International Food Research Journal 30(5): 1285 - 1296 (October 2023)
Journal homepage: *http://www.ifrj.upm.edu.my*

In vitro anti-inflammatory and comparative cytotoxicity studies on methanolic extract of *Enicostemma hyssopifolium*

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Article history

Received:
4 January 2022
Received in revised form:
15 January 2023
Accepted:
27 June 2023

Keywords

anti-inflammatory, cytotoxicity, Enicostemma hyssopifolium, hyaluronidase inhibition, lipoxygenase inhibition

Abstract

Enicostemma hyssopifolium is a perineal herb widely used as an antidiabetic agent in folklore medicine. The present work was focused on the assessment of its anti-inflammatory and cytotoxic properties. Its methanolic extract was evaluated for anti-inflammatory activity using murine monocytic macrophage RAW 264.7 cells, and screened for its cytotoxic property in different cancer cell lines. The methanolic extract was able to potentially inhibit the bacterial lipopolysaccharides-induced inflammatory response in RAW 264.7 cells. Results of the cytotoxicity studies revealed that the methanolic extract effectively induced the cytotoxicity at considerably lower concentration in MCF-7, A-549, and COLO-205 cell lines, while the viability of HeLa, CasKi, and HT-29 cells were inhibited at comparatively higher concentrations. Results thus indicated that *E. hyssopifolium* possessed potent anti-inflammatory and cytotoxic properties. This necessitates further exploration of bioactive phytochemical compounds responsible for these properties for therapeutic applications.

DOI
https://doi.org/10.47836/ifrj.30.5.16

Introduction

Inflammatory response is one of the parts of immunological orchestra which focuses on eliminating the preliminary causes of cell damage. The chronic inflammatory responses in human system have been reported to induce several disorders/diseases including Alzheimer's, cardiovascular diseases, osteoarthritis, cancers, and rheumatoid arthritis (Soonthornsit *et al.,* 2017). The key inflammation-associated elements such as cytokines and free radicals, which are released in excessive levels during chronic inflammation, are known as the major contributors to the pathogenesis of various diseases (Elisha *et al.,* 2016; Gunathilake *et al.,* 2018). Currently, the inflammatory responses are being monitored or treated mainly using various classes of drugs such as non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, and disease-modifying anti-rheumatic drugs (DMARDs). However, these anti-inflammatory drugs have certain side effects such as gastrointestinal (gastric mucosa, belching, gastric ulceration, and bleeding), cardiovascular diseases, and impairment of renal and hepatic functions (Reddy *et al.,* 2014; Gunathilake *et al.,* 2018).

Chronic inflammation, oxidative stress, and free radicals are some of the major elements contributing to the formation of cancerous cells within the body. According to the GLOBOCAN 2018 report, deaths due to cancers worldwide are estimated to be 9.6 million, among them, lung cancer (18.4%) has been the top-ranked cause, followed by breast cancer (11.6%), colorectal cancer (9.2%), and cervical cancer (7.5%) (Ferlay *et al.,* 2019). Although there are several therapies such as chemotherapy, radiation therapy, hormonal therapy, and surgery available to treat and manage cancers, due to their unaffordability and life-threatening side effects, there have been enormous research being carried out to investigate and identify drugs which could overcome the shortcomings and side effects imposed by the

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present available cancer therapies (Sateesh and Mohsin, 2014; Kalebar *et al.*, 2020b).

Published reports indicate that natural dietary supplements possess wide range of phytoconstituents, mainly polyphenols and flavonoids, which exhibit various biological activities (Pan *et al.*, 2010). *Enicostemma hyssopifolium* belongs to the Gentianaceae family, and is a traditionally-used medicine among many of the tribal regions of South Asia to treat various diseases such as diabetes, fever, stomachache, dyspepsia, malaria, and also as an antipyretic and laxative agent (Vyas *et al.*, 1979; Murali *et al.*, 2002). *Enicostemma hyssopifolium* is a clustered herbal flower that grows to a height of 5 - 20 inches. The herb is widely known for its hypoglycaemic, antioxidant, and hypolipidaemic properties due to the presence of various phytoconstituents such as phenols, catechins, tannins, steroids, flavonoids, glycosides, saponins, anthraquinones, and sterols in its tissue (Vasu *et al.*, 2005; Patel and Mishra, 2011; Mathur 2013). Though *E. hyssopifolium* has gained importance as traditional medicine by medicinal practitioners, considerably few reports are available on its *in vitro* pharmacological action and its clinical settings. The present work was thus carried out to determine the potential cytotoxic effect of *E. hyssopifolium* methanolic extract on various cancerous cell lines, and the anti-inflammatory action on murine monocytic macrophage cells to explore its medicinal property.

Materials and methods

Chemicals

Hyaluronidase (EC 3.21.35) from bovine testis, sodium-hyaluronate, disodium-cromoglycate (cromolyn), *p*-dimethyl amino benzaldehyde (DMAB), potassium tetraborate, 2 M borate buffer at pH 9.0, lipoxygenase, linoleic acid, Hammerstein casein, and trypsin were procured from SRL, India. The DMSO, foetal bovine serum (FBS), and penicillin-streptomycin were procured from Invitrogen. The cell lines such as HeLa, CasKi, A-549, HT-29, COLO-205, MCF-7, and RAW 264.7 were procured from NCCS, Pune, India. Analytical grade chemicals were used throughout. The chemicals such as indomethacin, curcumin, soybean trypsin inhibitor, lipoxygenase, hyaluronidase, and trypsin were procured from HiMedia.

