



# Bergapten inhibits chemically induced nociceptive behavior and inflammation in mice by decreasing the expression of spinal PARP, iNOS, COX-2 and inflammatory cytokines

Gurjit Singh<sup>1</sup> · Anudeep Kaur<sup>1</sup> · Jashanpreet Kaur<sup>1</sup> · Manpreet S. Bhatti<sup>2</sup> · Palwinder Singh<sup>3</sup> · Rajbir Bhatti<sup>1</sup> 

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## Abstract

In continuation with our previous studies on osthole, bergapten, a closely related furanocoumarin was investigated for its ameliorative effect on chemically induced neurogenic and inflammatory hyperalgesia and inflammation in mice. Chemical hyperalgesia and inflammation was induced by administration of formalin (intraplantar), acetic acid (intraperitoneal) and carrageenan (intraplantar) to different groups of animals. Pain responses were quantified and median effective dose (ED<sub>50</sub>) of bergapten was calculated. Lipopolysaccharide challenge was administered to study inflammatory cytokines which were analyzed in plasma using ELISA. The expression of poly ADP-ribose polymerase (PARP), cyclooxygenase (COX-2) and inducible nitric oxide synthase (iNOS) was quantified by immunofluorescence staining. Bergapten was found to ameliorate both neurogenic and inflammatory hyperalgesia precipitated by formalin, acetic acid induced writhing and carrageenan induced paw inflammation with ED<sub>50</sub> dose of 2.96 mg/kg. Bergapten also significantly decreased the levels of TNF- $\alpha$  and IL-6 and the expression of PARP, COX-2 and iNOS in the spine. It is concluded that bergapten is an interesting molecule with significant analgesic and anti-inflammatory activity emanating through the modulation of multiple pain mediating pathways.

**Keywords** Bergapten · COX-2 · Furanocoumarin · Hyperalgesia · iNOS · PARP

## Introduction

Coumarins are important secondary metabolites abundantly found in numerous plants of medicinal significance that protect the plants from environmental stresses and insults (Venugopala et al. 2013; Asif 2015). Furanocoumarins are known for pharmacological effects including analgesic/antipyretic, anti-inflammatory, antimicrobial, antiproliferative, antioxidative and antiviral actions (Tian et al. 2017). Bergapten (5-methoxypsoralen) present in the plants of rutaceae and umbelliferae family such as *Cnidium monnieri* is widely used for its medicinal values (Navarra et al. 2015;

Liao et al. 2018) such as anticoagulant (Mira et al. 2017), anti-inflammatory (Bose et al. 2011), antiproliferative (Hung et al. 2017) and anticancer activity (Yang et al. 2018). It has also been reported to have anticonvulsant effects (Luszczki et al. 2010). 8-Methoxypsoralen and 5-methoxypsoralen formulations are used in the clinical management of skin ailments like psoriasis, vitiligo and mycosis fungoides (Al-Asmari et al. 2017).

Pain is a physical and mental suffering, and often associated with co-morbidities including depression, anxiety and stress affecting the patient's daily activities (Stubbs et al. 2016; Pitcher 2018). Clinically available options for pain mitigation comprise of NSAIDs and opioids, while neuropathic pain is treated with anticonvulsants (Slater et al. 2010). However, all these drugs suffer from certain limitations necessitating the need to explore new chemical moieties for the treatment of pain. Since plant medicines are known to have relatively fewer adverse effects than synthetic analogs probably because they are naturally synthesized in plants and hence the living cells have better tolerability towards these molecules, the phytochemicals offer interesting leads in new drug development. After the successful

✉ Rajbir Bhatti  
rbhatti75@gmail.com

<sup>1</sup> Department of Pharmaceutical Sciences, Guru Nanak Dev University, Amritsar, Punjab 143005, India

<sup>2</sup> Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, India

<sup>3</sup> Department of Chemistry, Guru Nanak Dev University, Amritsar, India

investigations for analgesic activity of osthole (Singh et al. 2018), the current study was undertaken to explore the medicinal potential of another natural product bergapten, for which to the best of our knowledge, comprehensive studies on antihyperalgesic effect and its modulation of PARP, COX-2 iNOS and inflammatory cytokines are not reported. Therefore, the current investigation was focused to explore the analgesic and anti-inflammatory activity of bergapten and its role in modulation of PARP, COX-2, iNOS and inflammatory cytokines.

## Materials and methods

### Chemicals

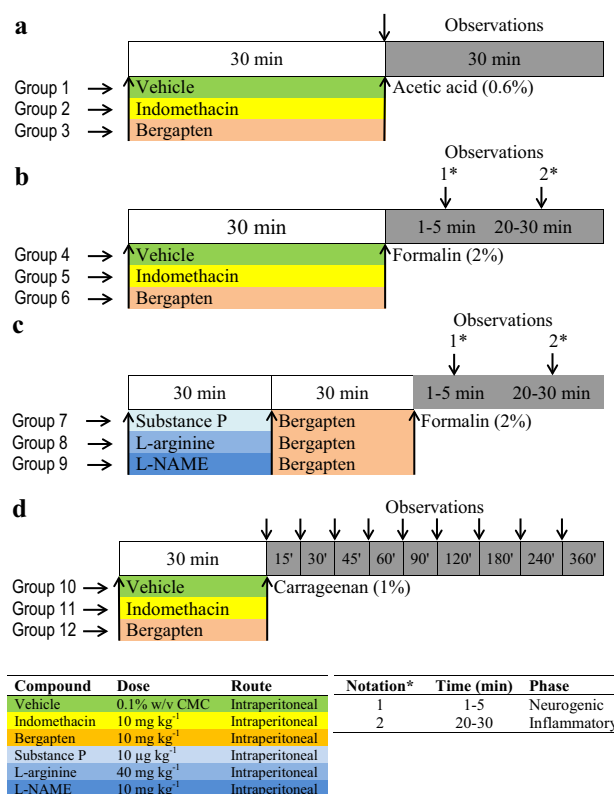
Indomethacin, substance-P, L-arginine, L-NAME were purchased from Sigma-Aldrich while all other analytical grade chemicals and reagents were procured from authorized local suppliers. Bergapten was a gift sample from Sugam Herbal Creation, Nanital, Uttarakhand and the structure of the procured sample was established by NMR and LC-MS spectrometry. TNF- $\alpha$  and IL-6 (mouse cytokine) kits were purchased from Krishgen Biotech, Mumbai, India. Mouse monoclonal anti-iNOS antibody was procured from Sigma Aldrich, USA and rabbit polyclonal COX-2, PARP antibodies were obtained from Santa Cruz Biotech, USA. Goat anti-mouse IgG1 secondary antibody, Alexa Fluor 546 and goat anti-rabbit IgG (H+L) secondary antibody, Alexa Fluor 546 were supplied by Invitrogen, USA.

