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Evaluation of Anti-Inflammatory Potential of Turmimax® an *in vivo* and *in vitro* Study

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Abstract: The main objective of this study was to investigate the *in vivo* and *in vitro* anti-inflammatory effects of the Turmimax® bio-enhanced turmeric formulation. By comparing the relative fluorescence intensities of the interleukins IL-8 (Interleukin-8) and IL-12 (Interleukin-12) cytokines in stimulated murine Raw 264.7 macrophages, Turmimax® was evaluated for its anti-inflammatory *in vitro* efficacy. Turmimax® was administered orally in two doses, 100 and 200 mg/kg, to assess its *in vivo* anti-inflammatory potential in ear edema induced by croton oil in mice and paw edema induced by histamine in albino rats. Diclofenac sodium 10 mg/kg p.o. is the standard drug. The results demonstrated that Turmimax® significantly and dose-dependently decreased the production of the inflammatory mediator's interleukin-8 and IL-12. According to the current investigation results, Turmimax® has shown strong anti-inflammatory effects.

Keywords: Turmimax®, Raw 264.7 Cell Line, IL-8 and IL-12, Flow Cytometry and Anti-Inflammatory Activity

Introduction

Inflammation, which develops in any tissue in response to injury, infection, or toxic stimuli, is often life-sustaining and leads to tissue healing, recovery, and repair. However, inflammation persists and causes diseases and problems if the targeted repair and removal of damaging stimuli are not regulated appropriately. Numerous anti-inflammatory drugs are on the market, including the most popular steroidal and non-steroidal anti-inflammatory medications. Since these medications have substantial side effects, research is constantly being done to create safe and efficient anti-inflammatory treatments.

Turmeric, also known as *Curcuma longa* Linn., is a member of the Zingiberaceae family and has long been used in medicine, food, and cosmetics worldwide (McCaleb *et al*., 2000; Jayaprakasha *et al*., 2005) because of its ability to promote health, food is considered as a functional food. (Trinidad *et al*., 2012). The traditional medicine, *C. longa* rhizome, has anti-inflammatory, anti-carcinogenic, anticoagulant, anti-mutagenic, antioxidant, anti-diabetic, anti-fertility, antifungal, antiprotozoal, antiulcer, antibacterial, antiviral, antivenom, anti-fibrotic, hypotensive and hypocholesterolemic properties (Jacob *et al*., 2013). An important component of turmeric known as curcumin has attracted the interest of scientists in recent years due to its potent anti-inflammatory and antioxidant properties and low risk of adverse effects. Numerous studies demonstrated curcumin's anti-inflammatory properties and its ability to slow the course of arthritis (Dai *et al*., 2018; Daily *et al*., 2016; Dou *et al*., 2018; Zhang *et al*., 2016). Tumor Necrosis Factor- (TNF-), interleukin (IL)-6, and IL-1 are the primary cytokines implicated in the inflammatory cascade of arthritis (Hata *et al*., 2004). Several studies have shown that curcumin reduces levels of key inflammatory markers such as TNF-α, nuclear factor-κB (NF-κB), Cyclooxygenase-2 (COX-2), mammalian Target of Rapamycin (mTOR), and interleukins. In illnesses like arthritis, psoriasis, inflammatory bowel disease, and asthma, curcumin can reduce inflammation (Aggarwal *et al*., 2013).

In addition to affecting the activity of enzymes, growth factor receptors, cofactors, and other molecules, curcumin is a highly pleiotropic chemical with a variety of targets and modes of action. Curcumin's capacity to bind to either lipoxygenase or phosphatidylcholine micelles and block lipoxygenase (Skrzypczak-Jankun *et al*., 2003). Additionally, curcumin prevents angiogenesis and tumor invasion via reversibly binding to CD13. (Shim *et al*., 2003).

Due to its quick degradation and elimination, extremely poor water solubility, and insufficient tissue absorption, curcumin has a restricted bioavailability, which significantly reduces its therapeutic efficacy (Anand *et al*., 2007; Szwed and Miłowska, 2012; Tiwari *et al*., 2014).

