Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
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ORIGINAL ARTICLE

Hypericin, an Anthraquinone Derivative of *Hypericum hookerianum* Wight and Arn. (Hypericaceae) of Palni Hills, South India, Exhibits Anti-Inflammatory Property in Lipopolysaccharide – Stimulated RAW 264.7 Macrophages

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Submitted: 20-10-2017 Revised: 04-12-2017 Published: 14-08-2018

ABSTRACT

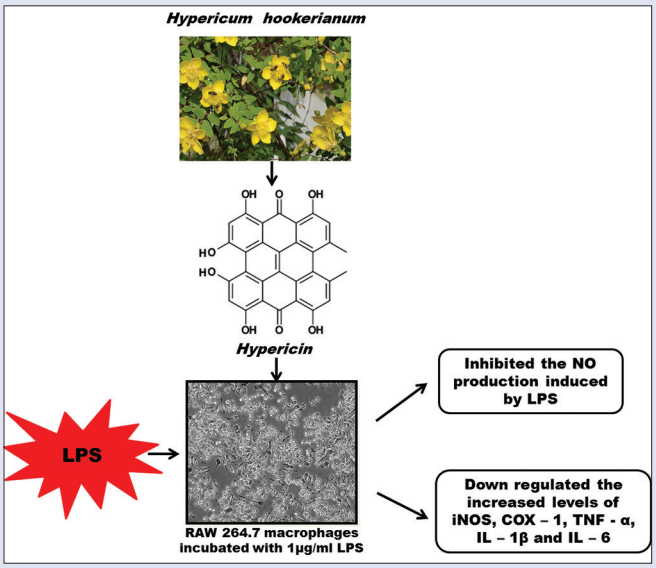
Background: Hypericin is an anthraquinone derivative of *Hypericum hookerianum*, a shrub from Palni hills of Southern India which possess various medicinal values. **Aim:** The present study was aimed to evaluate the anti-inflammatory activity of hypericin using RAW 264.7 macrophage cell line. **Materials and Methods:** The anti inflammatory property was determined by assessing the inhibitory action on lipopolysaccharide (LPS) stimulated nitric oxide (NO) production and pro inflammatory mediators/cytokines. RAW 264.7 macrophages cell line was used as in vitro inflammatory model. Cytotoxicity was evaluated using methylthiazolyldiphenyl tetrazolium bromide assay and NO estimation was carried out using Griess method. Gene expression of pro–inflammatory cytokines such as inducible nitric oxide synthase, cyclooxygenase 2, tumor necrosis factor α, interleukin–1 β (IL–1 β), and IL–6 was also evaluated. **Results:** The results exhibited that hypericin significantly suppressed LPS induced NO production with a concomitant decrease in the levels of pro–inflammatory cytokines. **Conclusion:** The study demonstrates the potential of hypericin as an effective anti–inflammatory agent.

Key words: *Hypericin,* inflammation, nitric oxide, pro–inflammatory cytokines, RAW macrophages

SUMMARY

- In the present study, we have evaluated the anti – inflammatory activity of Hypericin, an anthraquinone derivative of *Hypericum hookerianum* using RAW macrophages. Anti – inflammatory activity was evaluated by estimating the inhibitory potential of hypericin against LPS induced NO production. Hypericin significantly down regulated the pro – inflammatory cytokines such as iNOS, COX – 1, TNF - α, IL – 1β and IL – 6 that was increased due to inflammation.

Abbreviations used: NSAID: Nonsteroidal anti-inflammatory drug; NO: Nitric oxide; IL – 6: Interleukin – 6; IL – 1β: Interleukin – 1β; TNF – α: Tumour necrosis factor – α; iNOS: inducible Nitric oxide synthase; COX – 2: Cyclooxygenase - 2; NF-κB: nuclear factor kappa B; PGE2: prostaglandin E2; LPS: Lipopolysaccharide; DMEM: Phenol free Dulbecco's modified Eagle medium; MTT: methyl thiazolyl diphenyl tetrazolium bromide; DMSO: Dimethyl sulfoxide; PBS: phosphate buffer saline; RNA: ribonucleic acid; q-PCR: quantitative polymerase chain reaction.



