Proposal Number: 002/Pharmac/IAEC/2021

Form B (per rule 8(a)* for Submission of Research Protocol (s)

Application for Permission for Animal Experiments

Application to be submitted to the CPCSEA, New Delhi after approval of Institutional Animal Ethics Committee (IAEC)

Section –I

1.	Name and address of establishment	**Department of Pharmacology, MGM Medical college,N-6, Cidco Aurangabad, Maharashtra.**	
2.	Registration number and date of registration.	Registration Number	**CPCSEA Reg. No. 1777/PO/Re/S/14/CPCSEA**
		Date of registration	**28-08-17**
3.	Name, address and registration nu mber of breeder from which animal s acquired (or to be acquired) for experiments mentioned in parts B & C	Name	**1. National institute of Biosciences**
		Address	**1. Pune, Maharashtra.**
		Registration No.	**Registration No. 1091/GO/Bt/S/07/CPCSEA**
4.	Place where the animals are present ly kept (or proposed to be kept).	**Animal house, Department of pharmacology, MGM medical college Aurangabad.**	
5.	Place where the experiment is to be performed (Please provide CPCSEA Reg. Number)	**Animal house, Department of pharmacology, MGM medical college Aurangabad.(CPCSEA Reg. No. 1777/PO/Re/S/14/CPCSEA)Date:-28-08-17**	
6.	Date and Duration of experiment.	Date	**Feb 2022 to April 22**
		Duration	**6 weeks**
7.	Type of research involved (Basic Research / Educational/ Regulatory / Contract Research)	**Basic research**	

Signatures	
Name of Investigator	**Dr. Deepali Jaybhaye**
Designation of Investigator	**Associate professor**
Signature	
Date	**27/12/2021**
Place	**MGM Medical College & Hospital, Aurangabad.**

Proposal Number: 002/Pharmac/IAEC/2021

Section -II

Protocol form for research proposals to be submitted to the Institutional Animal Ethics Committee/ CPCSEA, for new experiments or extensions of ongoing experiments using animals.

1	**Project / Dissertation / Thesis Title:**	**The study of renoprotective effect of Citrus limon juice and Emblica officinalis extract on renal toxicity induced by carbon tetrachloride in wister rats.**
2	**Principal Investigator / Research Guide / Advisor**	
	Name	**Dr. Deepali Jaybhaye**
	Designation	**Associate professor Department of pharmacology, MGM Medical college Aurangabad**
	Dept / Div/ Lab	**Department of pharmacology, MGM Medical College Aurangabad**
	Telephone No.	**0240 – 6601100 Ext: 1502 , 1501.**
	E-mail Id	**deepalijaybhaye@rediffmail.com**
	Experience in Lab animal Experimentation (Years/Months)	**12 years**
3	**List of all individuals authorized to conduct procedures under this proposal.**	
	Investigator 1	
	a) Name	**Dr. Deepak Bhosle**
	b) Designation	**Professor and HOD**
	c) Dept / Div/ Lab	**Department of pharmacology, MGM Medical College, Aurangabad**
	d) Telephone No.	**0240 – 6601100 Ext : 1502 , 1501.**
	e) E-mail Id	**drdeepakbhosle@gmail.com**
	f) Experience in Lab animal Experimentation (Years/Months)	**17 Years**
4	**Funding Source / Proposed Funding Source with complete address**	**MGM Institute of Health Sciences, Navi Mumbai.**
	(Please attach the proof)	Yes
5	**Duration of the animal experiment.**	
	a) Date of initiation (Proposed)	Jan 22
	b) Date of completion (Proposed)	Jan 2023
6	**Describe details of study plan to justify the use of animals (Enclose Annexure)**	We will use Wistar rats for this study. **Citrus limon and Emblica officinalis has strong antioxidant property. But study of renoprotective activity is not available. So we want to explore this property of above plant.**
7	**Animals required**	
	a. Species	Rats of either sex.
	Strain	Wistar (Rat),

	b. Age	8- 20 weeks.
	Weight	150-250 gms,
	c. Gender	Both
	d. Number to be used	Rat-36 <table><tr><td></td><td>Available</td><td>Procure</td></tr><tr><td>Rat</td><td>08</td><td>28</td></tr></table>
	(Year-wise breakups and total figures needed to be given in tabular form)	<table><tr><th>Year</th><th>Number of Animals</th></tr><tr><td>Feb – 2022 to Feb-2023</td><td>Rat-36</td></tr></table>
	e. Number of days each animal will be housed.	Procurement to life time
8	**Rationale for animal usage**	
	a) Why is animal usage necessary for these studies?	As animal study is not available. So we have to do it as primary study to see the renoprotective effect. (lemon and Amla having strong antioxidant property and in renal failure the causative agent is oxidative stress but still not a single study is available as renoprotective drug. To explore the renoprotective property we want to do this study.)
	b) Whether similar study has been conducted on in vitro models?	Not Applicable
	If yes, describe the leading points to justify the requirement of animal experiment.	Not Applicable
	c) Why are the particular species selected?	As it is best demonstrated in rats.
	d) Why is the estimated number of animals essential?	For the statistical significant result.
	e) Are similar experiments conducted in the past in your establishment?	No
	f) If yes, justify why new experiment is required?	-
	g) Have similar experiments been conducted by any other organization in same or other in vivo models?	-
	If yes, enclose the reference.	-
9	**Describe the procedures in detail:**	
	a) Describe all invasive procedures that animals will be subjected to in the course of the experiments	Handling of animals and giving standard and test dose of drugs by oral route. (detail Performa attached)
	Describe all potentially stressful non-invasive procedures that animals will be subjected to in	-

	the course of the experiments			
	b) Furnish details of injections schedule Substances	Doses	CCl4- intraperitoneal injection of 1.5 ml/kg of 20% CCl4 dissolved in olive oil, Acetylcystine 950 mg/kg, Citrus limon 6 ml/kg/oral route for 6 wks, Emblica officinalis 700mg/kg/oral route for six wks.	
		Sites	IP and oral	
		Volumes	-	
	c) Blood withdrawal Details:	Volumes	1 ml	
		Sites	retro-orbital plexus	
	d) Radiation	Dosage	Not Applicable	
		Schedules	Not Applicable	
	e) Nature of compound/Broad Classification of drug/NCE:	CCl4 , Acetylcystine, Citrus limon , Emblica officinalis		
10	Does the protocol prohibit use of anesthetic or analgesic for the conduct of painful procedures?	NO		
	If yes, justify.	Not Applicable		
11	Will survival surgery be done?	No		
	If yes, the following to be described.			
	a) List and describe all surgical procedures (including methods of asepsis)	-----		
	b) Personnel involved in surgical procedure	Name 1	-----	
		Qualification	----	
		Experience in such surgeries	----	
		Name 2	----	
		Qualification	----	
		Experience in such surgeries	----	
		Name 3	----	
		Qualification	----	
		Experience in such surgeries	----	
	c) Describe post-operative care	------		
	d) Will major survival surgery is to be performed more than once on a single animal?	NO		
		If Yes, Justify: ------		
12	**Describe post-experimentation procedures.**			
	a) Scope for Reuse	No		

	b) Rehabilitation (if reuse is ti	-	
		-	
	c) Describe method of Euthanasia (If required in the protocol)	After giving CO_2 (70 %) animal will be scarified and kidney will be remove for histopathology. (detail of experiment synopsis is attached)	
	d) Method of carcass disposal after euthanasia.	Common biomedical waste facility affiliated to AMC.	
14	**Will extra-institutional transport is envisaged?**		YES
	If yes, Describe animal transportation methods	Through AC vehicle along with adequate food & water.	
15	**Use of hazardous agents:** (use of recombinant DNA-based agents or potential human pathogens requires documented approval of the Institutional Biosafety Committee (IBC). For each category, the agents and the biosafety level required, appropriate therapeutic measures and the mode of disposal of contaminated food, animal wastes and carcasses must be identified).		
	Does your project involved use of any of the below mentioned agent?		
	(a) Radionucleotides (AERB)		NO
	(b) Microorganisms / Biological infectious Agents (IBSC)		NO
	(c) Recombinant DNA (RCGM)		NO
	(d) Any other Hazardous Chemical / Drugs		NO
	Have you ticked "Yes" in either of above four hazardous agents? **If so, copy of the approval certificates of the respective agencies:**		
	Certificate attached		Not applicable

Investigator's declaration.

1. I certify that the research proposal submitted is not unnecessarily duplicative of previously reported research.
2. I certify that, I am qualified and have experience in the experimentation on animals.
3. For procedures listed under item 10, I certify that I have reviewed the pertinent scientific literature and have found no valid alternative to any procedure described herein which may cause less pain or distress.
4. I will obtain approval from the IAEC/ CPCSEA before initiating any changes in this study.
5. I certify that performance of experiment will be initiated only upon review and approval of scientific intent by appropriate expert body (Institutional Scientific Advisory Committee / funding agency / other body).
6. I certify that I will submit appropriate certification of review and concurrence for studies mentioned in point 14.
7. I shall maintain all the records as per format (Form D) and submit to Institutional Animal Ethics Committee (IAEC).
8. I certify that, I will not initiate the study before approval from IAEC/ CPCSEA received in writing. Further, I certify that I will follow the recommendations of IAEC/ CPCSEA.
9. I certify that I will ensure the rehabilitation policies are adopted (wherever required).

Signatures	
Name of Investigator	Dr. Deepali Jaybhaye
Signature	
Date	27/12/2021

CERTIFICATE

This is to certify that,

Project proposal no	002/Pharmac/IAEC/2021
Entitled	---
submitted by Dr./ Mr. / Ms	Dr. Deepak Bhosle and Dr. Deepali Jaybhaye

has been approved/recommended by the **IAEC MGM Medical College & Hospital Aurangabad** in its meeting held on **08-01-2022** (date) and **Rat-36,** have been sanctioned under this.

Authorized by	Name	Signature	Date
Chairman:	Dr. Deepali Jaybhaye		
Member Secretary:	Dr. Sangita Phatale		
Main Nominee of CPCSEA:	Dr. Shrikant Satale		

Annexure: Study Plan / Outline of Reasearch

<table>
<tr><td>Title</td><td>The study of renoprotective effect of Citrus limon juice and Emblica officinalis extract on renal toxicity induced by carbon tetrachloride in wistar rats.</td></tr>
<tr><td>Animal species / strain</td><td>Wistar rats</td></tr>
<tr><td>How do this animal relates to human in terms of test item nature</td><td>Wistar rats ideal model for human kidney study.</td></tr>
<tr><td>Study design
(In all the six groups renal injury will be induce by CCl4 IP injection of 1.5 ml/kg of 20% CCl4 dissolved in olive oil and then give standard drug Acetylcystine and test drugs i.e Citrus limon and Emblica officinalis while group I will serve as control group only distilled water will be given in this)</td><td>
<table>
<tr><td>Group</td><td>Test item</td><td>Dose</td><td>No of animals</td></tr>
<tr><td>Group I</td><td>CCl4</td><td>1 ml Distilled water /Oral route</td><td>6</td></tr>
<tr><td>Group II</td><td>Acetylcystine</td><td>950 mg/kg/ oral</td><td>6</td></tr>
<tr><td>Group III</td><td>Citrus limon</td><td>6 ml/kg/oral</td><td>6</td></tr>
<tr><td>Group IV</td><td>Emblica officinalis</td><td>700mg/kg/oral</td><td>6</td></tr>
<tr><td>Group V</td><td>Citrus limon + Emblica officinalis</td><td>6 ml/kg + 700 mg /kg/oral</td><td>6</td></tr>
<tr><td>Group VI</td><td>Citrus limon + Emblica officinalis + Acetylcystine</td><td>6 ml/kg + 700 mg /kg + 950 mg /kg/ oral</td><td>6</td></tr>
</table>
</td></tr>
<tr><td>Rationale for dose selection</td><td>Drug dose is selected as per the previous literature.</td></tr>
<tr><td>Duration, dosing schedule, route, other details</td><td>Duration of study – 6 weeks
Drugs and doses- CCl4 IP injection of 1.5 ml/kg of 20% CCl4 dissolved in olive oil and Acetylcystine 950 mg/kg/ oral , Citrus limon 6 ml/kg/oral route, Emblica officinalis 700 mg/kg/oral route for 6 wks</td></tr>
<tr><td>Parameters to be investigated</td><td>BUN and Serum Creatinine levels along with activities of antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT) in a homogenized renal tissue will be determined using ELISA kits, on the kit guidelines. This parameters will be taken after inducing the renal injury by CCl4. And after giving standard and test drugs.
Kidney histopathology will be done at the end of study.</td></tr>
<tr><td>Result interpretation criteria</td><td>Renoprotective effect of drugs.</td></tr>
<tr><td>How will you correlate/ translate these results to human</td><td>Improvement in markers of oxidative stress and BUN and serum Creatinine level.</td></tr>
</table>

What is the use of your obtained results? How it will be taken forward? How it will be used for humans	As Citrus limon i.e lemon and Emblica officinalis i.e Amla is easily available in India having no adverse effects in higher doses. If we got positive result the drug will be available for the treatment of kidney failure in less cost.

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Research Article

Anti-Inflammatory Effect of *Emblica officinalis* in Rodent Models of Acute and Chronic Inflammation: Involvement of Possible Mechanisms

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Emblica officinalis, commonly known as amla in Ayurveda, is unarguably the most important medicinal plant for prevention and treatment of various ailments. The present study investigated the anti-inflammatory activity of hydroalcoholic extract of *Emblica officinalis* (HAEEO). Acute inflammation in rats was induced by the subplantar injection of carrageenan, histamine, serotonin, and prostaglandin E_2 and chronic inflammation was induced by the cotton pellet granuloma. Intraperitoneal (i.p.) administration of HAEEO at all the tested doses (300, 500, and 700 mg/kg) significantly ($P < 0.001$) inhibited rat paw edema against all phlogistic agents and also reduced granuloma formation. However, at the dose of 700 mg/kg, HAEEO exhibited maximum anti-inflammatory activity in all experimental models, and the effects were comparable to that of the standard anti-inflammatory drugs. Additionally, in paw tissue the antioxidant activity of HAEEO was also measured and it was found that HAEEO significantly ($P < 0.001$) increased glutathione, superoxide dismutase, and catalase activity and subsequently reduced lipid peroxidation evidenced by reduced malondialdehyde. Taken all together, the results indicated that HAEEO possessed potent anti-inflammatory activity and it may hold therapeutic promise in the management of acute and chronic inflammatory conditions.

1. Introduction

Inflammation plays a major role in rheumatoid arthritis and osteoarthritis [1]. In clinics, the nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed for pain relief in arthritic conditions. However, their continual use is associated with serious adverse effects like gastric mucosal damage, occult blood loss and elevation of serum hepatic transaminases, salt and water retention, and also exacerbation of asthma [2]. In order to circumvent these adverse effects associated with conventional NSAIDs, novel selective COX-2 inhibitors are in progress. However, the development of serious adverse reactions, like cardiovascular events with rofecoxib and Stevens-Johnson syndrome with valdecoxib, has compelled their withdrawal from use [3]. Additionally, the clinical uses of the remaining drugs in this class have been prescribed with caution and have consequently decreased [4].

