fig. (1). The lipid peroxidation level of malondialdehyde formed in rat brains exposed to physalis juice and CCl_4 for 12 weeks. Values are the mean \pm SEM (n=7). ^a $p < 0.05$, significant change with respect to group I; ^b $p < 0.05$, significant change with respect to group II.

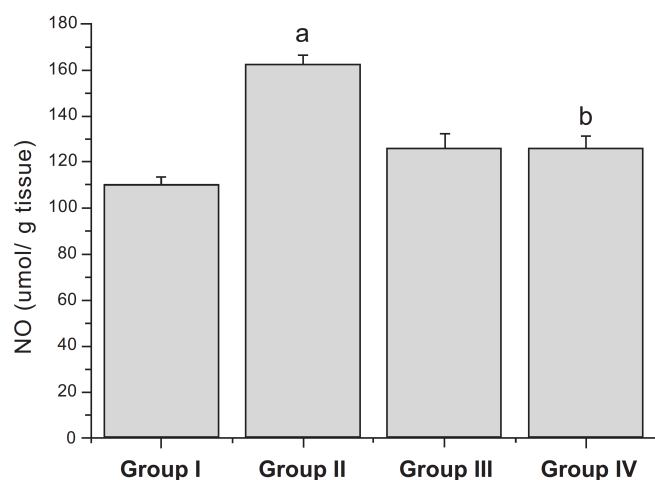


Fig. (2). The nitric oxide level in rat brains exposed to physalis juice and CCl_4 for 12 weeks. Values are the mean \pm SEM (n=7). ^a $p < 0.05$, significant change with respect to group I; ^b $p < 0.05$, significant change with respect to group II.

The data recorded in Table 1 and Fig. (3) showed that i.p injection of CCl_4 for 12 weeks significantly reduced ($p < 0.05$) the activities of GR, GST, GPx and GSH content compared to the control group. In the combined treated group (physalis and CCl_4), the activities of the antioxidant enzymes were increased significantly ($p < 0.05$) compared to the CCl_4 treated group, while a non-significant change was found compared to the control rats. This reflects the ameliorative effect of physalis against CCl_4 -induced oxidative damage in the brain. Moreover, physalis supplementation alone significantly ($p < 0.05$) demonstrated an increase at $p < 0.05$ in the all tested parameters.

One-way ANOVA demonstrated that, the injection of rats with CCl_4 exhibited a significant ($p < 0.05$) inhibition in SOD and CAT activities in brain tissues. Meanwhile, the concurrent treatment with physalis and CCl_4 produced a significant ($p < 0.05$) increase in the activity of SOD and CAT compared to the control and CCl_4 groups (Fig. 4). Our results indicate a marked protection by physalis juice against CCl_4 -induced neuronal damage. Moreover, the supplementation of

physalis alone showed a significant ($p<0.05$) elevation in both enzymes compared to the control levels.

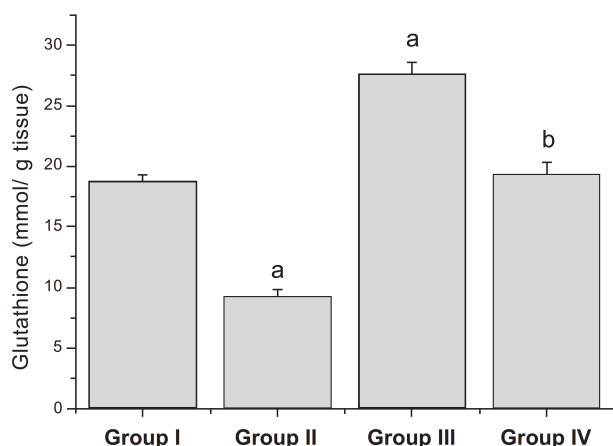


Fig. (3). The GSH content in rat brains exposed to physalis juice and CCl_4 for 12 weeks. Values are the mean \pm SEM ($n=7$). ^a $p<0.05$, significant change with respect to group I; ^b $p<0.05$, significant change with respect to group II.