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DOI: 10.4103/pm.pm_485_17

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INTRODUCTION

Inflammation is the body's immune response to various stimuli such as physical, chemical, microbial, and immunological reactions resulting in the pathological manifestation of acute or chronic pain, swelling, redness. Even though this condition does not require emergency attention, imminent care is required to assuage the condition and improve the way of living because over a period, prolonged inflammation condition leads to other disorders such as rheumatoid arthritis, diabetes, atherosclerosis, and cancer.[1] Long-term inflammation, otherwise termed as chronic inflammation is caused by the production of pro-inflammatory cytokines such as interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α),

Cite this article as: Mahendrakumar M, Seeni S, Perinbam K. Hypericin, an anthraquinone derivative of *Hypericum hookerianum* wight and Arn. (Hypericaceae) of Palni Hills, South India, exhibits anti-inflammatory property in lipopolysaccharide — stimulated raw 264.7 macrophages. Phcog Mag 2018;14:378-82.

IL-1α, and IL-1 β.[2] Macrophages are the target inflammatory cells that are involved in the instigation of inflammatory reactions and possess key role in the pathogenesis of various inflammatory disorders.[3] Lipopolysaccharide (LPS), a Gram-negative bacterial endotoxin can activate immunological responses through various signaling cascades, namely, mitogen-activated protein kinases, nuclear factor-kappa B (NF-κB) signaling, and janus kinase/signal transducers and activators of transcription pathways.[4,5]

Nonsteroidal anti-inflammatory drug (NSAID) is the classical treatment strategy engaged for inflammatory pains. NSAID drugs reduce inflammation and its associated painkiller drugs act by interacting with enzymes and inflammatory proteins but not without side effects. Alternative medicinal plant-derived anti-inflammatory bioactive small molecule comes with the blessing of zero side effects with maximum potential against various disorders.[6] Hypericin is an anthraquinone derivate compound derived from *Hypericum* species, including *Hypericum hookerianum*. Anti-inflammatory potential of hypericin is reported in some studies but falls short of in-depth analysis.[7,8] While we have already reported the pharmacognistical, phytochemical, and physicochemical properties of *H. hookerianum* dried leaves extract,[9] the present study is aimed to exclusively evaluate the anti-inflammatory potential of hypericin using RAW 264.7 macrophages.

MATERIALS AND METHODS

Chemicals

LPS, Phenol-free Dulbecco's modified Eagle medium (DMEM), methylthiazolyldiphenyl-tetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO), phosphate buffer saline (PBS), and antibiotic-antimycotic solution (100U penicillin, 100 µg streptomycin, and 0.25 µg amphotericin B per ml) were purchased from Sigma-Aldrich. Fetal bovine serum was purchased from GIBCO/BRL Invitrogen.

Cell culture

Macrophage RAW 264.7 cells were obtained from the NCCS, Pune with Passage no 16. Cells were cultured in phenol red-free DMEM supplemented with 100units/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated fetal bovine serum at 37°C with 5% CO_2. Cells were washed with DMEM medium and detached with 0.25% trypsin-EDTA. The cells were resuspended in DMEM medium at a density of 2×10^6 cells/ml.

Cell proliferation assay or methylthiazolyldiphenyl-tetrazolium bromide assay

Cytotoxicity of the compound hypericin was assessed independently by MTT assay. Cells were plated in 96-well plate at a concentration of 5×10^4 cells/well 24 h after plating. After 24 h of cells incubation, the medium was replaced with 100 µl medium containing only hypericin at different concentrations (5, 10, 20, 40, 80, 160, and 320 µM/well) incubated for 24 h. Untreated cells served as control and received only 0.1% DMSO in which the compound was prepared. At the end of treatment period, media from control, 0.1% DMSO, LPS (1 µg/ml) and hypericin alone-treated cells was discarded, and 50 µl of MTT (5 mg/ml PBS) was added to each well. Cells were then incubated for 4 h at 37°C in CO_2 incubator. MTT was then discarded, and the colored crystals of produced formazan were dissolved in 150 µl of DMSO and mixed effectively by pipetting up and down. The spectrophotometrical absorbance of the purple-blue formazan dye was measured using an ELISA reader (BIORAD) at 570 nm. The optical density of each sample was compared with control optical density and graphs were plotted. $IC_{50}$of hypericin was calculated using linear regression analysis. Further assays were carried out based on the IC_{50} value.