In milieu of these observations patients as well as health care providers prefer to use alternative therapeutic agents as they are considered to be safe and effective in alleviating inflammation associated with arthritis. Several Indian medicinal plants were reported as an important source of new chemical moieties with potential therapeutic effects [5]. The studies on plants with substantiated folkloric use as anti-inflammatory agents are viewed as a productive and logical research strategy in the search for new anti-inflammatory drugs

Emblica officinalis Gaertn. (*Euphorbiaceae*) commonly known as amla grow in the tropical areas of South-East

Asia. The fruit of the plant is one of the most important medicinal ingredients used in Ayurveda, Siddha, Unani, Arabic, Tibetan, and various other folk systems for the management of myriad chronic ailments [6]. Experimental studies have shown potent antioxidant, analgesic, antipyretic, adaptogenic, immunomodulatory, and antiulcerogenic activities of the fruit of *Emblica officinalis* [6–8].

The fruits are reported to contain thermostable vitamin C, minerals, amino acids, tannins, flavonoids, and other important phytochemicals which are believed to possess diverse pharmacological and biological effects [9]. Earlier studies have shown that the leaf extract possesses anti-inflammatory activities in the carrageenan and dextran-induced rat hind paw edema [10]. However, studies on the fruit extract which is the most used part of amla have never been performed. Therefore, the present study was carried out to evaluate the anti-inflammatory activity of the hydroalcoholic extract of the fruit of *Emblica officinalis* (HAEEO) in both acute and chronic models of inflammation in rats. Further, in order to understand the possible underlying mechanism, the effect of extract on the oxidative stress produced by carrageenan was also studied in the rat paw.

2. Methodology

2.1. Plant Extract. The standardized lyophilized hydroalcoholic extract of the fruit of *Emblica officinalis* (HAEEO) was procured from Sanat Products Limited, India (A WHO-GMP and ISO 9001 Accredited Herbal Extract Manufacturer Company). The voucher specimen of lyophilized extract of the fruits of *Emblica officinalis* (number EO 0114) was deposited at Department of Pharmacology, All India Institute of Medical Sciences, New Delhi, India. The phytochemical analysis was done by using HPLC (Waters, Milford Massachusetts, USA). The extract obtained was of the highest purity with 28.26% w/w of hydrolysable tannins emblicanin A and emblicanin B on dried weight basis.

2.2. Drugs and Chemicals. Carrageenan, histamine, 5-hydroxytryptamine (serotonin), chlorpheniramine, cyproheptadine, prostaglandin E_2 (PGE_2), and bovine serum albumin were purchased from Sigma Chemicals, St. Louis, MO, USA. Indomethacin was procured from Cipla, India. All other chemicals and reagents were of analytical grade.

2.3. Experimental Animals. All experimental procedures described were reviewed and approved by the Institutional Animal Ethics Committee and care of animals was taken as per guidelines of CPCSEA, Ministry of Environment and Forest, Government of India. Wistar albino rats of either sex weighing 180–200 g were used for the study. The animals were procured from the central animal facility in All India Institute of Medical Sciences, New Delhi. The rats were group-housed in polypropylene cages with no more than four animals per cage. They were maintained under standard laboratory conditions with natural dark-light cycle and were allowed free access to standard pellet diet (Golden Feeds, India) and tap water *ad libitum*. All the experiments were carried out using five groups, each containing 6 animals (Groups I–V) except carrageenan-induced paw edema where Groups I–VI were used.

2.4. Determination of Anti-Inflammatory Activity of HAEEO on Acute Inflammation

2.4.1. Carrageenan-Induced Hind Paw Edema in Rats. Acute inflammation was produced by injecting 0.1 mL of carrageenan (1% in saline) locally into the plantar aponeurosis of the right hind paw of the rats [11, 12]. Group I served as normal control, where no inflammation was induced. This group was used for evaluation of biochemical parameters. Groups II and III received vehicle (saline 1 mL/kg, i.p.) and standard drug indomethacin (10 mg/kg, p.o.), respectively, and served as vehicle and positive controls. HAEEO (300, 500, and 700 mg/kg, i.p.) was administered to Groups IV, V, and VI, respectively. The HAEEO or vehicle was administered 30 min prior to injection of carrageenan and indomethacin was orally administered 1 h prior to the injection of carrageenan. The pedal volume up to the ankle joint was measured using a digital plethysmometer (Ugo Basile, 7140 Comerio, Varese, Italy) at 0 h (just before carrageenan injection) and then at 3 h. The different timing was chosen because of the different route of drug administration. The % inhibition of edema volume between treated and control groups was calculated as follows: % Inhibition = $V_c - V_t \times 100/V_c$, where V_c and V_t represent the mean increase in paw volume in control and treated groups, respectively.

2.4.2. Autacoids-Induced Hind Paw Edema in Rats. This experiment was conducted according to the method described by Singh and Pandey [13]. The autacoids serotonin (1 mg/mL), histamine (1 mg/mL), and prostaglandin E_2 (1 μg/mL) were employed as phlogistic agents. The effect of HAEEO (300, 500, and 700 mg/kg, i.p.) and vehicle was tested individually against each autacoid. The anti-inflammatory effect of HAEEO was compared with that of standard drugs against each autacoid: phenylbutazone (PBZ, 100 mg/kg, p.o.) against prostaglandin E_2, chlorpheniramine (CPM, 3 mg/kg, p.o.) against histamine, and cyproheptadine (CPH, 3 mg/kg, p.o.) against serotonin. Right hind paw edema was induced by the subplantar injection of 0.1 mL of different phlogistic agents in the respective groups. HAEEO was administered i.p. 30 min prior to inflammatory insult and standard reference drugs were administered p.o. 1 h prior to the inflammatory insult. The pedal volume was measured just before (0 h) and then at 3 h after the phlogistic challenge.

2.5. Determination of Anti-Inflammatory Activity of HAEEO on Chronic Inflammation

2.5.1. Cotton Pellet-Induced Granuloma in Rats. The cotton pellet-induced granuloma in rats was studied according to the method of D'Arcy et al. [14]. The animals were divided into five groups with six animals in each group. The rats were anaesthetized and sterile cotton pellets weighing 10 ± 1 mg were implanted subcutaneously into both sides of the groin

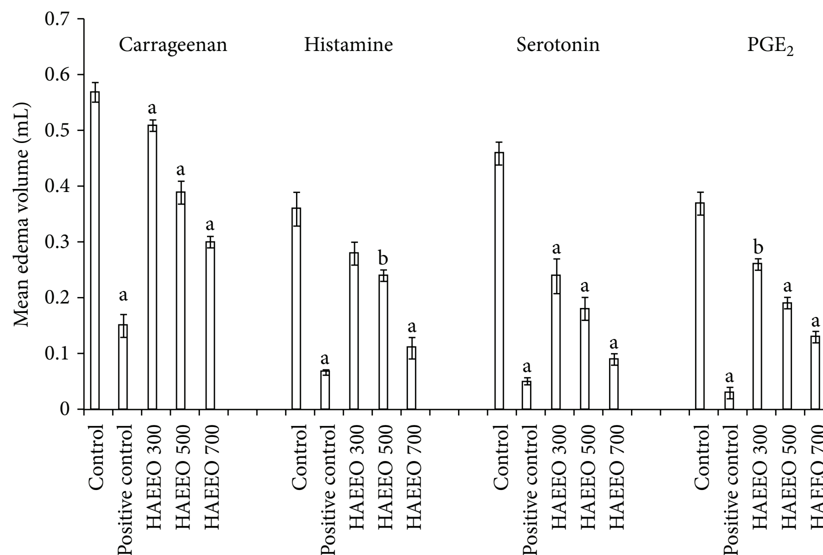

Figure 1: Effect of HAEEO on carrageenan- and autacoids-induced hind paw edema in rats. Each value represents the mean ± S.E.M. (n = 6). $^{a}P < 0.001$ and $^{b}P < 0.01$ compared to control. Positive control carrageenan (indomethacin 10 mg/kg), histamine (chlorpheniramine 3 mg/kg), serotonin (cyproheptadine 3 mg/kg), and PGE_2 (phenylbutazone 100 mg/kg). HAEEO: hydroalcoholic extract of *Emblica officinalis*.

region of each rat. Group I served as control and received the vehicle. HAEEO in the doses of 300, 500, and 700 mg/kg, i.p. was administered to animals in groups II, III, and IV for seven consecutive days from the day of cotton pellet implantation. Group V received indomethacin (10 mg/kg, p.o.) for the same period. On day 8, the animals were anaesthetized and the pellets together with the attached granuloma tissue were carefully removed and freed from extraneous tissues. The wet pellets were weighed and then dried in an oven at 60°C for 24 h to a constant weight; after that the dried pellets were weighed again. Increment in the dry weight of the pellets was taken as a measure of granuloma formation.

2.6. Determination of Levels of Oxidative Stress Parameters. The biochemical markers of oxidative stress were determined in the carrageenan-induced rat paw edema model. Animals were euthanized 3 h after measurement of paw volume and the inflamed paw tissue was removed and processed for the estimation of oxidative stress. Paw tissue samples were thawed and homogenized with 10 times (w/v) ice-cold 0.1 M phosphate buffer (pH 7.4). Aliquots of homogenates from paw tissue were used to determine the malondialdehyde (MDA) [15] and glutathione [16]. The remaining homogenates were centrifuged at 7000 rpm for 30 min at 4°C temperature and the supernatant was used for estimation of superoxide dismutase (SOD) [17], catalase [18], and protein [19].

2.7. Statistical Analysis. Data were expressed as mean ± S.E.M. Statistical differences between the treatment and the respective control groups were evaluated by one-way ANOVA followed by Tukey-Kramer post hoc test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Carrageenan-Induced Hind Paw Edema in Rats. The mean increase in paw edema volume was 1.0 ± 0.02 mL in the vehicle-treated control rats. All the three doses of HAEEO (300, 500, and 700 mg/kg, i.p.) produced a dose-dependent significant ($P < 0.001$) reduction in the mean paw edema volume (Figure 1). The percentage inhibition in paw edema volume as compared to the vehicle treated group was 48.9, 60.2, and 70.0% for HAEEO, respectively. The standard drug, indomethacin (10 mg/kg, p.o.), exhibited maximum anti-inflammatory activity with 84.27% inhibition.

3.2. Effect of HAEEO on Changes in Tissue Levels of MDA, GSH, SOD, and Catalase. Carrageenan injection into the subplantar tissue of the rat paw decreased the tissue GSH, catalase, and SOD levels (Table 1). Both HAEEO and indomethacin produced a significant increase in the endogenous antioxidants in a dose dependent manner to maintain oxidative homeostasis. Carrageenan injection produced significant lipid peroxidation, as evidenced by a marked increase in the levels of MDA. Both HAEEO and indomethacin produced a significant decrease in the levels of MDA. HAEEO at 700 mg/kg dose most effectively stabilized the oxidative stress parameters.

3.3. Autacoid-Induced Hind Paw Edema in Rats. A dose-dependent effect of HAEEO on hind paw edema was observed. The 700 mg/kg dose of HAEEO was the most effective (Figure 1). It significantly ($P < 0.001$) inhibited hind paw edema induced by histamine (68.47%), serotonin (79.26%), and PGE_2 (64.00%). Phenylbutazone (100 mg/kg, p.o.), chlorpheniramine (3 mg/kg, p.o.), and cyproheptadine

Table 1: Effect of HAEEO on oxidative stress parameters in carrageenan-induced paw edema in rats.

Treatment	GSH ($\mu g\ g^{-1}$ tissue)	MDA (nmol g^{-1} tissue)	SOD (U mg^{-1} protein)	Catalase (U mg^{-1} protein)
Normal control	32.91 ± 2.13	27.14 ± 2.96	40.54 ± 2.23	57.19 ± 2.48
Carrageenan control (vehicle treated)	13.33 ± 1.39[a]	88.45 ± 4.79[a]	15.19 ± 1.21[a]	14.48 ± 0.75[a]
Indomethacin (10 mg kg^{-1})	26.66 ± 1.66[b]	28.54 ± 6.85[b]	31.96 ± 1.08[b]	49.30 ± 1.86[b]
HAEEO (300 mg kg^{-1})	19.16 ± 1.53	63.18 ± 4.51[d]	21.18 ± 1.80	27.44 ± 1.66[b]
HAEEO (500 mg kg^{-1})	22.29 ± 2.80[d]	49.14 ± 5.83[b]	24.87 ± 0.98[d]	36.1 ± 0.83[b]
HAEEO (700 mg kg^{-1})	26.25 ± 2.18[c]	35.10 ± 2.78[b]	29 ± 1.66[b]	41.82 ± 1.41[b]

Values given are mean ± S.E.M. ($n = 6$). [a]$P < 0.001$ compared to normal control and [b]$P < 0.001$, [c]$P < 0.01$, and [d]$P < 0.05$ compared to carrageenan control. HAEEO: hydroalcoholic extract of *Emblica officinalis*; GSH: glutathione; MDA: malondialdehyde; SOD: superoxide dismutase.

Table 2: Effect of HAEEO on cotton pellet-induced granuloma in rats.

Group	Weight of cotton pellet granuloma (mg)	Protection percentage
Control (vehicle treated)	53.81 ± 1.94	—
Positive control (indomethacin 10 mg kg^{-1})	18.96 ± 2.18[a]	64.76
HAEEO (300 mg kg^{-1})	35.23 ± 1.48[a]	34.52
HAEEO (500 mg kg^{-1})	30.30 ± 0.94[a]	43.69
HAEEO (700 mg kg^{-1})	25.63 ± 1.29[a]	52.36

Each value represents the mean ± S.E.M. ($n = 6$). [a]$P < 0.001$ compared to control. HAEEO: hydroalcoholic extract of *Emblica officinalis*.

(3 mg/kg, p.o.) also significantly ($P < 0.001$) inhibited hind paw edema induced by PGE_2 (92.00%), histamine (82.06%), and serotonin (89.56%), respectively (Figure 1).

3.4. Cotton Pellet-Induced Granuloma. The study of HAEEO on proliferative phase of inflammation indicated that HAEEO (300, 500, and 700 mg/kg, i.p.) significantly ($P < 0.001$) and dose-dependently reduced the granuloma formation (Table 2). Indomethacin (10 mg/kg, p.o.) exhibited significant ($P < 0.001$) and maximum inhibition on granuloma formation.

4. Discussion and Conclusion

In the present study, it was observed that *Emblica officinalis* possessed potent anti-inflammatory activity both in acute and chronic rat models of inflammation. Inflammation is part of the host defense system and is triggered by a variety of noxious stimuli. It involves a complex interplay between cell-cell, cell-mediator, and tissue interactions [20]. Carrageenan-induced rat paw edema model is a well-established model for evaluating anti-inflammatory drugs [21]. The edema and inflammation induced by carrageenan are a biphasic event. In the initial 1 h after carrageenan administration, the edema and inflammation are mediated by histamine and serotonin. Later, the increased vascular permeability is maintained by the release of kinins up to about 2.30 h. Thereafter from 2.30 h to 6 h, inflammation is mediated by prostaglandins and is also associated with migration of leucocytes into the inflamed site [22].

Carrageenan-induced paw edema model in rats is known to be sensitive to cyclooxygenase (COX) inhibitors and has been used to investigate the effect of nonsteroidal anti-inflammatory agents [23]. The result of the present study indicated that HAEEO afforded protection against the carrageenan-induced acute inflammation in dose dependent manner. HAEEO at a dose of 700 mg/kg exhibited significant anti-inflammatory activity with 70.0% inhibition of paw edema and was comparable to the indomethacin group. In autacoid-induced models of inflammations (against serotonin, histamine, and PGE_2), HAEEO produced significant inhibitory activity. The present study exhibited HAEEO's anti-inflammatory action by means of inhibiting the synthesis, release, or action of inflammatory mediators like histamine, serotonin, and prostaglandins that are involved in inflammation. In earlier study on the anti-inflammatory activity of leaf extracts of *Emblica officinalis* in carrageenan- and dextran-induced rat paw edema models, it was reported that the extracts did not inhibit the synthesis of the lipid mediators LTB_4, TXB_2, or PAF [24]. Therefore, it is quite possible that a composite effect may have been responsible for the observed protection against autacoids-induced inflammation.