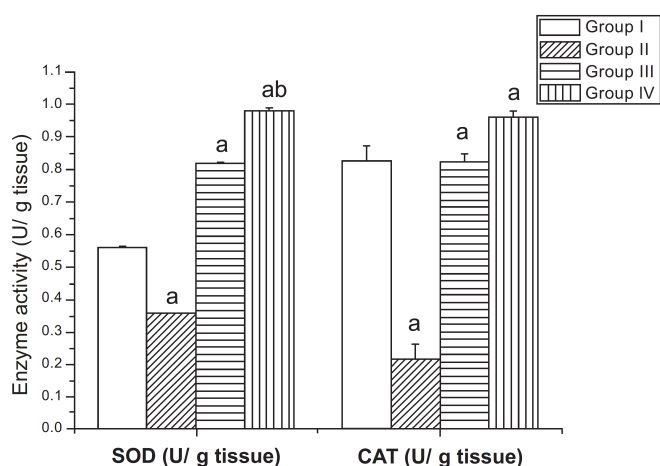


Fig. (4). SOD and CAT activities in rat brains exposed to physalis juice and CCl_4 for 12 weeks. Values are the mean \pm SEM ($n=7$). ^a $p<0.05$, significant change with respect to group I; ^b $p<0.05$, significant change with respect to group II.

The immunohistochemistry results showed that CCl_4 significantly downregulated Bcl-2 (anti-apoptotic protein) expression in different brain regions, namely the cerebellum, striatum and cerebral cortex (Fig. 5). However, physalis juice supplementation during CCl_4 treatment markedly upregulated CCl_4 -induced decreases in Bcl-2 expression. Physalis juice supplementation alone did not change Bcl-2 expression when compared with the control sections.

DISCUSSION

The brain is highly sensitive to oxidative stress [15, 16]. In the brain, low concentrations of the endogenous antioxidant component GSH and the antioxidant enzyme CAT, a high metabolic rate, and a high proportion of polyunsaturated fatty acids make this organ particularly susceptible to oxidative damage [16, 17]. In the present study, the neuronal damage induced by CCl_4 administration was evaluated. The possible protective role of physalis juice against CCl_4 -induced toxicity was examined by measuring the levels of antioxidant enzymes and oxidative stress markers in the brain tissues of adult male albino rats. Treatment with CCl_4 was associated with a disturbance in the oxidative status in the brain tissues. This was noticeable by the inhibition of the enzyme activities of GR, GST, GPx, SOD and CAT, and decreases the content of GSH. It was also accompanied by increased levels of MDA and NO.

CCl_4 requires bioactivation by the cytochrome P450 enzyme in the liver to yield a trichloromethyl free radical, which subsequently generates a trichloromethyl peroxy radical. PUFA are quite reactive toward free radicals and readily undergo LPO, which may cause cell membrane damage and alteration in enzyme activity [18]. MDA arises from the breakdown of lipid peroxy radicals, and it is commonly used as a biomarker of lipid peroxidation. It is one of the indicators of oxidative stress [19]. Cellular dehydration, whole cell deformity and cell death are observed in MDA accumulation [20]. Dani *et al.* reported that the liver is not the only target organ of CCl_4 ; the administration of CCl_4 also induced LPO and oxidative protein damage in the brain [21].

The cellular antioxidant defense system includes enzymes such as SOD, CAT, GPx, GR and GST. These antioxidant enzymes play a vital role during the process of scavenging ROS and preventing their formation [22]. Ohkawa *et al.* [10] demonstrated that the enhanced LPO associated with the depletion of antioxidants in the tissues is a characteristic observation in CCl_4 -intoxicated rats. Previous studies have reported that CCl_4 treatment caused a significant reduction in GSH content and SOD, CAT, GST and GPx activities in the liver, kidneys and brain of rats [23, 24]. Soliman and Fahmy [25] found that the administration of CCl_4 elevated the level of LPO and decreased the content of GSH and GST activity in the rat brain. The decreased enzymatic and non-enzymatic molecules might have contributed to the elevated protein and lipid oxidation levels during CCl_4 toxicity. The decrease in antioxidant enzyme activities after CCl_4 administration may have also resulted from inactivation caused by lipid peroxides which, led to tissue damage and failure of the antioxidant defense

Table 1. Antioxidant enzyme activity in rat brains exposed to physalis juice and CCl_4 for 12 weeks.

