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Evaluation of Pharmacokinetics, Antibacterial and Anti-Inflammatory Activities of Chrysin in Rat

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The pharmacokinetics, antibacterial and anti-inflammatory activities of Chrysin (100 mg/kg) were studied following intramuscular administration in rats. Drug concentration in rat plasma was determined using High Performance Liquid Chromatography (HPLC). The mean peak plasma drug concentration of 0.24 ± 0.01µg/mL was achieved at 0.25 h. The pharmacokinetic parameters like elimination half-life ($t_{1/2\beta}$), apparent volume of distribution (Vd_{area}) and total body clearance of Chrysin were 0.52±0.03 h, 338.63±13.39 L/kg and 456.20±15.62 L/h/kg respectively were determined. *In vitro* and *in vivo* antibacterial activity of Chrysin was determined by microbroth dilution technique against different bacterial pathogens and in neutropenic rat intraperitoneal infection model, respectively. In the present study, Chrysin was found to have no *in vitro* antibacterial activity in range of 10 - 0.07 mg/mL. In *in vivo* bacterial colony count between test drug and positive drug (Chloramphenicol) indicated that Chrysin had no protective activity against *Staphylococcus aureus* in neutropenic rat intraperitoneal infection model. In the present study, Chrysin found to inhibit LPS induced nitric oxide production on RAW 264.7 macrophage cell line and COX-2 enzyme through ELISA method but significantly ($p<0.01$) lower to Meloxicam. In addition to this Chrysin (100 mg/kg) was found to be effective (34.67 ± 1.55 %) in carrageenan-induced paw edema assay in rat after 4h following intramuscular administration.

Introduction

Chrysin is a naturally present flavone found in various herbs, mushroom and propolis (Premratanachai and Chanchao, 2014). It possess anti-inflammatory (Yao *et al.*, 2016), antiaging (Souza *et al.*, 2015), antiviral (Wang *et al.*, 2014) antioxidant (Freitas and Gaspar, 2016) antidiabetes (Samarghandian *et al.*, 2016), antiaromatase (Oliveira *et al.*, 2012) and anticancer (Zhang *et al.*, 2016) activities. However, it has poor bioavailability following oral administration (Noh *et al.*, 2016) as parent compound. Despite the great potential

data on its intramuscular pharmacokinetic are completely lacking and it's *in vitro* and *in vivo* antibacterial and anti-inflammatory activity of Chrysin as pure compound are limited. Looking to above facts, present study was undertaken to study pharmacokinetic of Chrysin following single intramuscular administration (100mg/kg b.wt.) in rats and evaluate *in vitro* and *in vivo* antibacterial and anti-inflammatory activities.

Materials and Methods

Experimental animals

The experiment was conducted on male albino wistar rats weighing between 300 to 400 grams. Rats were kept under constant observation for two weeks before the commencement of the experiment and subjected to clinical examination to exclude possibility of any diseases. The animals were divided into groups and kept in cages. Standard ration and water was provided *ad libitum.* The experimental protocol was approved by Institutional Animal Ethics Committee.

Drug and chemical

Pure chrysin, iodonitrotetrazolium chloride, meloxicam sodium (>98%), Lambda (λ) carrageenan, Lipopolysacharide (LPS) were obtained from Sigma-Aldrich, St. Louis, USA. Dimethysulfoxide (DMSO), PEG200, Methanol, Acetonitrile, Glacial acetic acid, Ortho-Phosphoric acid, Normal Saline (NS) and Sodium Nitrite were purchased from Merck Specialities Private Limited, Mumbai. Ethanol was used from store of College of Veterinary Science and A.H., N.A.U., Navsari after triple distillation. Gentamicin sulphate, Cyclophosphamide, Chloramphenicol, Dulbecco's modified Eagle's medium (DMEM), Penicillin, Streptomycin, Sulfanilamide, Naphthyl ethylene diaminedihydrochloride (NED), 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Himedia Laboratories Private Limited, Mumbai. Murine macrophage cell line RAW 264.7 was purchased from National Centre for Cell Science (NCCS), Pune. COX (ovine) inhibitor screening assay kit (Item No.560101) was purchased from Cayman Chemical Company, Ann Arbor, MI 48108.

