

## STANDARDIZED SUPERCRITICAL CO<sub>2</sub> EXTRACT OF *ACANTHUS ILICIFOLIUS* (LINN.) LEAVES INHIBITS THE PRO-INFLAMMATORY CYTOKINE TUMOR NECROSIS FACTOR- $\alpha$ IN LIPOPOLYSACCHARIDE-ACTIVATED MURINE RAW 264.7 MACROPHAGE CELLS

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### ABSTRACT

**Objective:** *Acanthus ilicifolius* Linn. (Acanthaceae) is a medicinal mangrove plant used in the treatment of inflammation. Previous phytochemical studies have identified 2-benzoxazolinone (BOA) from the leaves of *A. ilicifolius*. In the present study, we attempted to standardize the supercritical CO<sub>2</sub> leaf extract of *A. ilicifolius* (SCFE-AI) for BOA content and investigate the tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) inhibitory effect of SCFE-AI and BOA on the lipopolysaccharide (LPS)-induced inflammation in RAW 264.7 macrophages. The acute oral toxicity of SCFE-AI and BOA was also established.

**Methods:** SCFE-AI was standardized for BOA content using high-performance thin-layer chromatography (HPTLC) method. The cytotoxicity of SCFE-AI and BOA was evaluated using MTS colorimetric method. The *in vitro* anti-inflammatory effect of SCFE-AI and BOA on TNF- $\alpha$  production in LPS-activated RAW 264.7 cells was quantified using ELISA method. Acute oral toxicity studies were performed following the Organization for Economic Co-operation and Development test guideline No. 423.

**Results:** The amount of BOA was found 0.8% w/w of SCFE-AI. The RAW 264.7 cell viability was unaffected by SCFE-AI and BOA treatments within a concentration range <1000 mg/ml after 24 h incubation. SCFE-AI decreased the production of TNF- $\alpha$  in a dose-dependent manner compared to BOA. The LD<sub>50</sub> value for SCFE-AI was found to be >2000 mg/kg and ranges from 300 to 2000 mg/kg with BOA.

**Conclusion:** The HPTLC chromatogram could serve as an analytical tool for authentication and quantification of BOA content. The anti-inflammatory mechanism of *A. ilicifolius* might be through the inhibition of TNF- $\alpha$  production.

**Keywords:** *Acanthus ilicifolius*, 2-benzoxazolinone, Pro-inflammatory cytokine, Tumor necrosis factor- $\alpha$ , Anti-inflammatory, RAW 264.7 macrophage.

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### INTRODUCTION

*Acanthus ilicifolius* Linn. (Acanthaceae) is a perennial medicinal mangrove plant, commonly known as holy leaved mangrove [1]. The traditional use, biological activities, and chemical constituents of *A. ilicifolius* were reviewed [2]. This plant is employed in the preparation of an Ayurvedic medicine named Sahachara, prescribed in the treatment of rheumatic complaints [1]. It is found to possess anti-inflammatory and analgesic effects [3,4]. The leaves have been previously reported to contain 2-benzoxazolinone (BOA), a major bioactive phytochemical constituent [5,6]. Plant extracts standardized with the biologically active molecules are widely used as medicines and have tremendous commercial value than crude extracts or powders. In recent years, plant extracts obtained using supercritical CO<sub>2</sub> extraction technique has been widely accepted since it employs extraction of bioactive molecules at low temperature, allowing the thermolabile substance to be extracted without destruction, and results in the production of high-quality extracts that are free toxic chemical solvents. Earlier reports indicated that BOA and its structurally related analogs [7-9] were present in this species and the synthetic derivatives of BOA were claimed for their anti-inflammatory and antinociceptive effects [10-12]. BOA is also toxic allelochemical produced in plants to protect them against herbivores [13]. Although herbal medicines are considered to be efficacious in treating various ailments, their safety continues to be a major concern in the safe use of these medicines in humans. Unlike synthetic medicines, herbal medicines have to be tested for their safety, efficacy, and quality before being marketed in the market [14]. Acute oral toxicity studies in laboratory animals could provide the preliminary information on the toxic nature of a compound. It also allows determining the defined dose exposure ranges where the lethality of the animal is to be expected [15].