The role of excess generation of nitric oxide (NO) in inflammatory response is well studied. Inflammation or tissue damage leads to induction of iNOS (inducible nitric oxide synthase); consequently large amounts of NO are generated at the site of inflammation [25]. NO reacts with superoxide anion to form peroxynitrite, an oxidizing molecule capable of eliciting lipid peroxidation. In lipid peroxidation there is oxidative deterioration of polyunsaturated lipids to form radical intermediates that causes cellular damage [26]. MDA is a major end product of this reaction and an index of lipid peroxidation that is measurable by estimating as thiobarbituric acid reactive substance (TBARS) [27]. The present study showed that both HAEEO (500 and 700 mg/kg) and indomethacin (10 mg/kg) decreased the levels of MDA.

The infiltrating inflammatory cells also generate reactive oxygen species (ROS) and free radicals. The most common

ROS include the superoxide anion, hydroxyl radical, singlet oxygen, and hydrogen peroxide. The enzyme superoxide dismutase catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. The activity of SOD reduces during severe inflammation as well as in the presence of oxidative stress [28]. The large quantities of hydrogen peroxide generated are then taken care of by catalase and glutathione peroxidase (GPx) to water. Excessive production of lipid hydroperoxide may also lead to reduced activity of GPx in inflammatory conditions [29]. Besides the enzymatic antioxidants, the level of glutathione, a nonenzymatic reducing agent that traps free radicals and prevents oxidative damage, is also diminished in inflammatory conditions [30]. Both HAEEO (700 mg/kg) and indomethacin (10 mg/kg) maintained the oxidative homeostasis, and the levels of reduced glutathione and activities of catalase and SOD were comparable to the control animals.

Experimental studies have shown the potent antioxidant property of the fruit of *Emblica officinalis* [31]. Various phytochemical constituents of the plant such as emblicanins A and B, gallic acid, and ellagic acids have been identified as powerful free radical scavengers [9]. Moreover, other phytochemicals with NO scavenging properties like Geraniin, Corilagin, and Furosin have been reported in the *Emblica officinalis* fruit extract [32]. Recently, it has also been reported that the superoxide scavenging properties of *Emblica officinalis* extract approximate those of L-ascorbic acid, a well-established antioxidant [33].

In order to assess the efficacy of HAEEO against chronic inflammation, the cotton pellet granuloma model in rats was employed. HAEEO at all doses tested significantly ($P < 0.001$) reduced the granuloma formation. The maximum effect was observed at the dose of 700 mg/kg with 52.36% inhibition in granuloma formation as compared to the control group. Although the exact mechanism of anti-inflammatory activity of HAEEO on proliferative phase of inflammation in this model is not known, it may be hypothesized that both the antioxidant and the immunomodulatory properties of the plant may have been responsible for the protective action of the extract. *Emblica officinalis* extract has been reported to inhibit NF-κB activation, a key transcription factor involved in chronic inflammatory response and ageing [34]. The inhibition of NF-κB leads to reduction in the iNOS and COX-2 enzyme levels.

The main adverse effect of nonsteroidal anti-inflammatory drugs is their ability to produce gastric lesions [35]. Furthermore, Sairam et al. [36] demonstrated the ulcer protective potential of *Emblica officinalis* in different acute gastric ulcer models in rats induced by aspirin, ethanol, cold restraint stress, and pyloric ligation and healing effect in chronic gastric ulcers induced by acetic acid in rats. The antiulcerogenic activity of *Emblica officinalis* is definitely complementary to the good anti-inflammatory and antioxidant activity observed in the present study. Further, it has been shown that *Emblica officinalis* was well tolerated in mice even at the dose of 2.5 g/kg [37].

In conclusion, the present study clearly demonstrated that HAEEO possessed potent anti-inflammatory activity and also scientifically validated the traditional use of this plant for treating inflammatory disorders in the folk medicine. The advantages of HAEEO, namely, better and safer anti-inflammatory profile with potent antiulcerogenic activity, deserve further studies to establish the therapeutic value and elucidate the mechanism of action in the treatment of different inflammatory diseases.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Citric Acid Effects on Brain and Liver Oxidative Stress in Lipopolysaccharide-Treated Mice

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ABSTRACT Citric acid is a weak organic acid found in the greatest amounts in citrus fruits. This study examined the effect of citric acid on endotoxin-induced oxidative stress of the brain and liver. Mice were challenged with a single intraperitoneal dose of lipopolysaccharide (LPS; 200 μg/kg). Citric acid was given orally at 1, 2, or 4 g/kg at time of endotoxin injection and mice were euthanized 4 h later. LPS induced oxidative stress in the brain and liver tissue, resulting in marked increase in lipid peroxidation (malondialdehyde [MDA]) and nitrite, while significantly decreasing reduced glutathione, glutathione peroxidase (GPx), and paraoxonase 1 (PON1) activity. Tumor necrosis factor-alpha (TNF-α) showed a pronounced increase in brain tissue after endotoxin injection. The administration of citric acid (1–2 g/kg) attenuated LPS-induced elevations in brain MDA, nitrite, TNF-α, GPx, and PON1 activity. In the liver, nitrite was decreased by 1 g/kg citric acid. GPx activity was increased, while PON1 activity was decreased by citric acid. The LPS-induced liver injury, DNA fragmentation, serum transaminase elevations, caspase-3, and inducible nitric oxide synthase expression were attenuated by 1–2 g/kg citric acid. DNA fragmentation, however, increased after 4 g/kg citric acid. Thus in this model of systemic inflammation, citric acid (1–2 g/kg) decreased brain lipid peroxidation and inflammation, liver damage, and DNA fragmentation.

KEY WORDS: • ***antioxidant activity*** • ***citric acid*** • ***cytokines*** • ***dietary supplementation*** • ***peripheral infection*** • ***systemic inflammation***

INTRODUCTION

OXIDATIVE STRESS IS the term used to indicate the imbalance between reactive oxygen species and antioxidant defense mechanisms. Under physiological conditions, reactive oxygen species play integral roles in intracellular signaling, physiological immunological responses, and gene expression. Reactive oxygen metabolites can be generated in excess from many sources. The most important source is the leakage of electrons from the mitochondrial electron transport chain to generate superoxide radical ($O_2^{\bullet -}$). Other sources are xanthine oxidase, NADPH oxidases, activated phagocytes, and nitric oxide synthases (NOSs). When excessively produced, however, these species could result in potential cellular and tissue damage. Being highly unstable molecules with unpaired electrons, reactive oxygen metabolites, such as superoxide radical and hydroxyl radical, react with the cellular membrane polyunsaturated fatty acids to form lipid peroxides, oxidize and cross-link proteins including enzymes, or oxidize DNA, with the potential to produce a harmful or even lethal event.[1–3] Cellular defenses against free radicals and reactive oxygen species include enzymes, such as catalase, glutathione peroxidase (GPx), and superoxide dismutase, as well as nonenzymatic antioxidant mechanisms, for example, glutathione (GSH), ascorbic acid, carotenoids, and vitamin E.[2,4] Oxidative stress occurs when redox homeostasis is tipped toward an overbalance of free radicals, due to either their overproduction or deficiencies in antioxidant defense.[5] Oxidative stress has been implicated in the pathogenesis of numerous diseases, such as diabetes mellitus, cardiovascular disease, and neurodegenerative and psychiatric disorders.[6,7] The brain is considered particularly vulnerable to oxidative damage because of its high oxygen utilization and hence generation of free radical byproducts, the high content of polyunsaturated lipids, the biomacromolecules most susceptible to oxidation, its modest antioxidant defenses, and the presence of redox-catalytic metals, such as iron and copper.[7,8]

Citric acid (2-hydroxy-1,2,3-propane-tricarboxylic acid) is a weak organic acid found in the greatest amounts in citrus fruits, such as lemon, grapefruit, tangerine, and orange. Lemon and lime juices are rich sources.[9] It is used as a natural preservative and also to add an acidic (sour) taste to foods and soft drinks.[10] Being a component of the tricarboxylic acid or Krebs cycle, citric acid is found in all animal

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tissues as an intermediary substance in oxidative metabolism. Studies indicated that citrate decreases lipid peroxidation and downregulates inflammation by reducing polymorphonuclear cell degranulation and attenuating the release of myeloperoxidase, elastase, interleukin (IL)-1β, and platelet factor 4.[11–13] *In vitro*, citrate improved endothelial function by reducing the inflammatory markers and decreasing neutrophil diapedesis in hyperglycemia.[14] Moreover, citric acid has been shown to reduce hepatocellular injury evoked in rats by carbon tetrachloride.[15] Citric acid might thus prove of value in decreasing oxidative stress.

Thus, in view of the antioxidant and anti-inflammatory effects for citrate reported just now and since citrate anticoagulation has been employed in the critically ill patients, it looked pertinent to study the effect of citric acid administration on oxidative stress and tissue injury in a model of systemic inflammatory illness caused by intraperitoneal (i.p.) lipopolysaccharide (LPS) administration in mice. LPS is a constituent of the cell walls of gram-negative bacteria. When given systemically, LPS potently stimulates the immune cells in the periphery (through plasma membrane proteins, *e.g.*, the toll like receptor 4 [TLR4] and CD14) to release proinflammatory cytokines, such as necrosis factor-alpha (TNF-α), IL-1β, and IL-6 in the periphery and brain. This results in the development of systemic and neuroinflammation.[16–19] LPS-induced endotoxemia is a well-established model for infection with gram-negative bacteria and is widely used to study endotoxin effects on peripheral tissue/organs and the influence of systemic inflammation on the brain.

MATERIALS AND METHODS

Animals

Swiss male albino mice that weigh 22–25 g (age 5–6 weeks) were used. Mice were obtained from animal house colony of the National Research Centre. Standard laboratory food and water were provided *ad libitum*. Animal procedures were performed in accordance with the Ethics Committee of the National Research Centre and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

Drugs and chemicals

A purified, lyophilized *Escherichia coli* endotoxin (Serotype 055:B5; Sigma) was used; it was dissolved in sterile physiological saline, aliquoted, and frozen at −20°C. The same stock solutions were used for all experiments. Citric acid and all other chemicals were of analytical grade and were obtained from Sigma. The dose of LPS (200 μg/kg) and the time for tissue sampling were based on previous studies.[20]

Study design

Mice were randomly divided into five equal groups (six mice each). Mice were treated with either 0.2mL of: sterile physiological saline (group 1) or citric acid at doses of 1, 2, and 4 g/kg, orally (groups 2–4). Treatments were given just prior to endotoxin administration (LPS: 200 lg/kg, injected intraperitoneally, 0.1 mL). The fifth group received just the vehicle, no LPS (negative control). Mice were euthanized after 4 h of LPS or vehicle injection by decapitation under ether anesthesia, where the brain and liver of each mouse were then removed, washed with ice-cold phosphate-buffered saline (PBS; pH 7.4), weighed, and stored at −80°C until the biochemical analyses. The tissues were homogenized with 0.1 M PBS at pH 7.4, to give a final concentration of 0.1g/mL for the biochemical assays. Reduced GSH, malondialdehyde (MDA), nitric oxide (nitrite), GPx, and paraoxonase 1 (PON1) activity was determined in brain and liver tissues. TNF-α was measured in brain tissue. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and DNA fragmentation were measured in liver tissue.

Determination of lipid peroxidation, reduced GSH, and nitrite levels

Lipid peroxidation was assayed by measuring the level of MDA in brain tissue using the method of Ruiz-Larrea *et al.*[21] Reduced GSH was determined in tissue by Ellman's method.[22] Nitric oxide measured as nitrite was determined by using Griess reagent, according to the method of Moshage *et al.*[23]

Determination of GPx activity

GPx activity in supernatants was determined spectrophotometrically at 340 nm by the analysis of NADPH oxidation using glutathione peroxidase kit (Biodiagnostics).[24] One unit of GPx activity is defined as the amount of protein that oxidized 1 mM NADPH per minute. The activity of GPx is expressed as mU/mL.

Determination of paraoxonase activity

Arylesterase activity of paraoxonase was measured spectrophotometrically in supernatants using phenyl acetate as a substrate.[25,26] In this assay, arylesterase/paraoxonase catalyzes the cleavage of phenyl acetate resulting in phenol formation. The rate of formation of phenol is measured by monitoring the increase in absorbance at 270 nm at 25°C. The working reagent consisted of 20 mM Tris/HCl buffer (pH 8.0) containing 1 mM calcium chloride and 4 mM phenyl acetate as the substrate. Samples diluted 1:3 in buffer are added and the change in absorbance is recorded following a 20 s lag time. Absorbance at 270 nm was taken every 15 s for 120 s using a UV-Vis Recording Spectrophotometer (Shimadzu Corporation). One unit of arylesterase activity is equal to 1 μM of phenol formed per minute. The activity is expressed in kU/L, based on the extinction coefficient of phenol of 1310 M/cm at 270 nm, pH 8.0, and 25°C. Blank samples containing water are used to correct for the spontaneous hydrolysis of phenyl acetate.

Determination of TNF-α, DNA fragmentation, and liver enzymes

Tissue TNF-α was determined in brain tissue according to Chen *et al.*[27] by enzyme-linked immunosorbent assay using TNF-α kits (Biosource International) and microtiter plate reader (Fisher Biotech). Quantitation of DNA fragmentation in liver tissue was done according to the method described by Gercel-Taylor.[28] ALT and AST activities in liver were measured using commercially available kits (BioMérieux).[29,30]

Histological assessment of liver injury

The liver from each mouse was rapidly removed and fixed in freshly prepared 10% neutral buffered formalin, processed routinely, and embedded in paraffin. Sections of 5-μm thick were cut and stained by hematoxylin and eosin (H&E) for histopathological examination. All sections were investigated by the light microscope.

Immunohistochemistry for caspase-3 and inducible nitric oxide synthase

Paraffin-embedded liver sections were deparaffinized, and hydrated. Immunohistochemistry was performed with a mouse monoclonal caspase-3 and inducible nitric oxide synthase (iNOS) for detection of the caspase cleavage and iNOS activity. The paraffin sections were heated in a microwave oven (25 min at 720 W) for antigen retrieval and incubated with either anti-caspase or iNOS antibodies (1:50 dilution) overnight at 4°C. After washing with PBS, followed by incubation with biotinylated goat-anti-rabbit-immunoglobulin G secondary antibodies (1:200 dilution; Dako Corp.) and streptavidin/alkaline phosphatase complex (1:200 dilution; Dako) for 30 min at room temperature, the binding sites of antibody were visualized with DAB (Sigma). After washing with PBS, the samples were counterstained with H&E for 2–3 min, and dehydrated by transferring them through increasing ethanol solutions (30%, 50%, 70%, 80%, 95%, and 100% ethanol). Following dehydration, the slices were soaked twice in xylen at room temperature for 5 min, mounted, examined, and evaluated by high-power light microscope.[31]

Statistical analysis

Data are expressed as mean ± standard error. Data were analyzed by one-way analysis of variance, followed by Duncan's multiple-range test for *post hoc* comparison of group means. Effects with a probability of $P < .05$ were considered to be significant.