Pharmacokinetic study and data analysis

Animals (n=30) were divided into six groups and each group comprise of five animals. A single dose of Chrysin was given by intramuscular route in each group of animal at dose rate 100 mg/kg B.W. Blood samples (250 µl) were collected from treated rat in K3EDTA vials, at different time interval i.e., 0 (before drug administration), 0.08 (5 min), 0.25 (15 min), 0.5 (30 min), 1, 2, 4, 6, 8, 12, 18 and 24hours from retro orbital plexus under light anesthesia. Multiple numbers of rat were used for serial collection of blood at alternating time point. Blood samples were subjected to centrifugation at 5000 rpm for 10 minutes and separated plasma samples were transferred to cryo-vials to store at -20^{o}C. Samples were analyzed within 24-48 h to quantify Chrysin levels using High Performance Liquid Chromatography (HPLC). Chrysin was assayed in plasma by adopting procedure with minor modifications as described by Bruschi *et al.*, (2003). The High Performance Liquid Chromatography (HPLC) apparatus of Shimadzu (Japan) comprised of binary gradient delivery pump (model LC – 20AP), Diode Array Detector (model SPD M20A), Auto Sampler (model SIL 20A**)** and reverse phase C18 column (250 x 4.6 mm ID).For plasma protein precipitation, Acetonitrile and Glacial acetic acid mixture (9:1 ratio) was added in plasma (1:1 ratio) in a clean micro centrifuge tube and subjected to a vortex mixer for 1 minute. It was followed by

centrifugation for 15 minutes at 8000 rpm. The clean supernatant was transferred into inserts (automatic sampler vial) from which 20 µL of supernatant was injected into HPLC system. The mobile phase consisted of a mixture of ACN and water (70:30).Mobile phase was filtered by 0.2 µ size filter (Axiva N66) and degassed by ultra-sonication. The mobile phase was pumped into column at a flow rate of 1.0 mL/min at ambient temperature. The effluent was monitored at 257 nm wavelength. Various pharmacokinetic parameters were calculated from plasma concentration of Chrysin using software PK solution (Version 2.0). For plasma validation of HPLC method, initial stock solution of Chrysin was prepared by dissolving 2 mg pure Chrysin in 2 mL DMSO and PEG200 in 1:1 ratio. Final standards were prepared in drug-free rat plasma. The mean correlation coefficient (R^2) was 0.99 for calibration curves. The precision and accuracy of the assay were assessed using samples at concentration of 12.50, 1.56, 0.39 and 0.09 µg/mL. At all concentration studied, the C.V. of Chrysin was less than 6.78 %.

In vitro antibacterial activity of chrysin

Minimum inhibitory concentrations (MICs) of Chrysin was determined in range of 10 - 0.07 mg/ml for different organisms like *Staphylococcus aureus* (ATCC25923), *Escherichia coli* (ATCC25922), *Salmonella typhimurium* (ATCC23564), *Pseudomonas aerugonosa* (ATCC27853), *Streptococcus pyogenus* (ATCC8668), *Proteus mirabilis* (NCIM2241) and *Bacillus subtillis* (ATCC9372) by micro broth dilution technique.

In vivo antibacterial activity of chrysin

In vivo antibacterial efficacy of chrysin was evaluated in neutropenic rat intraperitoneal infection model. Bacterial suspensions of *Staphylococcus aureus* was prepared in sterile broth and adjusted to 1×10^8 CFU/mL (McFarland 0.5 standard) by measuring the OD of solutions at 620 nm, from overnight grown bacteria. CFUs were verified by plating serial dilutions of each inoculum onto nutrient agar. For induction of neutropenia in albino wistar rats, Cyclophosphamide was inject intraperitoneally on day 1 (150 mg/kg) and day 4 (100 mg/kg). On day 5 neutropenic condition was confirmed by determination of total leucocyte count from all animals by Blood Auto Analyzer (Exigo, USA). After confirmation the rats were infected by intraperitoneal injection of 0.2 ml of inoculum ($1x10^8$cfu/mL) on same day. Chrysin was administered intramuscularly at 2 h and 8 h post infection. After 24 h, peritoneal fluid samples (100 µL) were collected following euthanasia and inoculated on nutrient agar plates. Nutrient agar plates were incubated overnight at 37°C and bacterial colonies were enumerated by colony counter. Rats were divided into four groups (n=6). Group I animals were treated with bacterial suspension (0.2 mL, $1x10^8$cfu/mL, IP) and Chloramphenicol (50 mg/kg, IM) (positive control), Group II animals were treated with bacterial suspension (0.2 mL, $1x10^8$ cfu/mL, IP) (growth control), Group III animals were treated with bacterial suspension (0.2 ml, $1x10^8$cfu/mL, IP) and vehicle (0.2 mL, IM) (vehicle control), Group IV animals were treated with bacterial suspension (0.2 mL, $1x10^8$ cfu/mL, IP) and Chrysin (100 mg/kg, IM).