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a major pro-inflammatory cytokine that influences various cell signaling and metabolic pathways. TNF- $\alpha$  inhibitors are emerging drug targets in the treatment of a wide range of inflammatory and neurodegenerative disorders [16]. Our research laboratory is focused on searching for anti-inflammatory compounds from mangroves. In earlier *in silico* studies, we predicted the dual inhibitory effect of BOA and its selected derivatives on COX-2/5-LOX active sites [17]. Several studies focus on whole plant extract and single compounds isolated from plants as potential anti-inflammatory drugs, particularly with regard to cytokine suppression [18]. Although the anti-inflammatory activities of the *A. ilicifolius* leaf extract were demonstrated *in vivo* [3], the molecular mechanisms responsible for the anti-inflammatory properties of the extract remain unknown. In the present study, for the first time, we attempted to standardize the supercritical CO<sub>2</sub> extract of *A. ilicifolius* leaves (SCFE-AI) for BOA content by employing high-performance thin-layer chromatography (HPTLC) method and the acute oral safety was established the following Organization for Economic Co-operation and Development (OECD) test guideline 423 (acute toxic class method). In addition, the preliminary *in vitro* anti-inflammatory effects of *A. ilicifolius* and BOA in terms of inhibition of TNF- $\alpha$  production were investigated in lipopolysaccharide (LPS)-activated murine RAW 264.7 macrophage cells.

### METHODS

#### Chemicals and reagents

Analytical grade toluene, hexane, ethyl acetate, and formic acid were obtained from Sisco Research Laboratories Pvt., Ltd., India. BOA (>98.0% purity) was obtained from TCI Chemicals (India) Pvt., Ltd. Pre-coated silica gel 60 F<sub>254</sub> on aluminum plates (Merck KGaA, Darmstadt,

Germany) was obtained commercially from local suppliers. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, phosphate-buffered saline (pH 7.4), and other tissue culture reagents were purchased from Gibco BRL, Grand Island, USA. LPS and indomethacin were purchased from Sigma-Aldrich, St. Louis, USA. CellTiter 96 Aqueous One Solution cell proliferation assay kit (MTS; colorimetric) was purchased from Promega, Madison, USA. TNF- $\alpha$  ELISA kit was purchased from eBioscience, San Diego, USA.

#### Collection of plants and supercritical fluid extraction (SCFE)

The fresh leaves of *A. ilicifolius* were collected during September 2012 from Pichavaram Mangrove forest, Tamil Nadu, India. After cleaning, the leaves were shade dried and were made into a coarse powder using a pulverizer. 5 kg of dried leaf powder of *A. ilicifolius* was extracted by SCFE under optimized CO<sub>2</sub> conditions.

#### HPTLC analysis of BOA in SCFE-AI

##### Sample preparation

Stock standard solutions of SCFE-AI (50 mg/ml) and BOA (500  $\mu$ g/ml) were dissolved in methanol, sonicated until they get dissolved, and filtered using Whatman filter paper. Working standards were prepared by serial dilutions of BOA and were made with methanol to achieve the desired concentration ranging from 1, 2, 3, 4, to 5  $\mu$ g/5 $\mu$ l. The resulting solution was used for HPTLC analysis.

#### Experimental chromatographic conditions

The following HPTLC chromatographic conditions were maintained during the experiment. Stationary phase: Aluminum pre-coated plates with silica gel F<sub>254</sub>; mobile phase: toluene:ethyl acetate:formic acid (6:5:0.75 v/v; 15ml); developing chamber: Twin trough glass chamber (20 $\times$ 10); developing mode: Ascending mode; chamber saturation time: 1h; instrument: HPTLC version 1.3.4 (CAMAG, Switzerland); sample applicator: Linomat 5 equipped with a 100  $\mu$ l syringe; scanner: TLC scanner 3; scanning wavelength: 254 nm and 366 nm; photodocumentation chamber: Reprostar 3; and environmental conditions: Room temperature and relative humidity (30–70%).

#### HPTLC method

5  $\mu$ l of BOA standard solution (1–5  $\mu$ g; track 1–5) and SCFE-AI (4–8  $\mu$ l; track 6–8) was applied in a 5 mm wide band on pre-coated HPTLC plate using the sample applicator in a space of 5 mm. The plate spots were dried at a temperature (60–65°C) using hot air oven for ~1–2min. The developing chambers were pre-saturated with mobile phase vapor ½ h before the development of plates. The plates were developed in an ascending mode until the mobile phase has reached the premarked solvent front (8 cm) from the point of the sample application (1 cm). The plates were dried in the similar fashion. The eluted bands were scanned in remission-absorbance mode at 254/366 nm using TLC scanner and were photo documented. The R<sub>f</sub> values and peak areas were calculated, and the calibration curve of BOA and linear regression (concentration vs. area) was calculated using CAMAG winCATS planar chromatography manager software (version 1.4.3).