RESULTS

Effect of citric acid on LPS-induced oxidative stress

Lipid peroxidation. The administration of LPS resulted in a significant increase in the level of MDA in brain and liver tissues by 140.3% (23.1 ± 1.0 vs. 55.5 ± 2.7 nmol/g tissue) and 62.9% (66.8 ± 3.8 vs. 41.0 ± 2.2 nmol/g tissue), respectively, compared with the saline control group (Fig. 1A, B).

Brain MDA was significantly decreased by 40.4% and 58% after treatment with 1 and 2 g/kg citric acid, respectively, compared with the LPS control group (33.1 ± 1.9 and 23.3 ± 1.4 vs. 55.5 ± 2.7 nmol/g tissue). The higher dose of citric acid (4 g/kg) resulted in 33.3% inhibition of brain MDA (Fig. 1A).

In contrast, no significant effect on liver MDA has been observed after treatment with citric acid (1–4 g/kg; Fig. 1B).

Reduced GSH. Following LPS challenge, the level of GSH decreased in brain and liver tissues by 72.1% (1.21 ± 0.07 vs. 4.1 ± 0.28 μmol/g tissue) and 46.9% (4.16 ± 0.29 vs. 7.83 ± 0.36 μmol/g tissue), respectively.

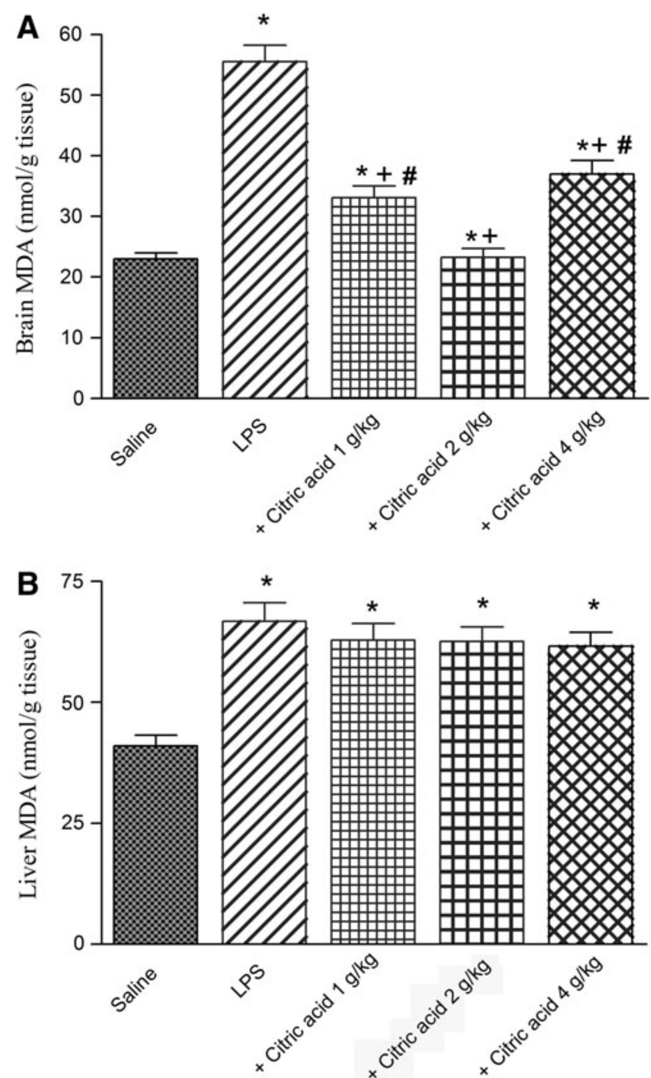

FIG. 1. **(A)** Brain and **(B)** liver tissue concentrations of malondialdehyde (MDA: nmol/g tissue) in mice given lipopolysaccharide (LPS) or LPS + citric acid (1–4 g/kg, p.o.). $^*P < .05$ versus saline control. $^+P < .05$ versus LPS control group. $^\#P < .05$ versus LPS + 2 g/kg of citric acid. p.o., per os.

Treatment with citric acid (1–4 g/kg) had no significant effect on brain or liver GSH (Fig. 2A, B).

Nitrite. Marked and significant increase in brain nitrite was observed after treatment with LPS compared with the vehicle-treated group (93.0 ± 4.6 vs. 31.0 ± 1.8 μmol/g tissue; Fig. 3A). Similarly, the level of liver nitrite was significantly increased by 86.3% after LPS administration compared with vehicle-treated group (123.7 ± 8.6 vs. 66.4 ± 4.1 μmol/g tissue; Fig. 3B).

In LPS-treated mice, the level of nitrite in brain tissue was markedly inhibited by 74.6% and 82.8% by citric acid at 1–2 g/kg (23.6 ± 1.2 and 16.0 ± 0.63 vs. 93.0 ± 4.6 μmol/g tissue). Nitric oxide decreased by 48.1% after citric acid at 4 g/kg, compared with the LPS-only group (Fig. 3A).

In the liver, nitrite decreased significantly by 17% by citric acid given at 1 g/kg compared with the LPS control group. The higher doses of citric acid, however, failed to significantly alter nitrite in liver tissue (Fig. 3B).

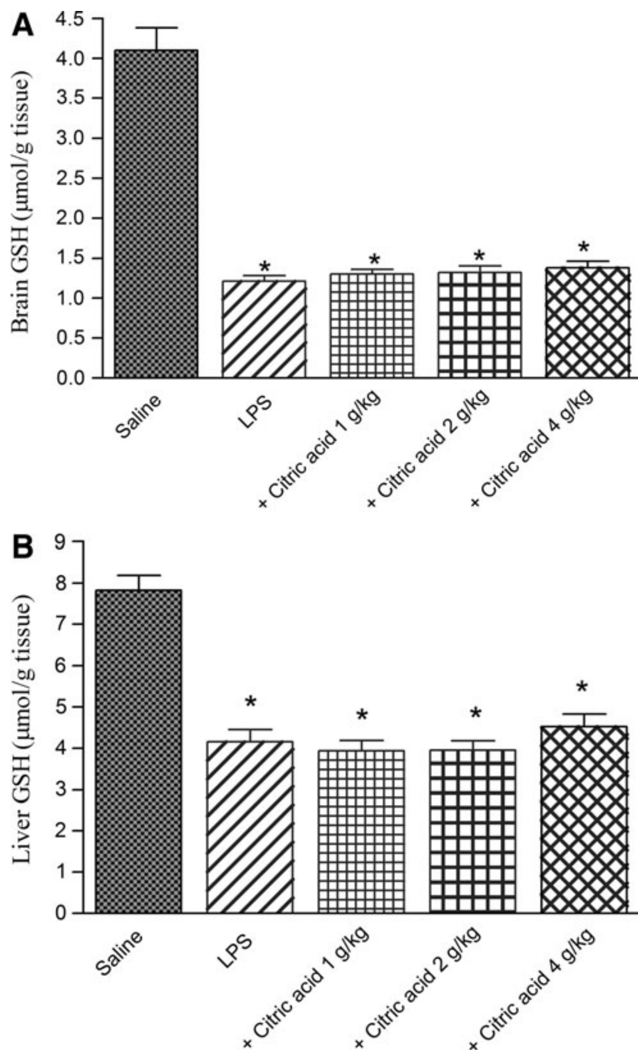

FIG. 2. **(A)** Brain and **(B)** liver tissue concentrations of reduced glutathione (GSH: μmol/g tissue) in mice given LPS or LPS + citric acid (1–4 g/kg, p.o.). $^*P < .05$ versus saline control.

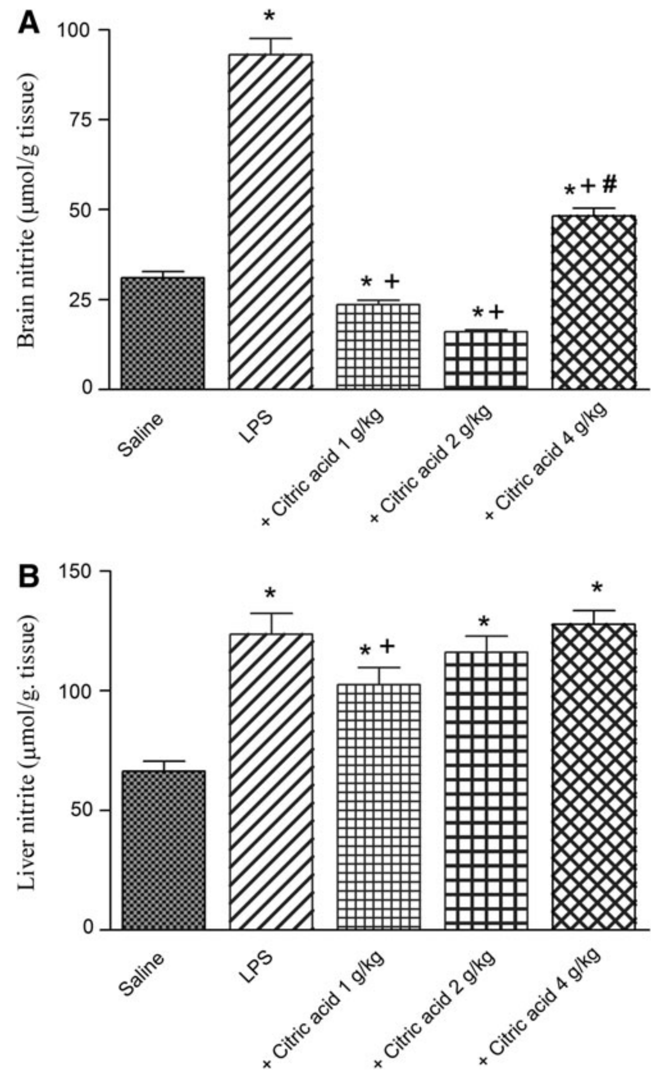

FIG. 3. **(A)** Brain and **(B)** liver tissue concentrations of nitrite (μmol/g tissue) in mice after treatment with LPS or LPS + citric acid (1–4 g/kg, p.o.). $^*P < .05$ versus saline control. $^+P < .05$ versus LPS control group. $^\#P < .05$ versus LPS + 2 g/kg of citric acid.

GPx activity. GPx activity showed a significant decrease in brain (by 81.6%) and liver tissues (by 47.3%) after LPS challenge compared with the vehicle-treated group. Brain GPx activity increased by 82.6% after treatment with 1 g/kg citric acid ($P < .05$) compared with the LPS control group (0.80 ± 0.052 vs. 1.015 ± 0.061 U/g tissue). No significant effect was observed in brain GPx activity after treatment with citric acid at 2 or 4 g/kg (Fig. 4A). On the other hand, liver GPx activity significantly increased by 29.7%, 79.6%, and 56.5% after treatment with 1, 2, and 4 g/kg of citric acid, respectively (Fig. 4B).

Paraoxonase activity. Paraoxonase activity significantly decreased in brain and liver tissues by 54.2% (11.3 ± 0.7 vs.

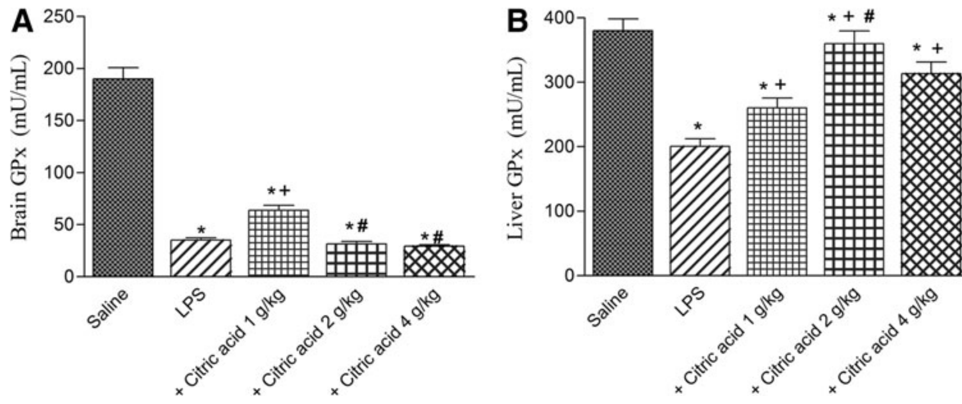

FIG. 4. Glutathione peroxidase (GPx) activity in **(A)** brain and **(B)** liver of mice after LPS or LPS + citric acid (1–4 g/kg, p.o.). $^{*}P<.05$ versus saline control. $^{+}P<.05$ versus LPS control group. $^{\#}P<.05$ versus LPS + 1 g/kg of citric acid.

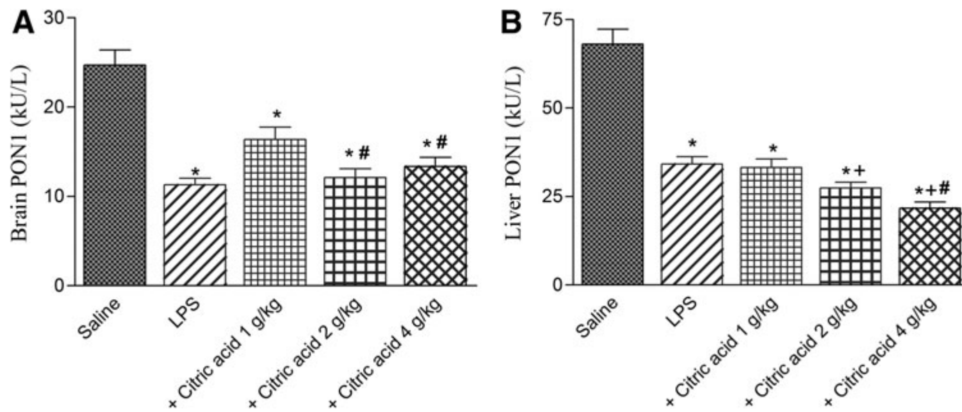

FIG. 5. Paraoxonase 1 (PON1) activity in mice **(A)** brain and **(B)** liver after treatment with LPS or LPS + citric acid (1–4 g/kg, p.o.). $^{*}P<.05$ versus saline control. $^{+}P<.05$ versus LPS control group. $^{\#}P<.05$ versus LPS + 1 g/kg of citric acid.

24.7 ± 1.8 kU/L) and 49.8% (34.2 ± 2.1 vs. 68.1 ± 4.2 kU/L), respectively, after LPS challenge (Fig. 5A, B). Brain PON1 activity increased by 44.9% following treatment with citric acid at 1 g/kg. Higher doses, however, failed to significantly alter PON1 activity (Fig. 5A). On the other hand, liver PON1 activity significantly decreased by 19.7% and 36.6% after treatment with citric acid at 2 and 4 g/kg, respectively, compared with the LPS control group (Fig. 5B).

TNF-α in brain tissue. A pronounced increase in TNF-α in mice brain was observed following i.p. injection of LPS (324.9% increase: 182.7 ± 6.2 vs. saline control value of 43.0 ± 2.7 pg/g tissue). TNF-α showed a significant decrease by 48.4% and 28.8% after treatment with citric acid at 1 and 2 g/kg (93.3 ± 3.8 and 130.0 ± 4.3 vs. LPS control value of 182.7 ± 6.2 pg/g tissue). The administration of citric acid at 4 g/kg failed to decrease the level of TNF-α (Fig. 6).

