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The anti-inflammatory effect of ethanolic ginger extract against carrageenan-induced rat paw edema

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ABSTRACT

Background*:** Ginger, the rhizome of *Zingiber officinale*, a commom constituent of diet worldwide, has some pharmacological activities. ***Objectives: The present study was undertaken to investigate the antiinflammatory effect of *ginger* extract against carrageenan-induced inflammation in the rat paw. ***Methods:*** One kilogram of air-dried rhizomes of *zingiber officinale* was extracted with 6L ethyl alcohol (96%). Thirty-six male Wister rats were divided into 6 groups (6 per each); the control group (C-Group): rats were injected intraperitoneally by DMSO (0.1 ml/kg). Ca-Group: rats were injected intraperitoneally with DMSO followed by subcutaneous injection with carrageenan solution [0.1 ml (1% w/v)]. Ce + Ca Group: rats were injected intraperitoneally with single dose of CeleCOXib (25 mg/kg) as NSAID. After that, it were injected subcutaneously by carrageenan solution [0.1 ml (1% w/v)]. G_{300}+Ca Group: rats were injected intraperitoneally by ethanolic ginger extract (300 mg/kg) followed by a subcutaneous injection with carrageenan solution. G_{600}+Ca Group: rats were injected intraperitoneally with ethanolic ginger extract (600 mg/kg) followed by a subcutaneous injection with carrageenan solution. G_{900}+Ca Group: rats were injected intraperitoneally with ethanolic ginger extract (900 mg/kg) followed by a subcutaneous injection with carrageenan solution. ***Results:*** Carrageenan treatment induced significant increases in the activities of COX-2 and LOX as well as the MDA level while caused significant decline in GSH level compared to the control group. The rats injected with ginger extract followed by carrageenan showed significant decline in the activities of COX-2 and LOX as well as the MDA level compared to the carrageenan-treated group. Our results indicated that the ginger at the higher doses were more effective. Our results concluded that the ginger extract causes inhibition of the edema in paw especially at the higher doses compared to the reference drug.

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INTRODUCTION

Inflammation is a fundamental biological process that is considered as the most frequent sign of disease [(1)]. Inflammation is an important process in the body's defense system, which acts to remove and repair the damaged tissue

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and/or neutralize the harmful agents [(2)]. Although, inflammation is a part of host defense, the host may suffer from tissue damage as a result of an inflammatory reaction [(3)]. Inflammation can be induced by physical or chemical agents and by pathogenic microorganisms [(4)]. Typical signs of inflammation are redness, color, heat, tumor (swelling), pain, and loss of function [(4)].

Inflammation is now well-known to play a key role in initiating and promoting cancer, while it can also contribute to both specific and innate tumor rejection [(5)]. Chronic inflammation has been linked to various steps involved in tumor genesis, including cellular transformation, promotion, survival, proliferation, invasion and metastasis [(6)]. In addition, it has been reported that inflammation is involved in a wide variety of human diseases such as atherosclerosis, ischemic heart disease and some neurodegenerative diseases [(7)]. Carrageenan-induced rat paw edema is a widely used test in the determination of the anti-inflammatory activity without any injury or damage to the inflamed paw [(8)]. A variety of chemical analogues and derivatives of drugs have been developed that have successful application in treating inflammatory diseases [(9)]. The conventional use of non-steroidal anti-inflammatory drugs (NSAIDs) is to control pain and inflammation. The aspirin is the oldest drug that was marketed as anti-inflammatory drug [(10)]. The principal targets for their conventional function are COX enzymes, which are membrane-associated proteins [(10)]. All of the NSAIDs are approximately equivalent in terms of anti-inflammatory efficacy but also cause untoward side effects like the gastrointestinal one and this frequently limits its therapy [(11)].

Natural products or natural product-derived compounds play a dominant role in the discovery of drugs for treating human diseases [(12)]. Many compounds have been described as anti-inflammatory agents because of their ability to affect the arachidonic acid metabolism or the induction of new proinflammatory agents and proteins [(13)]. Ginger obtained from the underground stems of rhizomes of *Zingiber officinale*, an herbaceous tropical perennial belonging to the family *Zingiberaceae*. Ginger (*Zingiber officinale*) is a non-toxic highly promising natural antioxidant compound having a wide spectrum of biological function antimicrobial, anti-inflammatory, antioxidant, immunomodulatory and anticarcinogenic [(14)]. This study aimed to investigate the anti-inflammatory effect of the crude ethanolic extract of ginger rhizomes using an *in vivo* model of inflammation. Also, this study estimated the effect of ginger extract on the cyclooxygenase and Lipoxygenase activities. We evaluate also the effect of ginger extract on GSH, MDA, liver function tests, kidney function tests, lipid profile, and blood glucose.