Collection and preparation of plant extract

Enicostemma hyssopifolium herb was collected from the surrounding area of Chitradurga district in Karnataka, and the plant voucher specimen was documented (GSC/2015/Eh/1). The whole plant was collected and shade-dried for 30 d. The dried plant material was powdered coarsely, and subjected to Soxhlet phytoextraction process using methanol as solvent. The methanolic extract was subjected to concentration using rota-evaporator (Buchi), and finally dried in a desiccator. The yield of the methanolic extract was 9 g/kg of plant material.

Anti-inflammatory assays

Hyaluronidase assay

The hyaluronidase assay was carried out according to Perera *et al.* (2018). Briefly, 120 µL of hyaluronidase was activated in 4.15 mg/mL of acetate buffer (0.1 M, pH 3.8) with 75 µL of sodium chloride prepared in 26.3 mg/mL acetate buffer (0.1 M, pH 3.8) at 37°C for 20 min. The activated enzyme solution was further incubated at 37°C for 20 min with different concentrations of *E. hyssopifolium* methanolic extract (200 µL), ranging from 10 - 320 µg/mL (10, 20, 40, 80, 160, and 320 µg/mL), and reference standard indomethacin (200 µL). Then, 150 µL of sodium hyaluronate prepared as 6 mg/mL in acetate buffer (0.1 M, pH 3.8) was added. This enzyme-substrate was further incubated for 40 min at 37°C. Finally, 100 µL of 0.4 N NaOH and 100 µL of 0.8 M potassium tetraborate was added to stop the reaction, and incubated for 3 min at 100°C. After incubation, the mixture was cooled, 10% DMAB (3 mL) was added, and incubated for 20 min at 37°C. The optical density (OD) at 585 nm was read using a spectrophotometer, and then the percent enzyme (hyaluronidase) inhibition was calculated using Eq. 1:

$$\% \text{ Inhibition} = \frac{(\text{OD of Control} - \text{OD of Sample})}{\text{OD of Control}} \times 100 \quad \text{(Eq. 1)}$$

Nitric oxide inhibition assay

The anti-inflammatory action was studied using the inflammatory stress-induced murine monocytic macrophage (RAW 264.7 cell line), passaged in DMEM with 10% (v/v) FBS for every 72 h using CO_2 (5%) incubator at 37°C. For anti-inflammatory study, 1×10^6 cells/mL seeded in 96-well plates were pre-incubated at 37°C under 5% CO_2 condition for 24 h. Then, 100 µL of *E. hyssopifolium*

methanolic extracts dissolved in DMSO (10 - 320 µg/mL) were added onto wells containing the murine cells. Cells were then induced with inflammatory stress by treating them with 10 µg/mL lipopolysaccharide (LPS of *Escherichia coli*) for 17 h by maintaining them at 37°C. After 17 h, the supernatant was transferred (100 µL) from each well into a 96-well microtiter plate, and added with equal volume of Griess reagent. After 10 min, the OD values at 550 nm were read using a microtiter plate reader (Tecan Device). The amount of nitrite formed as a result of NO formation in the murine cells was determined using the standard curve of sodium nitrite by regression analysis. The percentage inhibition of methanolic extract was then estimated from the inhibition of NO formation by treated cells as compared to the untreated control cells (DMSO added); here, control was measured as 0% inhibition. Curcumin was used as reference standard drug for this assay (Joo *et al.*, 2014).

Lipoxygenase inhibition assay

Lipoxygenase inhibition activity of *E. hyssopifolium* methanolic extract was performed according to Sari and Elya (2017) with slight modification. To 0.1 mL of the methanolic extract of *E. hyssopifolium* (10 - 320 µg/mL in borate buffer (2 M) of pH 9.0), 0.1 mL of lipoxygenase was added, and the mixture was stabilised for 5 min at room temperature. Then, 2.0 mL of 0.6 mM linoleic acid was added as substrate solution. After mixing thoroughly using vortex mixer, the OD values at 234 nm were read after 4 min. Vehicle control and basal reactions were maintained without extract inclusion. For reference standard, 300 µg/mL indomethacin dissolved in methanol (3%) was used. The assay was performed in triplicate, and mean values were taken. The percentage inhibition of lipoxygenase activity was calculated using Eq. 2:

$$\% \text{ Inhibition} = \frac{(\text{OD of Control} - \text{OD of Sample})}{\text{OD of Control}} \times 100 \quad \text{(Eq. 2)}$$

