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ORIGINAL ARTICLE

Cucurbita argyrosperma Seed Extract Ameliorating Oxidative Stress in H9c2 Cardiomyocytes through Suppression of Intracellular Reactive Oxygen Species Production

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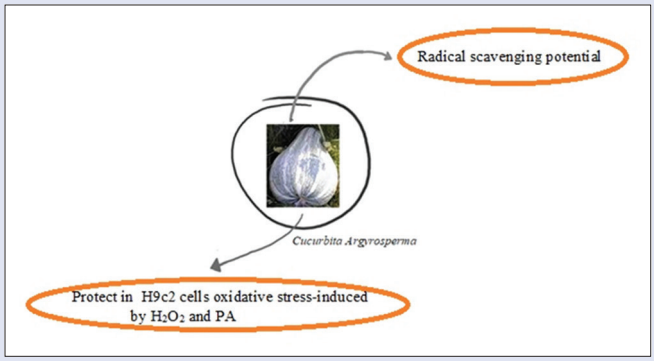
ABSTRACT

Background: Oxidative stress induced by the excessive reactive oxygen species (ROS) generation is involved in the pathogenesis of various diseases. Methanol extract from *Cucurbita argyrosperma* (CM) was used to test for antioxidant and antioxidative stress. **Materials and Methods:** The radical scavenging potential was determined using eight different *in vitro* assays: 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical, β-carotene-linoleic acid, chelating activity, ferric reducing antioxidant power (FRAP), hydroxyl radical, nitric oxide (NO) radical, and superoxide anion radicals, palmitate (PA) on H9c2 cells, and H_2O_2-induced cell damage on H9c2 cardiomyocytes. **Results:** CM showed antioxidant activity with DPPH radical values of EC_{50} 7.6 ± 5.38 mg/mL, β-carotene-linoleic acid EC_{50} of 279.4 ± 36.32 mg/ml, chelating activity EC_{50} of 301.2 ± 9.78 mg/ml, FRAP EC_{50} of 101.3 ± 3.10 mg/ml, hydroxyl EC_{50} of 42.6 ± 8.6 mg/mL, superoxide EC_{50} of 50.6 ± 8.66 mg/ml, NO EC_{50} of 101.2 ± 19.74 mg/ml, and ABTS EC_{50} of 13.2 ± 4.9 mg/ml. The methanol extract preincubation decreases the intracellular ROS induced by H_2O_2 and PA in H9c2 cells in a concentration-dependent manner. The methanol extract could also protect H9c2 cells and inhibit the activity of xanthine oxidase, malondialdehyde, and lactate dehydrogenase which increases cell viability, heme oxygenase-1, glutathione/oxidized glutathione, superoxide dismutase-1, catalase, and GSH-Px? levels. **Conclusions:** The finding suggests that methanol extract can protect the oxidative stress-induced cardiomyocyte damage through ROS regulation and can be used as a therapeutic agent for the improvement of oxidative stress in various diseases.

Key words: Antioxidant, *Cucurbita argyrosperma,* free radical, oxidative stress

SUMMARY

- *Cucurbita argyrosperma* seed showed antioxidant activity using different *in vitro* assays
- *C. argyrosperma* seed decreases the intracellular reactive oxygen species induced by H_2O_2 and palmitate in H9c2 cells
- *C. argyrosperma* seed increases cell viability, heme oxygenase-1, glutathione/oxidized glutathione, superoxide dismutase-1, catalase, and GPx levels
- *C. argyrosperma* seed inhibits the activity of xanthine oxidase, malondialdehyde, and lactate dehydrogenase.



Abbreviations used: ABTS radical: 2,2′-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; CAT: Catalase; DCFH-DA: 2′,7′-dichlorodihydrofluorescein diacetate; DMSO: Dimethyl sulfoxide; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DPPH: 1,1-diphenyl-2-picrylhydrazyl; DCFH-DA: 2′,7′-dichlorofluorescin diacetate; FRAP: Ferric reducing antioxidant power; GSH: Glutathione; GSH-Px: Glutathione peroxidase; HO-1: Heme oxygenase-1; OH^-: Hydroxyl; LDH: Lactate dehydrogenase; LOOH: Lipid peroxide; $LOO^•$: Lipid peroxyl; MDA: Malondialdehyde; NO: Nitric oxide; GSSG: Oxidized glutathione; O_2^{-1}: Oxygen; O_3 Ozone; PA: Palmitate; RO_2^-: Peroxyl; PBS: Phosphate-buffered saline; ROS: Reactive oxygen species; $O_2^•$: Superoxide; SOD: Superoxide dismutase; TFs: Total flavonoids; TP: Total phenolic; TCA: Trichloroacetic acid; XO: Xanthine oxidase.