2- Materials and methods

2.1. Chemicals

All the fine chemicals, used in this study, were purchased from Sigma Aldrich (St Louis, MO).

2.2. Plant material

One kilogram of air-dried rhizomes of ginger were purchased from a local herbal market in Alexandria, Egypt. The rhizomes were identified by the staff of the Botany department, faculty of science, Alexandria University, Egypt. The rhizomes were grinded into fine powder. The fine powder of the rhizome was extracted with 6L of 96% ethyl alcohol at the room temperature and left for 2 days of maceration [(15)]. The extract was filtered and the filtrate was concentrated to dryness under reduced pressure in a rotary evaporator. The viscous light brown extract was 120 g. The crude ginger

extract was kept at 4 ˚C until dissolved in DMSO for preparing of the different doses; 300, 600, and 900 mg/kg of rat weight.

2.3. Animals and experimental protocol

The experiment was carried out on 36 male Wister rats (165 - 200 g), obtained from the experimental animals breading center of the holding company for biological products and vaccines (Helwan, Cairo, Egypt). The rats were maintained under standard conditions of temperature (22-25˚C), relative humidity (40-60 %) and light /dark cycle, with free access to normal rodent food and water. The rats are randomized into 6 groups as follow:

C-Group (control group): 6 rats were injected intraperitoneally by DMSO (0.1 ml/kg), daily for 21 days.

Ca-Group: 6 rats were injected intraperitoneally with DMSO (0.1 ml/kg), daily for 21 days. After that, the rats were injected subcutaneously with 0.1 ml carrageenan in saline (1% w/v).

Ce+Ca Group: 6 rats were injected intraperitoneally with CeleCOXib (25mg/kg) as NSAID for only one single dose. After that, it were injected subcutaneously with carrageenan solution.

G_{300}+Ca Group: 6 rats were injected intraperitoneally with ethanolic ginger extract (300 mg/kg) daily for 21 days. After that, it were injected subcutaneously with carrageenan solution.

G_{600}+Ca Group: 6 rats were injected intraperitoneally with ethanolic ginger extract (600 mg/kg) daily for 21 days. After that, it were injected subcutaneously with carrageenan solution.

G_{900}+Ca Group: 6 rats were injected intraperitoneally with ethanolic ginger extract (900 mg/kg) daily for 21 days. After that, it were injected subcutaneously with carrageenan solution.

In order to examine the *in vivo* anti-inflammatory activity, carrageenan-induced paw edema was produced according to Olajide et al. [16]. Carrageenan-induced rat paw edema is a widely used test to determine the anti-inflammatory activity and constitutes a simple and routine animal model for evaluation of pain at the site of inflammation without any injury or damage to the inflamed paw [8]. One hour after the intraperitoneal administration of the last dose of the ginger extract (300, 600 and 900 mg/kg), the reference drug; CeleCOXib (25 mg/kg) was injected. the control group received the same volume of vehicle (DMSO). An injection of 0.1% solution of lambda carrageenan dissolved in 0.9% NaCl was injected into the planter region of the right hind limb of each rat under the sub-planter aponeuvrosis.

2.4. Measurements of the paw edema

Before any treatment, the average volume of the right paw of each animal was determined (V_o, basal volume), using a micrometer electronic digital caliber. The point of measurement should be premarked on the top of the foot for reference at subsequent measurements. Edema could also be assessed by the measurement of paw thickness in the dorsal plantar axis at the metatarsal level. Measurements of volume (v_t) ½,1,2,3,4 and 5 hours after carrageenan injection. The paw volume and the increase of paw volume due to edema was calculated. The change in paw volume was determined by duplicate measurements.

The percent of inflammation (edema) was calculated from the formula:

% Edema = $\mathbf{V_o / V_t \times 100}$**,** while Percentages of inhibition in our anti-inflammatory tests were obtained for each group using the following ratio:

% Inhibition = $\mathbf{(V_o - V_t / V_o) \times 100}$, where; V_o is the average volume of the

hind paw edema of the inflammation group at a given time and V_t is the average volume of the hind paw edema obtained for each treated group at a given time.