### Animals

Swiss albino mice (either sex) with body weight ranging from 30 to 40 g were taken from Central Research Institute, Kasauli. The animals were housed in central animal house of Guru Nanak Dev University and maintained as per the standard guidelines with free access to feed (Aashirwad Industries, Chandigarh) and water. The protocols for the animal studies were approved by the institutional animal ethics committee (226/CPCSEA 2015/11). All procedures were conducted between 09:00 and 17:00 h in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Forests and Climate Change, Govt. of India guidelines.

### Acetic acid-induced writhing

Acetic acid-induced writhing is a well-documented model of visceral pain (Pitcher et al. 2017). Acetic acid (0.6%) was



**Fig. 1** Experimental schemes for the animal studies **a** acetic acid-induced writhing, **b** formalin-induced nociception, **c** effect of substance-P, L-arginine and L-NAME pre-treatment on the effect of bergapten, **d** carrageenan-induced inflammation

administered intraperitoneally and the number of writhes, i.e., abdominal contractions was quantified for a period of 30 min post-acetic acid injection (Nogueira et al. 2003). The schematic presentation of treatment groups is summarized in Fig. 1a.

### ED<sub>50</sub> of bergapten

Median effective dose (ED<sub>50</sub>) of bergapten was calculated in the acetic acid model by varying the dose in a randomized manner with each dose group comprising of five animals and maximum dose of 20 mg Kg<sup>-1</sup>. Results were calculated in terms of percent anti-nociceptive activity. The response data obtained were fitted using analysis of variance (ANOVA). Best-fitted model having *p* value < 0.001 (highly significant) and non-significant lack of fit (*p* > 0.05) indicated strong signal as compared to model noise. The best-fitted regression equation is used for plotting one factor graph and the corresponding dose at which 50% anti-nociceptive activity is seen was determined.

## Formalin-induced nociception

Freshly prepared 2% formalin solution was administered to the animals via subcutaneous route under the plantar surface of the right hind paw. The animals were placed individually in the observation chamber and flinching response was monitored for a period of 30 min (Pinho-Ribeiro et al. 2003). The schematic presentation of treatment groups is summarized in Fig. 1b.

## Involvement of COX and NO pathways in the observed effect of bergapten

The involvement of cyclooxygenase (COX) pathway was studied by pre-treatment of the animals with substance-P which is a known modulator of COX and the involvement of nitric oxide (NO) pathway was studied using pre-treatment with L-arginine (NO precursor) and L-NAME (NO synthase inhibitor). The findings were further corroborated from immunofluorescence staining using antibodies for PARP, COX-2 and iNOS. The schematic presentation of treatment groups is summarized in Fig. 1c.

## Immunohistological expression studies

Mice were anaesthetized with ketamine (40 mg/kg, i.p) after 2 h of formalin injection and pre-perfusion with 4% paraformaldehyde phosphate buffer solution for 4 h was given. Post-perfusion, L4–L5 section of spinal cord was removed and immersed overnight in 30% v/v sucrose solution maintained at 4 °C. Ten random sections of 10- $\mu$ m sections of the spinal tissue were obtained using cryomicrotome (Leica Biosystems) and incubated separately with each of the primary antibodies including rabbit polyclonal PARP (1:200), mouse monoclonal anti-iNOS (1:100) and rabbit polyclonal COX-2 (1:150) for 48 h, followed by alternate washing with the PBS and PBST buffers. In the next step, the sections were incubated for 2 h with either goat anti-mouse IgG (1:2000) or goat anti-rabbit (1:2000). Repeated washings were given with the PBS as required. The imaging of the slides was done using confocal microscope (Nikon Eclipse Xi).

## Carrageenan-induced paw oedema

Anti-inflammatory activity was assessed using carrageenan induced paw oedema model (Hajhashemi et al. 2012). This method involves measuring acute inflammatory response on administration of 100  $\mu$ L of carrageenan (1%) w/v into the plantar surface of hind paw in each mouse. The extent of inflammation was expressed as paw oedema measured at a varied time interval between 15 and 360 min. The difference in the volume of both hind paws (including paw exhibiting oedema) was measured by plethysmometer (Medicaid Systems, India). The maximum possible effect (MPE) of

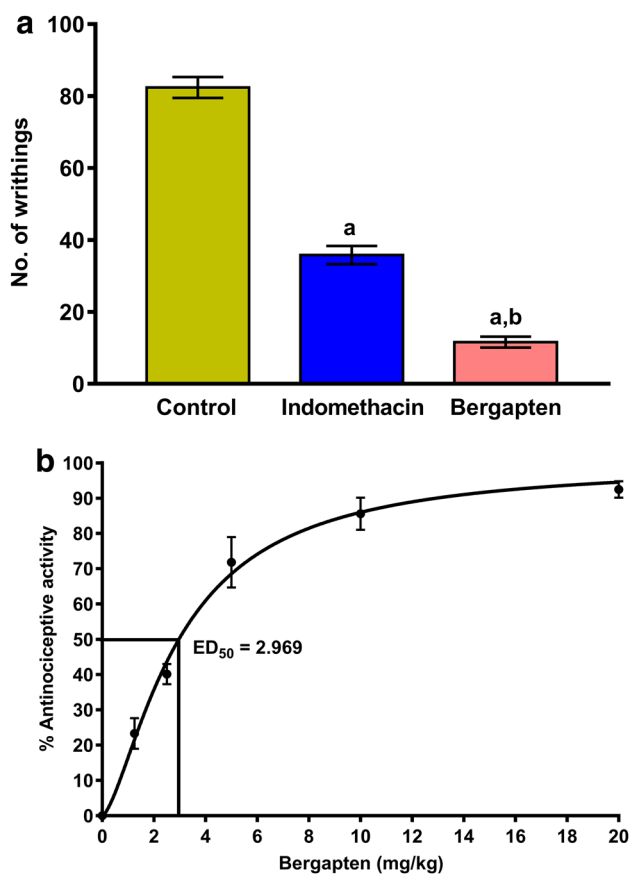
bergapten in carrageenan-induced paw oedema test was calculated using Eq. 1. The schematic presentation of treatment groups is summarized in Fig. 1d.