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INTRODUCTION

Reactive oxygen species (ROS) are products of normal metabolism. These species are known to play a dual role in biological systems, since they can be either harmful or beneficial to living systems. To encompass the harmful effects of ROS, the body has natural antioxidants that are the cell's defense mechanisms. Thus, there is an immense interest in natural antioxidants and their role in human

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health and nutrition.[1] The imbalance between ROS production and antioxidant defense results in oxidative stress and the overproduction of reactive ROS leading to lipid peroxidation, protein denaturation, and disruption of membrane fluidity, which can lead to cell damage and death.[2] ROS consist mainly of free radicals such as nitric oxide (NO), superoxide ($O_2^{\bullet}$), lipid peroxyl (LOO•), peroxyl (RO_2–), hydroxyl (OH^-) and different forms of activated oxygen such as lipid peroxide (LOOH), ozone (O_3), and oxygen (O_2^{-1}). Dietary antioxidants may influence lipid metabolism, inhibit low-density lipoprotein oxidation, reduce blood pressure, inhibit platelet aggregation, as well as enhance endothelial function.[3] In response to the demand by consumers of food supplements free of synthetic antioxidants with toxic potential, there is an overwhelming tendency to search natural sources of antioxidants.[4]

Pumpkin (*Cucurbita argyrosperma*) is a member of the Cucurbitaceae family that is widely cultivated in Mexico and has been used as food since ancient times.[5] Scientific studies indicated that several pumpkins (*Cucurbita pepo, Cucurbita moschata, Cucurbita maxima, and Cucurbita ficifolia)* have a wide range of pharmacological activities, such as anthelmintic properties,[6] anti-gastrointestinal nematodes,[7] anticancer, anti-inflammatory, and antioxidant activities,[8] amelioration dyslipidemia, hypertension, and blood sugar in type 2 diabetes.[9]

C. argyrosperma, commonly known as calabaza in Mexico, is a member of Cucurbitaceae family and has been widely cultivated and used in folk medicine and as food since ancient time. Previous studies have shown that *C. argyrosperma* seeds can ameliorate, prostate diseases, diabetes, and inflammation diseases.[10] The seed oil has pharmaceutical uses for its bioactive components including sterols, unsaturated fatty acids, carotenoids, tocopherols, and squalene. Despite this, there is an insufficient scientific study on this type of pumpkin in the popular consumption *C. argyrosperma* seeds. The aim of the present study was to establish the antioxidant potential of *C. argyrosperma* seeds.

MATERIALS AND METHODS

Plant material

Fresh *C. argyrosperma* was collected in Amecameca, Mexico State. Voucher specimen (6728) is being kept in the Herbarium of the National School of Biological Sciences, Mexico (ENCB, IPN).

Preparation of the plant extract (CM)

Thousand grams of *C. argyrosperma* seeds were dried and powdered in a mechanical grinder that was exhaustively extracted with hexane (5 L) at room temperature. Then, 5 L of methanol was added, and the mixture was refluxed for 3 h and filtered using Whatman filter paper. The extraction was repeated twice. The filtrates were combined and concentrated using a rotary evaporator at 40°–50°C for complete removal of solvent to obtain an oil (41.8 g) that was weighed and kept in tubes wrapped with aluminum foil to reduce the risk of oxidation and stored in deep freeze.

Estimation of total phenolic and total flavonoid

The total phenolic (TP) of the different samples was determined using a modified colorimetric Folin–Ciocalteu method.[11] Gallic acid solution was used as reference substance for standard curve. TP in the samples was calculated from the standard curve, and the results were expressed as milligrams of gallic acid equivalents per gram (mg GAE/g) of extract. The total flavonoids (TFs) of the different samples were also determined using the aluminum chloride colorimetric assay and expressed as milligrams of rutin equivalents per gram (mg RE/g) of extract. All determinations were done in triplicates and averaged.[12]

In vitro antioxidant assays

The scavenging activities of methanol extract were evaluated at various concentrations of 10, 25, 50, 100, 200, and 300 mg/mL, namely 1,1-diphenyl-2-picrylhydrazyl (DPPH),[13] 2,2'-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS radical),[14] β-carotene-linoleic acid,[15] chelating activity,[16] ferric reducing antioxidant power (FRAP),[17] hydroxyl radical,[18] NO radical,[18] and superoxide anion radical[19] assays.

Lipid peroxidation inhibition

Lipid peroxidation was determined using the method of Xie *et al.*[20] Briefly, phosphate-buffered saline (PBS, pH 7.4, 0.2 M) and egg yolk (1:25, v/v) were stirred vigorously for 5 min. Then, 1.0 mL of CM solutions was mixed with 0.2 mL of $FeSO_4$ (25 mM) and 0.5 mL of egg homogenates and afterward incubated for 30 min at 37°C. Then, 0.1 mL of trichloroacetic acid was incorporated and allowed to stand for 5 min at room temperature followed by heat treatment for 15 min at 100°C and centrifugation at 8000 rpm for 5 min; the absorbance of mixture was recorded at 532nm, distilled water was used as the blank control, and ascorbic acid was used as a positive control.