Trypsin (protease) inhibitory activity

Enicostemma hyssopifolium methanolic extract was assessed for its anti-trypsin activity (Kunitz, 1947). Briefly, 1 mL of trypsin prepared in 0.1 M phosphate buffer (pH 7) at a concentration of 0.5 mg/mL, and 1 mL of *E. hyssopifolium* methanolic extract in concentration range of 10 - 320 µg/mL (w/v) were incubated at 37°C for 15 min. Then, 1% Hammerstein casein prepared in 0.1 M phosphate buffer was added, and again incubated for 30 min at 37°C. The reaction was terminated by adding 2.5 mL of TCA solution (0.44 M). During this reaction, the TCA soluble casein was liberated by the action of trypsin hydrolysis. The OD values of the supernatant collected after centrifugation (10,000 rpm, 15 min) were measured at 280 nm using a UV-Vis spectrophotometer. The peptide fraction of casein without the extract was considered as blank. In this assay, one unit decrease in the OD value of liberated casein was considered as one unit trypsin inhibitor activity. The trypsin inhibition action of the methanolic extract was expressed in the form of percentage inhibition. Soybean extract was used as standard reference for the assay.

$$\% \text{ Inhibition} = \frac{(\text{OD of Control} - \text{OD of Sample})}{\text{OD of Control}} \times 100 \quad \text{(Eq. 3)}$$

In vitro cytotoxicity studies of Enicostemma hyssopifolium by MTT assay

The following procedure was used for cytotoxicity evaluation using HeLa, CasKi, A-549, HT-29, COLO-205, and MCF-7 cancer cell lines separately according to Alley *et al*. (1986) with slight modification. The monolayer cultured cells (HeLa, CasKi, A-549, HT-29, COLO-205, and MCF-7 in DMEM with 10% FBS, 100 µg/mL streptomycin, and 100 IU/mL penicillin) were dissociated by trypsinisation, and adjusted to 2.5×10^5 cells/ml. The 96-well microtiter plate was seeded with 200 µL/well of 50,000 cells/well upon dilution, and pre-incubated (24 h) under 5% CO_2 condition in a humidified incubator at 37°C. After 24 h, the media was removed, and the adhered cells was washed with its respective medium, and then replaced by 200 µL of various concentrations of *E. hyssopifolium* methanolic extract in the range of 10 - 320 µg/mL prepared in the media. The plates were again incubated at 37°C under 5% CO_2 condition for another 24 h. For evaluation of the toxic effects of the *E. hyssopifolium* methanolic extract on normal cells, the murine RAW 264.7 cells were similarly incubated with varying concentrations up to 1,000 µg/mL (0, 50, 100, 200, 400, 800, and 1000 µg/mL) of *E. hyssopifolium* methanolic extract. After 24 h of incubation, the supernatant from each well was removed and discarded. Then, 100 µL of fresh MTT solution (prepared by dissolving 6 mg of MTT in 10 mL of PBS, pH 7.4) was added to each well, and

incubated for 4 h at 37°C under 5% CO_2 condition. This was followed by the removal of MTT solution from the plate, and then 100 µL of DMSO was added, and the plates were shaken gently to solubilise the formazan crystals. After thorough solubilisation, the OD values were read at 570 nm using a microplate reader. The experiment was repeated thrice. The percentage of cell viability was calculated using Eq. 4, and IC_{50} concentration (treatment concentration required to inhibit cell viability by 50%) of methanolic extract of *E. hyssopifolium* were predicted from the non-linear regression curve plotted for each cell line.

$$\% \text{ Inhibition} = \frac{(\text{OD of Control} - \text{OD of Sample})}{\text{OD of Control}} \times 100 \quad \text{(Eq. 4)}$$

Statistical analysis

All the experiments were conducted in triplicate, and the results were expressed as mean values ± SD. The IC_{50} values of the methanolic extract for various assays performed in the investigation were predicted from a non-linear regression curve fit plotted as a dose response curve from the plotted curve. Data were statistically analysed using SPSS v17 and Origin 2022b version software. A p-value ≤ 0.05 was considered significant.

Results

Hyaluronidase inhibition

The degradation of hyaluronic acid is catalysed by hyaluronidase. The inhibition of hyaluronidase activity is associated with arresting the process of inflammation. The efficiency of *E. hyssopifolium* methanolic extract in inhibiting the hyaluronidase activity was assessed. Inhibition of hyaluronidase activity by *E. hyssopifolium* methanolic extract was identified to be in a dose-dependent manner, showing increased inhibition with increasing concentration (Figure 1A). The IC_{50} values of methanolic extract and reference standard indomethacin (Figure 1B) were calculated to be 890.3 ± 1.2 and 29.84 ± 0.9 µg/mL, respectively (Table 1).

Nitric oxide inhibition

Free radicals were reported to induce oxidative stress in the tissue, and eventually contributing to the inflammation. Increased concentration of nitric oxide in the tissue potentially leads to activation of inflammatory mediators. In the present work, the effect of *E. hyssopifolium* methanolic extract in controlling the formation of the pro-inflammatory factor, nitric oxide, in murine monocytic macrophage RAW 264.7 cell line was studied. Results revealed that the methanolic extract of *E. hyssopifolium* had a good anti-inflammatory activity by reducing nitric oxide formation in the inflammation-induced cells with IC_{50} of 609.7 ± 1.5 µg/mL. The IC_{50} of reference standard curcumin was found to be 167.1 ± 1.1 µg/mL (Table 2 and Figures 2A - 2B).