Estimation nitric oxide

The presence of nitrite, a stable oxidized product of nitric oxide (NO), was determined in cell culture media using Griess reagent. In brief, 50 µl of supernatant from the test culture was mixed with 50 µl of 1% (w/v) sulfanilic acid in 5% (v/v) phosphoric acid in a 96-well plate, followed by incubation for 10 min at room temperature. After that, 50 µl 0.1% (w/v) N-1-naphthylethylenediamine HCl in distilled water was added and incubated for 10 min at room temperature. The optical density at 540 nm was measured with a micro plate reader. The NO concentration was calculated by comparison with a $NaNO_2$ (0–100 µM) standard curve. The final concentration of DMSO was adjusted to < 0.1% for all treatments. The results were expressed as inhibition of NO production compared to the control (LPS) using: $([\text{nitrite}]_c - [\text{nitrite}]_t)/[\text{nitrite}]_c$, where $[\text{nitrite}]_c$ and $[\text{nitrite}]_t$ are the nitrite concentration in the control and test sample, respectively.

RNA Isolation and quantitative real-time polymerase chain reaction analysis

RAW macrophages were treated with 30 µM, 60 µM, and 90 µM of hypericin with 1 µg/ml of LPS and incubated for 24 h. Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol, and 2 µg of RNA was used for complementary DNA synthesis using M-MLV reverse transcriptase (Promega, Madison, WI, USA). Quantitative real-time polymerase chain reaction (q-PCR) was performed in an ABI 7500 Real-time system with SYBR Green PCR Master Mix (Takara). Reactions were initiated with an initial incubation at 50°C for 2 min and 94°C for 10 min, followed by 40 cycles of 94°C for 5 s, 60°C for 15 s, and 72°C for 10 s. The relative gene expression levels were calculated using the 2–ΔΔCt method. The specific primer sequences used were given below:

INOS: Forward: 5′-ATGTCCGAAGCAAACATCAC-3′
Reverse: 5′-TAATGTCCAGGAAGTAGGTG-3′
COX-2: Forward: 5′-CAGCAAATCCTTGCTGTTCC-3′
Reverse: 5′-TGGGCAAAGAATGCAAACATC-3′
IL-1 β Forward: 5′-ATGGCAACTGTTCCTGAACTCAACT-3′
Reverse: 5′-TTTCCTTTCTTAGATATGGACAGGAC-3′
TNF-α Forward: 5′-ATGAGCACAGAAAGCATGATC-3′
Reverse: 5′-TACAGGCTT GTCACTCGAATT-3′

β-Actin was used as an internal reference gene between different samples.

Statistical analysis

Data obtained from the experiments was expressed as mean ± standard error of the mean. One-way ANOVA followed by Tukey's multiple comparison was performed for MTT and NO production assays. For gene expression studies, the statistical analysis of the difference between the groups was evaluated using Dunnett's following one-way ANOVA *Post hoc* comparisons in Graph Pad Prism 5.0 software version. $P < 0.001$, $P < 0.01$, and $P < 0.05$ were considered as statistically significant.

RESULTS

Effect of hypericin on % inhibition of cell proliferation in RAW 264.7 macrophages

The effect of hypericin on cell viability was evaluated in RAW 264.7 macrophages cells by MTT assay; thereby the non–toxic concentrations of hypericin can be determined. The results showed that 0.1% DMSO, 5 µM and 10 µM of hypericin did not affect the cell viability which exhibited cell viability percentage of ≤90%. At a maximum concentration

Table 1: Effect of hypericin on percentage viability of RAW 264.7 macrophages

Concentration	Percentage cell viability
Control	100.59±2.09
0.1% DMSO	99.53±0.81
Hypericin 5 μM	99.76±0.35
Hypericin 10 μM	99.02±0.53
Hypericin 20 μM	93.77±0.36
Hypericin 40 μM	89.60±0.38
Hypericin 80 μM	84.59±0.42
Hypericin 160 μM	80.25±0.28
Hypericin 320 μM	71.31±0.24