Curcumin exhibits a big promise as a therapeutic agent due to its properties, but its major limitation is low solubility because it is a moderately polar and non-lipophilic molecule. Therefore, it is crucial to create a highly biocompatible delivery method. Turmimax® is an innovative patented product that contains 95% of curcuminoids and 100% water dispersibility, which dissolves quickly and maintains solubility over time resulting in a product that is 35 x more bioavailable than standard curcumin. This study tested the anti-inflammatory effects of Turmimax® both *in vitro* and *in vivo*.

Materials and Methods

Preparation of the Turmimax®

Turmimax® is manufactured and registered by Star Hi Herbs Pvt. Ltd, Jigani, Bangalore, Karnataka, India [IP-202041031859] (Firoz *et al.*, 2022).

Cell Lines and Reagents

The NCCS, Pune, Maharashtra, India, supplied the Raw 264.7 Murine macrophage cell line. Antibiotic-Antimycotic solution (Cat No. A002) and DMEM with high glucose (Cat No. AL111), FBS (Cat No. RM10432) were purchased from Hi-Media laboratories, Mumbai, India. Hydrogen Peroxide (H_2O_2) and Lipopolysaccharide (LPS) were of molecular biology grade procured from Sigma Aldrich, USA. We bought the MTT reagent (M6494) from Thermo Fisher Scientific (Gibco, USA). BD Biosciences, USA, CA provided the flow cytometry-focused reagents PE Mouse Anti-Human IL-8 antibody (Cat No. 554720) and PE Mouse Anti-Human IL-12 antibody (Cat No. 559329).

Histamine and croton oil were bought from Hi Media in Mumbai, India. The common anti-inflammatory medication, diclofenac sodium, was purchased from Recon in Bangalore, India.

Treatment and Cell Culture

Raw 264.7 cell lines were cultured in 100 µL of antibiotic-antimycotic (100X) at 37°C in a CO_2 incubator with 20% oxygen and 5% CO_2 under saturated humidity. The medium was DMEM high glucose medium supplemented with 10% FBS. The medium was changed following a 24-h seeding event before Turmimax®, H_2O_2, or LPS treatment. To guarantee complete solubility, Turmimax® and H_2O_2/LPS were dissolved in DMSO and vortexed. Less than 0.1% of DMSO was added to the cell culture medium in total. From 6.25 to 100 µg/mL of Turmimax® is the final concentration in the cell culture medium. As a vehicle control, 0.1% DMSO concentration was utilized, which showed no cytotoxicity in Raw 264.7 cells.

MTT Assay

The Raw 264.7 cell line's viability was assessed using the MTT test. To find the ideal concentration of Turmimax®, 20,000 cells per well were seeded in a 96-well plate and treated for 24 h with 6.25, 12.5, 25, 50, and 100 µg/mL of Turmimax®. After the incubation period, the used media was removed, 100 µL of 0.5 mg/mL MTT reagent was added and formazan crystals were produced as a result of the reduction of MTT by metabolically active cells for 4 h at 37°C. 100 µL of DMSO was used to dissolve them when they were freed from the cells (Sigma-Aldrich, USA). In the MTT experiment, formazan accumulation reflects mitochondrial activity in living cells directly, providing an indirect indicator of cell viability (Mosmann *et al*., 1983). The plate was shaken on a gyratory shaker for 10 to 20 min and an ELISA microplate reader was used to measure the absorbance at 570 nm (Biotek, USA).

The formula below is used to determine the percentage of cell viability:

$$\%\ of\ inhibition = 100 - \left(Test\ Optical\ density\ /\ Non-treated\ Optical\ density\right) \times 100$$