Inhibitory activity of xanthine oxidase

The xanthine oxidase (XO) inhibitory activity of the CM extract was evaluated by the rate of hydroxylation of the xanthine (substrate) into uric acid.[21] The reaction mixture contained 150 µl of phosphate buffer (50 mM, pH 7.4), 10 µl of the extract dissolved in dimethyl sulfoxide (DMSO), as well as substrate 20 µl of 0.1 mM xanthine and 0.003 units of XO dissolved in 20 µl of buffer. Then, the mixture was incubated at room temperature for 10 min and the absorbance was measured at 295 nm.

Cell culture

H9c2 rat heart-derived embryonic myocytes were used in this study. CRL-1446 (American Type Culture Collection, Manassas, VA, USA) was cultured using 2-mM L-glutamine, 100-mg/ml streptomycin, 100-U/ml penicillin G, and Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum 10% (v/v). The cells were incubated with 95% air and 5% CO_2 at 37°C. All experiments were repeated three times.

Cell viability assay

The H9c2 cardiomyocytes were cultured at a density of 2000 cells per well in 96-well plates and incubated for 24 h. The solution of CM (0.1-, 0.5-, and 1-µg) was prepared in deionized sterile water and further filtered through a 0.22 µm Millipore filter. Stress conditions in cardiomyocytes were induced with 500-µM H_2O_2. Cardiomyocytes cells were seeded in wells and preincubated for 24 h with or without different concentrations of CM, followed by incubation with H_2O_2 for 24 h. The number of viable cells was determined with the adition of 0.5-mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis MO, USA) cell proliferation reagent and incubated for 2 h at 37°C. The supernatants were then removed and the formazan crystals were solubilized with 150-µl DMSO. Absorbance was read at 490 nm on a microplate reader (Thermo Fisher Scientific, FL, USA). Viability was expressed as the percentage in each treatment group with reference to that of the nontreated control.

Detection of reactive oxygen species generation in cardiomyocytes with stimulation of palmitate

Evaluation of free radicals in H9c2 cells after palmitate (PA) stimulation was measured using 2',7'-dichlorodihydrofluorescein diacetate (DCFHDA) assay kits (Cosmo Bio, USA). After the H9c2 cells were left for adherence for 12 h, CM was added at concentrations of 1, 5, and 10 μg/ml 1 h before 500-μM PA addition in all assays. Then, the cells were stimulated, washed with PBS, and incubated in fresh culture medium containing 2-μM DCFH-DA at 37°C for 30 min and were washed twice. Then, was collected in 1-ml PBS, and the fluorescence intensity was recorded by microplate reader (Thermo Fisher Scientific, FL, USA) with excitation wavelengths at 535 nm and emission at 488 nm (UV-1800, Shimadzu, Kyoto, Japan).[22]

Glutathione/oxidized glutathione evaluation

The H9c2 cells were stimulated with 500-μm PA for 10 h. After cells were lysed, 30 μl of collected proteins was used to measured reduced glutathione (GSH)/oxidized glutathione (GSSG) using a GSH/GSSG assay kit (Abcam, Cambridge, UK) according to the manufacturer's instruction.

Effect of CM on levels of superoxide radical (superoxide dismutase) and heme oxygenase-1 in cardiomyocytes incubated with palmitate

In the supernatants, levels of heme oxygenase-1 (HO-1) and superoxide dismutase (SOD) were detected using ELISA kits according to the manufacturer's instruction (Cayman Chemical, MI, USA). The absorbance was measured at 450 nm with a microplate reader (Thermo Fisher Scientific, FL, USA).

Detection of reactive oxygen species generation in the cardiomyocytes under oxidative stress induced by H_2O_2

In addition, 1×10^5 cells per well in 96-well plates were treated with 500-μM H_2O_2 alone or pretreated for 24 h with 1, 5, and 10 μg/ml of CM followed by the addition of 500-μM H_2O_2. After 24 h, cells were treated with 1-μM 2',7'-dichlorofluorescin diacetate (DCFH-DA, Sigma-Aldrich, St Louis MI, USA) at 37°C for 30 min. The fluorescence intensity was calculated at excitation and emission wavelengths of 488 nm and 515 nm.

Evaluation of superoxide dismutase, glutathione peroxidase, catalase, and malondialdehyde activities

After treatment, the cells were washed twice with cold PBS, collected with 0.25% trypsin, and centrifuged for 10 min at 1000 ×g. The cell pellet was sonicated in cold PBS and the supernatant was discarded. After centrifugation for 5 min at 800 ×g, the supernatant was immediately evaluated for SOD, glutathione peroxidase (GSH-Px), malondialdehyde (MDA), and catalase (CAT) using commercial kits (Cayman Chemical, MI, USA) according to the manufacturer's instructions.