$$\% \text{ MPE} = \frac{[\text{PV}]_{\text{C}} - [\text{PV}]_{\text{T}}}{[\text{PV}]_{\text{C}}} \times 100, \quad (1)$$

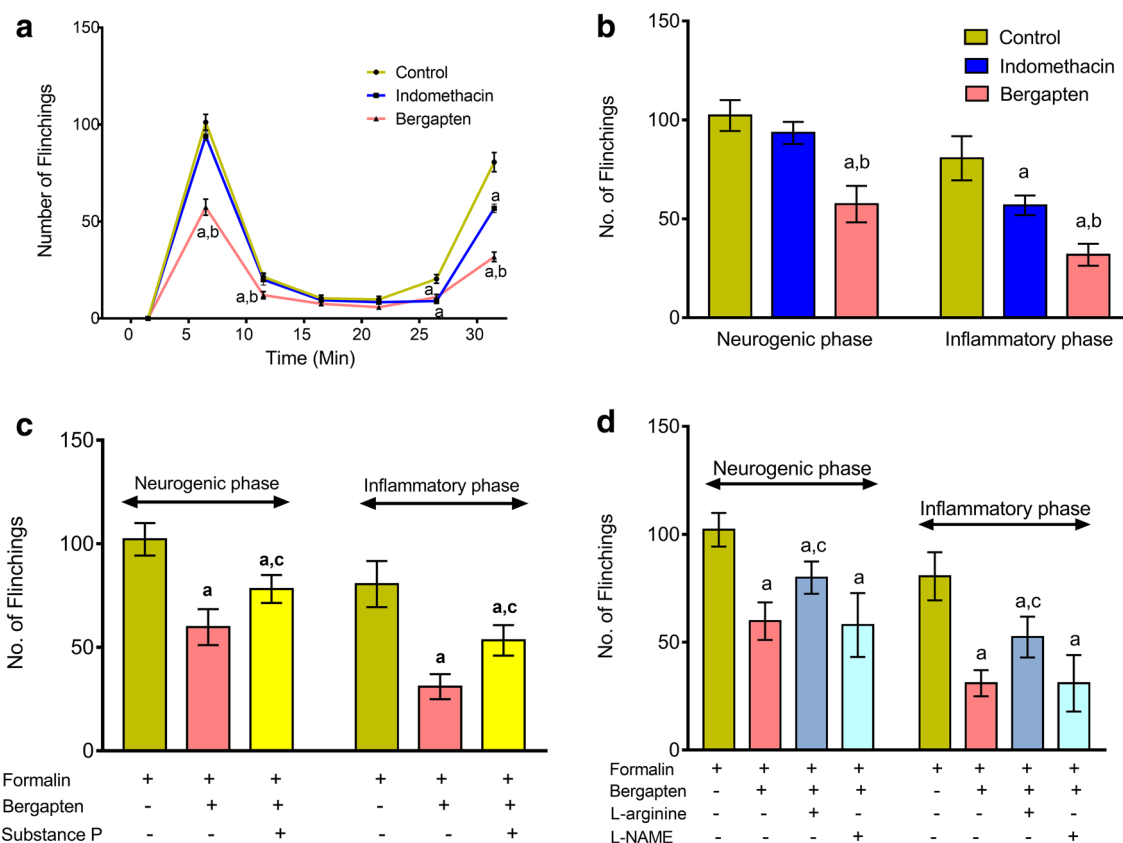
where  $[\text{PV}]_{\text{C}}$  is the Paw volume of control group;  $[\text{PV}]_{\text{T}}$  is the Paw volume of test group.

## Measurement of TNF- $\alpha$ and IL-6

Animals were divided into five groups. Group-1 is normal control and was administered vehicle, i.e., 0.1% carboxymethyl cellulose (CMC) intraperitoneally. Group-2 was lipopolysaccharide (LPS) treated control group, where LPS was administered i.p. at a dose of 1 mg/kg. The remaining three groups were pre-treated with bergapten at doses 5, 10, 20 mg/kg, respectively. LPS was administered 30 min following the bergapten treatment. Blood samples for TNF- $\alpha$  estimation were collected by cardiac puncture under



**Fig. 2** **a** Effect of bergapten, **b** median effective dose (ED<sub>50</sub>) of bergapten in acetic acid-induced writhing test. All values are expressed as mean  $\pm$  SEM. Statistical differences were determined by one-way ANOVA followed by Tukey's test <sup>a</sup> $p < 0.05$  vs. control, <sup>b</sup> $p < 0.05$  vs. indomethacin



**Fig. 3** **a** Effect of bergapten on formalin-induced flinchings in mice. **b** Effect of bergapten on formalin-induced flinchings in neurogenic and inflammatory phases in mice. **c** Modulation of the analgesic effect of bergapten by substance-P pre-treatment. **d** Modulation of the

analgesic effect of bergapten by L-arginine, L-NAME pre-treatment. All values are expressed as mean  $\pm$  SEM. Statistical differences were determined by two-way ANOVA followed by Tukey's test. <sup>a</sup> $p < 0.05$  vs. control, <sup>b</sup> $p < .005$  vs. indomethacin, <sup>c</sup> $p < 0.05$  vs. bergapten

anesthesia 2 h after LPS administration. The sampling for IL-6 was done 6 h post-LPS administration. Centrifugation of the samples was carried out at 5000 rpm for 10 min at 10 °C. Blood plasma was collected and stored at 20 °C until further estimations. Estimation of TNF- $\alpha$  and IL-6 was carried out using commercially available ELISA kits and the results were expressed as picogram per milliliter (pg/mL).

### Statistical analysis

All the results are expressed as mean  $\pm$  standard error mean (SEM). One-way and two-way ANOVA followed by Tukey's test was used to calculate statistical significance.