DNA fragmentation in the liver. DNA fragmentation in the liver was significantly and markedly increased by 633.5% after LPS injection compared with the vehicle-treated mice. It showed a 49.4% and 82.6% decrease after treatment with citric acid at 1 and 2 g/kg, respectively, compared with the LPS control value. However, a 92.5% increment in DNA fragmentation was observed after the highest dose of citric acid (4 g/kg; Fig. 7).

Liver transaminases. In LPS-treated mice liver, ALT and AST significantly increased by 145.4% and 204.8% compared with the saline-treated group. ALT significantly decreased by 22.5% after treatment with 1 g/kg of citric acid. The higher doses of citric acid, however, failed to

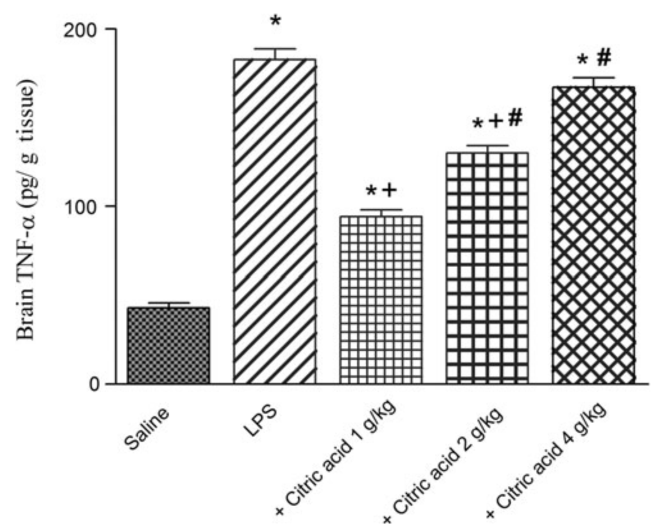

FIG. 6. Brain tissue tumor necrosis factor-alpha (TNF-α; pg/g tissue) in mice given LPS or LPS + citric acid (1–4 g/kg, p.o.). $^{*}P<.05$ versus the saline control. $^{+}P<.05$ versus LPS control group. $^{\#}P<.05$ versus the LPS + 1 g/kg of citric acid.

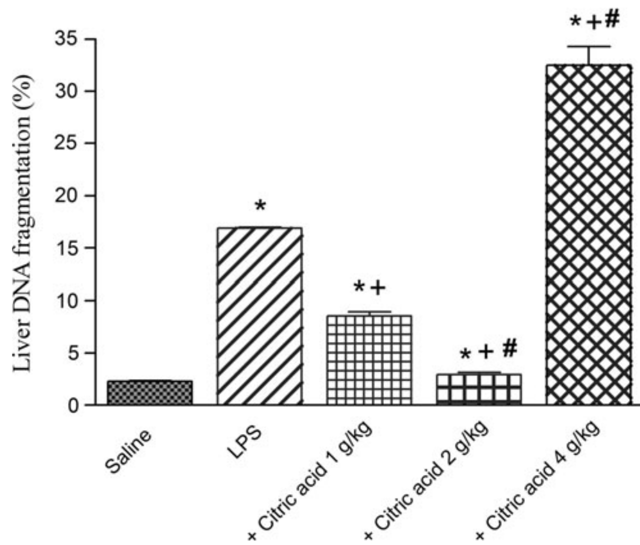

FIG. 7. DNA fragmentation (%) in mice liver after LPS or LPS + citric acid (1–4 g/kg, p.o.). $^{*}P<.05$ versus saline control. $^{+}P<.05$ versus LPS control group. $^{\#}P<.05$ versus LPS + 1 g/kg of citric acid.

significantly alter ALT in the liver of LPS-treated mice. Meanwhile, AST significantly decreased by 26.5% and 30.4% after treatment with 1 and 2 g/kg citric acid, respectively. The highest dose of citric acid, however, had no significant effect on liver AST in LPS-treated mice (Fig. 8A, B).

Histological results

The control livers showed normal hepatic architecture with distinct hepatic cells, sinusoidal spaces, and a central vein (Fig. 9A).

Histological examination of the liver from LPS-treated mice revealed mononuclear cell infiltrations, bile duct proliferation in the periportal areas, and minimal enlargement in the periportal areas. In the LPS group we also observed dilatation and congestion of the central vein and blood sinusoids that showed numerous Kupffer cells. Hepatocytes exhibited necrotic changes in the form of small pyknotic nuclei with condensed or marginated chromatin, lack of nucleoli, and strongly acidophilic cytoplasm (Fig. 9B, C).

On the other hand, histological examination of liver sections from mice treated with LPS + citric acid at 1 g/kg showed nearly normal hepatic architecture. The hepatic lobules appeared with prominent central vein with less sinusoidal dilatation and decreased number of Kupffer cells compared with the LPS-only-treated group (Fig. 9D).

The improvement in histological appearance was more pronounced after treatment with citric acid at 2 g/kg, evidenced in normal appearance of liver lobules with strains of hepatocytes compared with section of LPS groups (Fig. 9E).

In contrast, sections from mice treated with LPS and 4 g/kg of citric acid showed mild improvement with dilated portal areas. The hepatocytes exhibited some degree of histological regeneration with less sinusoidal dilatation and with decreased number of Kupffer cells and less necrotic cells (Fig. 9F).

Caspase-3 and iNOS immunoreactivity

Activated caspase-3 labeling was specific in delineating morphologically apoptotic cells. Caspase-3 and iNOS expression was localized in the cytoplasm of hepatocytes. There was negligible caspase-3 (Fig. 10A-i) and iNOS (Fig. 10A-ii) immunopositivity in the livers of vehicle-treated mice. After treatment with LPS strong expression of caspase-3 (Fig. 10B-i) and iNOS (Fig. 10B-ii) was observed compared with the vehicle control group. In these sections, caspase-3 and iNOS immunoreactivity was observed mainly around central vein.

Caspase-3 and iNOS immunopositivity decreased in the livers of LPS-intoxicated mice treated with 1 g/kg of citric acid (Fig. 10C) and 2 g/kg of citric acid (Fig. 10D), respectively. In contrast, citric acid in the high dose of 4 g/kg was not effective in reducing caspase-3 (Fig. 10E-i) and iNOS expression (Fig. 10E-ii).

DISCUSSION

In the present model of mild systemic inflammation caused by a subseptic dose of LPS endotoxin and associated with increased oxidative stress in brain and liver tissues,

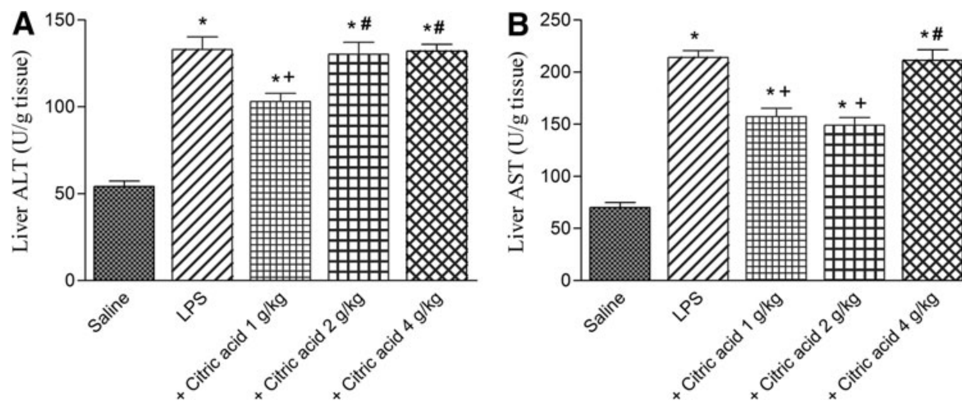

FIG. 8. **(A)** Alanine aminotransferase (ALT) and **(B)** aspartate aminotransferase (AST) activities in mice liver after LPS or LPS + citric acid (1–4 g/kg, p.o.). $^{*}P<.05$ versus saline control. $^{+}P<.05$ versus LPS control group. $^{\#}P<.05$ versus LPS + 1 g/kg of citric acid.

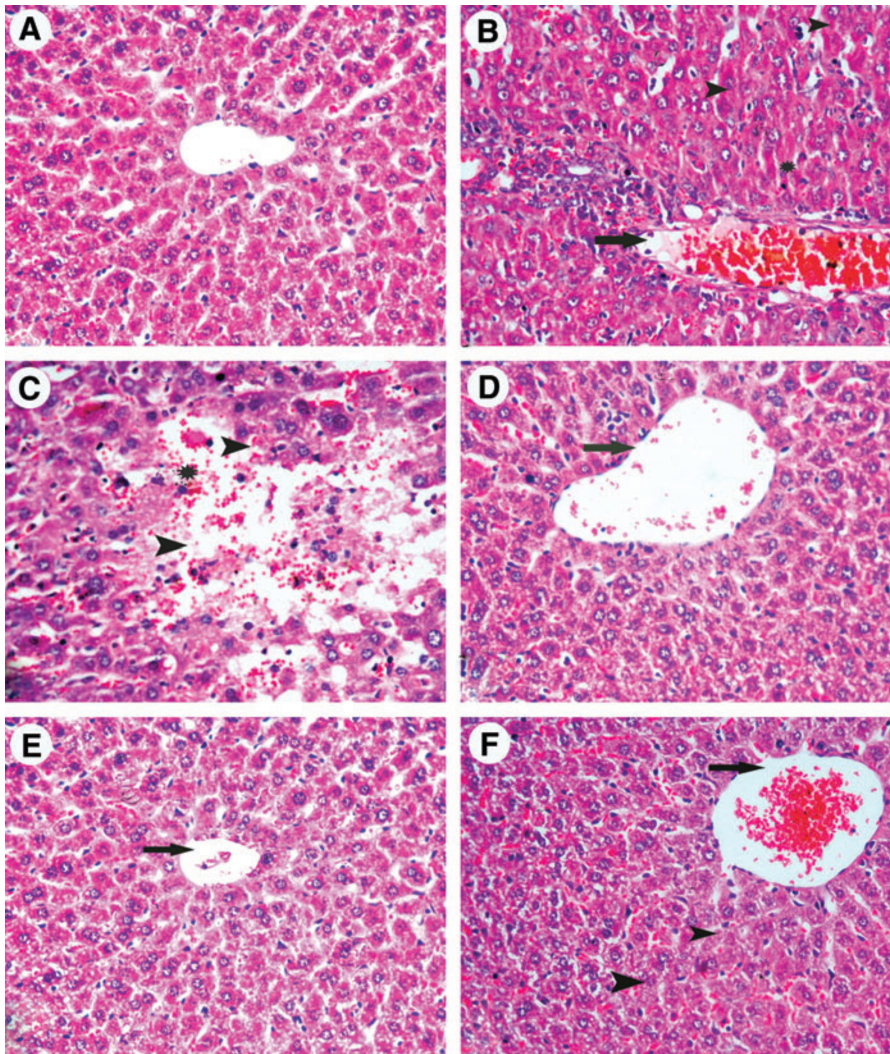

FIG. 9. Hematoxylin and eosin (H&E)–stained liver sections from mice treated with **(A)** saline (control). **(B)** LPS: inflammatory leukocytic cell infiltration around portal area (long arrow), necrosis (arrow head), dilated and congested blood sinusoids, and marinated nuclear chromatin (star). **(C)** LPS: focal necrotic area (arrow head), activated Kupffer cells, dilated and congested blood sinusoids, and pyknotic nuclei (star). **(D)** LPS + citric acid 1 g/kg: congestion of central vein (long arrow), dilated blood sinusoids, and few necrotic cells (arrowhead). **(E)** LPS + citric acid 2 g/kg: normal central vein (long arrow), minimally dilated blood sinusoids, and few Kupffer cells. **(F)** LPS + 4 g/kg of citric acid: congestion of central vein (long arrow), signs of degeneration of hepatocytes, dilated congested blood sinusoids, and few Kupffer cells (H&E × 400). Color images available online at www.liebertpub.com/jmf

citric acid exerted important pharmacological effects. A significant and marked decrease in lipid peroxidation (measured as MDA) was observed in brain tissue after treatment with citric acid, thereby suggesting decreased free radical attack on polyunsaturated fatty acids. In contrast, no significant effect on liver MDA has been observed after treatment citric acid. In both the brain and liver, however, citric acid displayed marked inhibitory effect on nitric oxide. Under physiological conditions, this free radical gas synthesized from the amino acid L-arginine by the enzyme NOS is important in neurotransmission, maintaining vascular tone, immune regulation, synaptic plasticity, and many other functions.[32,33] Increased levels of nitric oxide generated by glial cells, including astrocytes and microglia, due to action of inducible NOS, however, contributes to neuronal cell death in inflammatory, infectious, ischemic, and neurodegenerative diseases.[34] This is due to the ability of nitric oxide to react with other free radicals, especially with the oxygen radical superoxide (O_2^-), to form peroxynitrite ($ONOO^-$), decomposing to form the powerful and cytotoxic oxidants hydroxyl radical and nitrogen dioxide.[35,36]

In face of increased free radicals and reactive oxygen species, cells are equipped with a number of antioxidant mechanisms, such as catalases, GPxs, glutathione transferase, superoxide dismutase, and GSH.[3] The administration of LPS was associated with an increase in lipid peroxidation and a drop in GSH level and GPx activity in brain and liver tissues, which indicates increased generation of free radicals. In LPS-treated mice, brain and liver GSH were not altered by citric acid. Meanwhile, treatment with citric acid at 1 g/kg was associated with increased GPx activities in brain and liver tissues, possibly due to an antioxidant effect of citric acid. In the current study, decreased brain and liver PON1 activity was observed after the administration of LPS. PON1 enzyme that plays an important role in the metabolism of many xenobiotic compounds has recently drawn attention, for a possible role in protecting cellular membranes against lipid peroxidation.[25,26] In brain tissue, PON1 activity was improved by citric acid given at 1 g/kg. PON1 activity in liver tissue, however, decreased following higher doses of citric acid, possibly reflecting consumption or inactivation of the enzyme by increased free radicals with high concentration of citric acid.