Measurement of lactate dehydrogenase activity

Lactate dehydrogenase (LDH) activity was determined as LDH released in the culture medium. Briefly, after treatment, 0.2-ml culture medium of each group was evaluated for LDH activity using commercial kit (Cayman Chemical, MI, USA).

Statistical analysis

All data were expressed as mean ± standard deviation. Statistical analysis was performed by one-way ANOVA followed using Tukey's multiple comparison tests. Statistical analysis expressed as $P < 0.05$

RESULTS

Total phenolic and flavonoid content

The amounts of TP and TF in the methanolic extract of the CM extract are shown in Table 1. Pumpkin seeds are a good source of antioxidants due to the presence of phenolic compounds and tocopherols[23] that are used in traditional medicine. The values of TP (23.78 mg GAE/g CM) and TF (12.1 mg RE/g CM) found in our work [Table 1] are in agreement with previous studies of other *Cucurbita* species. In particular, some studies showed values to TP for 18.9 mg GAE/g and 10 mg RE/g for TF in squash pumpkin.[24] Another study was discovered that pumpkin had TPs of 25.3 mg GAE/g.[25] Bahramsoltani *et al.*[26] established 26.2 mg GAE/g for TP in *C. pepo*.

In vitro antioxidant activity

Table 2 shows a comparative antioxidant analysis of EC_{50} values of the CM with standards such as quercetin, ascorbic acid, ethylenediaminetetraacetic acid (EDTA), curcumin, trolox, butylated hydroxyanisole (BHA), and gallic acid using the DPPH radical, β-carotene-linoleic acid, chelating activity, reducing power, hydroxyl, superoxide, NO, and ABTS assays. The results clearly indicate that CM has a good potency toward free radical test compared to the standards.

Inhibition of lipid peroxidation

Figure 1a shows the effect of CM on lipid peroxidation inhibition capacity. Finding revealed that CM displayed inhibition of lipid peroxidation with increased concentration. CM showed the maximum inhibitory capacity of 68% at 300 mg/ml. The lipid peroxidation inhibition capacity of CM was less than the activity of the ascorbic acid used as a standard.

xanthine oxidase inhibitory activity

The methanol extract of *C. argyrosperma* exhibited a pronounced suppression of XO activity with a 54% enzyme inhibition at a concentration of 50 μg/ml with IC_{50} value of 49.6 μg/ml as compared to the drug allopurinol being used as reference with an IC_{50} = 117.2 μg/ml [Figure 1b]. Allopurinol, used as reference, is an inhibitor of XO that links to the active site of this enzyme, thereby producing its inhibition.

CM promotion the cytoprotective effect in H9c2 cardiomyocytes against palmitate-induced reactive oxygen species production

DCFH-HA after a concentration of 500 lM PA stimulates ROS production and increases oxidative stress in H9c2 cells. Pretreatment with CM extract at 1, 5, and 10 μg/ml significantly reduced ROS

Table 1: Total phenolic and flavonoid contents of CM

	Polyphenols (mg GAE/g CM)	Flavonoid (mg RE/g CM)
CM	23.78±7.06	12±2.13

CM: *Cucurbita argyrosperma* methanolic extract; GAE: gallic acid equivalents; RE: Rutin equivalent

Table 2: Antioxidant activity of *Cucurbita argyrosperma* extracts in the performed assays

Sample	DPPH radical EC_{50} (mg/mL)	β-Carotene-linoleic EC_{50} (mg/mL)	Chelating activity EC_{50} (mg/mL)	FRAP EC_{50} (mg/mL)	Hydroxyl EC_{50} (mg/mL)	Superoxide EC_{50} (mg/mL)	NO EC_{50} (mg/mL)	ABTS EC_{50} (mg/mL)
CM	7.6±5.38	279.4±36.32	301.2±9.78	101.3±3.10	42.6±8.6	50.6±8.66	101.2±19.74	13.2±4.9
Standard						EC_{50} (µg/mL)		
Quercetin	2.39±0.01	10.1±1.35	221.9±12.64	2.1±0.19	-	42.6±5.71	-	-
Ascorbic acid	4.5±0.19	849.5±23.19	2.7±24.19	7.5±2.34	-	-	-	-
EDTA	-	-	5.7±1.07	-	-	-	-	-
Gallic acid	-	-	-	-	16.7±4.21	7.9±1.78		-
Curcumin	-	-	-	90.23±12.28	-	-	91.9±23.16	-
Trolox	4.2±0.91	-	27.3±6.12	-	35.5±3.5	-	-	9.0±2.05
BHA	0.43±0.002	15.1±2.24	282±15.4	7.68±2.60	26.85±7.3	-	-	7.2±2.32

