

Original Article

IN VITRO ANTI-INFLAMMATORY AND HEPATOPROTECTIVE ACTIVITY OF TURMESAC®

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ABSTRACT

Objective: In this study, we investigated the hepatoprotective activity of Turmesac® on Human liver cells (HepG2 cell line) and anti-inflammatory effect on Murine macrophages (Raw 264.7 cell line) by flow Cytometry.

Methods: Cell viability of HepG2 and Raw 264.7 cells determined by the MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assay to identify a non-cytotoxic concentration of Turmesac® for the respective cell lines after 24 h exposure period. Further hepatoprotective effect of Turmesac® was performed in H₂O₂ treated liver cells using H₂DCF-DA staining by flow cytometry. The anti-inflammatory potency of Turmesac® was evaluated in Lipopolysaccharide (LPS 2µg/ml) stimulated Murine Raw 264.7 macrophages by measuring the relative fluorescence intensity of 2 cytokines, Interleukin-8(IL-8) and (Interleukin-12) IL-12 by flow cytometric analysis.

Results: Turmesac® concentrations of less than 50µg/ml did not show significant cytotoxicity on both HepG2 and Raw 264.7, cell lines following the treatment period of 24 h and selected 50µg/ml as the optimum concentration for hepatoprotective and anti-inflammatory models. The reactive oxygen species (ROS) study revealed that Turmesac® (50µg/ml) effectively suppressed the H₂DCF-DA expression in HepG2 cells. Secondly, Turmesac® significantly suppressed the anti-inflammatory cytokine expressions of IL-8 and IL-12 in LPS pre-stimulated cells categorising as a potentially potent anti-inflammatory drug. The mean fluorescence intensity percentage of IL-8 is control 8.86, LPS 50.49, Turmesac® 19.63 and IL12 is control 10.41, LPS 68.94, and Turmesac® 15.79 respectively.

Conclusion: This study highlighted that Turmesac® could be considered as a promising hepatoprotective and anti-inflammatory compound and a therapeutic agent in curing liver-related and inflammation-related diseases.

Keywords: Turmesac®, HepG2, Raw 264.7, MTT, Flow cytometry, IL-8, IL-12, Hepatoprotective, H2DCFDA and anti-inflammatory

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INTRODUCTION

Traditional medicine can be traced back centuries, with some of these still being used for treatments and have become the current focus of research in treating the various diseases and ailments seen today [1-2]. Turmeric is a spice that comes from the root *Curcuma longa*, a member of the ginger family Zingiberaceae. Curcumin has a long history of administration in the traditional medicine of India, China and Iran, and it has been used in different people for the treatment of many diseases such as diabetes, liver disease, rheumatoid diseases, atherosclerosis, infectious diseases and cancers [3]. In the ancient Indian medicine, Ayurveda, a topical agent made of turmeric paste has been used to treat common ocular infections and inflammations; it has also been used in wound dressing in conditions such as bites, burns, and some other skin diseases [4]. Curcuminoids are components of turmeric, which include mainly curcumin (diferuloylmethane), demethoxycurcumin, and bisdemethoxycurcumin. Curcumin, a polyphenol, has been shown to target multiple signaling molecules/pathways while also demonstrating activity at the cellular level, which has helped to support its multiple health benefits [5], metabolic syndromes [6], and to help in the management of inflammatory and degenerative eye conditions [7, 8]. In addition, it has been shown to benefit the kidneys [9]. While there appears to be countless therapeutic benefits to curcumin supplementation, most of these benefits are due to its antioxidant and anti-inflammatory effects [5, 10]. It has shown very strong antioxidant and anti-inflammatory properties that has vast research coverage in various human and animal cell lines, models and trials. Inflammation and oxidative activity are usually triggered during a stressful event or an imbalance in these systems. Reactive oxygen species (ROS) are a by-product of normal cellular respiration and is mainly involved in cellular damage. Previous *in vitro*, animal models and clinical trials have shown that curcumin is a potent nonsteroidal anti-inflammatory agent whose efficacy is as powerful

as known steroidal and nonsteroidal (indomethacin and phenylbutazone) agents [11, 12]. Curcumin has been shown to inhibit macrophage-derived cytokines like IL-8, monocyte inflammatory protein-1 and tumor necrosis factor α [13]. Therefore, the aim of this study, we investigated the hepatoprotective activity of Turmesac® on Human liver cells (HepG2) and anti-inflammatory effect on Murine macrophages (Raw 264.7) by flow cytometry.