2.5. Biochemical Investigations

After the final assessment of the paw volume. The rats were anaesthetized using diethyl ether and the heparinized and unheparinized blood samples were collected by retro-orbital plexus bleeding using glass capillary tube. The unheparinized blood was kept for 15 min at room temperature, then sera were separated by centrifugation at 3000 rpm for 20 min using centrifuge and stored at -20 ˚C until used for biochemical analyses. The heparinized whole blood was set for investigation of complete blood count determination.

At the end of the experimental period, rats were sacrified by cervical dislocation and paws were removed by cutting at the tibio-tarsal level. The paws were weighed and rinsed in a Tris buffer (PH 7.4) to remove any red blood cells and clots. Then, the paws were degloved to remove the bone. The tissues were immediately frozen in liquid nitrogen and stored at -80 ˚C until needed. After that, the activities of cycloxygenase-2, cycloxygenase-1, lipoxygenase were estimated. The TBARs and GSH levels were also assayed.

2.6. Determination of TBARs level

MDA, a product formed due to the peroxidation and decomposition of polyenic fatty acids in the lipids, was determined by thiobarbituric acid (TBA) test [(17)]. Thiobarbituric acid reactive substances (TBARS), including lipid hydroperoxides and aldehydes are expressed in terms of MDA equivalents.

2.7. Determination of GSH

The concentration of GSH was determined as described by Richardson and Mwephy [(18)]. Ellman's reagent [5, 5', Dithiobis (2-nitrobenzoic acid), DTNB] was reacted with GSH giving 2-nitro-5-thiobenzoic acid, a yellow colored product with a maximum absorbance at 412 nm.

2.8. Determination of cycloxygenase enzyme activity

The cycloxygenase (EC 1.14.99.1) assay kit was purchased from Caymen Chemicals Company (Ann Arbor, Michigan, USA). It measures the peroxidase activity of cyclooxygenase. The peroxidase activity was assayed colorimetrically by monitoring the appearance of oxidized N,N,N',N'-tetramethyl-*p*-phenylenediamine [TMPD] at 590 nm. The kit includes isozyme-specific inhibitors for distinguishing COX-2 activity from COX-1 activity.

2.9. Determination of lipoxygenase enzyme activity

Lipoxygenase (EC 1.13.11) is a dioxygenase that catalyzes, as a primary reaction the hydroperoxidation by molecular oxygen of linoleic acid, arachidonic acid and other polyunsaturated lipids that contain a cis, cis – 1,4-pentadiene moiety. The reaction could be followed by observing the increase in absorption at 234 nm arising from the conjugated double bonds formed during the reaction. One-unit lipoxygenase enzyme was defined as the quantity the enzyme catalyzing the formation of one µmol of hydroperoxyarachidonate (HPA) per min /mg protein under the assay conditions [(19)].

2.10. Statistical analysis

Data were analyzed as a completely randomized design and statistical significance of the difference in values of the control and treated animals was calculated by (F) test at 5% significance level. Data of the present

study were statistically analyzed by using LSD Multiple Range Test [20].

3. Results and discussion

3.1. The edema weight of the studied groups: -

The results of the animal experiments are shown in Table (1). Carrageenan-induced rat paw edema is used widely as a working model of inflammation in the search for a new anti-inflammatory drug. Carrageenan-treated group showed significant ($P<0.01$) elevation in the edema weight compared to the control group. The anti-inflammatory activity of the ethanolic ginger extract was evaluated by carrageenan-induced rat paw edema method (Winter et al., 1962; Adeyemi et al., 2002). The extract was tested at three different dose levels. The results showed that the ethanolic extract with a concentration of 900 mg/kg b.w. showed 21.2% of inhibition on carrageenan-induced rat paw edema. This result indicated that the ethanolic extract at this concentration showed a maximum anti-inflammatory activity as compared to the reference drug celeCOXib, which showed only 3.1% inhibition of carrageenan induced rat paw edema.

