International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.6, No.3, pp 1028-1035, July-Aug 2014

In vitro Anti inflammatory and Flowcytometric analysis of Poly herbal extract

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Abstract:
Background: We aimed at exploring the anti-inflammatory effect of EPHE against experimentally induced inflammation by assessing human red blood corpuscles (HRBC) membrane stabilization. In addition, we also evaluated the apoptotic mechanism of the EPHE. **Methods:** Hypotonicity induced heamolysis of HRBC membrane was used to assess membrane stabilization. The test solution consisted of stock erythrocyte (RBC) suspension 0.030ml mixed with 5ml of hypotonic solution (154mM NaCl in 10mM Sodium Phosphate Buffer at pH 7.4) containing different concentration of ethanolic poly herbal extract (EPHE) ranging from 200-1000 µg/ml. The control solution consisted of 0.030ml RBC suspension mixed with hypotonic buffer alone. The standard drug acetylsalicylic acid was treated similar as test at 200 and 400 µg/ml concentrations. The effect of EPHE on $HepG_2$/MCF7 cell cycle was investigated by flowcytometry for ascertaining its anticancer property. **Results:** EPHE significantly ($p<0.001$) inhibited lysis of HRBC induced by hypotonic solution and was observed to be 17.16 ± 0.022, 26.44 ± 0.027, 27.89 ± 0.029 and 29.62 ± 0.029 for doses of 400, 600,800 and 1000 µg/ml respectively. $HepG_2$ cells treated with IC_{50} concentration of EPHE displayed a dose-dependent accumulation of the G_0-G_1 region (i.e. loss of fragmented DNA) as an increase of dose from 40µg to 45µg/mL for 72.64%, 81.23% and an increase from 31.25µg to 45µg/mL for 16.57%, 16.75% of the S region for MCF7cells respectively. **Conclusions**: The result shows that EPHE has exhibited a significant anti-inflammatory activity studied by *in-vitro* membrane stabilization method and also produced remarkable effect on cell apoptosis in flowcytometric analysis. **Keywords:** HRBC, Membrane stabilization, Apoptosis, Poly herbal extract.
Keywords: Flowcytometric analysis, Poly herbal extract, In vitro Anti inflammatory.

Introduction

Inflammation is a normal protective response to tissue injury and it involves a complex array of enzyme activation, mediator release, fluid extravasations, cell migration, tissue breakdown and repair (1).The effect of synthetic and herbal anti-inflammatory agents on the stabilization of erythrocyte membrane exposed to hypotonic solution has been studied extensively. The erythrocyte membrane resembles lysosomal membrane and as such, the effect of drugs on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane (2). Programmed cell death or apoptosis is critical not only during development and regulation of cellular homeostasis but also in the pathogenesis of a variety of diseases including cancer, autoimmune disease, stroke and neurodegenerative disorders (3, 4).

The plant *Vitextrifolia* L., (Verbenaceae) is commonly known as common chaste tree English), *Nochi* (Kannada) and *Jalanirgundi* (Sanskrit). Leaves are commonly used as poultice for rheumatic pains, inflammations, sprains and fever. Roots are used as febrifuge and in the treatment of painful inflammations, cough and fever. Flowers are used against fever and fruits to correct amenorrhoea. This plant is known to possess various active constituents viz., essential oil, halimane-type diterpenes, vitetrifolins and several pharmacological properties have been studied viz., antipyretic, antibacterial, antiasthmatic and antiallergic and the leaf extract possesses anti-cancerous as well as anti oxidant activity (5, 6, 7, 8) *Vernoniacinerea* (L.) belongs to the *Asteraceae* family is an annual herb that grows in India, Bangladesh, Sri Lanka and Malay island. It is commonly known as 'little ironweed' in English, 'joanbeer', 'kukshim' in Bengali, 'puvamkurunnel' in Malayalam and 'sahadevi' in Sanskrit and Hindi. The plant is reported to be used in traditional medicine as tonic, astringent, diaphoretic, antirheumatic, anthelmintic and antidiarrheal, antimicrobial, antibacterial, antioxidant, anti-inflammatory, analgesic, antipyretic, antiflautulent,anti cancer, antispasmodic and antidiuretic properties (9,10). *Ocimum basilicum* (L.) belongs to the *Labiatae* family. It is popularly known as "Kali Tulsi" in Hindi, is a widely grown plant of Hindus. Different parts of the plant have been claimed to be valuable in wide spectrum of diseases. It is a small perennial, tropically growing shrub of Asian origin. Medicinal plants from *Labiatae* family are reported to possess anti-oxidant property. It is closely related with the prevention of degenerative illness, such as cardiovascular, neurological diseases, cancer and oxidative stress dysfunctions. And also it has been proved for its antipyretic, antiemetic, diuretic, and cardiotonic properties (11, 12, 13).

Materials and Methods

Chemicals

All chemicals used in this study were of analytical grade. They were procured from Sigma Chemical Co and Merck India Ltd, Mumbai, India.

Plant material and preparation of plant extract

Leaves of *Vitex trifolia*(L.), *Vernonia cinerea*(L.) and *Ocimum basilicum*(L.) were collected in the month of September 2010 from the surrounding area of Chennai, Tamilnadu, India. The plant material was taxonomically identified and confirmed by Botanist Dr.P.Jayaraman, Director, Plant Anatomy Research Centre, Chennai, Tamil Nadu. The collected leaves were cleaned dried under shade at room temperature and powdered using a mechanical grinder. 100g of coarse dried leaf powder of each plant was extracted separately by continuous hot extraction using Soxhlet apparatus with Ethanol. The step was repeated with fresh powder and solvent until the required quantity was achieved. The extract was concentrated in a rotary evaporator (yield 10.0% w/w).

