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Kaempferol-3-o-β-D-glucuronate exhibit potential anti-inflammatory effect in LPS stimulated RAW 264.7 cells and mice model

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ABSTRACT

Kaempferol-3-O-β-D-glucuronide (K3G) having various pharmacological effects was explored for its anti-inflammatory effect in LPS induced RAW 264.7 cells and mice model. K3G significantly inhibited various pro-inflammatory mediators like IL-1β, NO, PGE2, and LTB4. It upregulated the secretion of anti-inflammatory cytokine IL-10. K3G is found to reduce inflammation when studied for parameters like phagocytic index, carrageenan induced paw edema in mice and organ weight. It reduced inflammation in a dose dependent manner both *in-vitro* and *in-vivo*. Further molecular insights into the study reveal that K3G blocks the phosphorylation of NF-kB which is key regulator of inflammation, thereby exhibiting anti-inflammatory potential. Hence, this study permits further investigation to develop K3G as anti-inflammatory drug.

1. Introduction

Inflammation is the act of self-protection by body in response to injury being caused by harmful stimuli, including damaged cells, irritants, or pathogens. However, prolonged inflammation leads to pathogenesis of a number of diseases including arthritis, asthma, multiple sclerosis and many more [1,2]. Macrophages play a crucial role in the immune system as they provide an immediate defense against foreign agents. The detection of pathogenic substances by macrophages is through pattern-recognition receptors (PRRs), thereby subsequent regulation of inflammatory responses mediated by pro-inflammatory mediators, such as TNF-α, IL-6, IL-1β, NO, PGE2, and LTB4 [3]. LPS is a component of Gram negative bacterial cell membrane and can trigger inflammatory response and immune dysfunction [4]. LPS stimulated macrophages subsequently lead to the activation of NF-κB thereby inducing the pro-inflammatory mediator expression including IL-1β and inducible nitric oxide synthase (iNOS) [5,6]. All these mediators play significant roles in the inflammatory process. Targeting the regulation of these mediators is a major goal of inflammation treatments. Eicosanoid lipid mediators, prostaglandins and leukotrienes also play a key role in pathological conditions, like rheumatoid arthritis [7–16]. The lipid chemoattractant leukotriene B4 (LTB4) is an early mediator of inflammation playing a major role in pathological conditions, such as rheumatoid arthritis and endotoxin shock [17–22]. NO synthesized by inducible nitric oxide synthase (iNOS) play an important role in various physiological and pathological conditions [23]. However excessive NO causes tissue injury either directly, through damaging proteins, lipids and DNA or indirectly, through the modulation of leukocyte activity [24]. An early inflammatory response requires signal transduction mediated by NF-κB as it regulates the expression of various proteins associated with immunity and inflammation, such as NO, PGE2 and IL-1β [25,26]. In resting state, NF-κB is localized in the cytosol. In response to a stimuli, such as lipopolysaccharide (LPS), NF-κB translocates into the nucleus and results in target gene transcription [27,28].

Cell culture data always needs to be validated in *in-vivo* model as there is only one type of cell in *in-vitro* experiments, but multiple cells exist in an animal model. Hence the *in-vivo* effect of kaempferol-3-o-β-D-glucuronate (K3G) was also studied in LPS induced inflammatory mice. Moreover, other inflammatory mediators may influence the cytokine response [29]. Phagocytosis removes the microorganisms, dead and injured cells [30] and its evaluation is crucial to develop anti-inflammatory drug. K3G has been shown to have various pharmacological effects including anti-inflammatory, antioxidant activity on MLP stimulated neutrophils [31], anti HIV-1 activity from *Cyclocarya paliurus*

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[32]. K3G was found ineffective against various microorganisms and behaved like the other glycosides of quercetin and kaempferol [33].

Thus, in this study, we investigated the anti-inflammatory activities of K3G in LPS-induced RAW 264.7 cells in mice and determined its molecular mechanism of action.

2. Materials and methods

2.1. Cell line and reagents

Rolipram, Phosphate Buffer Saline (PBS), Ficoll–Hypaque, DMEM and carrageenan were purchased from Sigma-Aldrich. Lipopolysaccharide (LPS) *E. coli* serotype 0111:B4, (3-(4,5,di-methylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Calbiochem. Fetal bovine serum was obtained from GIBCO Invitrogen Corporation. All the ELISA kits, Mouse IL-10, Mouse IL-1β, Mouse PGE2 and Mouse LTB4 kits were bought from Invitrogen. Griess Reagent was purchased from Promega. Precoated TLC plate was from E. Merck, 60F-254. Hexane used was of HPLC grade, purchased from Merck. Heptane and anisaldehyde sulphuric acid were of AR grade, purchased from Rankem.