3.2. Effect of ethanolic ginger extract injection on edema volume at time intervals through 5 hrs in rats: -

Table (2) showed that the ethanolic extract of ginger significantly ($P < 0.01$) changed the paw volume after 5 hrs among the different studied groups. The edema volume (mm) was measured after different time intervals (0.5,1,2,3,4,5) in the studied groups G_{300}+Ca, G_{600}+Ca and G_{900}+Ca, respectively. Where, at 0.5 hrs. the average change in the edema volume showed a higher level in Ca group and Ce+Ca group. While, the volume of paw in the G300+Ca, G600+Ca and G900+Ca treated groups showed a lower volume in paw volume. On the other hand, at 1 hr, the edema volume in the ginger-treated groups (G_{300}+Ca, G_{600}+Ca and G_{900}+Ca) were significantly lower than the control group and Ce+Ca group. However, after 2 hrs, the paw volume showed a higher level in the Ca and Ce+Ca treated groups, while the ginger-treated groups showed lower paw volume. Also, similar results were observed after 3, 4 and 5 hrs of the experiment in which a higher paw volume was observed in the Ca and Ce+Ca-treated groups and the lower edema volume was observed in the ginger treated groups (G_{300}+Ca, G_{600}+Ca and G_{900}+Ca). These results concluded that, the ethanolic ginger extract caused asignificant decrease in the edema volume compared to the other treatments.

3.4. Effect of ethanolic ginger extract on % of edema through 5 hrs. in rats: -

Table (3) showed significant differences ($P < 0.01$) among the different groups in the incidence percentage of the edema after 5 hrs. It has been shown that at 0.5 hrs., the higher incidence of edema (%) in the Ce+Ca-treatedgroup followed by G_{300}+Ca treated group and G_{600}+Ca-treated group. While, the lower % of edema was observed in the G_{900}+Ca-treated group. Also, after 1, 2, 3, 4 and 5 hrs, the incidence percentage of the edema was increased in the Ce+Ca group, followed by G_{300}+Ca, G_{600}+Ca. While, the G_{900}+Ca-treated group showed a lower incidence percentage and could reached to the level of its percentage in the control group. Our results indicated that the higher dose of ginger decreased the incidence of edema than the other treated groups especially through the long period of ginger-treatment.

3.5. Effect of ethanolic ginger extract injection on the (%) inhibition of edema after 5 hrs in rats: -

Table (4) explained the significant differences ($P < 0.01$) among the different groups in the percentage of the inhibition

of edema after 5 hrs in rats. The results indicated that the higher level in inhibition was observed in the groups treated with G_{900}+Ca, G_{600}+Ca and G_{300}+Ca at all the periods of the experiment (0.5, 1, 2, 3, 4 and 5 hrs.).While, the lower level of the inhibition of the paw edema was observed in the Ce+Ca group at all the periods of the experiment (0.5, 1, 2, 3, 4 and 5 hrs.).

Table (5) showed that the carrageenan treatment induced a significant ($P<0.01$) increase in the activities of COX-2 and LOX compared to control group. The rats injected with "CeleCOXib" before injected with carrageenan in Ce+Ca group showed a significant ($P<0.01$) decline in the activities of COX-2 and LOX compared to the carrageenan-treated group. The rats injected with ginger extract followed by carrageenan-treatment showed significant ($P<0.01$) decline in the activities of COX-2 and LOX compared to the carrageenan-treated group. Our results indicated that the ginger at higher doses were more effective.

3.6. Effect of ginger extract on GSH and MDA levels: -

The administration of carrageenan caused significant ($P < 0.01$) decline in GSH level while induced significant ($P < 0.01$) elevation in MDA level compared to the control group. The rats injected with CeleCOXib followed by carrageenan (Ce+Ca group) showed a significant ($P < 0.01$) increase in the GSH Content by about 69.6% and a significant ($P < 0.01$) decline in the MDA level by about 13.9% compared to Ca group. The rats injected with ginger extract then administrated with carrageenan showed a significant ($P < 0.01$) increase in GSH level by about 106.8 %, 135.9 %, 168.3 % for groups G300+Ca, G600+Ca and G_{900}+Ca, respectively compared to the Ca group. While, these groups showed significant ($P < 0.01$) reductions in the MDA level by about 23.1.5 %, 39.5 %, 47.1 % for groups G300+Ca, G600+Ca and G_{900}+Ca, respectively compared to the Ca group. Our results indicated that, the ginger at higher doses increased the level of GSH while reduced the MDA level than the control groups.