CM: Methanol extract from seeds of *C. argyrosperma*. The EC_{50} is the concentration of a drug that gives half-maximal response

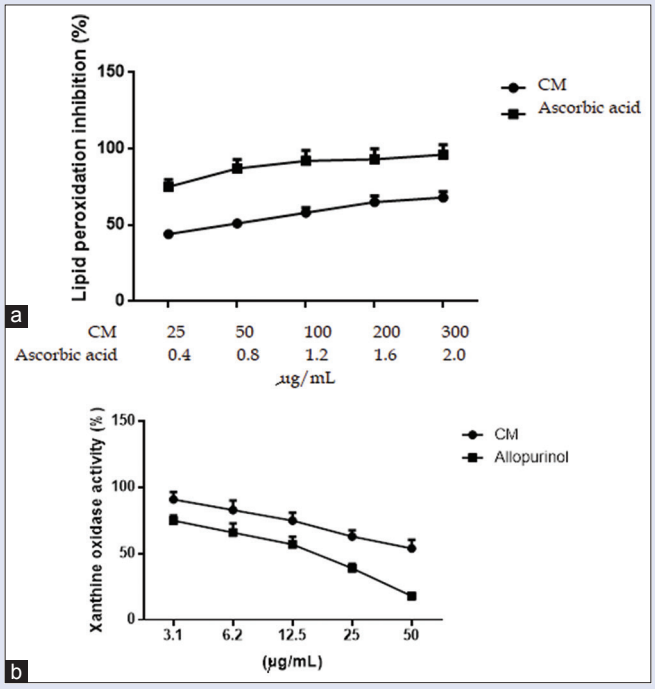

Figure 1: Lipid peroxidation inhibition ability (a); inhibitory effect of CM extract xanthine oxidase (b). Values were performed as means of three replicate determinations ± standard deviation (SD)

production by 25%, 39%, and 48% compared to those of the control [Figure 2a].

Increase in superoxide dismutase, heme oxygenase-1, and glutathione/oxidized glutathione in association with the cytoprotection of CM against palmitate-induced reactive oxygen species

CM increased the level of SOD production in supernatant of the treated H9c2 cells treated at concentrations of 1, 5, and 10 µg/ml which were found to be 2.8, 3.9, and 4.3 times, respectively [Figure 2b]. However, level of the HO-1 in cells treated at concentrations of 1, 5, and 10 µg/ml was found to be 1.22, 1.34, and 1.39 times than those of the control [Figure 2c]. The reduced/oxidized GSH rate was evaluated effect of CM on antioxidant variables in the *in vitro*. The results of the effect of MC on GSH/GSSG at concentration of 10 µg/ml are displayed in Figure 2d.

CM suppressing H_2O_2-induced oxidative stress in H9c2 cardiomyocytes

CM was assayed at different concentrations of 1, 5, and 10 µg/ml against H_2O_2-induced cell apoptosis [Figure 3a]. Without CM, only 47% H9c2 cells survived at 500-µM H_2O_2 compared with H_2O_2-treated group. Nevertheless, pretreatment with 1-, 5-, and 10-µg/ml CM for 24 h significantly increased the viability of H_2O_2-treated H9c2 cardiomyocytes in a dose-dependent manner with values of 72.1%, 84.6%, and 95.8%, respectively. At 10 µg/ml of CM, the percentage of viable cells (95.8%) was significantly ($P < 0.05$) higher than that of the H_2O_2 control group. The results are shown in Figure 3a which indicates that CM avoids oxidative stress-induced cell mortality by H_2O_2 without presenting cytotoxic effects. After preincubation of CM at different concentrations of 1, 5, and 10 µg/ml for 2 h, the H9c2 cells were exposed for 10 min to H_2O_2 and then subjected a DCFH-DA test. The finding reveals that H_2O_2 challenge markedly increases intracellular ROS content to 142.3% ($P < 0.001$) as compared with the control [Figure 3b]. The preincubation of CM at a concentration in a dependent manner reduces the increase in intracellular ROS induced by H_2O_2 132.6%, 105.4%, and 91.4%, respectively.

Effect of CM on lactate dehydrogenase release and malondialdehyde activity

LDH release increases with H_2O_2 exposure leading to severe cell damage [Figure 3c]. LDH activity in H9c2 cells with H_2O_2 exposure was significantly higher than that in the control group ($P < 0.05$). CM pretreatment significantly inhibited the levels of LDH in a dose-dependent manner. CM pretreatment reduced MDA activity [Figure 2d] and also improved the loss of antioxidant enzymes caused by H_2O_2 exposure, such as CAT, SOD, and GSH-Px with increase in a dose-dependent manner [Figure 4a-c, respectively].