Values are expressed mean±SEM (n=3). DMSO: Dimethyl sulfoxide; SEM: Standard error of the mean

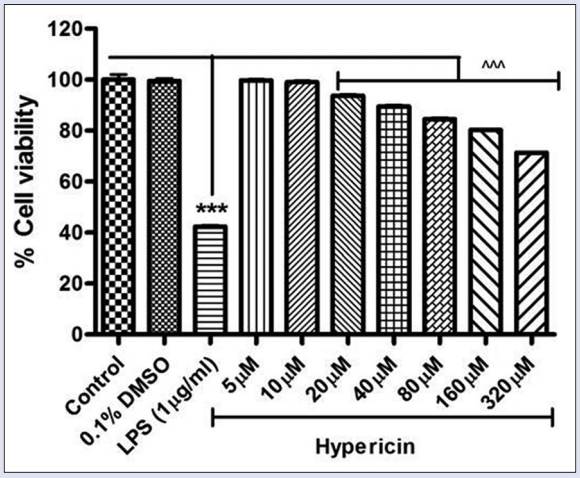

Figure 1: Effect of hypericin on the percentage of cell viability in RAW 264. 7 macrophages. After 24 h incubation with 0.1% dimethyl sulfoxide, lipopolysaccharide 1 μg/ml and hypericin, percentage of cell viability was assessed by methylthiazolyldiphenyl-tetrazolium bromide assay. Control cells were treated with media alone. Values are the mean ± standard error of the mean of three independent experiments. ***P < 0.001, **P < 0.01, *P < 0.05 indicates Control versus lipopolysaccharide; ^^^P < 0.001, ^^P < 0.01 and ^P < 0.05 indicates control versuss. hypericin treatment

of 320 μM, the cell viability was 71% [Table 1]. The other tested concentrations of hypericin showed significant (P < 0.001) percentage of decrease in cell viability when compared to control group [Figure 1]. IC_{50} of hypericin was calculated based on linear regression analysis and was found to be 67.62 μM. Based on the IC_{50} value, three different concentrations of 30 μM, 60 μM, and 90 μM were selected for gene expression studies.

Effect of hypericin on nitric oxide production

To examine the inhibitory effect of hypericin on LPS stimulated NO production in RAW 264.7 macrophages, cells were co–treated with different concentrations of hypericin (5–320 μM) and LPS (1 μg/ml) for 24 h. After 24 h of incubation, the nitrite levels in the supernatants were measured. As given in Figure 2a, the NO production was substantially decreased in a dose-dependent manner by hypericin. Considering the percentage of NO production in LPS group as 100%, the NO production in hypericin treated groups were calculated. The NO production was in the range of 96% to 17% at the concentrations of 5 μM–320 μM. The LPS group showed a significant (P < 0.001) increase in NO production when compared to normal control group. The hypericin treated cells at different concentrations of 10 μM–320 μM exhibited significant (P < 0.001) attenuation of NO production induced by LPS stimulation, while the 5 μM hypericin treatment did not show any inhibition against LPS stimulation.

The NO production in RAW 264.7 macrophage cells is synthesized by the enzyme inducible NO synthase (iNOS), and therefore, the effect of hypericin on iNOS expression in the transcriptional level have been examined. As shown in Figure 2b, iNOS gene expression level was remarkably elevated by LPS stimulation and it was shown in the results that hypericin has significantly attenuated the expression of iNOS, as analyzed using the qPCR analysis, in a dose-dependent manner. These results suggest that the inhibitory effects of hypericin on LPS-induced NO production was caused by the suppression of iNOS.

Hypericin inhibited cyclooxygenase 2, tumor necrosis factor-α, interleukin 1 β and interleukin 6

The results in Figure 3a demonstrated that cyclooxygenase 2 (COX-2) mRNA expression level in LPS–stimulated macrophages was elevated by ~38-fold compared to the control group. However, treatment with three concentrations of hypericin (30 μM, 60 μM, and 90 μM, respectively) exhibited significant (P < 0.001) decrease in the COX-2 expression when compared to LPS–stimulated group. Further, the effect of hypericin on pro-inflammatory cytokines TNF–α, IL–1 β and IL–6 were evaluated at mRNA level in LPS-stimulated RAW macrophages cells. As demonstrated in Figure 3b-3d, the gene expression levels of TNF–α, IL–1 β, and IL–6 have been significantly decreased when compared to the LPS-treated group.