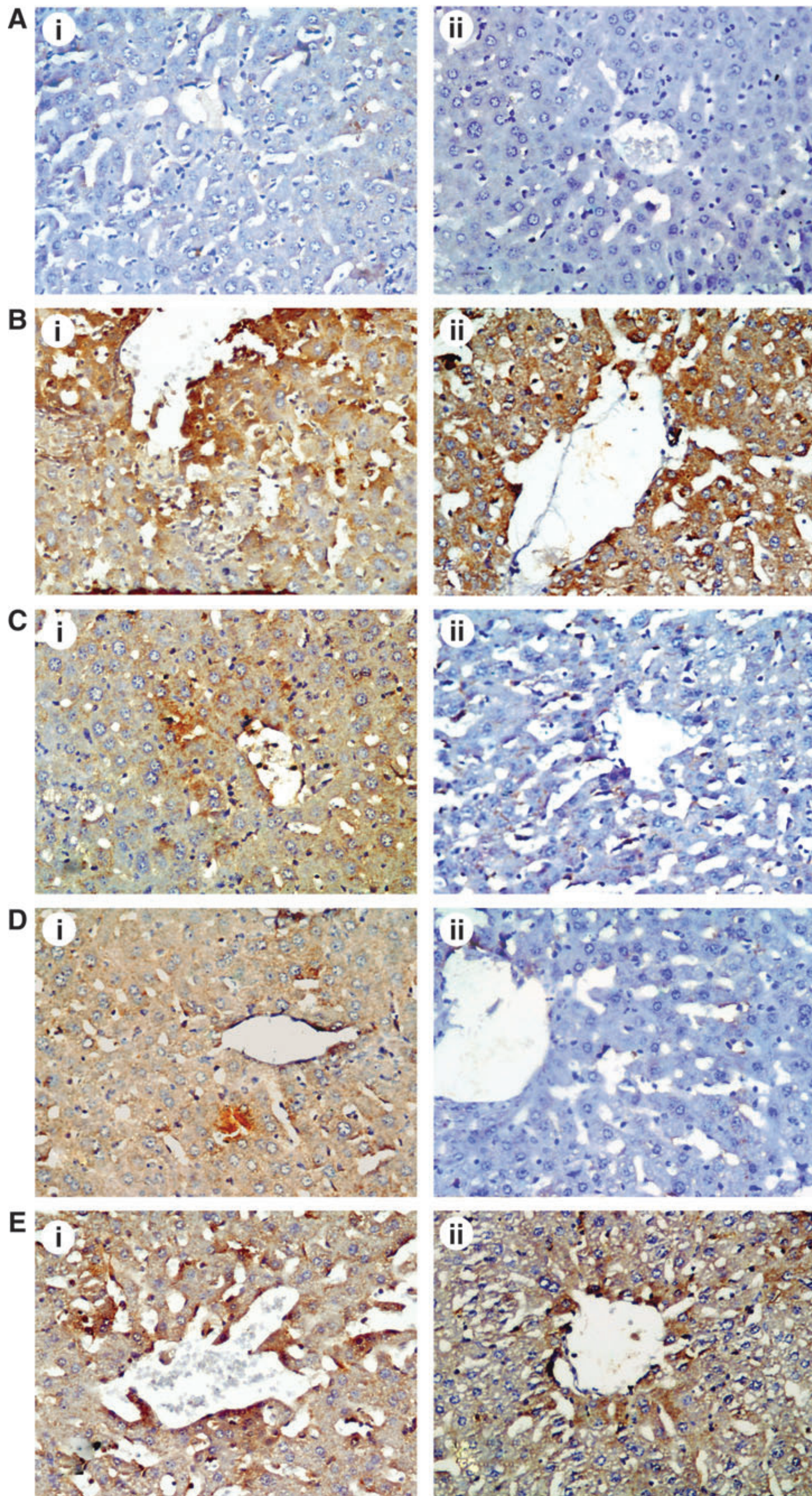

FIG. 10. The effect of LPS and citric acid treatment on hepatic caspase-3 (**i**) and inducible nitric oxide synthase (iNOS) (**ii**) immunostaining: **(A)** control liver; **(B)** LPS; **(C)** LPS + citric acid 1 g/kg; **(D)** LPS + citric acid 2 g/kg; **(E)** citric acid 4 g/kg (caspase-3 and iNOS immunohistochemistry, hematoxylin counterstain ×400). Color images available online at www.liebertpub.com/jmf

One potent proinflammatory cytokine is TNF-α, which is produced in the brain by glial cells in response to various stimuli and induces astrocytes and microglial cells to secrete several inflammatory mediators, such as chemokines, lipid mediators, nitric oxide, and other free radicals. TNF-α has been demonstrated to play an important role in central nervous system neuroinflammation-mediated cell death in various neurodegenerative conditions.[37,38] In the present study, the cytokine was markedly increased in brain tissue after LPS administration. Here we demonstrate that citric acid treatment was associated with marked inhibitory effect on TNF-α production within brain tissue after LPS challenge. This ability of citric acid to decrease pathological TNF-α production in the brain might be of value in relevance to neurodegenerative diseases. TNF-α expression appears to be upregulated in several neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis, and pharmacological manipulation of TNF-α within the brain has been proposed as one potential target in the treatment of these conditions and may represent a valuable target for intervention.[39–41]

The present data indicate that citric acid can act directly on brain cells to inhibit their production of TNF-α and nitrite. LPS acts on TLR4 receptors on macrophages, dendritic cells, and other immune cells to release proinflammatory cytokines, such as TNF-α and IL-1β, which might gain direct access to the brain via the blood–brain barrier or signal to the brain via the vagus nerve, the so-called gut–brain immune communication.[42,43] Thus it is also possible that the effects of citric acid on brain are accounted for by modulating the release of inflammatory mediators from leucocytes in the periphery.

Differences in results for the same markers in liver and brain tissues were observed. Thus, in contrast to the effects of citric acid in decreasing lipid peroxidation in the brain, no significant effect on liver MDA was observed. Moreover, PON1 activity in liver tissue decreased following citric acid at high doses. This might be due to the particular metabolic pathway interrelationships within each so different organ. Nevertheless, DNA fragmentation, serum transaminase elevations, caspase-3 and iNOS expression, and histological damage were all attenuated by 1–2 g/kg of citric acid. These data clearly indicated a protective effect for citric acid administration within this dose range on hepatic damage during endotoxemia. Citric acid intake, therefore, is likely to have a beneficial effect on the liver under toxic and inflammatory conditions. Citric acid might prevent liver injury through (1) reducing polymorphonuclear cell degranulation and attenuating the release of myeloperoxidase, elastase, IL-1β, and platelet factor; (2) stimulation of glycolysis and the tricarboxylic acid cycle; (3) increased production of bicarbonate with improvement of tissue acidosis in inflammatory conditions and therefore maintains tissue and cellular integrity; and (4) stimulation of vagal sensory afferents involved in signaling hepatic protection.[11–13,15,44,45]

It should be noted, however, that some of the beneficial effects observed for citric acid in brain and liver tissues were only in the dose range of 1–2 g/kg. This protective effect is lost when the dose is increased to 4 g/kg; for example, GPX and PON1 activities were increased only with 1 g/kg and TNF was decreased only by 1–2 g/kg of citric acid; the doses that were most effective in inhibiting brain nitrite. The protective effects on the liver were also lost with the higher dose of 4 g/kg, which also increased liver DNA fragmentation. Since citric acid is found in all animal tissues as an intermediate in the Krebs cycle, no limit has been set on the acceptable daily intake for humans for either the acid or salt.[46] It is possible, however, that at higher concentrations, citric acid acts as a pro-oxidant. Several antioxidants show pro-oxidant effects at higher doses/concentrations, for example, carotenoids,[47] vitamin E, and vitamin C.[48,49] Natural compounds also display double-edged effects on inflammatory reactions, depending potentially on their concentrations: physiologic doses leading to beneficial effects whereas high doses may result in harmful effects.[50]

In summary, the present data suggest an antioxidant and anti-inflammatory effect for orally given citric acid at 1–2 g/kg in brain tissue. Citric acid also demonstrated a beneficial hepatic protective effect at this dose range. Given that both increased brain oxidative stress and chronic inflammation have been linked to the development of neurodegenerative diseases, citric acid might thus prove of clinical benefit in such conditions. The present study suggests that citric acid might find utility in treatment of toxic and inflammatory conditions of the brain and liver tissues. This can take the form of supplementation as nutraceutical citric acid. Meanwhile, citric acid is naturally concentrated in citrus fruits with lemon juice and lime juice being rich sources of citric acid and intake of these has been suggested as an effective means of treating oxalate stones.[1,51,52] These studies have addressed the utility of dietary intervention with fruits and fruit juices with high citrate content (orange juice and lemonade) as an alternative to potassium citrate in increasing urinary pH and citrate, but the combination of citrate supplementation and fruit juices was not evaluated. This latter approach might prove a useful one combining the advantages of both classes of food additives. The presence of flavonoids and vitamin C in citrus fruits and juices makes the latter option an attractive one.

AUTHOR DISCLOSURE STATEMENT

The authors declare that there are no conflicts of interest.

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Lemon juice has protective activity in a rat urolithiasis model

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Abstract

Background: The use of herbal medicines (medicinal plants or phytotherapy) has recently gained popularity in Europe and the United States. Nevertheless the exact mechanism of the preventive effects of these products is still far to be clearly established, being its knowledge necessary to successfully apply these therapies to avoid stone formation.

Methods: The effect of oral lemon juice administration on calcium oxalate urolithiasis was studied in male Wistar rats. Rats were rendered nephrolithic by providing drinking water containing 0.75% ethylene glycol [v/v] (EG) and 2% ammonium chloride [w/v] (AC) for 10 days. In addition to EG/AC treatment, three groups of rats were also gavage-administered solutions containing 100%, 75% or 50% lemon juice [v/v] (6 μl solution/g body weight). Positive control rats were treated with EG/AC but not lemon juice. Negative control rats were provided with normal drinking water, and were administered normal water by gavage. Each group contained 6 rats. After 10 days, serum samples were collected for analysis, the left kidney was removed and assessed for calcium levels using flame spectroscopy, and the right kidney was sectioned for histopathological analysis using light microscopy.

Results: Analysis showed that the rats treated with EG/AC alone had higher amounts of calcium in the kidneys compared to negative control rats. This EG/AC-induced increase in kidney calcium levels was inhibited by the administration of lemon juice. Histology showed that rats treated with EG/AC alone had large deposits of calcium oxalate crystals in all parts of the kidney, and that such deposits were not present in rats also treated with either 100% or 75% lemon juice.

Conclusion: These data suggest that lemon juice has a protective activity against urolithiasis.

Background

Kidney stone formation or urolithiasis is a complex process that is a consequence of an imbalance between promoters and inhibitors in the kidneys [1]. The recurrence of urolithiasis represents a serious problem as patients who have formed one stone are more likely to form another. Not all standard pharmaceutical drugs used to prevent urolithiasis are effective in all patients, and many have adverse effects that compromise their long-term use [2].

Renal calculi can be broadly classified in two large groups: tissue attached and unattached [3]. Attached calculi are mainly integrated by calcium oxalate monohydrate (COM) renal calculi, with a detectable attachment site to the renal papilla and basically consisting of a core located near to the attachment site (concave zone) and radially striated concentrically laminated peripheral layers. Unattached calculi, with no detectable site of attachment to papilla, are developed in renal cavities of low or reduced urodynamic efficacy and can exhibit diverse composition and structures. Several reports have been published since Randall's first description of papillary calcifications and their possible active role in the genesis of COM papillary calculi [4-6]. At present, it seems clear that renal epithelial cell injuries play a decisive role in such a type of renal calculi development [7,8], and in fact the lithogenic effect caused by ethylene glycol (EG) must be mainly attributed to the oxidative damage caused by the high level of oxalate generated by EC. Thus, although EC rat model can be questioned as a general model to study renal stone formation, it must be considered as an interesting model to evaluate renal papillary stone development, at least for those stones which genesis is linked to oxidative cell damage. Thus, the first studies on experimental EC renal lithiasis appeared in the 60' decade [9,10] but the importance of the oxidative damage caused by hyperoxaluria was not clearly proposed until the end of the century [11]. From this last period it appeared several prophylaxis proposals on EC induced nephrolithiasis using herbal extracts and antioxidants [12-19]. In all these papers the effects of these compounds did not seem to be mediated by diuretic or other urinary biochemical changes and positive effects on calcium oxalate lithiasis are most likely due to antioxidative effects.

To further investigate the potential of lemon juice as a therapy for lithiasis, the present study examined the effect of lemon juice on experimentally EG-induced calcium oxalate (CaOx) nephrolithiasis in rats.

Methods

Animals

Thirty male Wistar rats weighing approximately 280 g were acclimated for 3 days in cages before experiments commenced. Experiments were conducted in accordance with internationally accepted standard guidelines for the use of animals. Rats had *ad libitum* access to standard chow and tap water, and were kept under a controlled 12 h light/dark cycle at 22 ± 2°C.

Ethylene glycol-induced urolithiasis

The thirty rats were divided into five groups comprising six animals per group. Each group underwent a different treatment protocol for 10 days. Group 1: negative control, *ad libitum* access to regular food and drinking water, and administered 6 μl distilled water per 1 g of body weight by gavage (intra-gastric administration). Groups 2, 3, 4 and 5: *ad libitum* access to regular food, and *ad libitum* access to drinking water containing 0.75% [v/v] ethylene glycol (EG) and 2% [w/v] ammonium chloride (AC) in order to promote hyperoxaluria and CaOx deposition in the kidneys. Groups 2, 3 and 4 were also administered 6 μl lemon juice solution/g body weight by gavage at the following concentrations: Group 2, 100% lemon juice; Group 3, 75% [v/v] and Group 4, 50%. Group 5 rats were administered 6 μl distilled water/g body weight by gavage (positive control). All rats were weighed daily.

Assessment of antiurolithic activity

Kidney and serum analysis

After the 10-day experimental period, rats were anaesthetized and blood was collected from the retro-orbital region, centrifuged at 10,000 × g for 10 min [20], and the serum collected and analyzed for calcium, phosphorus, urea and creatinine using an automated system (Cobas Integra 400 plus). The rats were then sacrificed by cervical dislocation, the abdomen opened and both kidneys removed. The left kidney was dried in an oven at 100°C for 24 h, after which the kidney was weighed and then minced in a beaker containing 7 ml 0.5 N nitric acid. The mixture was then heated until the liquid became transparent. After calibration using a standard calcium solution, the calcium content of the mixture was determined using flame spectroscopy. The amount of calcium is expressed as μg/g dry kidney. The right kidney was fixed in bouin liquid [21,22], soaked in paraffin, cut at 3–4 μm intervals, and the slices stained using hematoxylin and eosin [21]. Tissue slices were photographed using optical microscopy under polarized light (Olympus BX41).

Statistical analysis

Results are presented as mean ± standard error (S.E.). A one-way ANOVA was used to determine the significance of differences among groups. Student's *t*-test was used to assess differences between means. Conventional Windows software was used for statistical computations. A P value < 0.05 was considered to indicate a significant difference.

Results

Serum analysis

Serum analysis showed that urea and creatinine levels were higher in Groups 2, 3, 4 and 5 compared to Group 1 (Fig. 1). These data indicate marked renal damage in the EG/AC-treated rats. The data also showed that urea, creatinine, calcium and phosphorus levels were lower in rats treated with lemon juice (Groups 2, 3 and 4) compared to rats treated with EG/AC alone (Group 5, positive control).

Body weight

EG/AC-treated rats (Groups 2, 3, 4 and 5) weighed less than the negative control rats (Group 1) at the completion of the experiment (Fig. 2).

Calcium levels in the kidneys

The left kidneys were assessed for calcium levels. EG/AC treatment alone (Group 5) resulted in increased kidney calcium levels compared to the negative control rats, while the administration of 100% lemon juice reduced this calcium accumulation (Group 2) (Fig. 3).