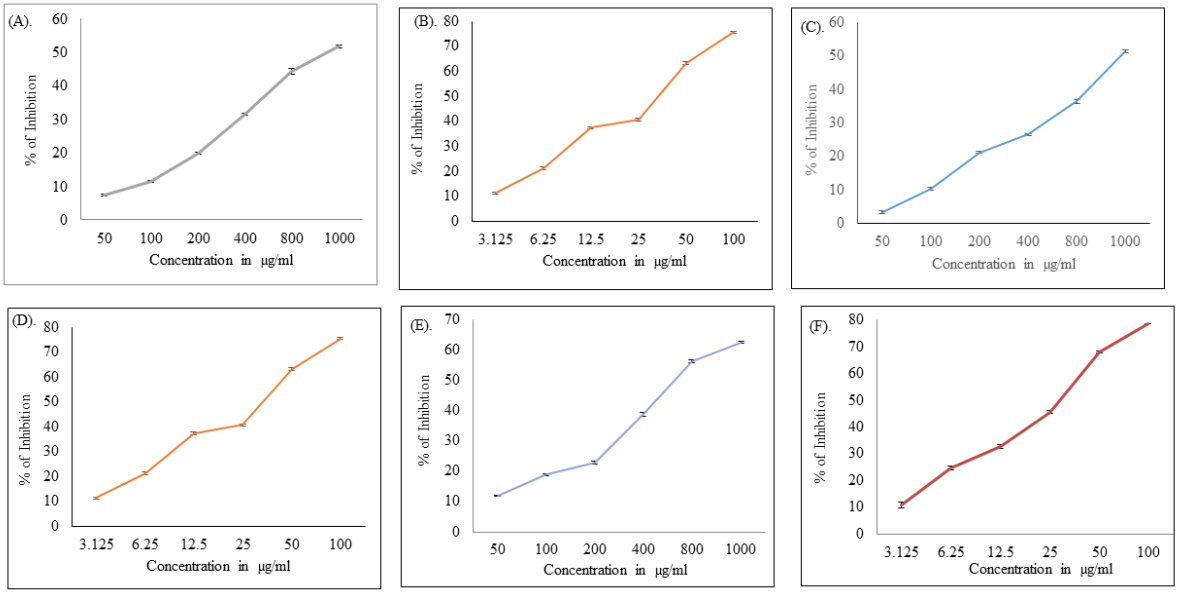

Figure 1. Hyaluronidase inhibition assay (HIA) of *Enicostemma hyssopifolium* **(A)** and indomethacin (standard) **(B)**. Lipoxygenase inhibition assay (LIA) of *Enicostemma hyssopifolium* **(C)** and indomethacin (standard) **(D)**. Trypsin inhibition assay (TIA) of *Enicostemma hyssopifolium* **(E)** and soybean (standard) **(F)**. Results are from three independent experiments. Values are mean ± SD of triplicates (n = 3). $p \leq 0.05$.

Table 1. Percentage inhibition of hyaluronidase inhibition activity (HIA), lipoxygenase inhibition activity (LIA), and trypsin inhibitory activity (TIA).

Conc. µg/mL	% of inhibition of standard			Conc. µg/mL	% of inhibition of extract		
	HIA	LIA	TIA		HIA	LIA	TIA
	Indomethacin	Indomethacin	Soybean		*Enicostemma hyssopifolium*	*Enicostemma hyssopifolium*	*Enicostemma hyssopifolium*
3.125	11.24 ± 0.35	11.24 ± 0.35	10.80 ± 1.06	50	7.21 ± 0.28	3.44 ± 0.42	11.84 ± 0.24
6.25	21.38 ± 0.07	21.38 ± 0.07	24.68 ± 0.83	100	11.35 ± 0.26	10.35 ± 0.49	18.90 ± 0.27
12.5	37.65 ± 0.12	37.65 ± 0.12	32.85 ± 0.73	200	19.84 ± 0.21	21.29 ± 0.28	22.87 ± 0.38
25	40.96 ± 0.12	40.96 ± 0.12	45.66 ± 0.69	400	31.51 ± 0.29	26.60 ± 0.37	38.83 ± 0.64
50	64.31 ± 0.83	64.32 ± 0.83	68.08 ± 0.19	800	44.29 ± 0.78	36.44 ± 0.51	56.37 ± 0.41
100	75.9 ± 0.23	75.9 ± 0.23	78.60 ± 0.03	1,000	51.80 ± 0.44	51.49 ± 0.41	62.53 ± 0.27

Values are mean ± SD of triplicates (n = 3).

Table 2. Anti-inflammatory effect of *Enicostemma hyssopifolium* by nitric oxide inhibition assay.

Conc. µg/mL	% of inhibition in curcumin (standard)	% of inhibition of extract
10	9.28 ± 0.03	6.50 ± 0.39
20	14.7 ± 0.24	10.00 ± 0.45
40	22.23 ± 0.15	17.93 ± 0.46
80	31.67 ± 0.18	21.14 ± 0.40
160	38.73 ± 0.09	23.56 ± 0.43
320	61.06 ± 0.15	38.0185 ± 0.37
640	65.6 ± 0.15	53.47 ± 0.41

Values are mean ± SD of triplicates (n = 3).