2.2. Instrumentation and HPTLC conditions

The HPLC system consisted of Waters instrument equipped with a binary pump, an autosampler, an automatic electronic degasser, an automatic thermostatic column oven, a diode array detector, and Chemstation software (version 06.03 [509]) for data analysis. The LC separations were optimized using RP-18, Merck column (4 × 250 mm, 5 μm) where mobile phase consisted of a mixture of methanol and 1.5% acetic acid in water. Elution was achieved with a flow rate of 0.8 mL/min using a gradient from 92:8 (1.5% aqueous acetic acid: methanol) for 5 min, followed by a linear increase in the organic mobile phase to 50% at 25 min, 75% at 60 min with a hold of 5 min (until 65 min) followed by a linear decrease in the organic percentage to 8% at 70 min, which was sustained for further 5 min with a total run time of 75 min. The mobile phase was filtered through 0.45 μm filter (Millipore) before use. The column temperature was maintained at 30 °C to provide sharpness to the eluting peaks. The UV chromatograms were recorded at 335 nm. Purity of the isolated compound was evaluated by HPLC. K3G showed a pure peak at a retention time of 24.9 min in the LC-UV (DAD) chromatogram respectively (Fig. 2).

2.3. Test compound

K3G (Fig. 1) was provided by the Natural Product Chemistry Division, IIIM, Jammu.

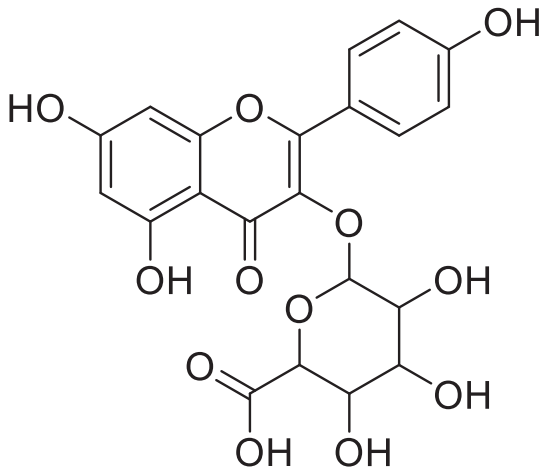


Fig. 1. Kaempferol-3-o-β-D-glucuronate (K3G).

2.4. Experimental animals

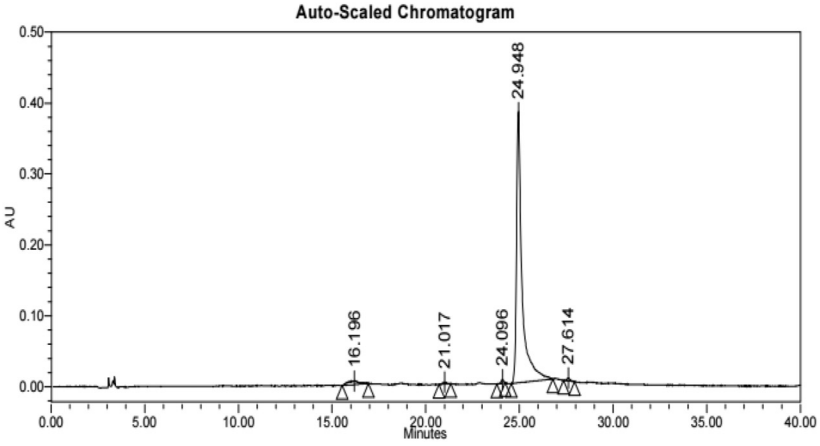
Female Balb/C mice 10–12 weeks old and weight is 20–25 g were housed under standard laboratory conditions, 23 ± 1 °C, 55 ± 10% relative humidity, 12/12 h light/dark cycles and fed with pellet diet (Lipton India Ltd) and water *ad libitum*. Experiments were designed in such a way so as to use minimum number of animals. The experimental protocols were approved by Institutional Animal Ethic Committee (IAEC reg. no. 66/81/2/16). After experimentation, animals were subjected to euthanasia with high inhalation dose of diethyl ether and disposed off by incineration. All the drugs used for the study were dissolved in 100% DMSO and the working concentration of DMSO in animals is below 1%.

2.5. Cell culture

RAW 264.7 cells, murine macrophages were cultured in DMEM medium containing 10% Fetal Bovine Serum (FBS), penicillin-streptomycin (GIBCO) at 37 °C in a humidified 5% CO_2 atmosphere. Drugs used for the study were dissolved in 100% DMSO but the actual concentration of DMSO in treated plate is equal to or below 0.1%.

2.6. Effect of K3G on cytokine production in RAW264.7 cells

The inhibitory effect of K3G on the production of IL-1β and IL-10 were determined by mouse enzyme-linked immunosorbent assay kit (ELISA). RAW 264.7 cells were seeded at a density of 2 × 10^5 cells/well in 96-well plate and incubated overnight. The cells were treated with



	RT	Area (μV*sec)	% Area	Height (μV)
1	16.196	230927	2.83	5130
2	21.017	41492	0.51	2785
3	24.096	49232	0.60	4980
4	24.948	7788597	95.49	381332
5	27.614	46236	0.57	4032

Fig. 2. HPLC purity profile of K3G.