Histological examination

Examination of kidney paraffin sections showed that Group 5 rats (EG/AC alone, positive control) had the greatest amount of CaOx deposition, and this was present in all parts of all three major areas of the kidney. Intratubular and interstitial crystals were observed on the cortex (Figs. 4d and 4e). There was greater calcification on surface of the renal parenchyma (Fig. 5) and the papillary tip (Fig. 6) in Group 5 rats compared to the Groups 2, 3 and 4 rats (EG/AC and lemon juice). Longitudinal sections showed the papillary tips were encrusted with CaOx crystals (Figs. 6d and 6e). Analysis of portions of these crystalline deposits removed from the papillary tip showed they were composed of CaOx monohydrate and CaOx dihydrate. No papillary encrustations were seen in tissue from the negative control rats (Group 1) (Fig. 6a) or rats treated with EG/AC and 100% lemon juice (Group 2) (Fig. 6b). Major calcium deposits were observed on the surface of the papillary tips in 33% of the positive control rats (Group 5) and 17% of the rats treated with EG/AC and 75% lemon juice (Group 3). All positive control rats (Group 5) had major calcium deposits on the surface of the cortex and medulla, while no such deposits were

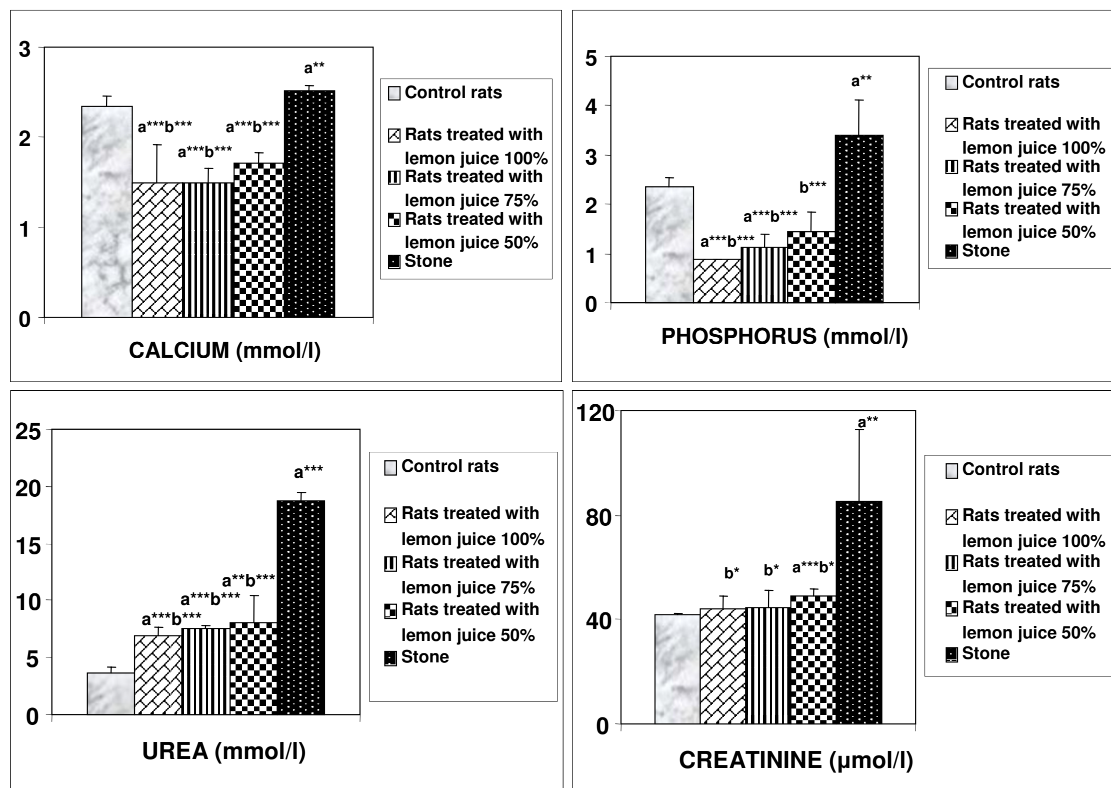

Figure 1
Serum biochemical data. Values represent mean ± SD for six animals in each group. [a] Values are significantly different from the negative control group: *p < 0.05, **p < 0.01, ***p < 0.001. [b] Values are significantly different from the positive control group: * p < 0.05, **p < 0.01, ***p < 0.001.

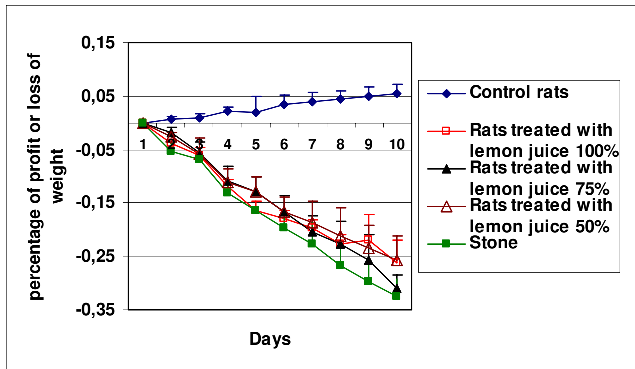

Figure 2
Changes in body weight in the various rat groups over the ten days of the experiment.

observed in the negative control rats (Group 1) (Tables 1 and 2). These morphological findings were consistent with the left kidney calcium level data.

Discussion

Urinary lithiasis is generally the result of an imbalance between inhibitors and promoters in the kidneys. Human kidney stones are usually composed of CaOx [1], and several studies have examined the effect of the citrus juices on calcium salt crystallization [23-27]. However, the conclusions from those studies were not consistent.

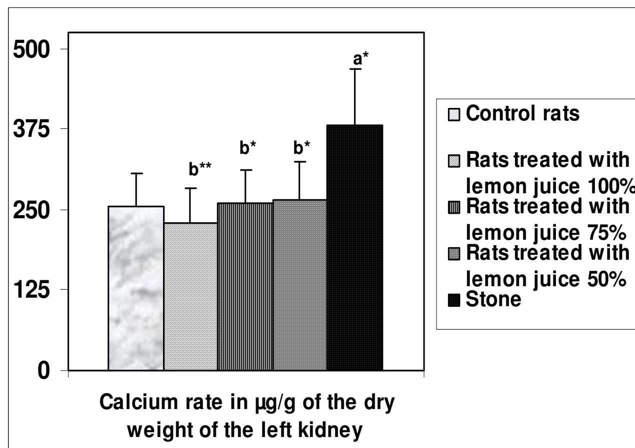

Figure 3
Amount of calcium in the left kidney. Values represent mean ± SD (µg/g) for six animals in each group. [a] Values are significantly different from the negative control group: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. [b] Values are significantly different from the positive control group: $* p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Many in vivo models have been developed to investigate the mechanisms involved in the formation of urinary stones, and to ascertain the effect of various therapeutic agents on the development and progression of the disease [28-33]. Rats are the most frequently used animals in models of CaOx deposition in the kidneys, a process that mimics the etiology of kidney stone formation in humans [28]. Rat models of CaOx urolithiasis induced by either EG alone or in combination with other drugs such as AC, are often used to study the pathogenesis of kidney crystal deposition [30]. Using the accelerated model [32], in the present study rats were treated with 0.75% EG and 2% AC for 10 days. All positive control rats (Group 5) developed CaOx depositions during that time.

The present study examined the effect of various lemon juice concentrations on the deposition of CaOx crystals within the rat kidney. Previous studies concluded that medicinal plants had little effect on the urinary chemistry of urolithiasis [34,35]. The current study analyzed body weight, kidney calcium level, serum concentrations of calcium, phosphorus, urea and creatinine, and the histopathology of the kidney. We found that Group 1 rats (negative controls) remained active and gained weight, while Group 2, 3, 4 and 5 rats lost weight over the 10 days of treatment. Microscopic examination using polarized light of kidney sections derived from nephrolithiasic rats showed intratubular and interstitial crystal deposits, consistent with the findings of others [36]. These crystals were intensely birefringent, polycrystalline, and arranged in a rosette characteristic of CaOx crystals. The presence of such deposits is evidence of adhesion and retention of particles within the renal tubules. These crystal deposits were observed in the kidneys of all Group 5 rats. Moreover, 33% of these rats showed major calcifications on the papillary tip. In contrast, no rats treated with lemon juice showed such papillary crystalline deposits. Rats treated with 100% or 75% lemon juice had far less kidney calcification and lower renal tissue calcium levels than the positive control rats (Group 5) (Table 1 and 2). No papillary encrustations were seen in 100%, 83% and 50% of rats treated with 100%, 75% and 50% lemon juice, respectively. Furthermore calcic parenchymatous deposits were not observed in 83% of rats treated with 100% and 75% lemon juice. These results clearly demonstrate the ability of the lemon juice to prevent the development of papillary and renal parenchymatous calcifications on the kidney, consequently preventing the development of papillary and parenchymatous calculi. All rats treated with 50% lemon juice showed fewer calcium deposits on the kidney surface than positive control rats (Group 5). While treatment with 100% and 75% lemon juice appeared to be more beneficial that treatment with 50% juice, this difference was not found to be statistically significant.

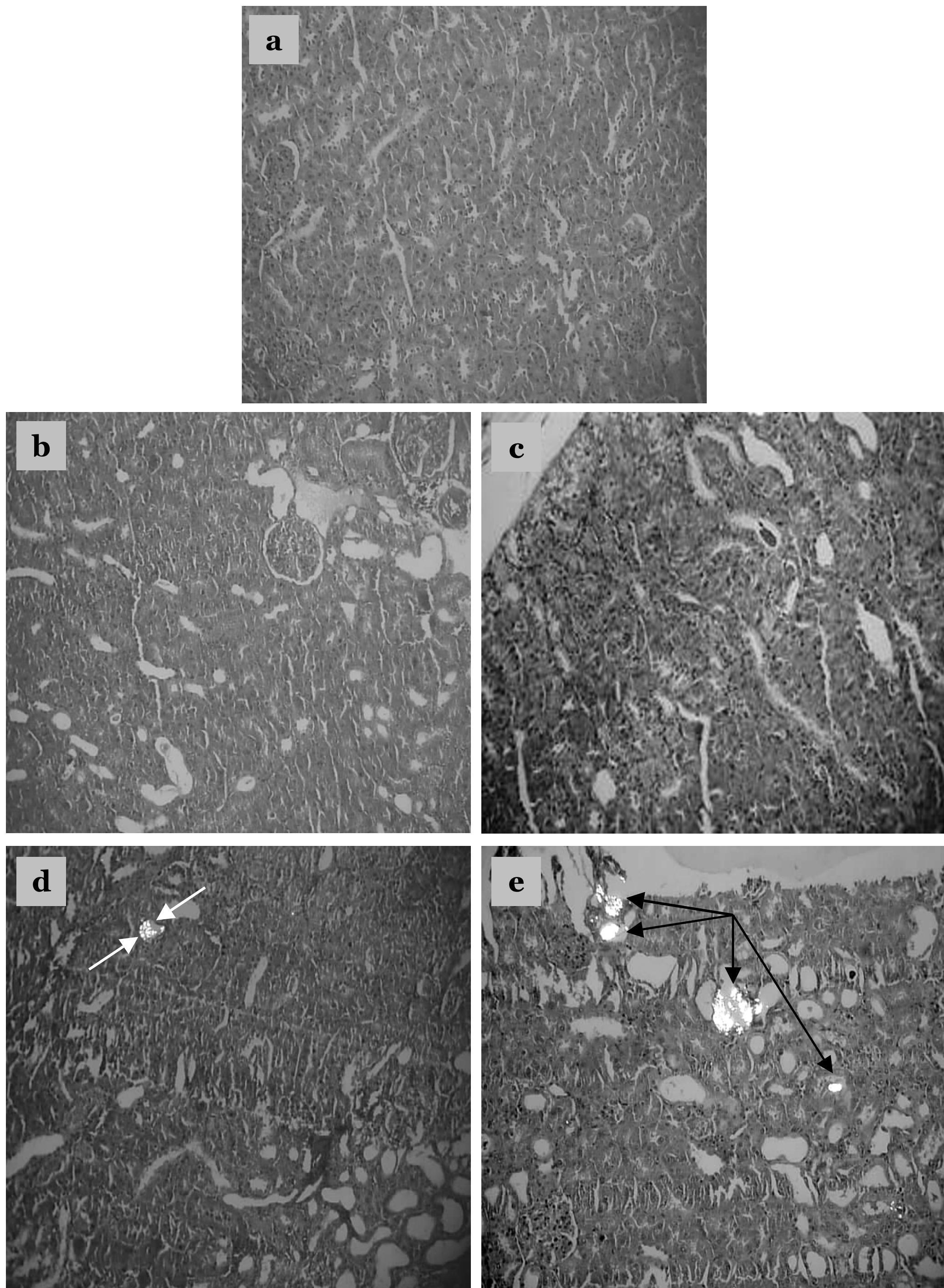

Figure 4
Crystalline formations in the rat kidney cortex. Sections were viewed using a BX41 optical microscope and polarized light. a: Tissue from negative control rats, b: Tissue from rats treated with ethylene glycol (EG), ammonium chloride (AC) and 100% lemon juice, c: Tissue from rats treated with EG, AC and 75% lemon juice, d: Tissue from rats treated with EG, AC and 50% lemon juice, e: Tissue from rats treated with EG and AC only (positive control). Crystalline formations in the renal cortex are indicated by arrows. Magnification ×100.

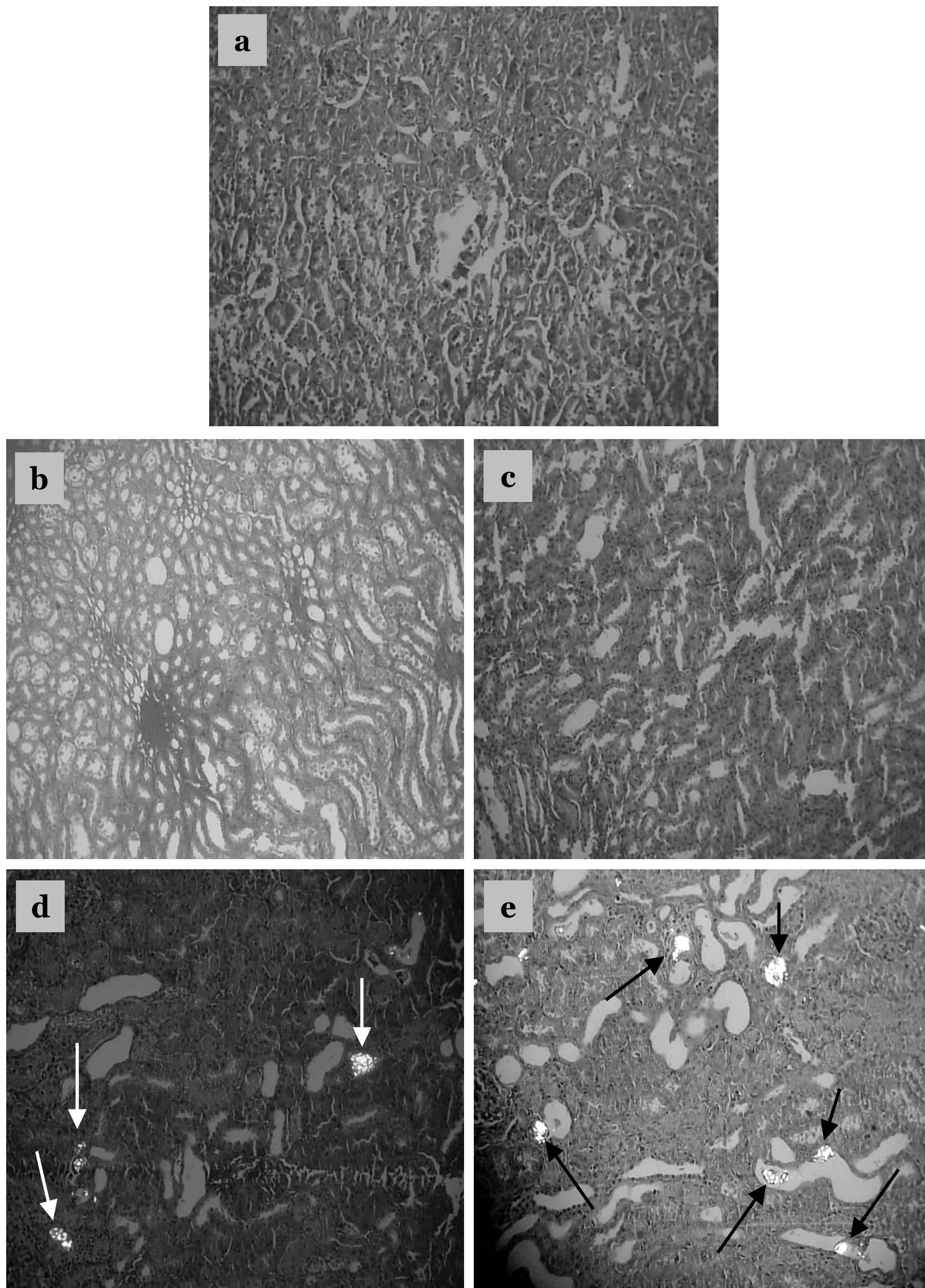

Figure 5
Crystalline formation in the renal parenchyma. Sections were viewed using a BX41 optical microscope and polarized light. a: Tissue from negative control rats, b: Tissue from rats treated with ethylene glycol (EG), ammonium chloride (AC) and 100% lemon juice, c: Tissue from rats treated with EG, AC and 75% lemon juice, d: Tissue from rats treated with EG, AC and 50% lemon juice, e: Tissue from rats treated with EG and AC only (positive control). Crystalline formations in the renal parenchyma are indicated by arrows. Magnification ×100.