In vitro Anti-Inflammatory effect of Turmimax® by Murine Raw 264.7 Cell Line

Raw 264.7 cells were grown in a 6-well plate under 3 distinct culture conditions: LPS (2 µg/mL), LPS (2 µg/mL) +Turmimax® (50 µg/mL), and untreated, which received no treatment. Briefly, the cells were first stimulated with 2 µg/mL of LPS for 3 h to generate inflammation and then either treated with 50 µg/mL of Turmimax® or stimulated with LPS alone (negative control) in 2 mL of DMEM media. Cells were cultured for 24 h, collected, and then centrifuged at 300xg for five minutes in a Remi: R-8°C centrifuge using centrifuge tubes from BD Biosciences. After that, D-PBS was used to wash them twice. The pelleted cells were then rinsed with a 0.5% bovine serum albumin solution (1 x PBS and 0.1% sodium azide) and incubated at room temperature with 0.5 mL of BD Cytofix/Cytoperm for 10 min. Cell Quest Pro software version 6 was used to analyze the data after the cells were incubated with 20 µL of a PE-mouse anti-human interleukin 8 (IL-8) antibody and an anti-human IL-12 antibody separately for 30 min in the dark at 25°C. Expression was then measured using a BD FACS Caliber flow cytometer (BD Biosciences).

Statistical Analysis

All the data were analyzed using Microsoft Excel 2007 version to create a graphical representation of the mean with calculated standard errors. Flow Cytometric data was analyzed using Cell Quest Pro software version 6. The tests were carried out three times. *$p<0.05$, ** $p<0.01$ were used to determine the statistical significance.

In vivo Anti-Inflammatory Effect of Turmimax®

Experimental Design

30 Swiss albino mice (25-35g each) and 30 male Wistar albino rats were used in the experiments (180–200 g). They were collected from the Bharathi College of Pharmacy in Mandya, Karnataka, India, from the animal house. With no more than six individuals per cage, the animals were housed in groups in polyacrylic cages that measured 38 by 23 by 10 cm. They were kept in these cages under conventional laboratory settings, which included a temperature of 25±2°C and a 12-h cycle of darkness and light. Before the experiment began, all of the animals spent a week getting used to the conditions in the lab. Before the experiment, IAEC (1135/PO/Re/S/07/CPCSEA), the institutional animal ethics committee, granted its approval.

Assessment of Turmimax® Anti-Inflammatory Properties

Croton Oil-Induced Ear Edema

The croton oil-induced ear edema method was used to assess Turmimax® anti-inflammatory efficacy. Throughout the experiments, 30 Swiss albino mice were used. The Hosseinzadeh *et al*. (2003) approach was used to carry out this experimental process. Five groups of six Swiss Albino mice, one of each sex, measuring 25–35 g were created. The following are the medication dosages that were given to the various groups: Diclofenac sodium was employed as the standard medication in Group I Control (10 mL/kg of normal saline), Group II Negative Control (2.5% Croton oil), Group III Diclofenac sodium (10 mg/kg), Group IV Turmimax® (100 mg/kg b.wt) and Group V Turmimax® (200 mg/kg b.wt). Applying 50 µL of croton oil to the right ear's inner surface caused edema in each mouse. The mice were killed by cervical dislocation 90 min after being daubed with croton oil and both ears were taken off and weighed (Chen *et al*., 1991).

Histamine-Induced Paw Edema

Five groups of six Wistar albino rats, one of each sex, measuring 180–200 g were created. Following are the drug dosages given to the various groups: Group I Control (10 mL/kg) normal saline; Group II Negative Control (histamine); Group III Diclofenac sodium (10 mg/kg); Group IV (100 mg/kg); and Group V (200 mg/kg) Turmimax® b.wt. The reference standard anti-inflammatory medication is diclofenac sodium. Rats received Turmimax® for 1 h before the inflammatory process was induced. The difference in paw volume between the control and 30 min, one hour, two hours, three hours, and four hours after the administration of the inflammatory agent inhibition was used to measure edema. To cause inflammation in rats, 0.1 mL of 0.1% histamine in normal saline was injected into the subplantar tissue of the animal's right hind paw. Rats received test medications for one hour before the production of inflammation. Oral distilled water in the amount of 10 mL/kg body weight was given to the control group. The difference in paw volume between the control and 30 min, an hour, two hours, and four hours after the administration of the inflammatory agent, inhibition, was used to measure edema:

$$\%\ of\ \ inhibition = \left(Control - Treated\right) \times 100$$
$$Control$$

Results

MTT Cell Viability Assay

Colorimetry was used to assess the Turmimax® MTT cytotoxicity research on the Murine macrophage cell line (Raw 264.7). The IC_{50} concentration was determined from the resulting dose-response curve after different dosages of Turmimax® were used to assess its toxicity. Figure 1 shows the relative Turmimax® cytotoxicity on Raw 264.7 cell lines. In Raw 264.7 cells, Turmimax® did not have any cytotoxic effects at doses of 6.25, 12.5, 25, and 50 µg/mL. Turmimax® demonstrated cytotoxicity on the Raw 264.7 cell line at a dose of 100 µg/mL.