## Results

### Effect of bergapten on acetic acid-induced writhing

Treatments with indomethacin (10 mg kg<sup>-1</sup>) and bergapten (10 mg kg<sup>-1</sup>) markedly attenuated acetic acid-induced writhing (Fig. 2a).

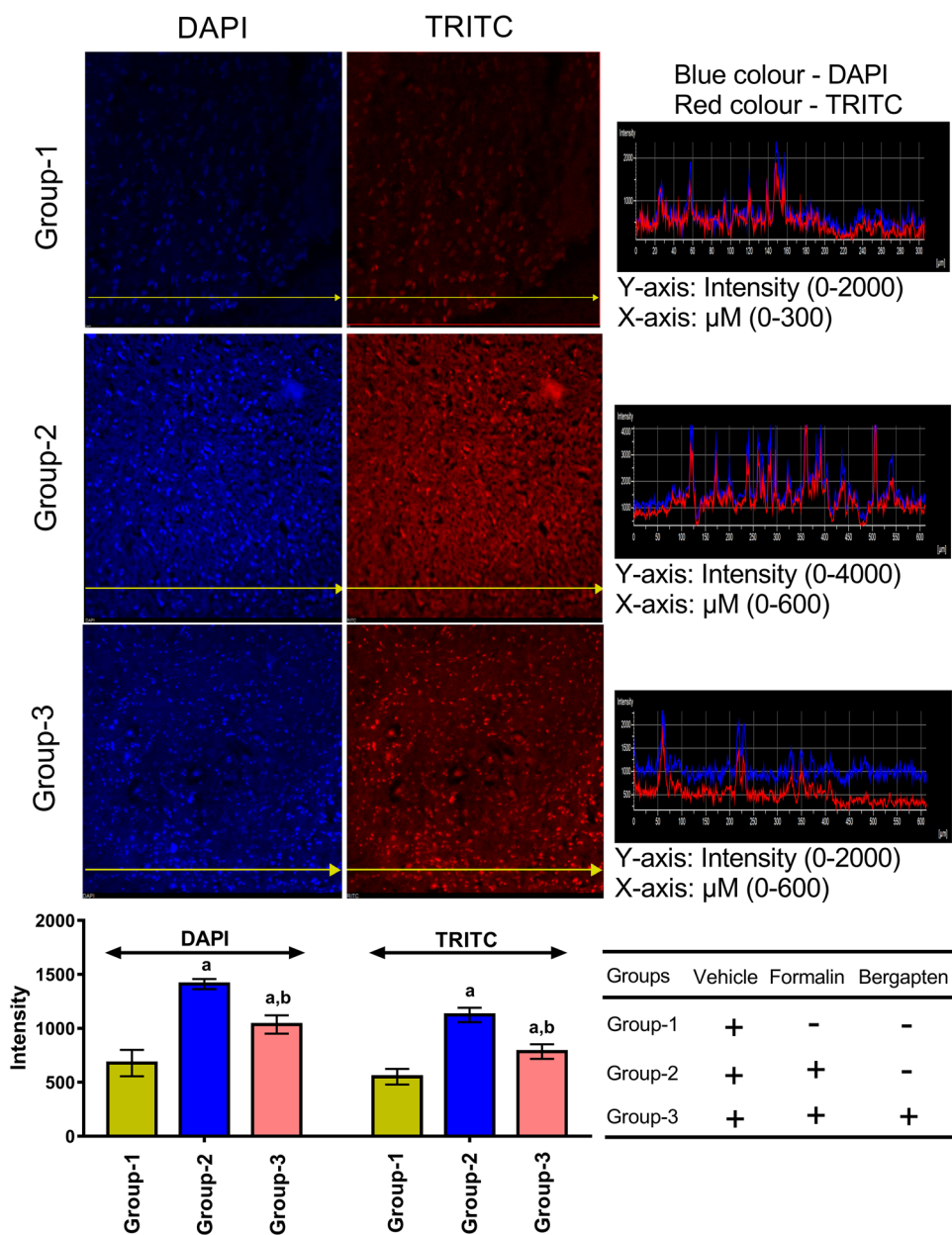
### ED<sub>50</sub> of bergapten

% Anti-nociceptive activity was found to be ranging from 23.3% at 1.5 mg/kg to 92.4% at 20 mg/kg. There was an increase in % anti-nociceptive activity up to the dose of 10 mg kg<sup>-1</sup>. However, further increase in the dose did not affect the response. Regression equation was numerically optimized to yield a 50% increase in antinociceptive activity and ED<sub>50</sub> was found to be 2.96 mg kg<sup>-1</sup> (Fig. 2b).

### Effect of bergapten treatment on formalin-induced hyperalgesia

Formalin-induced pain test was found to produce distinct biphasic response. First peak in nociceptive activity was evident in the first 5 min; whereas second peak was found at 20–30 min after the formalin injection (Fig. 3a). Treatment with indomethacin did not attenuate the first phase but markedly attenuated the second phase; whereas, bergapten treatment was found to attenuate both the phases significantly

**Fig. 4** Immunofluorescence staining of Group-1, Group-2 and Group-3 with PARP antibody showing DAPI-, TRITC-positive cells and average intensity in the transverse section of spinal cord (200× magnification). All values are expressed as mean ± SEM. Statistical differences were determined by one-way ANOVA followed by Tukey's test <sup>a</sup>*p* < 0.05 vs. control group and <sup>b</sup>*p* < 0.05 formalin-treated group



with 43.8% reduction in the first phase and 60.5% reduction in flinching in the second phase (Fig. 3b).