#### In vitro experiments

##### Cell culture

RAW 264.7 murine macrophage was purchased from American Type Culture Collection. They were cultured in DMEM supplemented with 10% FBS containing 100 U/ml of penicillin and 100  $\mu$ g/ml of streptomycin at 37°C in a 5% CO<sub>2</sub> humidified incubator.

##### MTS assay for cell viability

Cell viability was assessed by MTS assay [19] using CellTiter 96 Aqueous One Solution cell proliferation assay kit. Briefly, the cells were plated at a density 1 $\times$ 10<sup>4</sup> in 96-well plates and incubated at 37°C for 24 h. Stock solutions of SCFE-AI and BOA (100 mg/ml) were made using dimethyl sulfoxide (DMSO) and further diluted with DMSO to achieve the desired working concentration. The cells were treated with derived concentrations of SCFE-AI, BOA (1000, 100, 10, 0.1, and 0.01  $\mu$ g/ml), and vehicle. Final DMSO concentrations in the cells were  $\leq$ 1%. After 24, 48,

and 72 h of incubation at 37°C, 20  $\mu$ l of the MTS solution was added to each well and incubated under the same conditions for another 2 h. The absorbance was measured using a microplate reader at the wavelength of 490 nm. The percentage viability was calculated by comparing the optical density (OD) of the treated cells with vehicle treated cells using the formula: % Cell viability = [(OD<sub>490</sub> [test]/OD<sub>490</sub> [vehicle]) $\times$ 100]. The inhibitory concentration required for 25% (IC<sub>25</sub>), 50% (IC<sub>50</sub>), and 75% (IC<sub>75</sub>) cell death was calculated. Each experiment was done in triplicate.

#### TNF- $\alpha$ assay

The RAW 264.7 cells were treated with the concentration of SCFE-AI and BOA corresponding to IC<sub>25</sub>, IC<sub>50</sub>, and IC<sub>75</sub> as determined from cell viability assays. Different incubation period did not have any effect on cell viability/cytotoxicity, so 24 h of incubation was selected for TNF- $\alpha$  assay. RAW 264.7 macrophages were cultured in 24-well plates with different concentrations of SCFE-AI and BOA for 1 h and then incubated with 1  $\mu$ g/ml LPS for 24 h. The control cells were treated similarly without exposure to test compounds. Supernatants were obtained and frozen at 80°C until analysis. TNF- $\alpha$  in the culture medium was determined by ELISA kit according to the manufacturer's recommendations. TNF- $\alpha$  was measured in triplicates, and the ELISA plates were read using a microplate reader (Infinite M200, Tecan, USA). Indomethacin (1  $\mu$ M) was used as positive control. The ability of SCFE-AI and BOA to inhibit TNF- $\alpha$  production was calculated relative to the TNF- $\alpha$  production after vehicle treatment.

#### In vivo experiments

##### Experimental animals

Healthy Swiss albino male mice weighing 25–35 g (age: 8–10 weeks) were obtained from the Central Animal House, Raja Sir Muthiah Medical College and Hospital, Faculty of Medicine, Annamalai University, Annamalai Nagar, Tamil Nadu, India. All mice were housed in polypropylene cages and maintained at 12-h light/dark cycle at room temperature (21 $\pm$ 2°C) with relative humidity 30–70%. Animals were allowed to acclimatize to laboratory conditions for a week before starting the experiment. Portable drinking water and pelleted rodent feed (Sri Venkateswara Enterprises, Bengaluru, India) were provided *ad libitum* throughout the experiment, except for the short fasting period where the drinking water was still in free access, but no food supply was provided 4 h before treatment. The animals were housed, cared, and experimented in accordance with the Central Animal House guidelines and the guidelines laid by the Committee for the Purpose of Control and Supervision of Animals (CPCSEA), Government of India [20]. The Institutional Animal Ethical Committee of Annamalai University approved the study (Reg.No.160/1999/CPCSEA; Proposal No. 985 dated 07.02.2013).