Carrageenan-induced hind paw edema is the standard experimental model of acute inflammation. Carrageenan is the phlogistic agent of choice for testing the anti-inflammatory drugs devoid of apparent systemic effect and exhibits a high degree of reproducibility [(21)]. Carrageenan induced edema is a biphasic response; the first phase is mediated through the release of histamine, serotonin and kinins. Whereas, the second phase is related to the release of prostaglandin and slow reacting substances which peak at 3h [(22)]. Carrageenan induced paw edema and several nonsteroidal anti-inflammatory drugs have been shown to be active in the rat model [(23)]. The increase in the paw volume following carrageenan administration is comparable to the findings of Vadivelan et al. [(21)]. The development of edema in the paw of the rat after the injection of carrageenan is due to release of histamine, serotonin and prostaglandin like substances [(24), (22)]. In many countries, ginger and its products raise the immune system [(25)]. The high significant anti-inflammatory activity of the ethanolic extract of ginger (900 mg/kg) may be due to the inhibition of the mediators of inflammation such as histamine, serotonin and prostaglandin [(26)] (Habib et al., 2008). Our results suggested that ginger could possibly be used as anti-inflammatory agent to reduce inflammation and therefore relieve pain [(27)].

Although, the cyclooxygenase and lipoxygenase pathways play a role in the inflammatory process, the inhibition of cyclooxygenase is more effective in inhibiting carrageenan-induced inflammation than lipoxygenase inhibitors [(28)]. Our results showed that carrageenan

treatment induced a significant increase in the activities of COX-2 and LOX compared to the control group. Our results are consistent with the findings of Posadas et al. [28] who indicated that carrageenan induced activation of COX-2 which is responsible for the increased production of prostaglandins. The rats injected with ginger extract then administrated with carrageenan showed significant decline in the activities of COX-2 and LOX compared to carrageenan-treated group. Our results indicated that the ginger at the higher doses was more effective. Gingerol, shogaol, and other structurally-related substances in ginger inhibit prostaglandin and leukotriene biosynthesis through suppression of 5-lipoxygenase or prostaglandin synthetase [30]. Additionally, the compounds can also inhibit synthesis of pro-inflammatory cytokines such as IL-1, TNF-α, and IL-8 [30]. In addition, Pan et al. [31] showed that *zingiber shool* can down-regulate the inflammatory iNOS and COX-2 gene expression in macrophages. Furthermore, Mashhadi et al. [25] indicated that the hexane extract of *zingiber officinale* inhibited the excessive production of NO, PGE_2, TNF-alpha, and IL-1beta.

In the present study, the administration of carrageenan caused significant decline in GSH level while induced significant elevation in the MDA level compared to the control group. Our results indicated that, the higher doses of ginger caused elevation in the level of GSH and reduction in MDA level than the control group. Free radicals have been regarded to be responsible for various diseases including inflammation [32]. It is well known that the antioxidant activity offers anti-inflammatory action [33]. Since, inflammation elevates the levels of free radicals; the antioxidants are considered to possibly display anti-inflammatory effects [34]. The anti-oxidative properties of ginger and its components have been explored in various *in vitro* and *in vivo* tests. This antioxidant proports strengthens the body's defenses by improving the antioxidant status will undoubtedly protect human against many chronic diseases [25]. 6-Shogaol has exhibited the most potent antioxidant and anti-inflammatory properties in ginger, which can be attributed to the presence of α, β-unsaturated ketone moiety [35]. Animal modeling showed that ginger significantly lowered the induced lipid peroxidation and raised the levels of the antioxidant enzymes, together with serum glutathione [25].

Our results demonstrated that the rats administrated with "carrageenan" in Ca group showed decline in the leukocyte count. On the other hand, the ginger at higher doses caused increases in the leukocyte count. In conclusion, our results suggest that ginger could possibly be used to ameliorate carrageenan-induced paw edema and also be used as anti-inflammatory agent to reduce inflammation and therefore relieve pain.

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Table (1): The edema weight (%) of the experimental groups:

Parameter	Experimental groups					
	C	Ca	Ce+Ca	G_{300}+Ca	G_{600}-Ca	G_{900}+Ca
% of Edema weight	0.27±0.01[a]	0.33±0.01[b]	0.32±0.01[b]	0.29±0.01[c]	0.28±0.01[a]	0.26±0.01[a]

Values are expressed as means ± SE.