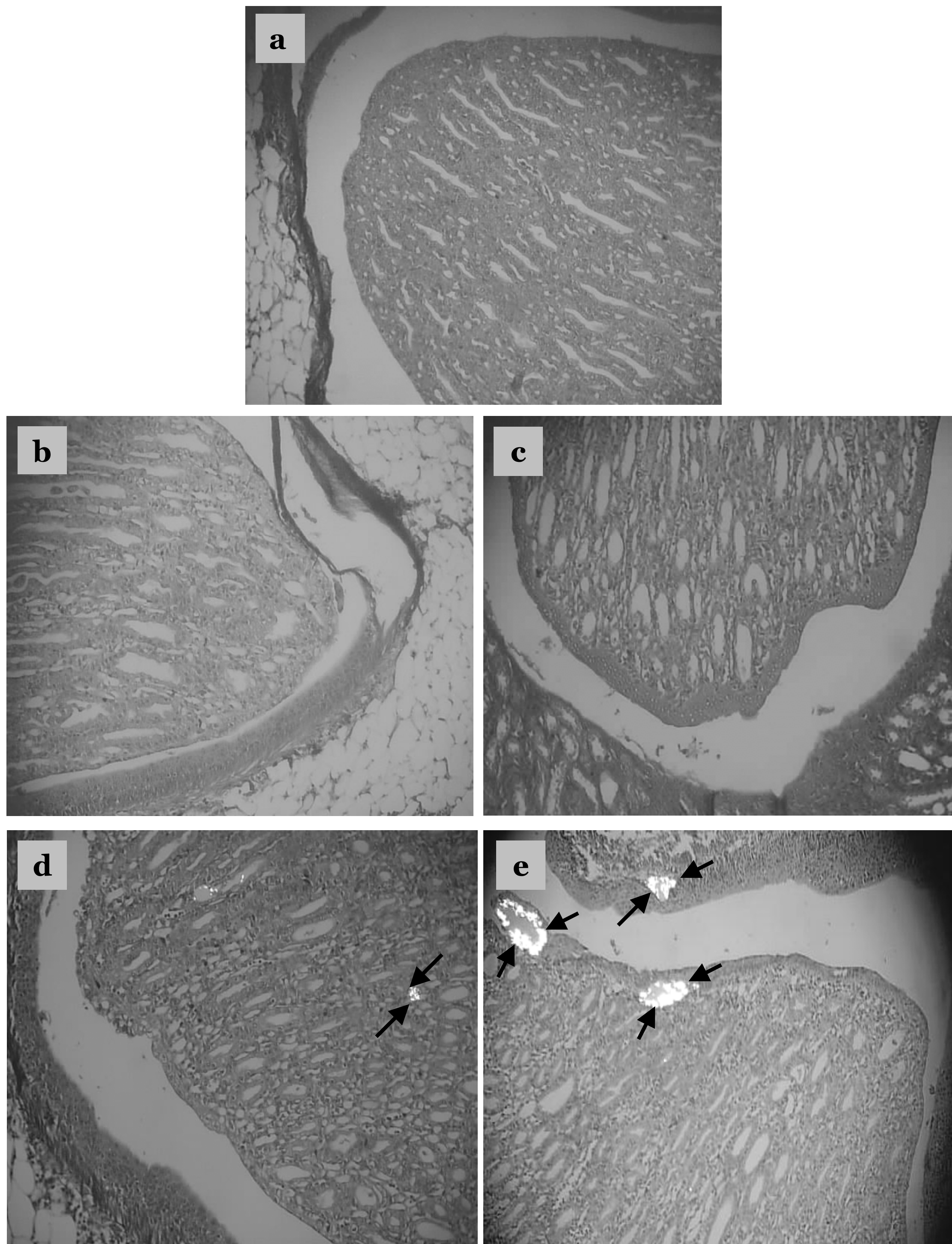

Figure 6
Crystalline formations in the renal papilla. Sections were viewed using a BX41 optical microscope and polarized light. a: Tissue from negative control rats, b: Tissue from rats treated with ethylene glycol (EG), ammonium chloride (AC) and 100% lemon juice, c: Tissue from rats treated with EG, AC and 75% lemon juice, d: Tissue from rats treated with EG, AC and 50% lemon juice, e: Tissue from rats treated with EG and AC only (positive control). Crystalline formations in the renal papilla are indicated by arrows. Magnification ×100.

Table 1: Number and type of calcifications observed

Groups	Percentage of rats with major calcifications on the papillary tip (> 90% of the papillary tip calcified)	Percentage of rats with some area of the papillary tip calcified	Percentage of rats with some calcified points on the papillary tip	Percentage of rats without calcifications on papillary tip
1. Negative controls	-	-	-	100
2. EG, AC and 100% lemon juice	-	-	-	100
3. EG, AC and 75% lemon juice	17	-	-	83
4. EG, AC and 50% lemon juice	-	-	50	50
5. EG and AC (positive controls)	33	33	17	17

The association of crystals with renal tubular cells is considered a potential factor in the process of renal stone formation. Indeed, calculations considering the rate of crystal growth even at its maximum speed and tubular fluid flow suggest that a single crystal would not become large enough to be retained and occlude the lumen during its normal transit through the nephron [28]. Furthermore, it is established that crystals, especially calcium oxalate monohydrate crystals, can be retained by attachment to the surface of renal epithelial cells and be internalized [28].

Lemon juice has a high antioxidant capacity due to the presence of citrate, vitamin C, vitamin E and flavonoids such as eriocitrin, hesperetin [37,38] and limonoids [39]. Vitamin E may prevent calcium oxalate crystal deposition in the kidney by preventing hyperoxaluria-induced peroxidative damage to the renal tubular membrane surface (lipid peroxidation) [40,41], which in turn can prevent calcium oxalate crystal attachment and subsequent development of kidney stones [41,42].

In urolithiasis, the glomerular filtration rate (GFR) decreases due to stones in the urinary system obstructing urine outflow. This leads to the accumulation of waste products in the blood, particularly nitrogenous substances such as urea, creatinine and uric acid. In addition, increased lipid peroxidation and decreased levels of antioxidant potential have been reported in the kidneys of rats supplemented with a calculi-producing diet [20]. In this context, oxalate has been reported to induce lipid peroxidation and to cause renal tissue damage by reacting with polyunsaturated fatty acids in cell membranes [20]. In the present study, the positive control calculi-induced rats (Group 5) were found to have marked renal damage, consistent with the elevated serum levels of creatinine and urea. The administration of lemon juice inhibited these changes that would otherwise promote new stone formation in the urinary system. In rats treated with lemon juice, we attribute the lower serum creatinine and urea levels to an enhanced GFR and the anti-lipid peroxidative property of lemon juice [20]. As commended, the lithogenic effects of EG must be mainly attributed to the oxidative damage caused by the high level of oxalate generated by this substance. For this reason, the presented studies were focused to evaluate the effects on renal papillary tissue through histological studies and the protective effects caused by the consumption of lemon juice. Previous studies evaluated the effects of citrate on renal lithiasis induced by EG [43,44]. Nevertheless, to attain an increase in citrate excretion it is necessary to induce metabolic acidosis in rats and to achieve this condition it is necessary to increase the doses of EG to 2%. In such case, urinary pH of EG treated rats was clearly inferior to urinary pH of control group, the treatment with high doses of potassium citrate significantly increased the urinary pH and, as a consequence, the urinary citrate excretion notably rose. Nevertheless, EG doses of 0.75% practically did not change the urinary pH value when compared with control group [36,44] and consequently the administration of citrate did not cause important changes in urinary citrate excretion [45].

Table 2: Cortex and medullar tissue data (see text for description of various groups)

Crystal deposits	Group 1 n = 6	Group 2 n = 6	Group 3 n = 6	Group 4 n = 6	Group 5 n = 6
None	6	5	5	-	-
Crystals: +	-	1	1	2	-
Crystals ++	-	-	-	4	-
Crystals +++	-	-	-	-	6

Conclusion

The present study found that the administration of lemon juice effectively prevented the development of urolithiasis in rats. These findings support the use of lemon juice as an alternative medicine to prevent urolithiasis. Further research is necessary to clarify the mechanism underlying this preventative effect of lemon juice.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

MT participated in this study by gavage of rats, measurement of body weight and analysis of kidney calcium levels. AL performed the statistical analysis. KE participated in the animal experiments. FL participated in laboratory management. IZ examined the histological samples. YE participated in analytical determinations. AO performed image processing. FG participated in the evaluation and discussion of the obtained results. AC participated in coordination. All authors read and approved the final manuscript.

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To study the renoprotective effect of Citrus limon juice and Emblica officinalis extract on renal toxicity induced by carbon tetrachloride in wistar rats.

Introduction-

Reactive oxygen species (ROS) are various forms of activated oxygen. A disproportionately large amount of ROS and the absence of their scavenging systems in cells lead to oxidative stress and increase the risk of several human diseases, including hepatic injury, carcinogenesis, and inflammation [1]. The liver plays a central role in the maintenance of systemic lipid homeostasis and is especially susceptible to ROS-induced damage. Carbon tetrachloride (CCl4) is widely used to develop experimental animal models of liver failure (caused by free radical production) that mimic human hepatic toxicity. Although the main target organ of CCl4 is the liver, toxic effects of CCl4 are also observed in other organs, including the kidneys, testis, and brain [2–5], and the nephrotoxic effect of CCl4 is also associate with free radical production [2].

To prevent the damage caused by ROS, living organisms have developed an antioxidant system, which includes nonenzymatic antioxidants and enzymes, such as catalase, superoxide dismutase, and peroxidase [6]. In addition to these natural antioxidants, other synthetic or natural ROS scavengers may reduce the incidence of free radical-mediated diseases. The use of antioxidants in the prevention and cure of various diseases is intensifying, and there is considerable interest in the study of antioxidant activities of molecules, such as Citrus limon and Emblica officinalis [6–8]. Antioxidants appear to act against disease processes by increasing the levels of endogenous antioxidant enzymes and decreasing lipid peroxidation [9, 10]

Citrus lemon (Citrus limon Burm.F) is a source of vitamin C, flavonoids and carotenoids [11]. Eriocitrin and hesperidin are the main flavonoids in lemon. The antioxidant activity of eriocitrin is more potent than other citrus flavonoids [12] , so we use the lemon as a rich source of antioxidant in present study.

Emblica officinalis Gaertn. (Euphorbiaceae) commonly known as amla. Experimental studies have shown potent antioxidant, analgesic, antipyretic, adaptogenic, immunomodulatory, and antiulcerogenic activities of the fruit of Emblica officinalis [13,14] As Amala has antioxidant property we included it in our study to see the nephroprotective activity.

Aim and objective-

Aim-

To study the renoprotective effect of Citrus limon juice and Emblica officinalis extract on renal toxicity induced by carbon tetrachloride in wistar rats.

Objectives-

1) To evaluate the renoprotective activity of Citrus limon in comparison with standard drug and in control group in wistar rats.

2) To evaluate the renoprotective activity of Emblica officinalis in comparison with standard drug and in control group in wistar rats.

3) To evaluate the mixture of Citrus limon and Emblica officinalis with standard drug.

<u>Materials and Methods-</u>

Citrus limon-

The fresh Citrus limon will properly identified and purchase. The Citrus limon will be authenticated from the botanist in Aurangabad. Juice will be collected and will be stored in jar.

Emblica officinalis -

Emblica officinalis will be purchase from market and will be authenticated from the botanist in Aurangabad.

<u>Preparation of extract Emblica officinalis</u>

The Emblica officinalis will be dried and powdered by using mixer . Emblica officinalis powdered (5.0 g) will be extracted with mixture of distilled water 25 mL and 75 ml of ethanol i.e hydroalcoholic extract will be prepared by using percolater. The extract will be dried in fan air and stored in cool and dry place.

Chemicals-

Acetylcistein (granules)

Carbon tetrachloride-

Animals –

Albino wistar rats of either sex of weight (150-250 gm) will be use for the study. Animal will housed in ventilated animal rooms having free supply of standard laboratory diet ad libitum and allowed free access to drinking water. The animals will also kept in 12:12 hour light/dark cycle. The experimental rats will be handled in strict compliance.

Experimental induction of CCl4 nephrotoxicity

CCl4-induced acute renal injury will be initiated by intraperitoneal injection of 1.5 ml/kg of 20% CCl4 dissolved in olive oil as described by Lu et al. (2002).[15] CCl4 will injected intraperitoneally in wistar rats to produce nephrotoxicity. Blood will be collect by retro-orbital plexus and sent for estimation of BUN and Serum Creatinine levels and Oxidative Stress Parameters - activities of antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT) in a homogenized renal tissue will be determined using ELISA kits, on the kit guidelines. The above test will be done before giving standard and test drug and after giving standard and test drugs.

.The animals will then randomly divided into six experimental groups as shown in below.

Groups	Drugs	Drug dose
Group I	CCl4	1 ml Distill water /Oral route
Group II	Acetylcystine	950 mg/kg
Group III	Citrus limon	6 ml/kg/oral route for six wks.
Group IV	Emblica officinalis	700mg/kg/oral route for six wks
Group V	Citrus limon + Emblica officinalis	6 ml/kg + 700 mg /kg/oral route for six wks.
Group VI	Citrus limon + Emblica officinalis + Acetylcystine	6 ml/kg + 700 mg /kg + 950 mg /kg/ oral route for six wks.

Only single dose of CCl4 will given in animals for induction of renal injury.

The dose of Citrus limon, Emblica officinalis , and acetyl cystine is selected as per it were use in previous literature. [16-18]

Histological Evaluation of kidney.- The animals will be sacrificed by giving CO2 and kidney will be removed for histological assessment, kidney tissue samples will be will be fixed in 10% formalin solution for one week. After embedding in paraffin, the tissues will cut into 3-4 μm sections. The sections will be mounted on the glass slides, stain with hematoxylin-eosin (H&E) reagent, and finally survey by a pathologist in a blinded way.

Statistical Analysis-

Results will be done by ANOVA test using SPSS.

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