#### Acute oral toxicity

The acute oral toxic effects of SCFE-AI and BOA were evaluated using mice in accordance with the procedures outlined in the OECD test guideline 423 [15]. The SCFE-AI and BOA were suspended in a vehicle (1% v/v Tween 80) individually. Following 4 h fasting period, the recent body weight was used to calculate the dose to be administered. A single oral dose of SCFE-AI (2000 mg/kg) or BOA (300 mg/kg) was administered to the fasted mice (n=3) using an oral feeding needle and control groups (n=3) received 1% v/v Tween 80 (10 ml/kg). The mice were observed for mortality, any indications of clinical signs of toxicity [21] during the first 4 h and 24 h of oral exposure, and thereafter observed for mortality for 14 days. Water and feed were provided to the mice approximately 4 h after treatment. Weekly body weight changes were recorded. The flow chart of Annex 2d of the OECD test guideline 423 describes the sequential testing procedure with three animals based on the mortality of animals during the experiment. The LD<sub>50</sub> range was calculated based on the mortality of animals as shown in Annex 2d of the OECD test guideline 423 [15].

#### RESULTS

Till date, the biological studies reported on *A. ilicifolius* were on the use of crude extracts obtained using toxic solvents. Extraction in supercritical

conditions using CO<sub>2</sub> constitutes a useful technique of isolating elements from plant materials. Hence, we optimized the conditions of SCFE method for the extraction of 2-BOA from *A. ilicifolius*. The optimal extraction conditions were determined as follows: Extraction pressure 350 g bar pressure, extraction temperature 45°C, CO<sub>2</sub> flow rate 3 L/h, extraction time 3h, the total volume of CO<sub>2</sub> consumed 7 kg, and the average yield of SCFE-AI was 0.5% w/w. An HPTLC fingerprint using hexane:ethyl acetate:formic acid (7:3:0.2 v/v) was developed (data not shown) initially, but the band separation was not satisfactory. Best separation of active constituents of SCFE-AI was achieved on HPTLC plates and pre-coated with silica gel 60F<sub>254</sub>, eluted with toluene:ethyl acetate:formic Acid (6:5:0.75 v/v) solvent system. HPTLC fingerprinting of SCFE-AI revealed major thirteen peaks with R<sub>f</sub> values in the range of 0.18–0.97 (Fig. 1). The amount of BOA in SCFE-AI could be achieved by constructing a linear graph using various concentrations of BOA. Using this solvent system, a compact band for BOA (R<sub>f</sub>=0.66) was obtained (Fig. 2).

The linear regression data for the BOA calibration plots (Fig. 3) showed a good linear relationship in the concentration range of 1–5 µg. The linear relationship was described by the equation:  $Y=2053.388+1713.213X$  (with  $r>0.98410$ ;  $SD=7.85\%$ ) where Y is the area under the curve and X is the amount of BOA. The amount of BOA content in SCFE-AI was 0.8%w/w.

The MTS assay was performed to determine the non-cytotoxic dose of SCFE-AI and BOA in RAW 264.7 macrophage cells. The RAW 264.7 macrophage cells were exposed to varying concentrations of SCFE-AI and BOA. After 24, 48, and 72 h incubation, the cells were harvested and cell viability was estimated using the MTS assay. The MTS results of SCFE-AI and BOA obtained at different time points are shown in Fig. 4. Analyzing the Fig. 4, it can be seen that the 100 µg/ml concentration of SCFE-AI and BOA did not affect the cell viability at all the time intervals. Similarly, a decrease in the cell viability was found with 1000 µg/ml for both the treatments. From the MTS results, the concentration (<1000 µg/ml) of BOA and SCFE-AI was selected for further studies.

RAW 264.7 cells were incubated with LPS (1 µg/ml) in the presence of SCFE-AI (100, 400, and 700 µg/ml) and BOA (50, 250, and

600 µg/ml) with IC<sub>25</sub>, IC<sub>50</sub>, and IC<sub>75</sub> concentrations for 24 h. From the Fig. 5, it is evident that SCFE-AI decreased the production of TNF-α comparatively better than BOA. At the highest concentration tested, SCFE-AI (820 µg/ml)-treated cells could decrease (~2.2 fold) the TNF-α production compared to BOA (1820 µg/ml)-treated cells and LPS-treated cells (2320 µg/ml).