DISCUSSION

Utilization of plant–derived natural products has increased extensively during the past few years, due to its low–toxicity, economy, and wide-array of pharmacological effects. The present study investigated the anti-inflammatory activity of hypericin, an anthraquinone derivative present in *Hypericum* species including *H. hookerianum*. Among, traditional medicinal plants, *Hypericum perforatum* have been widely used to relieve depression. Literature have previously reported the anti-inflammatory property of different species of *Hypericum* such as *Hypericum perforatum* and *Hypericum gentianoides*[10] in RAW 264.7 macrophages. In line with these data, the present study also evaluated the anti-inflammatory of hypericin, a compound characteristic of *H. hookerianum* and found that the compound possesses marked anti-inflammatory property against LPS stimulated inflammation.

In the present study, RAW 264.7 macrophage cell line was used as cell line model to investigate the anti-inflammatory property of hypericin. LPS stimulated RAW macrophages are considered as an appropriate *in vitro* model for inflammation and its related studies.[11-13] NO plays a vital role as essential mediator and regulator in many body functions, however, increased the amount of NO produced by activated macrophages due to transcription of iNOS lead to cytotoxicity, inflammation, and auto-immune disorders.[14,15] This, in turn, will activate intracellular signaling mechanism that is involved in immune dysfunctions. COX-2, a pro-inflammatory enzyme is also a critical mediator in chronic inflammatory conditions which produces prostaglandin E2 (PGE2).[16,17] Hence, inhibitors of iNOS, PGE2 or COX-2 may be considered as potent anti–inflammatory agents. The study showed that hypericin significantly down-regulates the expression of iNOSthereby suppressing the production of NO and COX-2 at transcriptional level, which corroborated with the given facts, suggesting hypericin as an effective anti-inflammatory agent.

Pro-inflammatory cytokines such as TNF-α, IL–1 β, and IL–6 are directly implicated in intracellular mechanisms such as NF–κB pathway that involves acute and chronic inflammation.[18] While

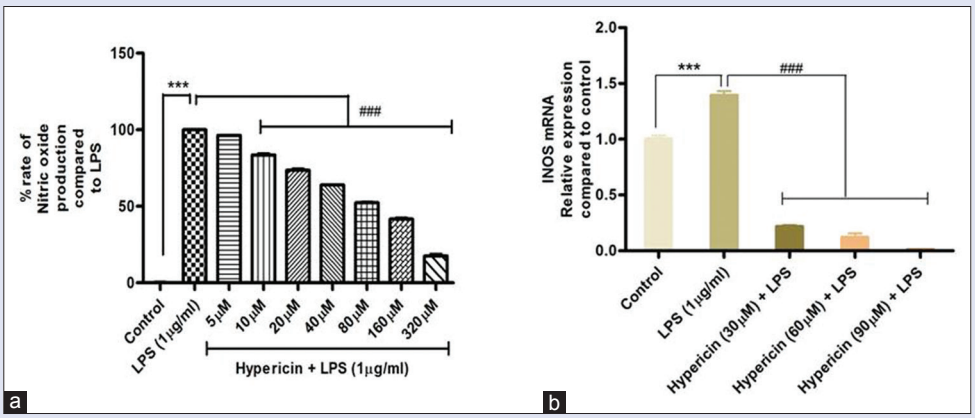

Figure 2: Effect of hypericin on (a) Nitric oxide production and corresponding (b) Inducible nitric oxide synthase mRNA expression in lipopolysaccharide–stimulated RAW 264. Seven macrophages. Cells (2×10^4 cells/well) in 96-well plates were co-treated with/without hypericin and lipopolysaccharide (1 μg/mL) for 24 h. The amount of nitric oxide production was evaluated using Griess method and mRNA expression of inducible nitric oxide synthase was determined by real-time polymerase chain reaction analysis. Each value is expressed as mean ± standard error of mean of three independent experiments. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ indicates control versus lipopolysaccharide; ###$P < 0.001$, ##$P < 0.01$ and #$P < 0.05$ indicates lipopolysaccharide versus hypericin treatment