MATERIALS AND METHODS

Preparation of sample

Turmesac® is manufactured and registered by Star Hi Herbs Pvt. Ltd, Jigani, Bangalore, Karnataka, India

Cell lines and chemicals

The Raw 264.7 Murine macrophage cell line and HepG2 Human hepatocellular carcinoma cell lines were procured from National Centre for Cell Science, NCCS, Pune, Maharashtra, India. DMEM with high glucose (Cat No. AL111), FBS (Cat No. RM10432), Antibiotic-Antimycotic solution (Cat No. A002) were purchased from Hi-Media laboratories, Mumbai, India. Lipopolysacchride (LPS) and Hydrogen Peroxide (H₂O₂) were of molecular biology grade procured from Sigma Aldrich, USA. H2DCFDA (D-399) and MTT reagent (M6494) purchased from Thermo Fisher Scientific (Gibco, USA). Flow Cytometry oriented reagents viz., PE Mouse Anti-Human IL-8 antibody (Cat No.554720) and PE Mouse Anti-Human IL-12 antibody (Cat No. 559329) were procured from the BD Biosciences, USA, CA.

Cell culture and treatment

Raw 264.7 and HepG2 cell lines grown in DMEM high glucose medium supplemented with 10% FBS, 100 µl of Antibiotic-Antimycotic (100X) in a CO₂ incubator at 37 °C with 20% oxygen and 5% CO₂ in saturated humidity. Post 24hr seeding event, the medium was changed prior to

treatment with Turmesac® and H₂O₂ or LPS. Turmesac® and H₂O₂/LPS were dissolved in DMSO and vortexed to ensure 100% solubility. The final concentration of DMSO added to the cell culture medium was below 0.1%. The final Turmesac® concentrations added to the cell culture medium ranging from 6.25-100µg/ml, respectively. A 0.1% DMSO concentration was used as a Vehicle control, which does not exhibit any cytotoxicity on both the cells.

MTT assay

An MTT assay was performed to evaluate the HepG2 and Raw 264.7 cell viability. 20,000 cells per well were seeded in 96 well plate and treated with 6.25, 12.5, 25, 50 and 100 µg/ml of Turmesac® for 24 h to determine the optimum concentration for further studies. Post incubation period, remove the spent media and add 100 µl of 0.5 mg/ml MTT reagent for 4 h at 37 °C, the formazan crystals generated due to the reduction of MTT by metabolically active cells. They were released from the cells and dissolved using 100 µl DMSO (Sigma-Aldrich, USA). In MTT assay, formazan accumulation directly reflects the mitochondrial activity in the live cells, which is an indirect measurement for the cell viability [14]. The plate was agitated on a gyratory shaker for 10-20 min and the absorbance was measured at 570 nm with an ELISA microplate reader (Biotek, USA). The percentage of cell viability is calculated using the below formula:

$$\% \text{ of viable cells} = \frac{\text{Absorbance sample} - \text{Absorbance blank}}{\text{Absorbance control} - \text{Absorbance blank}} \times 100$$

Hepatoprotective activity

Hepatoprotective effect of Turmesac® on HepG2 cells was evaluated by ROS activity through flow cytometric analysis. Cell-permeant 2, 7-dichlorodihydrofluorescein diacetate (H₂DCF-DA) is a widely used ROS indicator. The reduced non-fluorescent fluorescein H₂DCF-DA can be oxidized and converted into fluorescent 2, 7-dichlorofluorescein (DCF) by intracellular ROS. HepG2 cells were seeded at a density of 0.5 x 10⁶ cells/1 ml of medium per well in a 6-well plate (Biolite-Thermo) and incubated at 37 °C in a humidified incubator for 24 h. The medium was removed and washed with 1 x

PBS. Cells were pre-treated with H₂O₂ (200 µM) for 3 h to induce stress on cells and further treated with Turmesac® with the concentration of 50µg/ml for test group by leaving controls and incubated for 24 h at 37 °C. After the incubation period, collect the supernatant and cells into sterile tubes and centrifuged at 300 × g for 5 min at room temperature. The pellets were washed twice with 1 x PBS and cells were stained with 10µM of H₂DCF-DA and incubate for 30 min at 37 °C in the dark. Further cells are centrifuged at 300 × g and resuspend the pellet with 400 µl of DPBS. Fluorescence of H₂DCF-DA was measured by flow cytometry at a laser excitation of 485/535 nm using Cell Quest Pro software version 6.