Earlier reports on *A. ilicifolius* revealed that it is orally safe at 2000 mg/kg [22], and hence, we conducted directly a limit test at 2000 mg/kg for SCFE-AI. No information pertaining to the toxicity of the BOA was available; hence, we tested the toxicity using 300 mg/kg as starting dose. No mortality was observed until the experiment completion after oral administration SCFE-AI (2000 mg/kg) and BOA (300 mg/kg). The time interval of dosing within the same dose (step-1 and step-2) or the next dose was determined based on toxic clinical signs and mortality of mice. After 14 days of observation, BOA was tested at the next higher dose (2000 mg/kg). We observed a mortality of 2/3 mice after 72 h of BOA administration in each step testing. All the mice treated with SCFE-AI (2000 mg/kg) were in the analgesic state after 4 h of administration. No body weight changes were observed, and minor clinical signs (piloerection, soft stools, and repetitive scratching) were observed in fewer mice treated with either SCFE-AI (2000 mg/kg) or BOA (300 and 2000 mg/kg). However, these clinical signs were not observed after 24 h of administration.

## DISCUSSION

Our research laboratory is currently involved in the search for anti-inflammatory bioactive compounds from medicinal mangrove plants. *A. ilicifolius* is a medicinal mangrove plant known for its anti-inflammatory effect [3]. In the present study, we attempted to prepare a SCFE-AI leaves and standardize it for BOA content using HPTLC method. The acute oral safety and pro-inflammatory cytokine (TNF-α) inhibitory effect of SCFE-AI and BOA were investigated in the LPS-activated RAW 264.7 macrophage cells.

SCFE is a green technology, which uses CO<sub>2</sub> to extract bioactive compounds from plants. However, the efficiency of the extraction

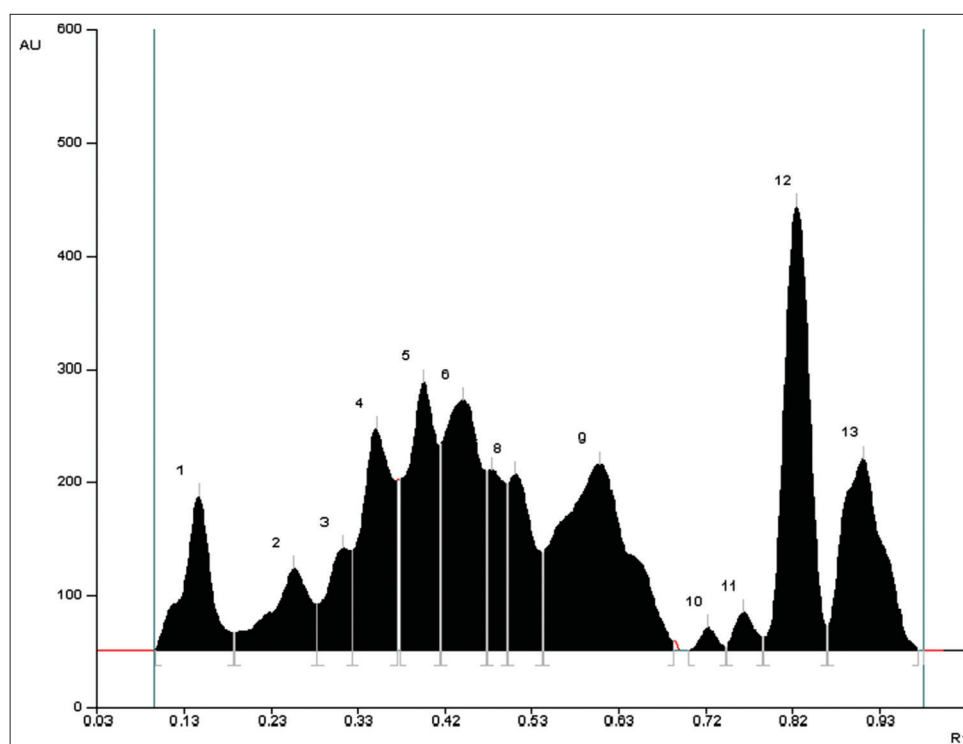


Fig. 1: High-performance thin-layer chromatography densitogram of supercritical CO<sub>2</sub> leaf extract of *Acanthus ilicifolius*