In vitro anti-inflammatory activity of chrysin

COX-2 enzyme inhibition assay

The chrysin and meloxicam were dissolved in 100% Methanol to prepare a stock concentration of 1mM/100mL. The test compound was tested in triplicates at different

concentrates (100µM, 50µM and 10µM) by using a commercial COX (ovine) inhibitor screening assay kit following procedure as recommended by the manufacturer. Cyclooxygenase catalyzes the first step in the biosynthesis of Arachidonic acid to PGH2 and thereafter PGF2α produced from PGH2 by reduction with stannous chloride was measured by enzyme immunoassay. This assay is based on the competition between PGs and a PG-acetyl cholinesterase conjugate (a PG tracer) for a limited amount of PG antiserum. The amount of PG tracer that is able to bind to the PG antiserum is inversely proportional to the concentration of PGs in the wells, since the concentration of the PG tracer is held constant while PG concentration varies. This antibody-PG complex binds to an anti-IgG antibody previously attached to the well. The plate was washed with a buffer solution and Ellman's reagent, which contains the substrate of acetylcholinesterase, was added to the well. The yellow product of this enzymatic reaction is determined spectrophotometrically in a Microplate Reader (Multiskan EX, Thermo scientific) at 450 nm.Results were expressed as percentage of inhibition of PGF2α production.

Determination of NO production

The murine macrophage cell line RAW 264.7 cells were grown and maintained in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 20% FBS, 100U/mL penicillin and 100 µg/mL streptomycin. The culture was incubated at 37°C in humidified atmosphere and 5% CO_2 until the cells were confluent.

The cells then washed and resuspended in DMEM. The cells were seeded in 12 well plate (1 x 10^6 cells per well) and incubated for 24 hours at 37°C in a humidified atmosphere and 5% CO_2 and were sub cultured twice before the experiment.

The medium (DMEM supplemented with 10% FBS and 100 U/mL penicillin and streptomycin) then washed and supplemented with 1600 µL growth medium and 200 µL Chrysin and Meloxicam (positive control) in different concentration (100 µM, 50 µM and 10 µM) then incubated for 2 hours. 200 µL LPS (1 µg/mL) was added into the medium and incubated for 24 hours at 37°C in a humidified atmosphere and 5% CO_2. After pre-incubation of RAW 264.7 cells with LPS (1 µg/ml) for 24 h, the quantity of nitrite accumulated in the culture medium was measured as an indicator of NO production based on the Griess reaction (Hevel and Marletta, 1994).100 µl of cell culture medium was mixed with 100 µL of Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylene diaminedihydrochloride in 2.5% phosphoric acid). The Mixture was incubated at room temperature for 10 min and the absorbance at 540 nm was measured in spectrophotometer (Halo DB-20, Dynamica). The quantitative estimation of nitrite is based on a sodium nitrite standard calibration curve. The assay was performed in triplicate.

In vivo Anti-inflammatory activity of Chrysin

The carrageenan-induced paw edema test was used with slight modification as described (Suebsasana *et al.,* 2009). Experimental animals were divided into four groups (n=6). All the animals were treated with 100µL of 1% lambda carrageenan solution in 0.9% normal saline subcutaneously into subplantar region of right hind paw. Half an hour before the carrageenan challenge, vehicle, test and positive control drugs were injected via intramuscular route. Group I animals act as carrageenan control, Group II animals were treated intramuscularly with 200 µL of DMSO: PEG200 (1:1) (vehicle control), Group III animals were treated with Meloxicam (5 mg/kg, IM), Group IV animals

were treated with Chrysin (100 mg/kg IM). Make a mark on the left hind paw and volume of the edematous paw was measured using a plethysmometer after carrageenan treatment at 0, 1, 2, 3, 4, 5 and 6 h. Edema was expressed as the increase in paw volume (mL) after carrageenan injection, in comparison to the pre-injection value for each animal. The results obtained for the Chrysin treated group was compared with the control for percent inhibition of edema.