In vitro Anti-Inflammatory effects of Turmimax®

Turmimax® significantly reduced inflammation by inhibiting IL-8 and IL-12 in LPS-stimulated macrophage cells. *In vitro* cell lines and animal models of LPS-induced inflammation serve as a typical pattern for investigating inflammation. The pro-inflammatory cytokines IL-8 and IL-12 are highly expressed in cells with increased inflammation. The current study examines the expression of the pro-inflammatory cytokines IL-8 and IL-12 to demonstrate Turmimax® capacity to have an anti-inflammatory effect on Raw 264.7 cells. Both cytokines' relative interleukin expression is relatively low in untreated cells (Fig. 2 and 3). LPS-stimulated cells alone displayed 6-7 times greater expression than the untreated group at the same time. IL-8 regulates an average of 8.86, LPS 50.49, Turmimax® 11.63, and IL-12 10.41, LPS 68.94, and Turmimax® 13.42. In LPS-stimulated macrophages, Turmimax® reduced the expression of pro-inflammatory cytokines.

In vivo Anti-Inflammatory Effect of Turmimax®

Croton Oil-Induced Ear Edema

Figure 1 displays the average ear weight and the amount of suppression of the inflammatory response, respectively, in Table 1 and Fig. 4. While the reference drug Diclofenac (10 mg/kg), which served as the positive control, significantly reduced the average ear weight in comparison to the negative control, topical application of Croton oil increased the weight of the right ear when

compared to the control left ear in the negative control group. Similar to the vehicle control group, Turmimax® at all tested doses (100 and 200 mg/kg b.w.t.) showed a significant decrease in the average ear weight.

Histamine-Induced Paw Edema

By injecting 0.1 mL of 0.1% histamine in normal saline into the subplantar tissue of the right hind paw, inflammation was elicited in rats. Rats received Turmimax® one hour before the production of inflammation. Normal saline, 10 mL/kg body weight, was given orally to the control group. Edema was measured as the difference in paw volume between the control and 0.5 h, 1, 2, and 4 h after the inflammatory agent, inhibition, was administered.

The study's usage of Turmimax® at doses of 100 and 200 mg/kg body weight resulted in a significant decrease in paw edema following histamine injection, suggesting that Turmimax® has a similar anti-edematous action as Diclofenac. According to data on these preclinical models, Turmimax® can cause an anti-inhibition, which leads to a reduction in prostaglandins. Our findings thus supported the theory that Turmimax® anti-inflammatory impact involves a reduction in prostaglandins through cyclooxygenase inhibition. The results are shown in Table 2 and Fig. 5.

Table 1: Effects of Turmimax® anti-inflammatory efficacy on croton oil-induced induced ear edema

	% Inhibition				
Treatment	0-hr	2-hr	4-hr	2-hr	4-hr
Croton oil	0.308±0.023	0.786±0.039	0.786±0.059	-	-
Standard (Diclofenac Sodium 10mg/kg p.o)	0.310±0.051	0.320±0.029**	0.326±0.019**	59.28%	58.52%
Turmimax® (100 mg/kg p.o)	0.310±0.032	0.425±0.091*	0.435±0.039*	45.92%	44.65%
Turmimax® (200 mg/kg p.o)	0.305±0.09	0.325±0.079**	0.335±0.029**	58.65%	57.25%

All values are expressed as mean ± SD; **= P<0.01, *= P<0.05 v/s croton oil control

Table 2: Effect of Turmimax® anti-inflammatory properties on rats' histamine-induced paw edema