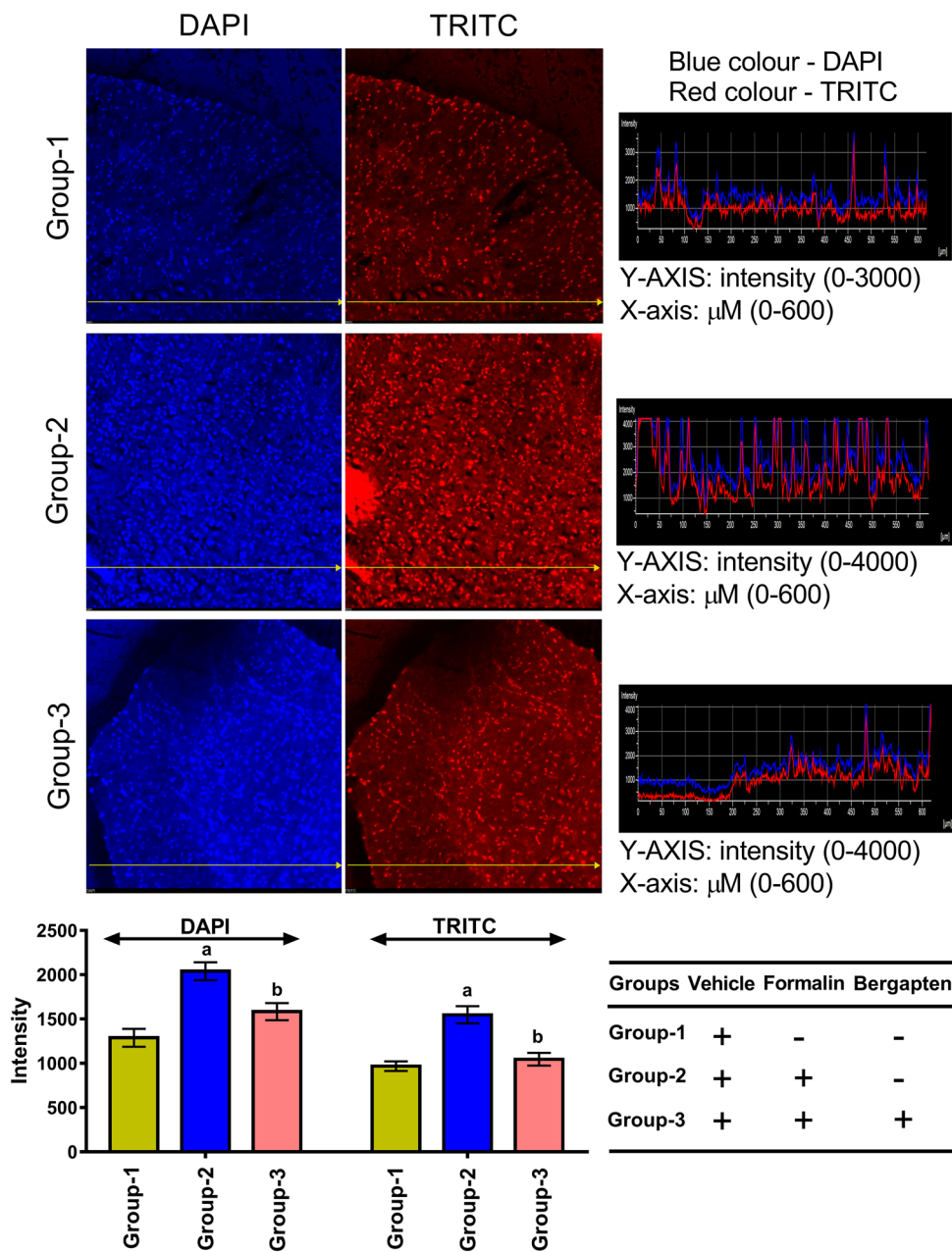
**Mechanistic studies for COX and NO pathway**

Substance-P pre-treatment completely prevented the antinociceptive effect of bergapten in both phases (Fig. 3c). L-arginine pre-treatment was found to reverse the antinociceptive effect of bergapten in both phases; whereas L-NAME pre-treatment did not alter the effect of bergapten (Fig. 3d).

**Immunohistological findings of PARP, COX-2 and iNOS expression**

Immunofluorescent staining with antibodies revealed a marked increase in the expression of PARP, COX-2 and iNOS in L-5 (lumbar) region of the spinal cord in formalin-treated control animals as compared to normal animals. Bergapten treatment showed a marked reduction in the expression of PARP (Fig. 4), COX-2 (Fig. 5) and iNOS (Fig. 6) in L-5 region of the spine as compared to formalin control group.

**Fig. 5** Immunofluorescence staining of Group-1, Group-2 and Group-3 with COX-2 antibody showing DAPI-, TRITC-positive cells and average intensity in the transverse section of spinal cord (200× magnification). All values are expressed as mean ± SEM. Statistical differences were determined by one-way ANOVA followed by Tukey's test <sup>a</sup>*p* < 0.05 vs. control group and <sup>b</sup>*p* < 0.05 formalin-treated group