Statistical analysis

Chrysin plasma concentration and pharmacokinetic parameters of different treatment groups were compared by students' "t" test and Duncan's New Multiple Range Test (DNMRT) at 1 per cent and 5 per cent level of significance.

Results and Discussion

Pharmacokinetics of Chrysin in rats

Pharmacokinetic parameters and semilogarithmic plot of drug concentration in plasma versus time following single dose intramuscular administration of Chrysin (100 mg/kg) in rats is depicted in table 1 and figure 1. In the present study following intramuscular administration of Chrysin (100 mg/kg) in rats, the mean peak (C_{max}) plasma drug concentration of0.24 ± 0.01µg/mL was achieved at 0.25 h (T_{max}). The drug concentration of 0.15 ± 0.01 µg/mL in plasma was detected at 1 h and beyond then the drug was not detected in plasma. Contrary to the present observation high peak plasma drug concentration of 32.08±7.98 µg/mL was observed in rats (Aishwarya and Sumathi, 2016) and low plasma drug concentration of 0.09±0.01µg/mL in rats (Tong *et al.,* 2012) and 0.01 µg/mL in human (Walle *et al.,* 2000) were reported following oral administration. Moreover Chrysin was not detected at all in plasma (Noh *et al.,* 2016).The elimination half-life ($t_{1/2\beta}$), apparent volume of distribution (Vd_{area}) and total body clearance of Chrysin following single dose intramuscular administration in the present study was 0.52±0.03 h, 338.63±13.39 L/kg and 456.20±15.62 L/h/kg respectively. However, longer elimination half-life of 1.75±0.16 h (Aishwarya and Sumathi, 2016) and 9.72±3.16 h and lower total body clearance of 2.72±0.67 L/h/kg (Tong *et al.,* 2012) in rats following oral administration were observed in rats. Following intravenous administration of Chrysin in rats, Noh *et al.,* (2016) observed shorter half-life (0.04 ±0.01 h), lower apparent volume of distribution (0.4±0.1L/kg) and lower total body clearance (7.40±1.30 L/h). The MRT values calculated following single dose intramuscular administration of Chrysin in present study was 0.83±0.05 h which was lower than MRT of 10.20±1.40 h observed following oral administration of Chrysin in rats (Tong *et al.,* 2012).

In vitro and *in vivo* antibacterial activity of Chrysin

In vitro and *in vivo*antibacterial activity of Chrysin was determined by microbroth dilution technique against different bacterial pathogens and in neutropenic rat intraperitoneal infection model, respectively and result shown in table 2. In the present study the Chrysin was found to have no *in vitro* antibacterial activity in range of 10-0.07 mg/mL. In *in vivo* bacterial colony count between test drug and positive drug (Chloramphenicol) indicated that Chrysin had no protective activity against *Staphylococcus aureus* in neutropenic rat intraperitoneal infection model. However, Nina *et al.,* (2015) observed MICs >50 µg/mL for Chrysin against methicillin-sensitive *Staphylococcus aureus* (ATCC 25923), methicillin-resistant *Staphylococcus aureus* (ATCC 43300), *Escherichia coli* (ATCC 25922), *Escherichia*

coli 121, *Escherichia coli* 122, *Escherichia coli* LM2, *Salmonella* sp. LM and *Proteus mirabilis* 94-2. Several scientists also observed antibacterial effect of crude extract containing Chrysin and other secondary metabolite on different bacterial organism (Darwish *et al.*, 2010; Liu *et al*., 2010; Wang *et al.*, 2011; Alves *et al.*, 2013).

The difference in *in vitro* activity of Chrysin as pure compound may be due hydrophobicity, method of susceptibility and interaction with other compounds in crude extract. Chrysin did not show *in vivo* antibacterial activity in neutropenic intra-peritoneal infection (*Staphylococcus aureus*) model which may be due to non-buildup of required drug concentrations in plasma or at site of infection after intramuscular administration because faster clearance of the drug and that may be due to rapid hepatic metabolism (Noh *et al.*, 2016).