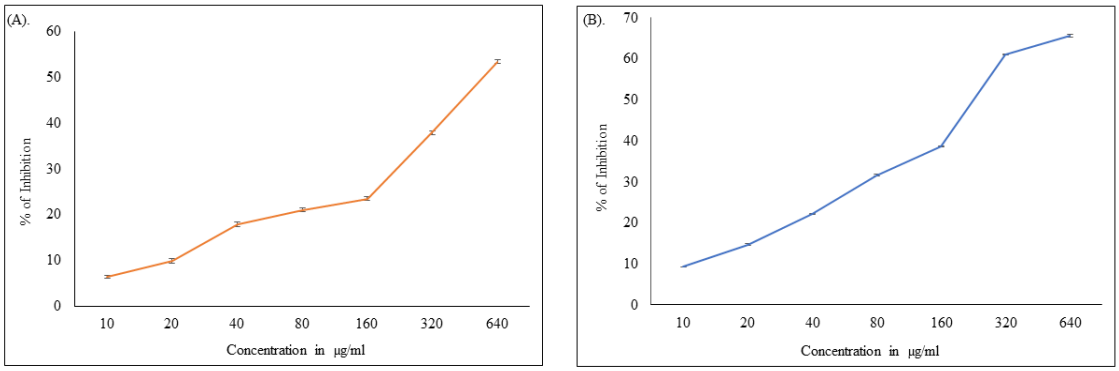

Figure 2. iNOs assay of *Enicostemma hyssopifolium* **(A)** and curcumin (standard) **(B)**. Results are from three independent experiments. Values are mean ± SD of triplicates (n = 3). $p \leq 0.05$.

Lipoxygenase inhibition

Lipoxygenase catalyses oxygenation of arachidonic acid which is precursor of prostaglandins. Prostaglandins are key inflammatory mediators in various diseases. *Enicostemma hyssopifolium* methanolic extract was also tested for its effects on lipoxygenase inhibition. Results indicated that the methanolic extract could successfully inhibit the lipoxygenases activity with IC_{50} of 904.9 ± 1.7 µg/mL. The IC_{50} of the reference drug used is 16.92 ± 1.2 µg/mL (Table 1 and Figures 1C - 1D).

Trypsin inhibitory activity

Trypsin inhibitors are reported to control the progression of inflammatory response (Mike *et al.*, 2001). The inhibitory action of the *E. hyssopifolium* methanolic extract against trypsin was studied, which showed an increased inhibition with gradual increase in the treatment concentration of 25 - 1,000 µg/mL with IC_{50} of 718 ± 0.9 µg/mL. The reference standard, soybean, showed IC_{50} of 28.84 ± 0.8 µg/mL (Table 1 and Figures 1E - 1F).

In vitro cytotoxicity effects

The cytotoxic action of *E. hyssopifolium* methanolic extract was assessed by MTT assay using six different cancer cell lines such as HeLa, CasKi, A-549, HT-29, COLO-205, and MCF-7. The cancer cell growth inhibition effects of the *E. hyssopifolium* methanolic extract was determined based on its action in controlling cell proliferation and inhibition of the cell viability, which was assessed by the decrease in formazan crystals formation due to the decrease in viable cells. In the present work, a gradual decrease in the cell viability was observed with the increase in the treated concentration of methanolic extract. Results also revealed that *E. hyssopifolium* methanolic extract potentially prevented the cell viability and proliferation of all these tested cancer cell lines. Among all the cancer cell lines, the breast cancer cell line (MCF-7) showed the highest susceptibility towards the tested plant extract with a relatively lower IC_{50} of 54.24 ± 0.9 µg/mL. Figure 3A describes the comparative levels of cell viability. The microscopic images of treated cell lines with lowest and highest doses are shown with growth inhibition in Figures 4A - 4C.

The effects of *E. hyssopifolium* methanolic extract on normal cells were also tested for toxic action using the normal murine macrophage RAW 264.7 cells with up to 1,000 µg/mL concentration by MTT assay (Figure 3B). The extract did not show

noticeable cytotoxicity on this normal cell, and the percentage inhibition of cells were below 5% only, even for treatment with 1,000 µg/mL, and the morphology changes are shown in Figure 4D. This proved that the plant extract was safe for *in vitro* treatment of normal cells.