DISCUSSION

In the present study, we have demonstrated that *C. argyrosperma* has a considerable antioxidant capacity and exhibits a very safe profile in H9c2 cardiac cells because it does not exert any cytotoxicity within the test concentration (0.1, 0.5, and 1 µg CM). The radical scavenging potential plays an important role in the damage caused by the free radicals produced in biological systems and foods. The main mechanisms through which antioxidants can act are single-electron transfer, hydrogen atom transfer, and metal chelation.[27,28] Therefore, it is important to evaluate plant extracts using several assays rather than single assay.[25] To determine the antioxidant capacity of *C. argyrosperma* methanol extract, the following analysis was performed:

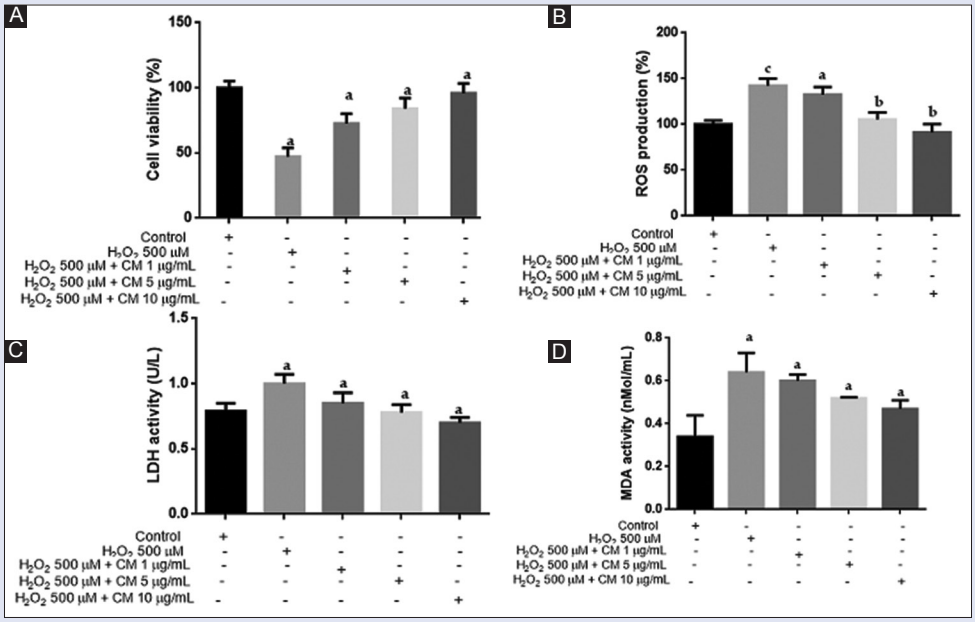

Figure 2: (A) Cell viability measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; (B) reactive oxygen species production measured using DCFH-DA assay; (C) lactate dehydrogenase activity; (D) malondialdehyde activity. (a) $P < 0.05$, (b) $P < 0.01$, (c) $P < 0.001$ compared to the control

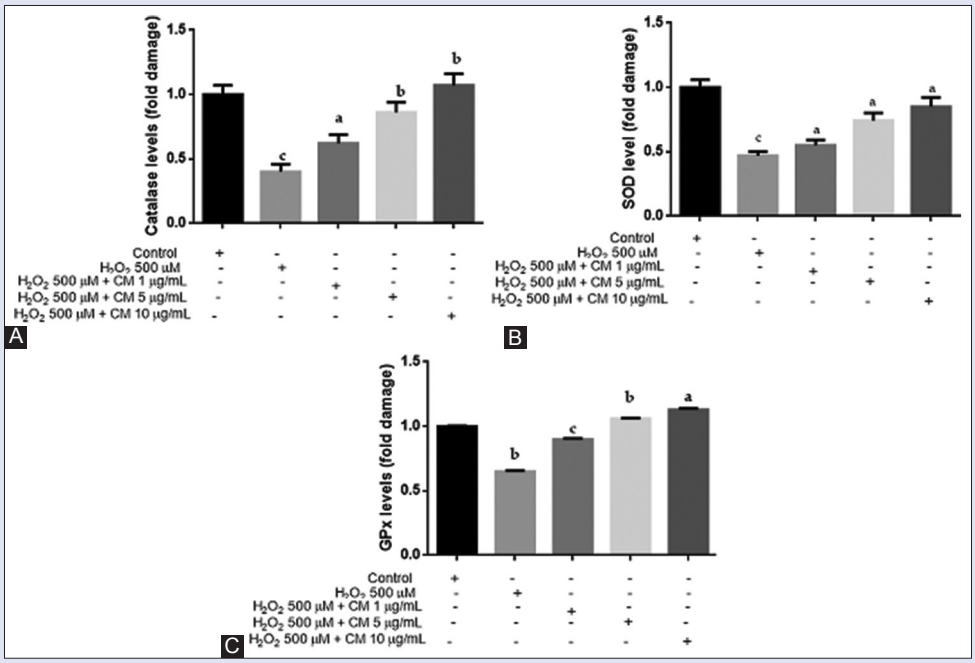

Figure 3: (A) Catalase levels; (B) superoxide dismutase levels; (C) GPx levels. (a) $P < 0.05$, (b) $P < 0.01$, (c) $P < 0.001$ compared to the control

DPPH radical, ABTS radical, β-carotene-linoleic acid, chelating activity, FRAP, hydroxyl radical, NO radical, and superoxide anion radical scavenging.