Table.1 Pharmacokinetic parameters of chrysin (100 mg/kg) following intramuscular administration in rats

Pharmacoki--netic Parameter	Rat Number							Mean ± S.E
	Unit	R1	R2	R3	R4	R5	R6	
α	h^{-1}	12.26	13.17	11.38	13.35	11.63	7.95	11.62±0.80
β	h^{-1}	1.43	1.27	1.21	1.11	1.39	1.76	1.36±0.09
$t_{½\alpha}$	h	0.057	0.053	0.061	0.052	0.060	0.087	0.06±0.01
$t_{½\beta}$	h	0.48	0.55	0.57	0.62	0.50	0.39	0.52±0.03
C_{max}	µg/mL	0.27	0.23	0.23	0.24	0.23	0.26	0.24±0.01
T_{max}	h	0.25	0.25	0.25	0.25	0.25	0.25	0.25 ±0.00
$AUC_{(0-\infty)}$	µg.h/mL	0.23	0.23	0.22	0.25	0.20	0.20	0.22±0.01
AUMC	$\mu g.h^2/mL$	0.18	0.20	0.20	0.24	0.16	0.13	0.18±0.02
$Vd_{(area)}$	L/kg	308.31	341.87	371.86	366.09	354.23	289.38	338.63±13.49
$Cl_{(B)}$	L/h/kg	441.79	434.59	450.74	407.21	493.71	509.19	456.20±15.62
MRT	h	0.78	0.85	0.90	0.98	0.79	0.65	0.83 ± 0.05

Table.2 *In vivo* activity of chrysin against *Staphylococcus aureus* in neutropenic rat intraperitoneal infection model

Group	Bacterial Colony Count (Log_{10} CFU/mL)						Mean ± S.E
	Rat Number						
	R1	R2	R3	R4	R5	R6	
Growth control	8.71	8.63	8.49	8.58	8.78	8.76	$8.66±0.05^b$
Vehicle control	8.70	8.66	8.60	8.76	8.56	8.58	$8.64±0.03^b$
Chrysin	8.81	8.78	8.69	8.71	8.59	8.74	$8.72±0.03b^b$
Chloramphenicol	4.48	4.52	4.45	4.38	4.57	4.60	$4.50±0.03^a$

Means bearing different superscripts within a column (between treatment groups) differ significantly ($p<0.01$)

Table.3 *In vitro* inhibition effect of chrysin on COX-2 enzyme

Group	Percent inhibition (Mean±SE) of COX-2 at different drug concentrations (µM)		
	100	**50**	**10**
Meloxicam	80.24 ± 0.39[b]	60.78 ± 0.70[b]	33.02 ± 0.70[b]
Chrysin	11.14 ± 0.48[a]	6.90 ± 0.77[a]	1.57 ± 0.34[a]

Means bearing different superscripts within a column (between treatment groups) differ significantly ($p<0.01$)