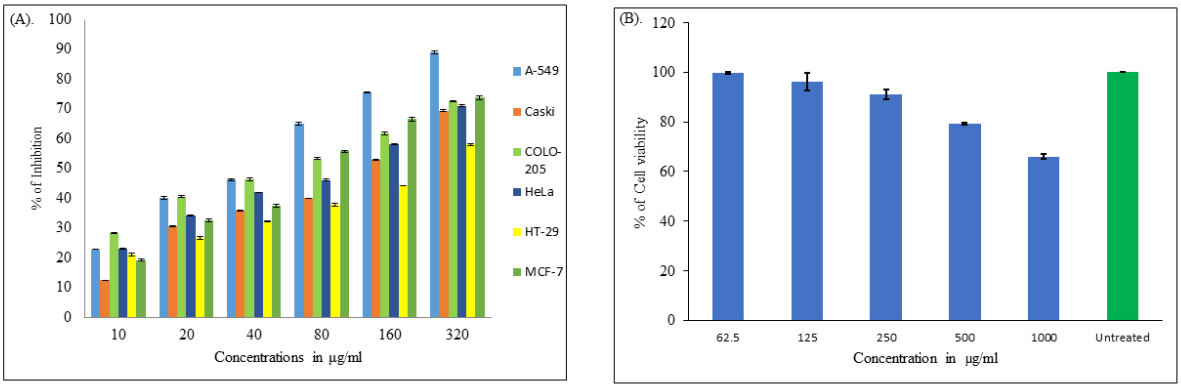

Figure 3. Cytotoxicity effect of methanolic extract of *Enicostemma hyssopifolium* on cancer cell lines **(A)** and on the proliferation of RAW 264.7 cells **(B)**. Results are from three independent experiments. Values are mean ± SD of triplicates ($n = 3$). $p \leq 0.05$.

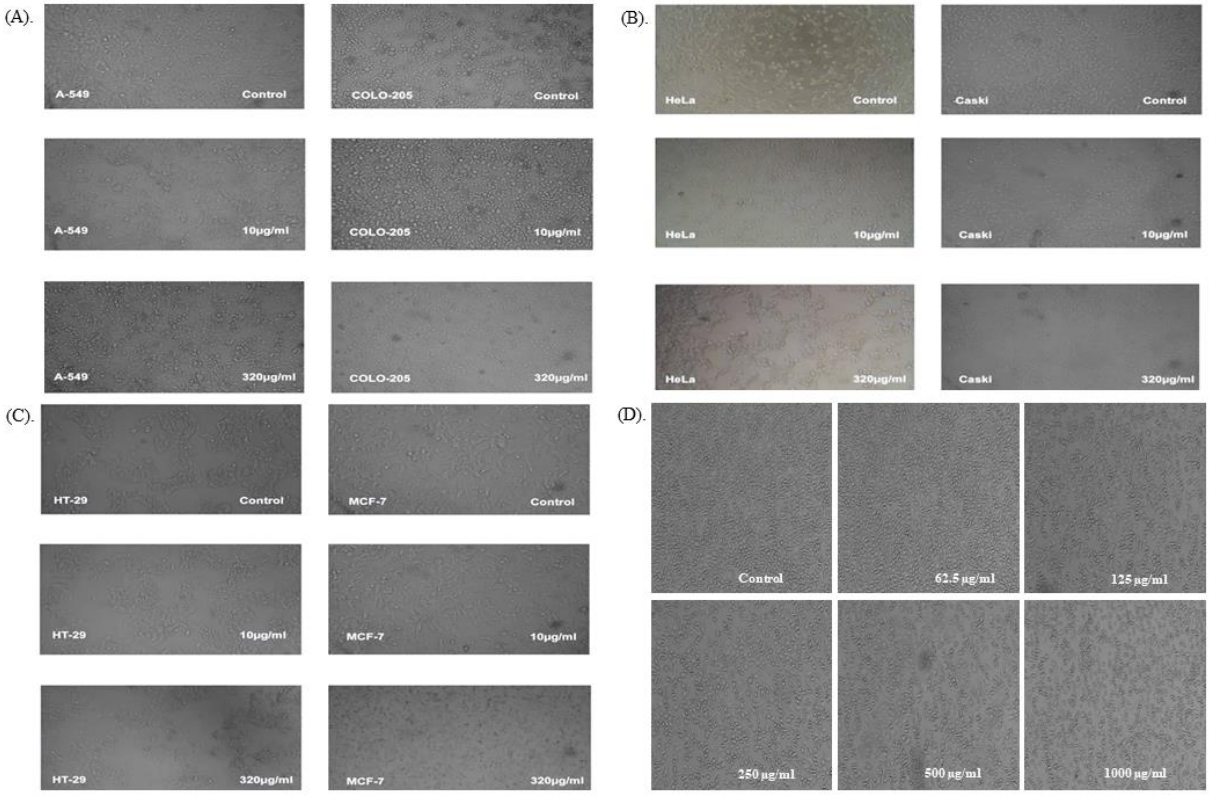

Figure 4. Cytotoxicity studies of methanolic extract of *Enicostemma hyssopifolium* on A-549 and COLO-205 cell morphology **(A)**, HeLa and Caski cell morphology **(B)**, HT-29 and MCF-7 cell morphology **(C)**, and RAW264.7 cell morphology **(D)** at 10× magnification. Scale bar = 20 µM.

Discussion

Inflammation is a complex biological phenomenon exhibited as defensive mechanism against any immunogen by the action of lysosomal enzymes, and also by imposing oxidative stress on it. These lysosomal enzymes and oxidative ions are the results of pivotal role displayed by the leukocytes and the macrophages (Shaikh *et al.*, 2016; Naz *et al.*, 2017). Among the several assays available for the evaluation of anti-inflammatory property of various agents, the present work was focused on four prominent assays to determine the anti-inflammatory effect of the *E. hyssopifolium* methanolic extract.