Anti-inflammatory activity

For the evaluation of anti-inflammatory activity, Raw 264.7 cells were cultured in a 6 well plate and treated with 3 different culture conditions viz., LPS (2µg/ml), LPS (2µg/ml)+Turmesac® (50µg/ml) and untreated without any treatment. Briefly, the cells were pre stimulated with 2 µg/ml of LPS for 3 h to induce inflammation and following stimulation, the cells were either treated with 50µg/ml of Turmesac® or LPS stimulated alone (negative control) with 2 ml DMEM medium. Cells were incubated for 24 h and harvested into centrifuge tubes (BD Biosciences) and centrifuged at 300 × g for five minutes in a Remi: R-8 °C centrifuge and were washed twice with D-PBS. The pelleted cells were incubated at room temperature with 0.5 ml BD Cytofix/Cytoperm for 10 min and washed with 0.5% Bovine Serum Albumin solution (1x PBS and 0.1% sodium azide). Cells were incubated with 20µl of PE-Mouse Anti Human interleukin 8 (IL-8) antibody/Anti Human IL-12 separately for 30 min in the dark at 25 °C and expression measured using a BD FACS Calibur flow cytometer (BD Biosciences) and data analyzed by Cell Quest Pro software version 6.

Statistical analysis

All the data were analyzed using Microsoft Excel 2007 version in creating graphical representation of the mean with calculated standard errors. Flow Cytometric data was analyzed using Cell Quest Pro software version 6.

Table 1: % of cell viability values turmesac® towards HepG2 and Raw 264.7 cells at 24 h as determined by MTT assay

Cell line/Concentration	0	6.25	12.5	25	50	100
HepG2	100	87.32246	91.21515	94.79221	98.79011	99.36875
Raw 264.7	100	97.56543	94.09617	90.44431	79.18442	63.42057

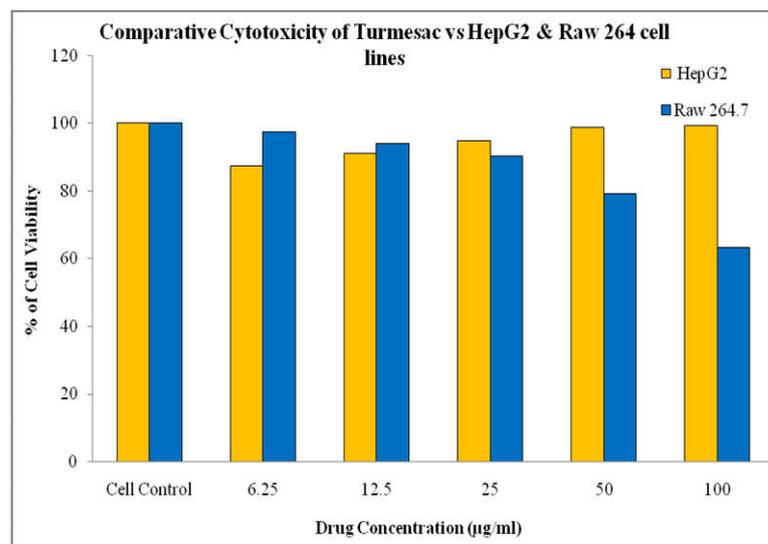


Fig. 1: The percentage viability of HepG2 and raw 264.7 cells treated with various concentrations of turmesac® for 24 h as determined by MTT assay