Treatment	0-hr	0.5-hr	1-hr	2-hr	4-hr
Group I (Vehicle Control)	1.9±0.3	1.9±0.9	1.9±0.9	1.9±0.9	1.9±0.9
Group II (Negative Control) Vehicle + histamine	1.9±0.6	2.6±0.2	3.2±0.4	3.8±1.4	3.8±1.4
Group III (Diclofenac sodium10mg/kg p.o + histamine)	1.9±0.7	2.3±0.4**	2.7±0.1**	2.2±0.6**	2.2±0.2**
Group IV (Turmimax® (100mg/kg p.o) + histamine)	1.9±0.6	2.6±0.5*	3.0±1.2*	2.9±0.9*	2.8±0.7*
Group V (Turmimax® (200mg/kg p.o) + histamine)	1.9±0.8	2.1±0.6**	2.8±0.8**	2.3±0.6**	2.3±0.2**

All values are expressed as mean ± SD; ** = P<0.01, *= P<0.05 v/s histamine control

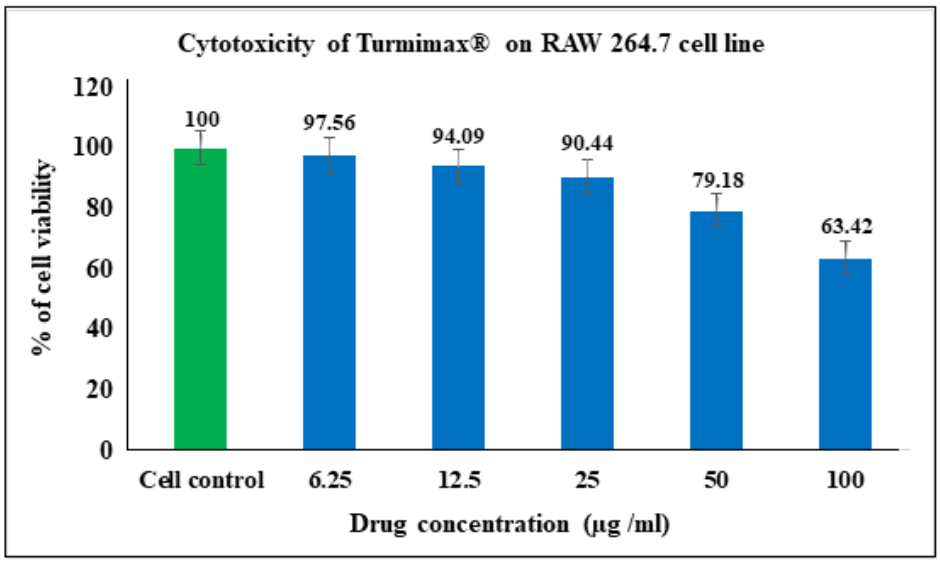

Fig. 1: The effect of Turmimax® on Raw 264.7 cell viability as determined by MTT assay with various concentrations. The data were shown as mean ± standard deviation of triplicate experiments (p<0.05)