Groups	GR ($\mu\text{mol/g Tissue}$)	GST ($\mu\text{mol/g Tissue}$)	GPx (U/g Tissue)
Group I	82.39 \pm 5.27	0.21 \pm 0.007	1482.16 \pm 83.96
Group II	42.77 \pm 4.68 ^a	0.13 \pm 0.003 ^a	775.82 \pm 60.61 ^a
Group III	108.51 \pm 8.95 ^a	0.24 \pm 0.002 ^a	1945.34 \pm 61.19 ^a
Group IV	73.68 \pm 7.09 ^b	0.19 \pm 0.006 ^b	1268.67 \pm 54.74 ^b

Values are the mean \pm SEM ($n=7$). ^a $p<0.05$, significant change with respect to group I; ^b $p<0.05$, significant change with respect to group II.

mechanisms [26]. This may explain the decrease in the tested antioxidant enzymes activities in the current study.

In the present study, the level of NO increased in CCl₄ intoxicated rats. Previous studies showed that treatment with CCl₄ enhanced the generation of NO in the rat brain [27, 28], which may be due to the upregulation of inducible nitric oxide synthase expression [24]. NO can exacerbate oxidative stress by reacting with ROS, particularly with superoxide to form the much more powerful oxidant peroxynitrite, causing inhibition in the key enzymes of energy metabolism, DNA damage and depletion of intracellular glutathione [29]. In addition, Prezedborski *et al.* [30] reported NO neurotoxicity under conditions of excessive production.

SOD and CAT play an important role in the antioxidant defense system. The inhibition of SOD and CAT may result in an increased flux of superoxide in cellular compartments which may be the reason for the increased lipid peroxidation indices in our study. GSH is a major a non-enzymatic antioxidant both intracellularly and extracellularly acting against xenobiotics and neutralizing ROS. Evidence is growing that glutathione plays an important role in the detoxification of ROS in the brain [31]. Therefore, the level of GSH in the body is considered an important indicator of its antioxidative capacity. Disturbance in the GSH status in the biological system have been reported to lead to serious consequences [19]. Oxidative stress is known to lower GSH content [32]. In the present experiment, the decrease in GSH content in CCl₄ treated rats was observed. This depletion in GSH content might have contributed to the enhanced lipid peroxidation.

In recent years, ample interest has focused on developing therapeutically effective agents from commonly available natural products [16, 33]. Polyphenols possess diverse biological activities and are thought to be beneficial in treating cell damage induced by free radicals. The antioxidant activity of polyphenols might be related to their ability to scavenge free radicals by their hydroxyl groups [34]. In addition, polyphenols are known transcriptional regulators, thus mediating long-term effects [35]. The present study showed that rats treated with physalis juice showed an increase in enzymatic and non-enzymatic molecules (GSH, GST, GPx, GR, SOD and CAT) and reductions in the enhanced MDA and NO levels were observed in CCl₄-intoxicated rats.

The active ingredients in physalis are not clear however, phytochemical analyses showed that *Physalis* species contain high levels of polyphenols, vitamins A and C, witanolides, physalins, calystegines, tropane and nortropane alkaloids. It is also possible that these active compounds may have biological significance in the elimination of reactive free radicals [36]. Antioxidants play an important role in the regulation and maintenance of the antioxidant defense system against free radicals [37, 38]. *Physalis* species also have the ability to suppress lipid peroxidation [39, 40]. In addition, our group and others demonstrated the inhibitory effects of physalis in scavenging NO radicals *in vitro* [4]. In the present study, the higher levels of GSH in the physalis juice treated group suggested that physalis might have protected the brain by neutralizing free radicals leading to the inhibition of CCl₄-induced neuronal damage.