Hyaluronic acid (HA) is one the constituent enzymes of the connective tissue, involved in maintaining the membrane integrity of the cell. Hence, HA is also an important factor during inflammation, wound healing, tissue repair, tissue regeneration, and tumorigenesis. Hyaluronidase is an enzyme which catalyses the breakdown of HA, thus leading to multiple impediments including exhibition of inflammation. In order to arrest its activity, agents such as hyaluronidase inhibitors are found to be profoundly effective which could eventually hamper the allergic and inflammation reactions. The hyaluronidase assay measures the amount of N-acetyl glucosamine produced due to the degradation of HA by the breakage of glycosidic bond (Mio *et al.,* 2000; Nawy *et al.,* 2001; Ticar *et al.,* 2017). The present work was carried out using *E. hyssopifolium* methanolic extract with increasing concentrations to determine its effect in inhibiting the hyaluronidase activity. Results obtained revealed that *E. hyssopifolium* methanolic extract potentially inhibited the hyaluronidase enzyme activity with a higher IC_{50} of 890.3 µg/mL, while the reference standard indomethacin significantly inhibited the hyaluronidase enzyme activity with a lower IC_{50} of 29.84 µg/mL. The inhibitory effect was observed at higher range of doses (50 – 1,000 µg/mL) of *E. hyssopifolium* as it was the crude extract when compared with the pure reference compound indomethacin which exhibited the inhibitory effect at lower range of doses (3.125 - 100 µg/mL), and the IC_{50} was reported by other studies as 21.16 µg/mL (Uzma *et al.,* 2022).

As earlier discussed, the ROS-induced oxidative stress in cells leads to inflammation in the tissue. But these oxidative ions in excess concentrations also play a role in tissue damages and other disorders. Nitric oxide (NO) is a free radical with very short half-life during which it mediates many physiological processes that include enhancement of the tumoricidal and microbicidal activities through the activated macrophages. Excessive formation of NO in the tissue could have detrimental effect, cause tissue damage, and also activate the pro-inflammatory mediators (Jorens *et al.,* 1995; Tripathi *et al.,* 2007; Zhang *et al.,* 2017). The murine monocytic macrophage (RAW 264.7) cell line was used in the present work to determine the anti-inflammatory effects of *E. hyssopifolium* methanolic extract by inhibiting the formation of excessive NO by the macrophage. Results shown an increasing inhibition of NO formation with increasing concentrations (10 - 640 µg/mL) of both standard drug-curcumin and the methanolic extract of *E. hyssopifolium* with IC_{50} of 167.1 and 609.7 µg/mL, respectively. Another study reported IC_{50} of 90.82 ± 4.75 µg/mL for curcumin (Habu and Ibeh, 2015). This implied that the methanolic extract of *E. hyssopifolium* had good NO inhibitory effect like pure curcumin though it was still in crude form.

The cytosolic lipoxygenase enzyme family catalyses the process of oxygenation of polyunsaturated fatty acids like arachidonic acid, and generates hydro-peroxides (prostaglandins, thromboxanes, and leukotrienes), which play a major role in inflammation and other disorders including cancers. The conversion of linoleic acid to 13-hydroperoxy linoleic acid by lipoxygenases is followed by the appearance of conjugated diene (Tamanoi and Bathaie, 2014; Wisastra and Dekker, 2014). *Enicostemma hyssopifolium* methanolic extract exhibited dose-dependent lipoxygenase inhibition activity in a higher concentration range of 50 - 1,000 µg/mL with IC_{50} of 904.9 µg/mL, while the indomethacin reference standard was effective in inhibiting lipoxygenases activity in a lower concentration range of 3.125 - 100 µg/mL with much lower IC_{50} of 16.92 µg/mL, which might have been due to its pure nature. Another study also shown 19.46 µg/mL as indomethacin IC_{50} (Uzma *et al*., 2022).

Protease-activated receptor-2 (PAR2) is a unique small transmembrane G protein which is activated by the cleavage of receptor itself at its N-terminus. The increased levels of PAR2 protein are reported to be associated with several inflammatory responses and other disorders namely rheumatoid arthritis (RA), osteoarthritis (OA), synovitis, cartilage degradation, subsequent bone erosion, and irritable bowel syndrome (IBS). These PAR2 are known to be expressed on eosinophils and many of the cells of endothelium, epithelium, smooth muscle, and tissues. PAR2 are mainly produced by eosinophil cells that are activated through proteolysis by trypsin (Mike *et al.,* 2001; Coelho *et al.,* 2002; McCulloch *et al.,* 2018). Based on literature survey, effect of trypsin can be suppressed by the action of a number of agents known as trypsin inhibitors which contribute to controlling the progression of inflammatory response (Mike *et al.,* 2001). The anti-inflammation effect of *E. hyssopifolium* methanolic extract in inhibiting the influence of trypsin was assessed in the present work

following the methods proposed by Kunitz (1947), with slight modification. Results indicated that the methanolic extract of *E. hyssopifolium* expressively suppressed the function of trypsin as its inhibitor, in a concentration dependent manner (25 - 1,000 µg/mL). Further, the IC_{50} was determined to be 718 µg/mL, which was higher than that of the reference treatment as it was still in crude form. The reference standard, soybean extract, however shown inhibition in the concentration range of 3.125 - 100 µg/mL with IC_{50} of 28.84 µg/mL. In another study, the IC_{50} of soybean extract was found to be 14 µM (Lin and Ng, 2008).