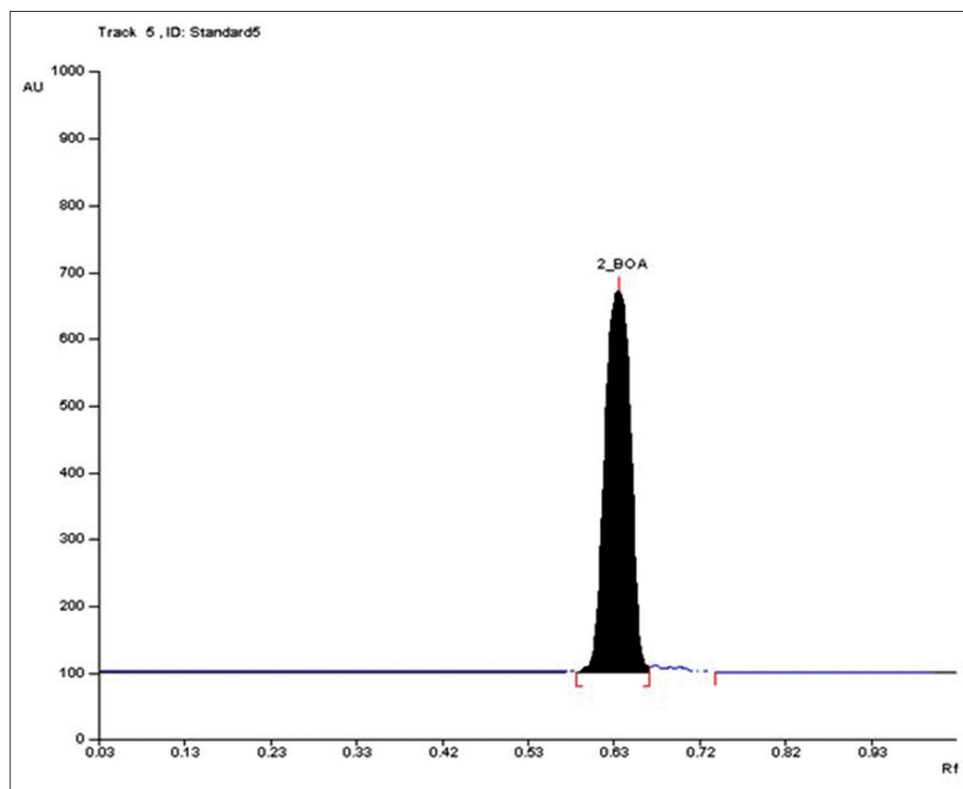


Fig. 2: High-performance thin-layer chromatography densitogram of standard 2-Benzoxazolinone

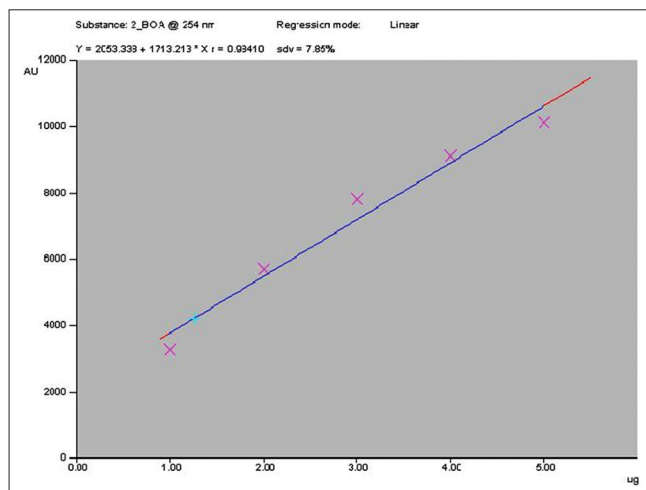


Fig. 3: Linear regression curve of standard 2-benzoxazolinone

technique relies on optimizing the processing parameters and chemistry of bioactive compounds of our interest [23]. In this present study, we have selected leaf part for extraction and BOA as the bioactive compound for standardization. To avoid deforestation of this species, we preferred to use leaves for extraction and earlier studies have also revealed the presence of BOA in the leaves [1,5]. To the best of our knowledge, this is the first report to be published on the use of SCFE method, phytochemical standardization, and biological evaluation of *A. ilicifolius* using standardized extracts.