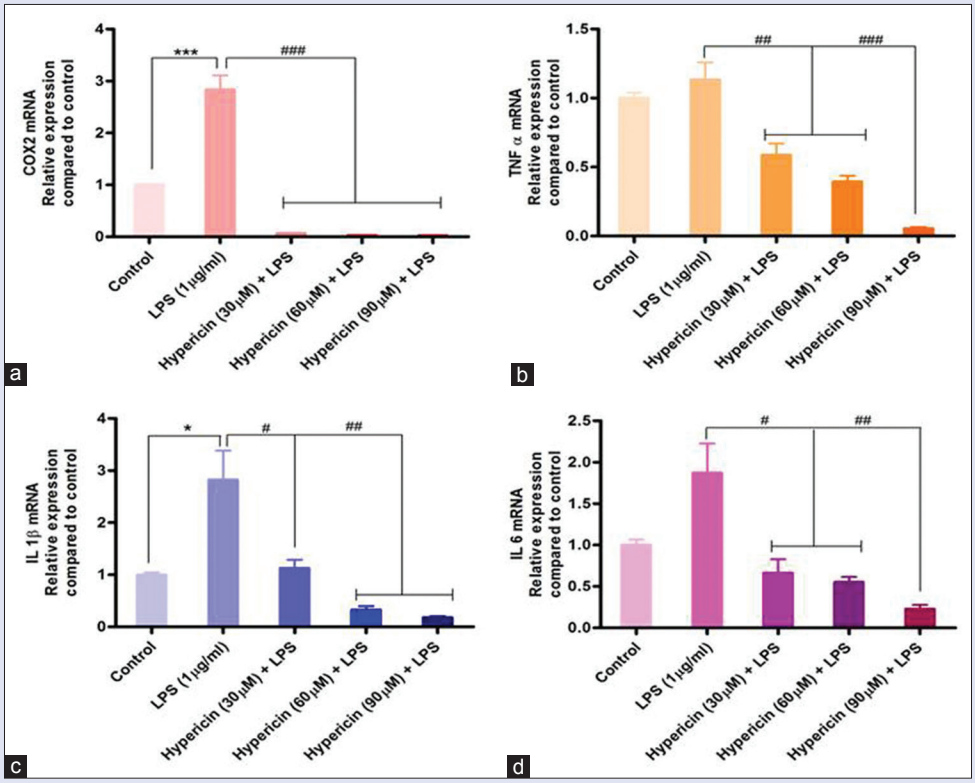

Figure 3: Effect of hypericin on (a) cyclooxygenase 2, (b) tumor necrosis factor –α, (c) interleukins–1 β and (d) interleukins–6 mRNA expressions in lipopolysaccharide–stimulated RAW 264.7 macrophages. Cells (2 × 104 cells/well) in 96 well plates were co treated with or without indicated concentrations of hypericin and lipopolysaccharide (1μg/mL) for 24 h. Untreated is negative control without lipopolysaccharide treatment. Each value is expressed as mean ± standard error of the mean of three independent experiments. ###$P < 0.001$, ##$P < 0.01$ & #$P < 0.05$ indicates lipopolysaccharide versus hypericin treatment.

TNF–α and IL–1 β are known to induce tissue damage and multiple organ failure through perpetuation of inflammation,[19,20] increased production of IL-6 results in chronic autoimmune inflammatory disease.[21] The results of the present study demonstrated that hypericin exhibited a dose-dependent response in inhibiting LPS–stimulated inflammation. Previous, reports showed that uliginosin A, compound isolated from *H. gentianoides* exhibited anti-inflammatory property by inhibiting PGE2, NO, TNF–α, and IL–1 β. A similar mechanism of action was observed in hypericin treated RAW macrophages suggested that an upstream inflammation regulator could be its molecular target.[10]

CONCLUSION

We have demonstrated that hypericin is an anthraquinone derivative of *H. hookerianum* and has substantial effect on NO inhibition associated

with down-regulated expression of iNOS, COX-2, TNF–α, IL–1 β, and IL–6. The findings suggest hypericin is a potent anti-inflammatory agent; however further studies are needed to validate the compound as anti-inflammatory drug.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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