The DPPH radical is the most commonly used test to measure of antioxidant experiments because it accepts a hydrogen radical or electron and has good stability at room temperature. CM demonstrated good antiradical effect, although lower than the activity of the standard controls. The possible mechanism of C. *argyrosperma* methanol extract could be due to the electron donation ability to the free radicals, completing the radical chain reaction increasing the half-life of natural antioxidants.[29]

C. argyrosperma antioxidant potential was also evaluated using decolorization of ABTS•+ cation radical which is an assay associated with lipid peroxidations. Has been widely used due to its applicability in lipid and aqueous phase.[25] Spectrophotometric assays have shown that *C. argyrosperma* has modest chelating ability toward ABTS•+ cation radical in comparison with BHA and trolox.

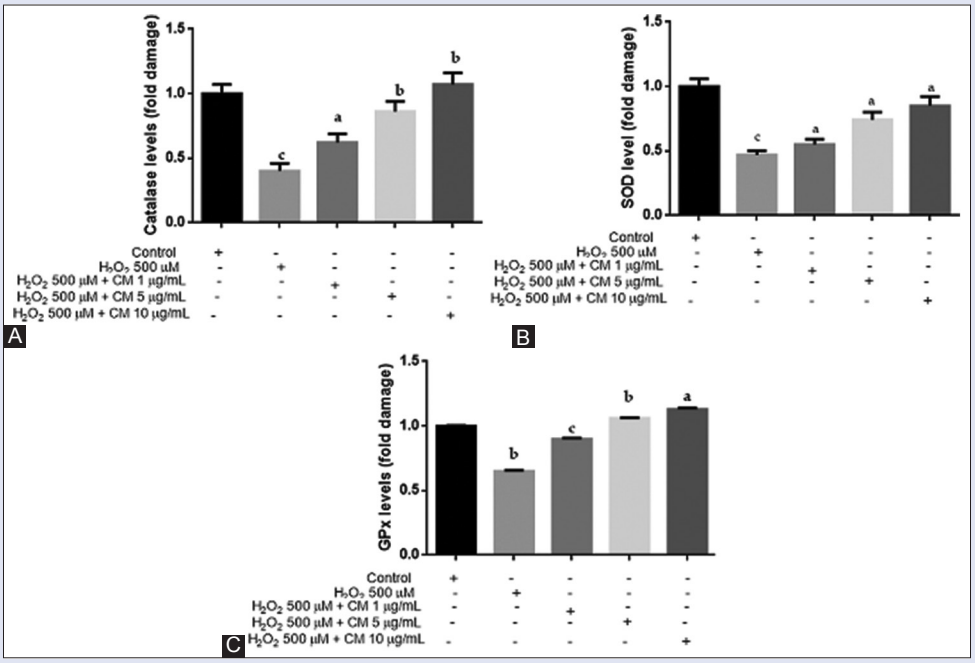

Figure 4: (A) Catalase levels; (B) superoxide dismutase levels; (C) GPx levels. (a) $P < 0.05$, (b) $P < 0.01$, (c) $P < 0.001$ compared to the control

Metal chelating ability is an antioxidant mechanism, which in the lipid peroxidation processes inhibits the transition-metal catalysis. In the Fenton reaction, Fe^{2+} interacts with H_2O_2 to generate ROS to accelerate oxidation process.[30] Poorly liganded or free iron is known to be a source of many diseases related to apoptosis and cell death. Thus, the chelating ability of CM toward ferrous ions was studied. CM has modest chelating ability toward ferrous ions in comparison with ascorbic acid, EDTA, and trolox; nevertheless, they demonstrated similar results to quercetin and BHA.

The hydroxyl radical (•OH) causes polymerization. auto-oxidation, and fragmentation of biological molecules. The plants that contain hydroxyl radical scavengers would be important for some disorder developed by oxidative stress. Therefore, hydroxyl radical scavenging activity of the methanol extract from *C. argyrosperma* is evaluated in this study.[31] Our results show that *C. argyrosperma* has the ability to inhibit hydroxyl radical, prevent the reaction with phospholipids and polyunsaturated fatty acid moieties of cell membrane, and avoid injury to cells. The extract showed a good *in vitro* antioxidant effect toward hydroxyl radical scavenging activity than the antioxidants such as gallic acid, trolox, and BHA at tested concentrations.