A previous study reported that pre-treatment with water extract of *Physalis peruviana* led to an increase in SOD, CAT and GPx activity in the liver of acetaminophne-treated rats [37]. The degradation of superoxide radicals can be neuroprotective. This has been shown with the transgenic overexpression of SOD [37]. Wang *et al.* [41] demonstrated that the overexpression of SOD and CAT genes plays a neuroprotective role in the brain against oxidative damage. In addition, the overexpression of GPx prevents neuron loss and reduces H₂O₂ accumulation and lipid peroxidation under oxidative stress conditions [41]. Moreover, the increase in GST activity prevents the development of oxidative injury [42].

Furthermore, previously published data from our group suggest that physalis juice has the ability to suppress matrix metalloproteinase-9 (MMP-9). The increased expression and activation of MMP-9 is likely to play critical roles in excitotoxicity-induced disruption of cell-matrix homeostasis and neuronal cell death. MMP-9 hyperactivity is associated with an increase in blood-brain barrier permeability, which results in brain edema and hemorrhage, and contributes directly to neuronal injury, apoptosis and brain damage after acute cerebral ischemia [43]. Moreover, MMP-9 may contribute to the pathogenesis of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis by inducing neuronal death [44, 45].

In this study, we also demonstrated that physalis juice upregulated the expression of Bcl-2 protein in the brains of rats exposed to CCl₄ that downregulated it in rats receiving CCl₄ alone, suggesting that oxidative stress caused by CCl₄ injection downregulated Bcl-2 protein content. CCl₄ is a common hepatotoxin used in liver injury experiments. Studies by other authors showed that the damage induced by CCl₄ in the liver and other organs is partly involved in the apoptosis pathway *in vivo* through the mitochondrial pathway. Mitochondria are known to be a vulnerable target of various toxins and oxidative stress. The mitochondrial apoptotic pathway is regulated by the Bcl-2 family of proteins [46]. Therefore, our results also confirm that physalis may inhibit apoptosis by increasing the expression levels of proteins associated with anti-apoptosis.

Furthermore, we found that CCl₄-induced neuronal damage displayed traits typical of cell death (data not shown). The pattern of cell death observed in the cerebral cortex of the CCl₄ treated rats included neurodegenerative features of neuronal necrosis with enlarged cell bodies and distorted membranes, and the degeneration of the cytoplasm and nuclear materials was also observed. Physalis juice improved the damage induced by CCl₄ administration, but some neurons still degenerated.

In conclusion, exposure to CCl₄ generates free radicals, which results in the elevation of neuronal lipid peroxidation and nitric oxide levels and reduces in the enzymatic and non-enzymatic antioxidant components. However, the oxidative stress elicited by CCl₄ intoxication is nullified by the protective effect of physalis juice by increasing the activities of antioxidant enzymes, thereby inhibiting lipid peroxidation and nitric oxide generation. Furthermore, physalis juice exerts its beneficial effects on CCl₄-induced apoptosis in the brain by modulating Bcl-2 expression.

AUTHORS' CONTRIBUTIONS

AA made a significant contribution to the conception and design of the study, the acquisition and analysis of data and drafting the manuscript. ME, RK, SA, MS and EA contributed to drafting the manuscript. All of the authors read and approved the revised manuscript.

LIST OF ABBREVIATIONS

CAT	= Catalase
CCl ₄	= Carbon tetrachloride
GSH	= Glutathione
GPx	= Glutathione peroxidase
GST	= Glutathione-S-transferase
GR	= Glutathione reductase
LPO	= Lipid peroxidation
MDA	= Malondialdehyde
NO	= Nitric oxide
ROS	= Reactive oxygen species
SOD	= Superoxide dismutase

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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