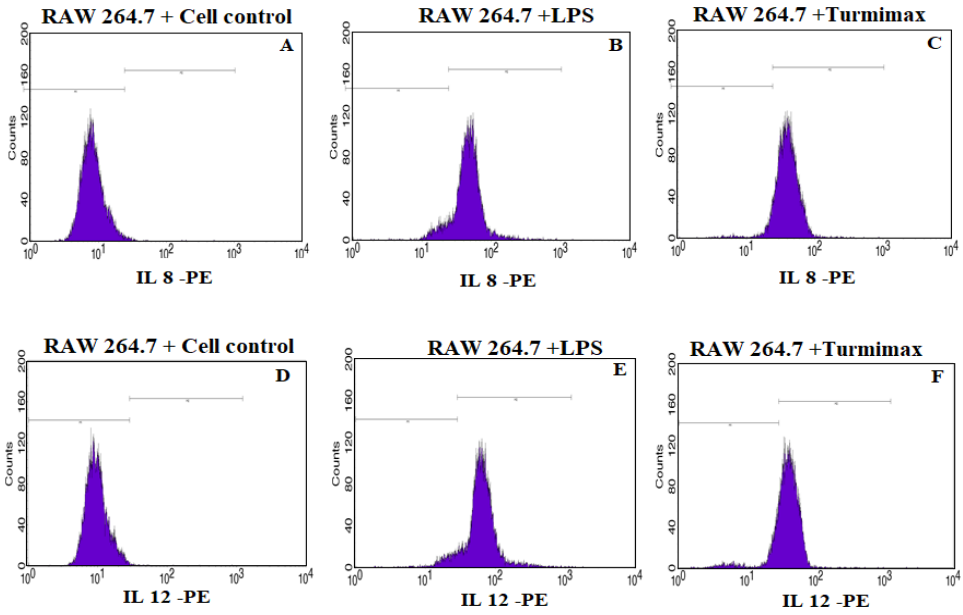

Fig. 2: Mean pro-inflammatory cytokine histograms in Raw 264.7 cells from flow cytometry pre-stimulated with LPS and then exposed to Turmimax® for 24 h. The X-axis denotes the fluorescence intensity of PE (Phycoerythrin) -Texas red and Y-axis denotes the count

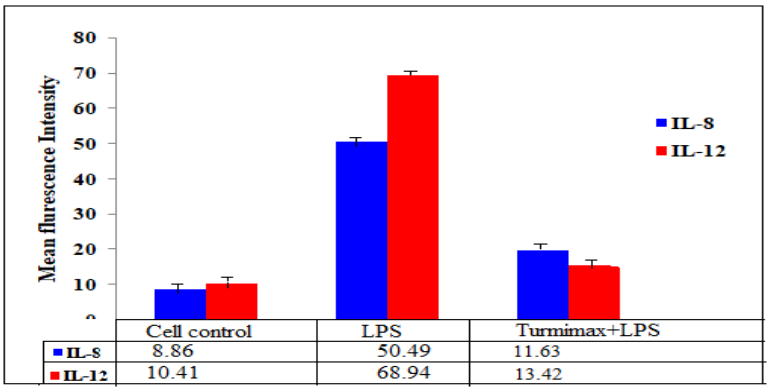

Fig. 3: IL-8 and IL-12, two pro-inflammatory cytokines, in Raw 264.7 cells pre-stimulated with LPS, then Turmimax® applied for 24 h. LPS used as a positive control (n = 3, mean ± standard error)

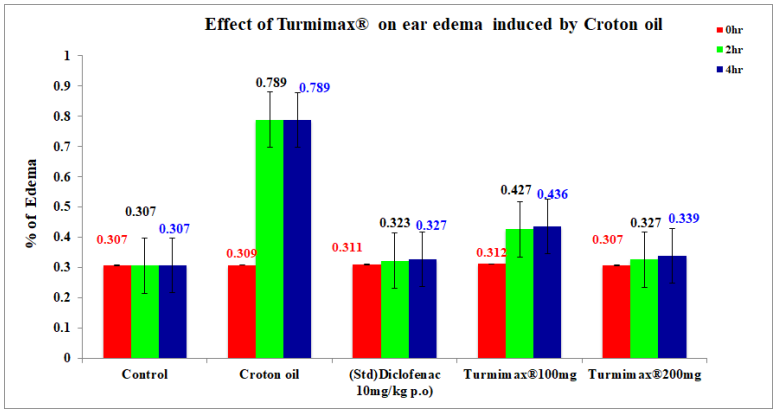

Fig. 4: Turmimax® anti-inflammatory effects on mice with Croton oil-induced ear edema