Overall, the anti-inflammatory study results shown that the crude phytochemical extract of *E. hyssopifolium* using methanol exhibited inhibitory effects on inflammation-mediating enzymes in LPO-induced inflammatory RAW 264.7 cells in a concentration range of 10 - 1,000 µg/mL with IC_{50} concentration range between 600 - 900 µg/mL. The methanolic extract of *E. hyssopifolium* showed better NO inhibition potency with IC_{50} of 609.7 µg/mL when compared with other anti-inflammatory actions. Hence, the anti-inflammatory effects of the plant studied might have been exhibited *via* NO inhibition pathway. In these studies, the pure standard reference anti-inflammatory compounds used showed inhibitory effects at lower concentration range of 3 - 100 µg/mL with IC_{50} concentration range between 16 - 167 µg/mL. Further exploratory studies on separated phytochemicals of the methanolic extract of *E. hyssopifolium* are warranted to identify the potent phytochemical compounds responsible for the anti-inflammatory actions. We have also reported that the anti-inflammatory activity of *E. hyssopifolium* might have been due to the presence of phytochemical compounds such as 2-methoxy-4-vinylphenol, 3,5-dimethoxyacetophenone, erythrocentaurin, pentadecanoic acid, phytol, 9,12-octadecadienoic acid, ergost-5-en-3-ol, (3.beta.), vitamin E, and stigmasterol as identified by GC-MS analysis (Komal *et al.*, 2020).

It is well known that cancer pathogenesis and tumour progression are majorly contributed by the inflammation mediators (Aggarwal *et al.*, 2009). With these promising results on anti-inflammatory effects of *E. hyssopifolium* methanolic extract, the present wok further explored the cytotoxicity properties of the extract. Cytotoxicity studies are preliminary tests carried out using the mammalian cells under *in vitro* conditions, where the effect of drugs on the cell viability mainly targeting its growth, multiplication, and morphogenesis occurring within the cells can be evaluated (Li *et al.*, 2015). For the cytotoxicity studies, six different cancer cell lines such as human cervical carcinoma (HeLa and CasKi), human lung carcinoma (A-549), human colon carcinoma (HT-29 and COLO-205), and human breast carcinoma (MCF-7) was used for the evaluation of cytotoxic effects on these cancer cells.

Amongst various assays reported for the determination of cytotoxic activity, MTT assay is one of the most widely accepted and used for cytotoxic assays to determine the drug toxicity of both crude extracts and isolated pure compounds on the target cancer cells. The assay assesses the level of the dehydrogenase produced by the metabolically active cells in reduction of tetrazolium salts, thus leading to the formation of purple colour formazan product, which is then measured spectrophotometrically (Kalebar *et al.*, 2020a). The methanolic extract was found to exhibit a profound anti-proliferative action on all the cancer cell lines used in the present work, and was more potent in inhibiting the viability of MCF-7, A-549, and COLO-205 cell lines at the concentration lower than 100 µg/mL, thus showing the IC_{50} of 54.24, 57.22, 63.74 µg/mL, respectively. The inhibition of HeLa, CasKi, and HT-29 cell viability was observed at comparatively higher concentration (more than 100 µg/mL) with IC_{50} of 106, 111.9, and 203.2 µg/mL, respectively, thus indicating comparatively lesser action. The difference in carcinogenesis pathways of these different types of cancer cell line may be the reason for varying cytotoxic potencies of the methanolic extract of *E. hyssopifolium.* Furthermore, the extract did not show significant (only less than 5%) cytotoxic effects on non-cancerous macrophage (RAW 264.7) cells with up to 1,000 µg/mL concentration by MTT assay. This implied that the extract was safe for normal cells.

The data obtained unmasked the potential cytotoxic properties of *E. hyssopifolium* methanolic extract against different cancer cell lines, thus making it beneficial for further exploration on its therapeutic applications. We have earlier reported (Komal *et al.*, 2020) the GC-MS results which indicated the presence of few phytochemical having anti-cancer potency such as 2-hydroxy-gamma-butyrolactone, 2-methoxy-4-vinylphenol, pentadecanoic acid, phytol, 2,6,10,14,18,22-tetracosahexaene,2,6,10,15,19,23-hexamethyl, (all-E)- or squalene, vitamin E,

stigmasterol, and γ-sitosterol/clionasterol, which may be the underlying reason for the cytotoxic activities on the cancer cell lines observed in the present work.

Conclusion

In the present work, the methanolic extract of *E. hyssopifolium* was found to possess anti-inflammatory properties, thus adding to its therapeutic applications in treating diseases associated with inflammatory response, as inflammation is also a major contributor for cancer pathogenesis. Further, the cytotoxic studies of *E. hyssopifolium* methanolic extract have demonstrated its ability in inhibiting the viability and proliferation of the cancer cells, thus affirming that the plant possesses many bioactive phytoconstituents whose identification and characterisation need to be executed. The present work would serve as a platform for development of safe and effective drug against cancer and its associated inflammation. Further pre- and clinical studies can be implemented in order to use it as a potential drug in treating various inflammatory diseases and cancers.

Acknowledgement

The authors are grateful to Prof. R. K. Rangaswmy, the head of the institution for assistance in completing the present work.

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