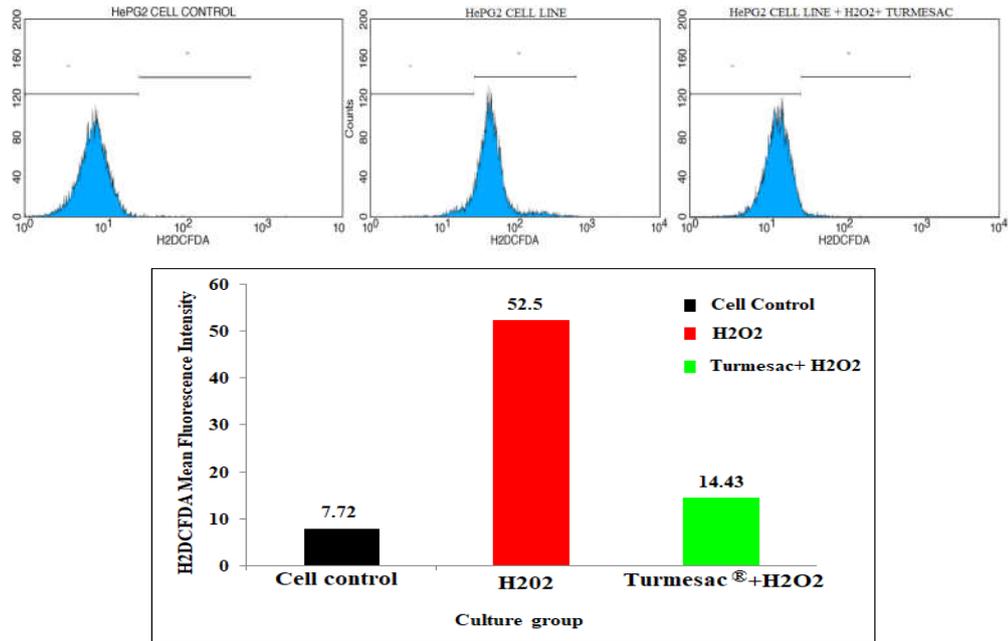


Fig. 2: Representative flow cytometry histograms of total intracellular ROS levels (H₂DCF-DA) in HepG2 cells prestimulated with H₂O₂ (24 h) and treated with Turmesac® for 24 h. H₂O₂ served as a positive control (n=3, mean±standard error)

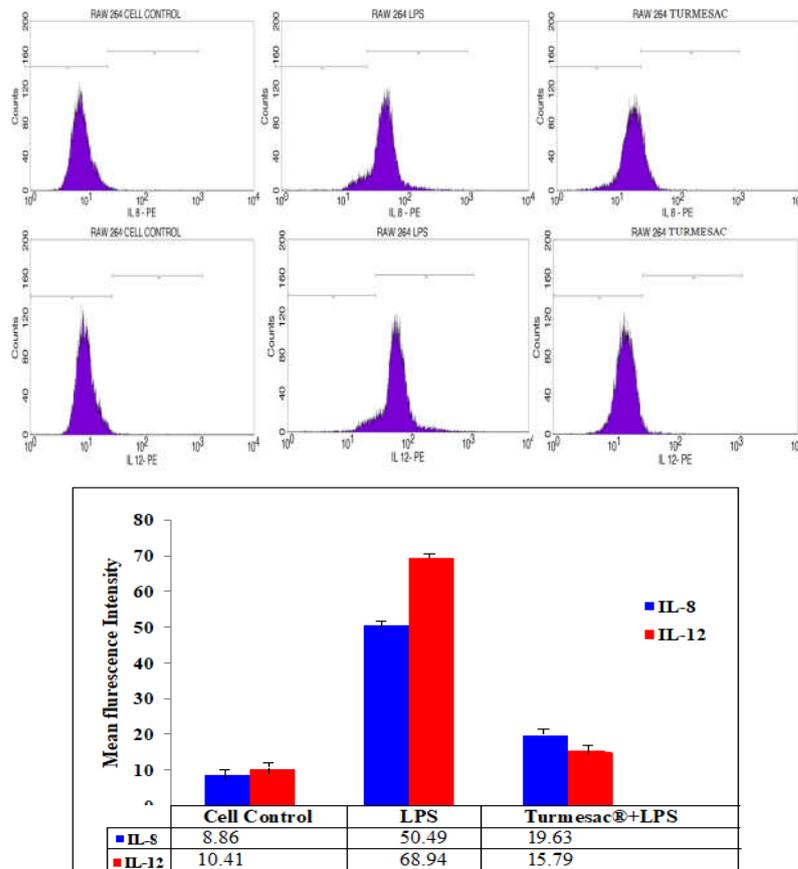


Fig. 3: Flow cytometry histograms of mean pro-inflammatory cytokines (IL-8 and IL-12) in Raw 264.7 cells pre-stimulated with LPS followed by 24 h exposure to Turmesac®. LPS served as a positive control (n=3, mean±standard error)

RESULTS AND DISCUSSION

MTT cell viability assay

The MTT cytotoxicity study of Turmesac® on human liver and murine cells lines was evaluated by colorimetry. Various concentrations were used to evaluate the toxicity of Turmesac® and the IC₅₀ concentration was calculated from the resultant dose-response curve. The relative Turmesac® cytotoxicity on HepG2 and Raw 264.7 cell lines are depicted in table 1. Turmesac® exhibited no cytotoxic effect on both HepG2 and Raw 264.7 cells at concentrations of and below 50µg/ml. On Raw 264.7 cell line, Turmesac® exhibited cytotoxicity at a concentration of 100µg/ml. Therefore, the non-cytotoxic concentration of 50µg/ml was selected for both cell lines for further mechanistic studies.