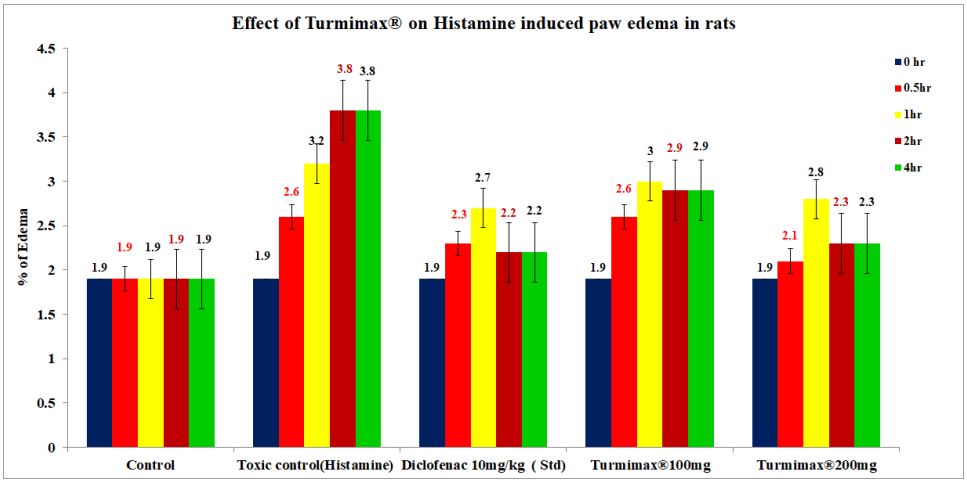

Fig. 5: Effect of Anti-inflammatory activity of Turmimax® on Histamine induced paw edema

Discussion

Turmeric has been used as an anti-inflammatory in Ayurveda for a very long time (Bagad *et al*., 2013). The primary bioactive ingredients in *C. longa* are turmerones and curcuminoids. Those have already shown promise as anti-inflammatory foods. Inflammation is typically used to describe heat, redness, swelling, and pain. Edema (swelling) is a helpful indicator of inflammation and can be used to quantify the degree to which phlogistic substances like croton oil cause skin inflammation. The major method for researching the inflammatory process of the skin and examining anti-inflammatory compounds that might be helpful in the treatment of skin problems is croton oil-induced ear edema (Winter *et al*., 1962; De Young *et al*., 1989). The predominant irritating agent in croton oil-induced ear edema in mice is 12-O-Tetracanoilphorbol-13-Acetate (TPA), along with other phorbol esters. Inducible nitric oxide synthase and cyclooxygenase 2 are two other enzymatic cascades that can be activated by the TPA (12-O-tetracanoilphorbol-13-acetate) (Aquila *et al*., 2009). This series of actions increases vascular permeability, vasodilation, migration of polymorphonuclear leukocytes, histamine and serotonin release and somewhat increases the production of inflammatory eicosanoids by the enzymes cyclooxygenase and 5-lipoxygenase (Murakawa *et al*., 2006). Curcumin's ability to reduce ear edema brought on by croton oil is likely due to lipophilic molecules that can pass through the epidermal barrier (Okoli *et al*., 2007).

In rats, histamine-induced inflammation was suppressed by Turmimax®, according to our study. An effective method for evaluating possible anti-inflammatory drugs is the paw edema assay. According to the current study, histamine exposure caused the extravasations of fluid from nearby microvessels to increase, which resulted in tissue swelling. Turmimax® ability to greatly reduce histamine-induced paw edema in rats was demonstrated by the results and this is attributable to the blockage of the H1 receptor or other signaling molecules that travel along its pathway. Vascular permeability is critical for controlling and is raised when the Histamine H1 receptor, which is expressed in endothelial cells of blood vessels, is active. Through the PI3K/Akt, PPAR-gamma, and NF-κB pathways, Turmimax® has been proven to decrease NF-κB activity. Additionally, Turmimax® modulates inflammatory reactions from the mRNA by manipulating its expression levels, which lowers the cytokine expression levels of IL-8 and IL-12 within a cell. The overall findings demonstrated that Turmimax® significantly reduced inflammation in various inflammation models.

Conclusion

In the current work, we examined the anti-inflammatory effect of Turmimax® *in vitro* using LPS-activated RAW264.7 cells and *in vivo* utilizing several animal models. We demonstrate that Turmimax® therapy reduces the inflammatory response as shown by a reduction in croton oil- and histamine-induced paw and ear edema. On croton oil and histamine-induced ear and paw edema, Turmimax® demonstrated maximum suppression of edema comparable to the common medication Diclofenac (10 mg/kg). *In vitro*, LPS-activated RAW264.7 cells produced IL-8 and IL-12 at much lower levels after taking Turmimax®. These data suggest that Turmimax® can reduce inflammation, supporting its usage in conventional medicine.

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Star Hi Herbs Pvt Ltd, Bangalore, Karnataka, India.

Author's Contributions

All authors equally contributed to this study.

Ethics

The final draught of this manuscript was read and approved by all authors. There are no ethical concerns to declare that might surface once this study is published.

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