An extract standardized for a bioactive chemical compound is widely accepted by pharmaceutical industries and by various regulatory authorities. Authentication could be achieved by comparing the colors of the bands, the number of peaks, and Rf values obtained under optimized chromatographic conditions. Ethanolic extract of *A. ilicifolius*

leaves was standardized using HPTLC method and claimed to contain 0.03% of BOA. We attempt to develop the HPTLC chromatogram for SCFE-AI initially with the reported solvent system [24], and by trial and error method, we arrived a new solvent system with maximum band separation. The yield of BOA in *A. ilicifolius* leaves extracted using optimized supercritical CO<sub>2</sub> extraction method was found to be higher than conventional liquid extraction method. This is the first report on standardization of SCFE-AI for BOA content. The developed HPTLC fingerprint chromatogram could be used for authentication and for estimation of BOA content in *A. ilicifolius*.

The CellTiter 96® Aqueous One Solution Cell Proliferation Assay Kit has been widely used to measure the cell viability. The ethanol leaf extract of *A. ilicifolius* was found to be cytotoxic toward lung fibroblast (L-929) cells in 72 h MTT assay, and the concentration required for 50% cell death was 18 µg/mL [25]; meanwhile, the methanol extract of this plant was cytotoxic to HeLa and κB cell lines [26]. From our MTS assay results, it was found that SCFE-AI and BOA of concentration <1 mg/mL are non-toxic and safe.

Plant extracts have shown to have greater biological activity than pure compounds at an equivalent dose [27]. *A. ilicifolius* extract (5 mg/kg i.p.) exhibited significant anti-inflammatory activity against carrageenan-induced edema in rats [3]. 4-hydroxy-2 (3H)-benzoxazolinone is a naturally occurring alkaloid of *A. ilicifolius* have shown to attenuate the increased TNF-α levels in an acute liver injured mice [8]. A similar effect was observed with indomethacin (1840 µg/ml) and BOA treatment on the TNF-α level. TNF-α inhibitory effect of certain BOA derivatives was compared using indomethacin as a positive control [28].

Agshikar *et al.* 1979 [4] reported that the extract did not show any mortality in mice in doses up to 1 g/kg. The ethanolic extract of *A. ilicifolius* showed no mortality and evidence of either hepatic or renal damage following a single dose of up to 5 g/kg [22]. BOA [29] and its structural analogs [30] have been earlier reported as plants secondary metabolites which are essential for a plant defense against herbivory.