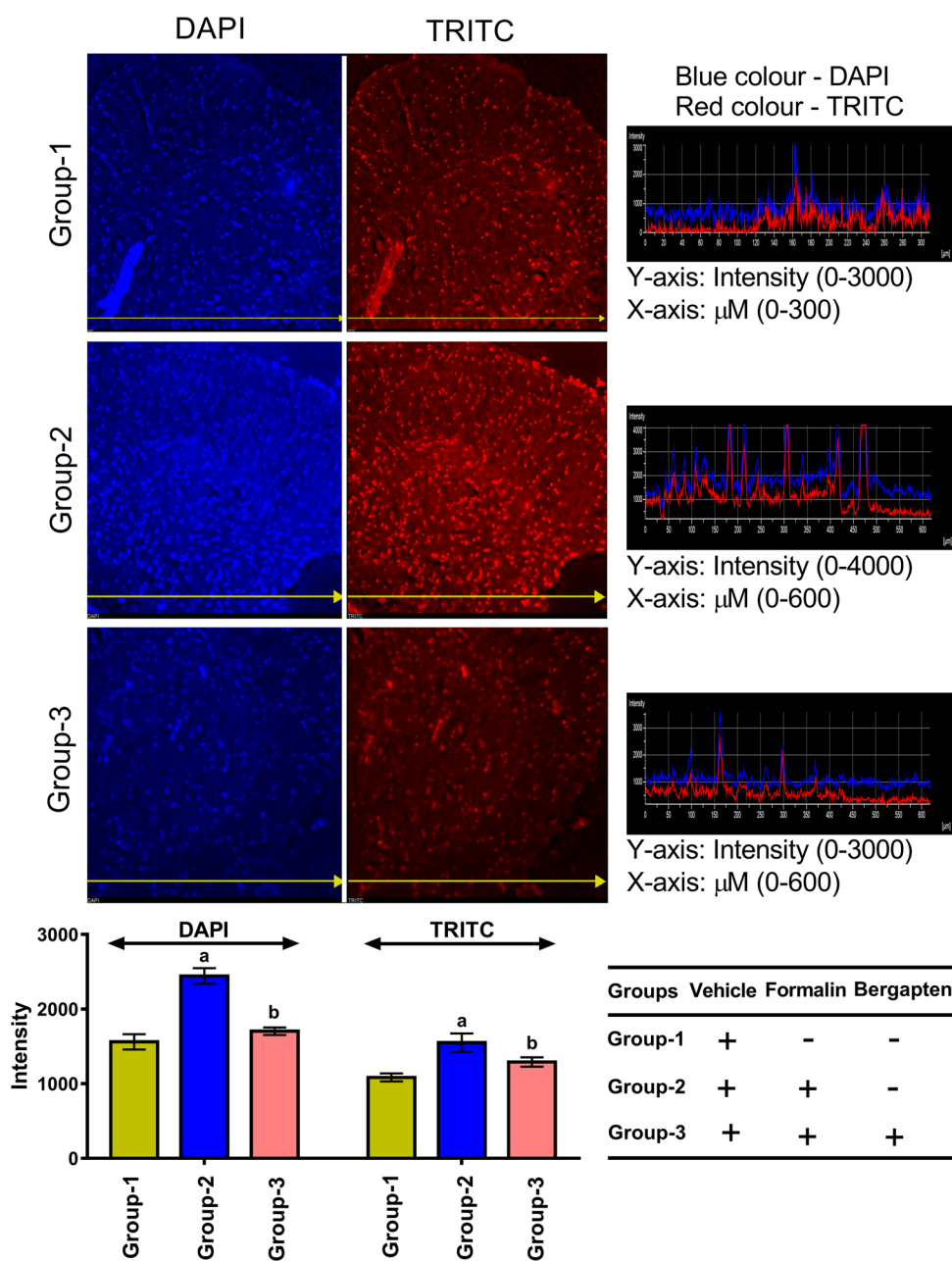
**Effect of bergapten on paw oedema induced by carrageenan**

Carrageenan injection was found to increase the paw volume steadily and peak oedema was evident after 45 min of carrageenan injection into the intraplantar region. Treatment with indomethacin (10 mg kg<sup>-1</sup>) and bergapten (10 mg kg<sup>-1</sup>) (Li et al. 2016) was found to reduce the paw oedema significantly and maximum anti-inflammatory effect was observed at 240 min of carrageenan injection (Fig. 7a, b).

**Effect of bergapten treatment on plasma cytokines**

LPS treatment enhanced the production of TNF-α and IL-6. However, bergapten produced a significant reduction in the level of both TNF-α and IL-6 in a dose-dependent manner. The doses of 5, 10, 20 mg kg<sup>-1</sup> diminished the level of TNF-α by 33.0, 66.3 and 86.0% (Fig. 8a) and IL-6 by 40.6, 63.3 and 82.6%, respectively (Fig. 8b). For determining IC<sub>50</sub> of bergapten, analyses were run by varying dose of bergapten from 5 mg/kg to 20 mg/kg in triplicates, and % inhibition of TNF-α and IL-6 was recorded as response.

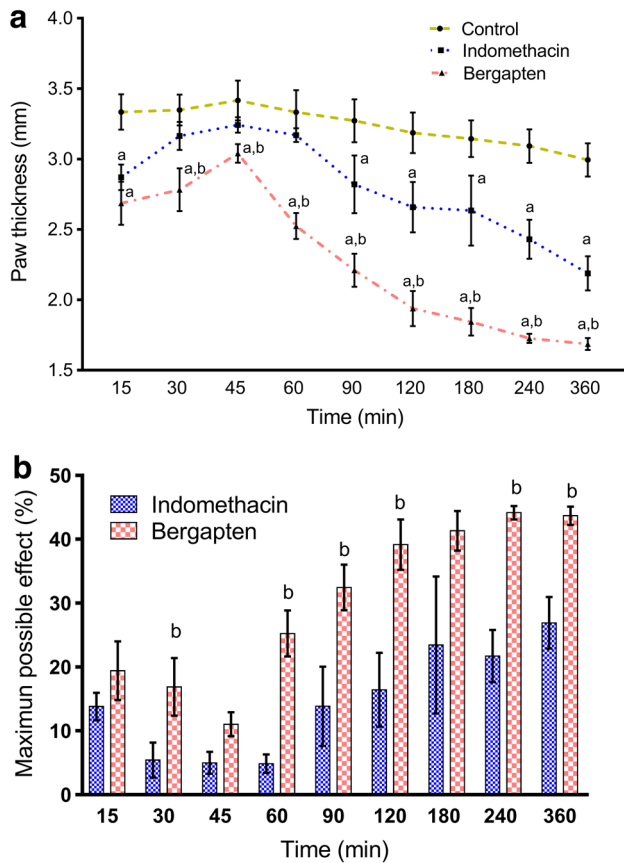
**Fig. 6** Immunofluorescence staining of Group-1, Group 2 and Group 3 with iNOS antibody showing DAPI-, TRITC-positive cells and average intensity in the transverse section of spinal cord (200× magnification). All values are expressed as mean ± SEM. Statistical differences were determined by one-way ANOVA followed by Tukey's test <sup>a</sup>*p* < 0.05 vs. control group and <sup>b</sup>*p* < 0.05 formalin-treated group



ANOVA modeling suggested second-order regression equation and explained 94.5% variance in the data (Coeff. of determination = 0.945). Fitted ANOVA model shows a highly significant model fitting at *p* value < 0.0001 along with the model statistics and regression equations which is given in Table 1. The one factor plot of bergapten vs. percentage inhibition of TNF-α indicated IC<sub>50</sub> of bergapten 7.27 mg/kg (Fig. 8c) and IL-6 was found 6.9 mg kg<sup>-1</sup> (Fig. 8d).