Table.4 Percent inhibition (Mean± SE) of NO production by chrysin

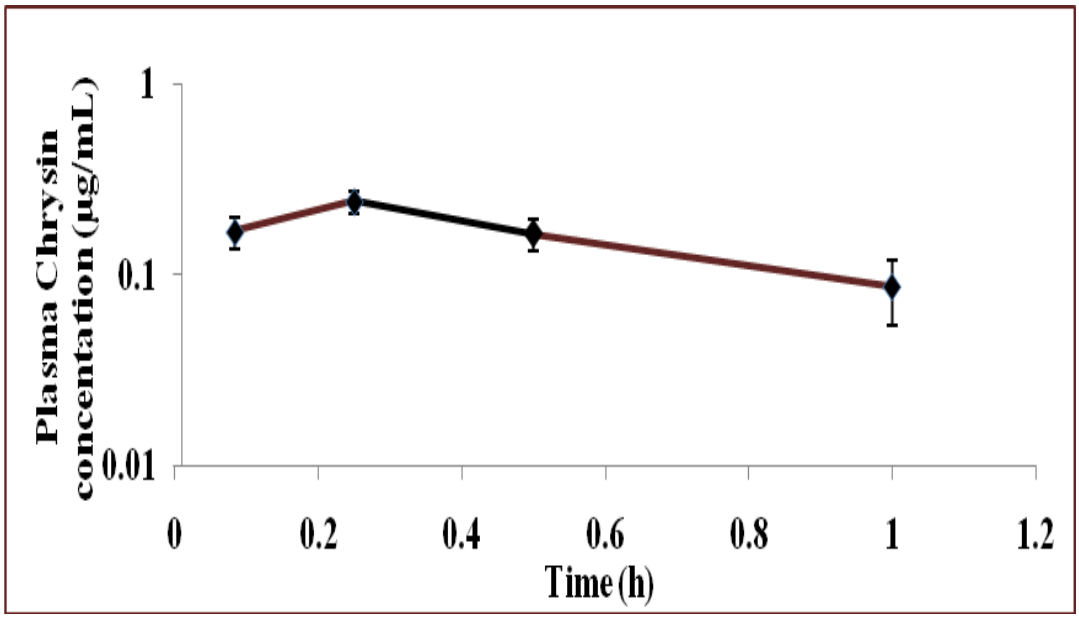
Group	Percent inhibition (Mean± SE) of NO production at different drug concentrations (µM)		
	100	50	10
Indomethacin	63.27 ± 3.40	-	-
Chrysin	53.88 ± 2.12	39.19 ± 3.35	24.52 ± 7.06

Table.5 Percent inhibition of edema by chrysin in rats

Group	Percent inhibition of edema (Mean±SE)					
	1 Hour	**2 Hour**	**3 Hour**	**4 Hour**	**5 Hour**	**6 Hour**
Meloxicam	23.99±3.41[b]	24.70±0.99[b]	36.97±2.02[b]	34.70±1.85[b]	39.46±1.82[b]	43.52±1.35[b]
Chrysin	14.82±2.32[a]	4.66 ± 3.87[a]	5.89 ± 4.30[a]	14.64±3.46[a]	26.38±2.76[a]	34.67±1.55[a]

Means bearing different superscripts within a column (between treatment groups) differ significantly ($p<0.01$).

Fig.1 Semilogarithmic plot of Chrysin concentration in plasma versus time following single dose intramuscular administration of Chrysin (100 mg/kg) in rats. Each points represents mean ± S.E.



In vitro and *in vivo*anti-inflammatory activity of Chrysin

In the present study, Chrysin found to inhibit LPS induced nitric oxide production on RAW 264.7 macrophage cell line and COX-2 enzyme through ELISA method but significantly ($p<0.01$) lower to Meloxicam and Indomethacin (Table 3 and 4). In addition to this Chrysin (100 mg/kg) was found to be effective (34.67 ± 1.55 %) in carrageenan-induced paw edema assay in rat after 4h following intramuscular administration (Table 5). Results of the present *in vitro* assay are in agreement with the results reported by several workers like Woo *et al.,* (2005) found significant suppression of LPS-induced COX-2 enzyme and mRNA expression in a dose-dependent manner; Ha *et al.,* (2010) observed significant inhibition of nitric oxide (NO) release, expressions of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) in lipopolysaccharide (LPS) stimulated microglia; Leeand Park, (2015) also observed significant inhibition the production of NO in polyinosinic-polycytidylic acid induced RAW 264.7 mouse macrophages; Kaidama and Gacche, (2015) exhibited significant inhibition in carrageenan-induced acute inflammation and chronic inflammation/cotton pellet granuloma in guinea pigs at 40 mg/kg following oral administration of Chrysinand Rauf *et al.,* (2015) observed significant reduction of mice paw edema and its maximum effect was observed between the 4hand 5hfollowing intraperitoneal injection of Chrysin. The *in vivo* anti-inflammatory activity observed from 4 h onwards in the present study may be due to inhibition of prostaglandin synthesis. *In vitro* COX-2 enzyme inhibition in the present study supports the observation of *in vivo* anti-inflammatory activity. The *in vivo* anti-inflammatory activity observed from 4 hour onwards in the present study may be due to inhibition of prostaglandin synthesis. *In vitro* COX-2 enzyme inhibition in the present study supports the observation of *in vivo* anti-inflammatory activity.

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Conflict of interest statement

Authors declare that they have no conflict of interest.

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