Means within the same row of different litters (a, b, c) are significantly different at (P<0.01).

Table (2): Average change in the edema volume at time intervals through 5 hrs. in the experimental groups:

Parameter	time	Experimental groups				
		Ca	Ce+Ca	G_{300}+Ca	G_{600}-Ca	G_{900}+Ca
Average change in edema Volume through 5 hrs.	0.5	0.45±0.01[a]	0.49±0.05[a]	0.29±0.03[b]	0.18±0.02[c]	0.14±0.01[c]
	1	0.65±0.07[a]	0.45±0.05[b]	0.37±0.04[c]	0.36±0.04[c]	0.32±0.03[d]
	2	1.13±0.12[a]	0.46±0.05[b]	0.45±0.05[b]	0.34±0.03[c]	0.29±0.03[d]
	3	1.21±0.13[a]	0.67±0.07[b]	0.47±0.05[c]	0.27±0.03[d]	0.23±0.02[d]
	4	1.59±0.17[a]	0.67±0.07[b]	0.59±0.06[c]	0.28±0.03[d]	0.07±0.01[e]
	5	1.55±0.17[a]	0.68±0.07[b]	0.28±0.03[c]	0.23±0.02[c]	0.03±0.01[d]

Values are expressed as means ± SE.

Means within the same row of different litters are significantly different at (P<0.01).

Table (3): The incidence percentage of the edema after 5 hrs among the different groups:

Parameter	time	Experimental groups				
		Ca	Ce+Ca	G_{300}+Ca	G_{600}-Ca	G_{900}+Ca
% Edema after 5 hrs.	0.5	-	108[a]	66[b]	41[c]	32[d]
	1	-	70[a]	56[b]	56[b]	49[c]
	2	-	41[a]	40[a]	31[b]	26[c]
	3	-	43[a]	39[b]	22[c]	19[d]
	4	-	42[a]	37[b]	18[c]	5[d]
	5	-	44[a]	18[b]	15[c]	2[d]

Values are expressed as means ± SE.

Means within the same row of different litters are significantly different at ($P<0.01$).

Table (4): Percentage inhibition of edema after 5 hrs among the different groups:

Parameter	time	Experimental groups				
		Ca	Ce+Ca	G_{300}+Ca	G_{600}-Ca	G_{900}+Ca
% inhibition of paw Edema after 5 hrs	0.5	-	0[a]	34[b]	59[c]	68[d]
	1	-	30[a]	44[b]	44[b]	51[c]
	2	-	59[a]	60[b]	69[c]	74[d]
	3	-	44[a]	61[b]	78[c]	81[d]
	4	-	58[a]	63[b]	82[c]	95[d]
	5	-	56[a]	82[b]	85[b]	98[c]

Values are expressed as means ± SE.

Means within the same row of different litters are significantly different at ($P<0.01$).

Table (5): The activities of COX-2 and Lox in the experimental groups:

Parameter	Experimental groups					
(µ mol/min/mg protein)	C	Ca	Ce+Ca	G_{300}+Ca	G_{600}-Ca	G_{900}+Ca
COX-2	0.02±0.001[b]	0.04±0.001[c]	0.03±0.001[d]	0.03±0.001[d]	0.02±0.001[b]	0.01±0.001[a]
LOX	1.33±0.07[a]	2.72±0.12[b]	2.26±0.08[c]	1.48±0.08[a]	1.47±0.07[a]	1.33±0.07[a]

Values are expressed as means ± SE.

Means within the same row of different litters are significantly different at (P<0.01).

Table (6): Effect of ginger extract on GSH and MDA levels:

Parameter	Experimental groups					
(µg/ml)	C	Ca	Ce+Ca	G_{300}+Ca	G_{600}-Ca	G_{900}+Ca
GSH	14.01±1.11[b]	10.79±0.42[c]	23.77±1.54[d]	28.98±1.37[a]	33.05±1.06[a]	37.60±0.66[e]
MDA	26.34±0.92[a]	53.10±2.17[b]	45.67±1.55[c]	40.86±1.41[d]	32.11±0.96[e]	28.10±1.07[ae]

Values are expressed as means ± SE.

Means within the same row of different litters are significantly different at (P<0.01).