### Discussion

The current investigation was undertaken to explore the analgesic, anti-inflammatory effect and mechanism of action of naturally occurring furanocoumarin, bergapten. Further, the effect of bergapten treatment on the expression of PARP, COX-2 and iNOS pathways and inflammatory cytokines (TNF-α and IL-6) was also studied. The anti-hyperalgesic effect was studied by a well-established model of acetic acid-induced writhing that mimics visceral pain (Pitcher 2018; Pinho-Ribeiro et al. 2003) and formalin-induced



**Fig. 7** Effect of bergapten on **a** paw volume, **b** % maximal possible effect in carrageenan-induced paw oedema. All values are expressed as mean  $\pm$  SEM. Statistical differences were determined by two way ANOVA followed by Tukey's test <sup>a</sup> $p < 0.05$  vs. control and <sup>b</sup> $p < 0.05$  vs. indomethacin

hyperalgesia. Intraperitoneal injection of acetic acid induces pain response quantified as number of abdominal constrictions or writhes for a period of 30 min. Similar response was observed in our study. Acetic acid treatment is documented to exhibit nociception by inducing the release of inflammatory cytokines such as TNF- $\alpha$ , IL-6, IL-1 $\beta$  and IL-8 (Satyanarayana et al. 2004).

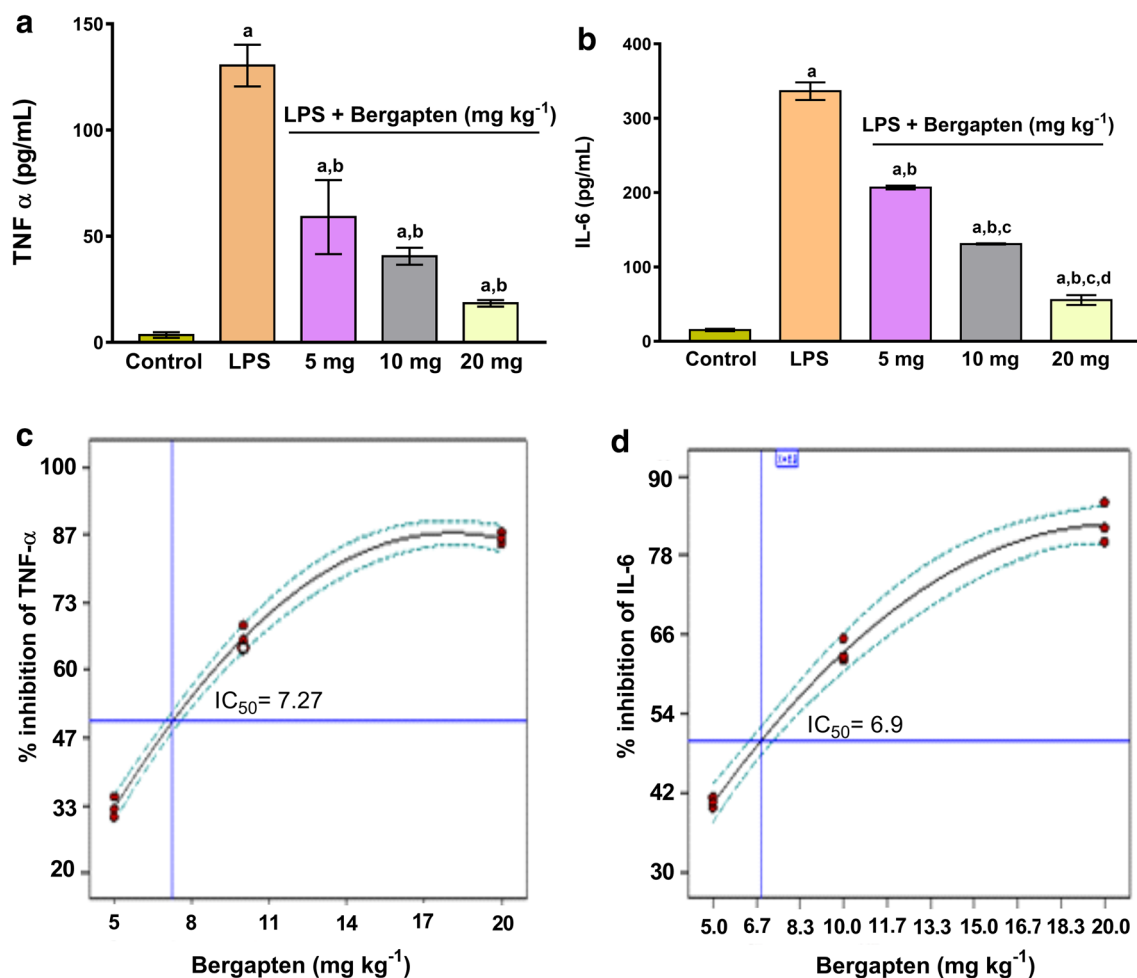
Formalin is known to induce a biphasic pain response comprising of an initial neurogenic phase mediated at the spinal level and a delayed phase of inflammatory pain which occurs due to combined inputs from the afferent and peripheral nociceptive pathways (Yi et al. 2011; Fischer et al. 2014). While opioid analgesics are known to block both the neurogenic and inflammatory phases of formalin hyperalgesia, NSAIDs, on the contrary, do not attenuate the neurogenic hyperalgesia but effectively blocks the inflammatory phase (McNamara et al. 2007). Bergapten was found to inhibit both acetic acid-induced writhing and the phases of formalin-induced hyperalgesia.

Furthermore, bergapten exhibited significant decrease in the carrageenan-induced paw oedema. Pretreatment with substance-P and L-arginine reversed the analgesic effect of bergapten; whereas L-NAME pretreatment produced no effect. As reported previously, increased expression of COX-2 and iNOS is involved in hyperalgesia in formalin-treated mice (Singh et al. 2018; Hwang et al. 2013). Substance-P, a neurokinin, is also reported to increase the expression of COX-2 (Gallicchio et al. 2006). The findings of the current study indicate a reversal of analgesic effect of bergapten on pretreatment with substance-P thereby suggesting a role of inhibition of COX-2 in the analgesic effect of bergapten. This contention is further supported by the decreased expression of spinal COX-2 in the spinal sections of formalin-treated mice.