NO is associated with several pathological conditions.[32] The extract has ability to counteract the formation of NO, by executing similar free radical scavenging activity against NO as suggested in its EC_{50} values compared to curcumin being used as standard.

Superoxide anions showed a limited chemical reactivity and a weak oxidative activity. However, they can generate dangerous species of hydroxyl radicals and singlet oxygen that combines with other biomolecules can indirectly induce lipid peroxidation[33] extract showing less activity against gallic acid and quercetin.

Discoloration of β-carotene is also used to determine the antioxidant activity of bioactive compounds. In this test, the oxidation of linoleic acid produces hydroperoxyl radicals that develop into volatile by-products, conjugated dienes, and lipid hydroperoxides which simultaneously react with the chromophore of the β-carotene, resulting in bleaching of the reaction emulsion.[33] The presence of CM hinders the degree of bleaching by neutralizing the linoleic hydroperoxyl radicals formed. The hydroperoxides formed in this system will be decomposed by the antioxidants content in the *C. argyrosperma* extract.

The transformation of Fe^{3+} into Fe^{2+} in the presence of CM was used to measure the reducing power ability which could be due to the presence of reducers that break the free radical chain by donating a hydrogen atom.[34] The antioxidant content in the CM results in the reduction of Fe^{3+}/ferricyanide complexes to the ferrous form, thus demonstrating the reducing power ability. The reductive ability of CM was lower than the activities of quercetin, ascorbic acid, and BHA. However, CM did not differ significantly in activity with curcumin.

XO is capable of producing ROS such as hydrogen peroxide and superoxide radicals that can led to oxidative stress.[35,36] The assay was used to evaluate the potential of CM to inhibit XO effect in comparison to allopurinol, which is a clinically used inhibitor in the treatment of XO-related disorders and gout. Thus, CM with antioxidant properties is effective in inhibiting ROS generated by XO.

Oxidative stress indicates an increased in ROS generation and/or a reduction in its elimination which can be produced as normal products of aerobic metabolism but can be in high quantities under pathophysiological conditions. Pretreatment of H9c2 cells with the *C. argyrosperma* extract resulted in a concentration-dependent protective effect against palmitic acid. PA is involved in various metabolic pathways in H9c2 cells. An increased palmitic acid allows it to cross the cell membrane and enter mitochondria, where it undergoes oxidative reactions.[37] The finding shows that palmitic acid produces a cytotoxic effect in H9c2 cardiomyocytes and decreases HO-1, SOD protein levels, and GSH/GSSG ratio. Interestingly, *C. argyrosperma* can increase HO-1, SOD protein levels, and GSH/GSSG ratio confirming it antioxidant capacity in H9c2 cells.

To confirm the antioxidant properties of CM, H_2O_2-induced oxidative damage on H9c2 cells was also evaluated. H_2O_2 exposure activates oxidative stress in cells, which is characterized by reduced intracellular antioxidant activity, cell viability, and increased intracellular ROS and lipid peroxidation. The results of this study indicated that CM significantly reduces cell death caused

by H_2O_2 in H9c2 cells *in vitro* and the protective effect exerted by CM may be due to its ability to improve the H9c2 potential cells to handle oxidative stress. In addition, CM blocks the generation of MDA and LDH leakage, which are indicators of oxidative stress damage. All these results directly reveal that CM protects cells from oxidative damage by reducing lipid peroxidation and increasing the intracellular antioxidant level.[38]

The activity of LDH reflects cell membrane damage caused by oxidative stress. H_2O_2 exposure produced by a severe increase of LDH activity leads to cell damage. Thus, the protective effects of CM were measured on LDH release in H9c2 cells exposed to H_2O_2. Pretreatment with CM significantly reduced the levels of LDH activity in a dose-dependent manner.

CAT, SOD, and GSH-Px play important roles in the endogenous antioxidant defense system, which are important free radical scavengers and are used to prevent ROS-mediated injury when abnormally high levels of ROS are generated, which can be decomposed by GPx and CAT into O_2.[39] Our study revealed that the addition of H_2O_2 to H9c2 cells significantly decreased GSH-Px, SOD, and CAT activities. The pretreatment of CM in H9c2 cells induced by in H_2O_2 inhibits ROS generation and increases the expression of antioxidant enzymes such as CAT, SOD, and GPx. The data indicated that *C. argyrosperma* significantly protects against the oxidative stress in H_2O_2-treated H9c2 cardiomyoblasts.

CONCLUSIONS

The finding indicated that *C. argyrosperma* exhibits good antioxidant activity compared to different standards. In addition, methanol extracted from *C. argyrosperma* protects H9c2 cardiomyocytes against oxidative stress damage by regulating apoptosis-related proteins, maintaining mitochondrial function, ameliorating antioxidant enzyme systems, and decreasing ROS generation.

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Conflicts of interest

There are no conflicts of interest.

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