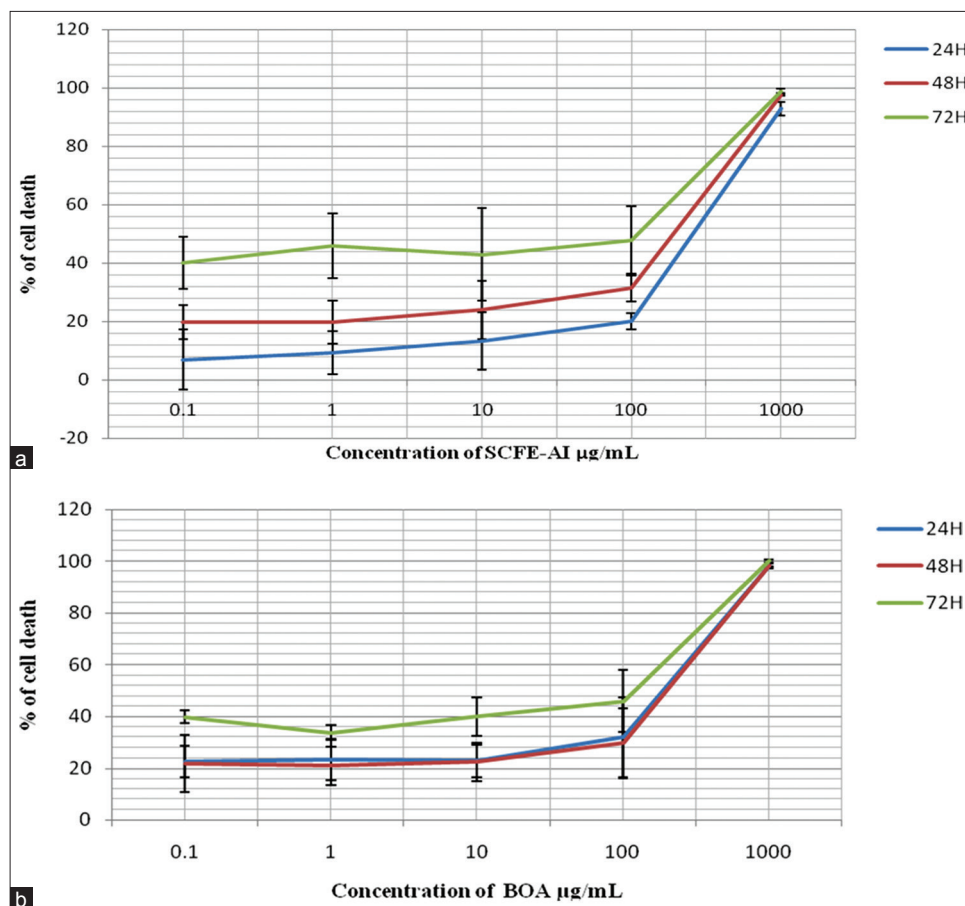


Fig. 4: Cell viability after 24, 48, and 72 h exposure of supercritical CO<sub>2</sub> leaf extract of *A. ilicifolius* (a) and 2-benzoxazolinone (b). Values are expressed as the mean±standard deviation of three independent experiments

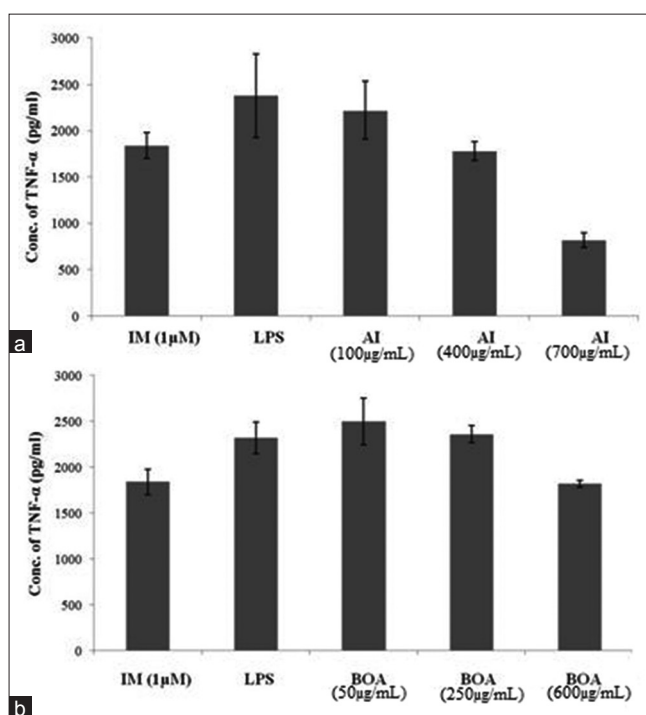


Fig. 5: The effect of supercritical CO<sub>2</sub> leaf extract of *A. ilicifolius* (SCFE-AI) (a) and BOA (b) on the tumor necrosis factor-α concentration of cell supernatant of RAW 264.7 macrophage cells induced with lipopolysaccharide. Data are expressed as mean±standard error of the mean (n=3). IM indicates indomethacin and AI indicates SCFE-AI

Hence, we believe that it is important to determine its acute oral safety. The oral acute toxic class method uses a sequential testing procedure with three animals per step at the defined dose levels (5, 50, 300, and 2000 mg/kg). This method recommends using 2000 mg/kg as starting dose when there is information available on the test compound indicating it is non-toxic. The starting dose of 300 mg/kg is recommended when no information or literature related to its toxicity are available [15]. Based on the mortality record and clinical sign observations, the LD<sub>50</sub> value of SCFE-AI was found to be >2000 mg/kg and ranges from 300 to 2000 mg/kg (orally in mice) with BOA.

#### CONCLUSION

From our results, we conclude that the HPTLC fingerprint profile could be used for standardization and identification of *A. ilicifolius* leaves. Standardized SCFE-AI (0.8% w/w of BOA) is comparatively safer than BOA on acute oral exposure. The preliminary anti-inflammatory study revealed the TNF-α inhibitory effect of SCFE-AI in LPS-stimulated RAW 264.7 macrophage cells. The use of standardized SCFE-AI in the management of inflammatory disorders and pain could be further investigated.

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#### AUTHOR'S CONTRIBUTION

Saranya AR and Ramanathan T conceived and designed the experiments. Saranya AR performed the experiments and wrote the manuscript. Ramanathan T supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

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