Nitric oxide is a gaseous transmitter that has been postulated to be generated at the sites of inflammation and mediates pain and inflammation (Fan et al. 2012). Increased expression of iNOS is known to play pivotal role in several inflammatory conditions such as neuropathic pain (Freire et al. 2009) and carrageenan-induced inflammatory pain in animals (Cury et al. 2011). L-arginine is a precursor of NO and is documented to accentuate formalin-induced hyperalgesia (Schmidtke et al. 2009). Tissue nitric oxide synthesis is catalyzed by the enzyme nitric oxide synthase (NOS) which has three isoforms. While neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed, inducible or iNOS is a non-constitutive enzyme expressed at the time of inflammation (Cury et al. 2011; Forstermann and Sessa 2012). Increased expression of iNOS is linked with several chronic inflammatory conditions such as rheumatoid arthritis, multiple sclerosis, dilated cardiomyopathy, asthma and ulcerative colitis and neuropathic pain (Choudhari et al. 2013; Khan et al. 2016). Inhibitors of NOS including L-NAME, which non-selectively inhibits NOS (Meena et al. 2011) and selective iNOS inhibitor GW274150 and GW273629, have been documented to confer analgesic effect in chemically induced pain in several studies (Koch et al. 2007; Wang et al. 2015; Wang et al. 2005). In the current investigation, pretreatment with L-NAME did not alter the effect of bergapten. Studies have revealed that the expression of iNOS is induced by oxidative insults, inflammatory cytokines and other types of tissue stresses such as hypoxia (Ohtsu et al. 2010). COX-2 and iNOS have been documented to be produced in large amounts under inflammatory conditions (Jean et al. 2008).

Ploy(ADP-ribose) polymerase (PARP), an enzyme responsible for nicking DNA and transferring ADP to nuclear proteins, has been documented to play a role in inflammatory conditions (Ahmad et al. 2014). Studies have revealed PARP inhibitors to have ameliorative effects in animal models of acute and chronic inflammation (Patcher and Szabo 2007; Bai and Virag 2012). PARP





**Fig. 8** Effect of bergapten on plasma levels of **a** TNF- $\alpha$  and **b** IL-6 in mice. All values are expressed as mean  $\pm$  SEM. Statistical differences were determined by one-way ANOVA followed by Tukey's test

<sup>a</sup> $p < 0.05$  vs. control group, <sup>b</sup> $p < 0.05$  vs. LPS treated group, <sup>c</sup> $p < 0.05$  vs. 5 mg kg<sup>-1</sup> and <sup>d</sup> $p < 0.05$  vs. 10 mg kg<sup>-1</sup>. **c** Median inhibitory concentration (IC<sub>50</sub>) of bergapten on TNF- $\alpha$ , **d** on IL-6

has been reported to play a role in ischemia reperfusion injury produced by peroxynitrite (Szabo et al. 2002). Furthermore, PARP has been shown to control the expression of nitric oxide synthase (Le Page et al. 1998). Studies have revealed that treatment with 3-aminobenzamide, a PARP inhibitor, ameliorates the expression of iNOS, COX-2 and inflammatory cytokines in adjuvant-induced arthritis in mice (Ahmad et al. 2014). Bergapten treatment was found to decrease the PARP expression in the spinal sections of formalin treated mice. Bergapten treatment also decreased

the level of inflammatory cytokines TNF- $\alpha$  and IL-6 in mice. These findings corroborate the hypothesis that anti-hyperalgesic and anti-inflammatory effects of bergapten might be mediated by the inhibition of iNOS COX-2 expression and the inflammatory cytokines.

In conclusion, the results of present studies provide useful insights into the analgesic, and anti-inflammatory potential of bergapten that is possibly emerging through the modulation of PARP, COX-2, iNOS expression and inflammatory cytokines.

**Table 1** (a) Experimental data, (b) ANOVA model for TNF- $\alpha$ , (c) ANOVA model for IL-6, (d) model statistics, (e) model equations by varying doses of bergapten

## (a) Experimental data

S. No.	Independent variable $X_1$	Response variable $Y_1$	Response variable $Y_2$
1	5	31	40
2	5	33	41
3	5	35	41
4	10	64	62
5	10	66	63
6	10	69	65
7	20	85	80
8	20	86	82
9	20	87	86

(b) ANOVA model fitting for TNF- $\alpha$ 

Source	SS	Df	MS	F value	p value Prob. > F
Model	4317.8	2	2158.9	612.5	<0.0001*
Dose	4220.9	1	4220.9	1197.6	<0.0001*
Dose <sup>2</sup>	481.5	1	481.5	136.6	<0.0001*

## (c) ANOVA model for IL-6

Source	SS	Df	MS	F value	p value Prob. > F
Model	2664.1	2	1332.0	316.0	<0.0001*
Dose	2658.1	1	2658.1	630.7	<0.0001*
Dose <sup>2</sup>	147.2	1	147.2	34.9	<0.0010*

## (d) Model statistics

	TNF $\alpha$	IL-6		TNF $\alpha$	IL-6
Std. Dev.	1.8	2.0	$R^2$	0.995	0.990
Mean	61.6	62.2	Adjusted $R^2$	0.993	0.987
C.V.%	3.0	3.3	Predicted $R^2$	0.989	0.978
PRESS	47.5	56.9	Adequacy precision	48.94	35.51

## (e) Fitted model equation

Response	Term	Equation
$Y_1$	Coded	$+ 77.13 + 26.52 \times \text{dose} - 17.78 \times \text{dose}^2$
$Y_2$	Coded	$+ 71.51 + 21.05 \times \text{dose} - 9.83 \times \text{dose}^2$
$Y_1$	Actual	$- 16.451 + 11.437 \times \text{dose} - 0.316 \times \text{dose}^2$
$Y_2$	Actual	$+ 9.118 + 7.175 \times \text{dose} - 0.174 \times \text{dose}^2$

Coded (Bergapten dose in coded units - 1 to + 1)

Actual (Bergapten dose is in mg kg<sup>-1</sup>)

SS sum of squares, Df degree of freedom, ms mean square,  $X_1$  bergapten dose (mg/Kg),  $Y_1$  % inhibition for TNF- $\alpha$ ,  $Y_2$  % inhibition for IL-6

\*Significant at p value < 0.01

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## Compliance with ethical standards

**Conflict of interest** Authors do not have any conflict of interest.

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