Hepatoprotective activity by reactive oxygen species (ROS)

Flow cytometry analysis using H₂DCFDA-based staining study of ROS level in HepG2 cells revealed that H₂O₂ caused oxidative stress in HepG2 cells by inducing production of high levels of ROS. The generation of ROS within cells plays a major role in apoptosis. Hence, we evaluated the Turmesac® dependent intracellular ROS generation in HepG2 cells. The relative mean DCF fluorescence intensity value was very high in the H₂O₂ alone treated cells (52.5) due to heavy cell damage/apoptosis. However, post H₂O₂ exposure followed by Turmesac® treatment exhibited lower mean DCF fluorescence intensity (14.43). The Turmesac® treated cells' observed mean DCF fluorescence intensity value was more than the control cells (7.72) and lower than the H₂O₂ treated cells (Fig.1 and 2). This suggests that Turmesac® having negligible influence in generating intracellular ROS but rather suppresses the toxic potency of H₂O₂. This finding indicates that Turmesac® might exert its protective potency via a ROS-independent mechanism in HepG2 cells [15]. Showed that L-6 myoblast cells treated with 4 µM curcumin completely suppressed oxidation of H₂DCF-DA. They had mentioned that, this indicates curcumin can easily diffuse through cellular membranes to elicit its effects by acting as a strong radical scavenging agent in the polar intracellular environment [15]. Nitric oxide production which is another contributor to oxidative stress [13, 16], can combine with superoxide to create a more damaging oxidant, peroxynitrate [17]. *In vitro* studies have shown that curcumin also strongly inhibits nitric oxidative stress [13, 16], can combine. Furthermore, a study by [18] showed turmeric had the highest scavenging activity when compared to other natural antioxidants, ginger, white saffron and temulawak. While curcumin alone had the second highest activity. We have previously shown that Turmesac® exhibited hepatoprotective properties in MCF-7 cells at a concentration of 235.04 µg/ml [19].

Anti-inflammatory activity

Turmesac® exhibits significant anti-inflammatory effect through IL-8 and IL-12 inhibition in LPS stimulated macrophage cells. LPS-induced inflammation, in *in vitro* cell lines and in animals, represents a standard paradigm for studying inflammation. IL-8 and IL-12 are the pro-inflammatory cytokines that are expressed drastically in cells exhibit more inflammation. The current study shows, the ability of Turmesac® to elicit anti-inflammatory effects on RAW 264.7 cells by evaluating pro-inflammatory cytokines, IL-8 and IL-12, expression. In the untreated cells, the relative interleukin expression is very low for both cytokines (fig. 3). On the other hand, LPS stimulated cells alone, exhibited 5-6 times higher expression than the untreated group. However, the Turmesac® treated cells following LPS stimulation were expressing lower expressions than the LPS alone treated cells. The mean fluorescence intensity percentage of IL-8 control 8.86, LPS 50.49, Turmesac® 19.63 and IL10 control 10.41, LPS 68.94 and Turmesac® 15.79. *In vitro* studies have shown that curcumin inhibits inflammation in mouse fibroblast cell line [13, 16]. Turmesac® showed a substantial inhibitory effect on pro-inflammatory cytokine production, IL-8 and IL-12. Curcumin has been shown to inhibit macrophage-derived cytokines like IL-8, monocyte inflammatory protein-1 and tumor necrosis factor α [13]. At a concentration of 20 µM, curcumin showed pro-inflammatory cytokine inhibition of IL-8 in adult peripheral mononuclear

monocyte cells and preterm lung inflammatory cells and IL-12, thereby preventing allergic encephalomyelitis in T-lymphocytes [12]. Keratinocytes treated with 6.7 µg/ml curcumin showed attenuated levels of IL-8 and the researchers believed that it could be used as a short-term treatment in type 2 diabetic nephropathy [5]. A study by [16] showed in LPS stimulated mouse splenocytes there was potent inhibitory release of IL-12 when treated with 0.8 to 100 µg/ml of curcumin extract. Human phase one trial has shown patients and animal models administered with a daily curcumin intake between 1125-8000 mg/day showed no cytotoxic effect and exhibited anti-inflammatory effects through various target molecules including IL-12 [2]. Thus Turmesac® inhibited the expression of pro-inflammatory cytokines in LPS stimulated macrophages.

CONCLUSION

In conclusion, Turmesac® possesses biological actions in cell proliferation, suppression of ROS expression in H₂O₂ treated HepG2 cells and inhibiting the expression of pro-inflammatory cytokines in LPS stimulated murine macrophages. This study's results significantly indicated that Turmesac® is a potent and promising natural compound for the treatment of liver and inflammatory related diseases. The current work innovatively illustrated the hepatoprotective and anti-inflammatory expression studies through flow cytometric analyses.

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AUTHORS CONTRIBUTIONS

All the authors have contributed for preparation and editing of the manuscript.

CONFLICTS OF INTERESTS

The authors declare no conflicts of Interest

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