Preparation of poly herbal extract

Ethanolic extract of equal quantity of leaves of *Vitex trifolia* (L.), *Vernonia cinerea* (L.), and *Ocimum basilicum* (L.) were mixed together and stored in a sterile air tight container under controlled temperature (28±1°C).

Cell culture and drug preparations

The Human Breast cancer cell lines (MCF-7) and Human Liver cancer cell lines (HEP-G_2) were purchased from National centre for cell sciences (NCCS) Pune. Cell lines were grown as monolayer cultures maintained in Dulbecco's modified Eagle's medium (GIBCO BRL) supplemented with heat inactivated 10% Fetal bovine serum (GIBCO BRL) and 2 mML-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin and maintained at 37°C in a atmosphere of 5% CO_2 incubator at 95% air humidified. The stock solution was prepared in DMSO and was stored at -20 °C until use. The dilutions were freshly made for each experiment with a final DMSO concentration of 0.1%. All the experiments were performed as three biological replicates for each concentration.

Screening of *In-vitro* Anti-Inflammatory Activity

In-vitro anti-inflammatory activity of EPHE was assessed by Human Red Blood Corpuscles (HRBC) membrane stabilizing method with slight modifications. The test sample consisted of stock erythrocyte (RBC) suspension 0.030ml mixed with 5ml of hypotonic solution (154mM NaCl in 10mM Sodium Phosphate Buffer at pH 7.4) containing different concentrations of EPHE ranging from 200-1000 µg/ml. The control solution consisted of 0.030ml RBC suspension mixed with hypotonic buffer alone. Acetyl salicylic acid was treated as standard at 200 and 400 µg/ml concentrations. The experiment was carried out in triplicate. The mixtures were

incubated for 10 minutes at room temperature, centrifuged for 10 minutes at 3000rpm and absorbance of the supernatant was measured spectrophotometrically at 540 nm (14).The percentage inhibition of haemolysis or membrane stabilization was calculated by following equation.

% Inhibition of haemolysis = 100 x [$A_1 - A_2 / A_1$]

Where: A_1 = Absorbance of control; A_2 = Absorbance of test /standard sample in hypotonic solution.

Flow cytometric analysis

To investigate the effect of EPHE on the cell cycle distribution, cells (1×10^5 cells/ml) were treated with IC_{50} concentrations of extract and cultured for 24h. The treated cells were harvested, washed with phosphate-buffer saline (PBS) and fixed in 75% ethanol at 4°C overnight. After washing twice with cold PBS, cells were suspended in PBS containing 40µg/ml propidium iodide (PI) and 0.1mg/ml RNase A followed by shaking at 37°C for 30min (15). Cells (10000) were analysed by flow cytometry (Becton-Dickinson San Jose, CA, USA) on the FL2-A detectors and analysed.

Statistical analysis:

Win MDI 2.9 computer software programme was used to assess the percentage of cells in apoptosis at different phases of cell cycle distribution. Data of anti inflammatory study was statistically analyzed by Student's t-test. $p<0.001$ vs. standard was considered to be statistically significant. The values are expressed as mean ±SD.

Results

Table 1: *In-vitro* anti-inflammatory effect of EPHE using HRBC method

S no	Treatment	Conc (µg/ml)	% Inhibition of haemolysis
1.	EPHE	200 400 600 800 1000	15.42 ± 0.021 17.16 ± 0.022* 26.44 ± 0.027* 27.89 ± 0.029* 29.62 ± 0.029*
2.	Acetyl-salicylic acid	200 400	31.65 ± 0.070 38.13 ± 0.101*

Values are mean ± SD, n=3, p<0.001, using Student's t-test.*

Effect of EPH E on hypotonicity induced haemolysis of HRBCs

Data from Table 1, shows that EPHE significantly ($p<0.001$) inhibited lysis induced by hypotonic solution. The percentage inhibition of haemolysis (17.16 ± 0.022, 26.44 ± 0.027, 27.89 ± 0.029 and 29.62 ± 0.029) was obtained for doses of 400, 600, 800 and 1000 µg/ml respectively. The inhibition of haemolysis was found to be dose dependent and was comparable with that of Acetyl-salicylic acid (Table 1).

Table 2. Percentage of cell Apoptosis at different cell cycle distribution of $HepG_2$ cell line

Sample ID: Control Gated Events: 9363			Gate: G_2 Total Events:10000	
Marker	Events	% Gated	% Total	Mean
All	9363	100.00	93.63	245.67
Sub G_0-G_1	156	1.56	1.56	150.12
G_0-G_1	6787	73.57	67.87	213.56
S	1564	15.84	15.64	312.78
G_2-M	856	9.03	8.56	423.45

Table 3.Effect of EPHE on percentage of cell apoptosis in $HepG_2$ at 40µg/ml

Sample ID: 40µg/ml			Gate: G_2	
Gated Events: 9437			Total Events:10000	
Marker	Events	% Gated	% Total	Mean
All	9437	100.00	94.37	245.95
Sub G_0-G_1	1756	17.86	13.56	134.78
G_0-G_1	7564	71.76	72.64	199.89
S	543	3.57	5.43	345.78
G_2-M	265	7.83	2.74	406.11

Table 4. Effect of EPHE on percentage of cell apoptosis in HepG2 at 45µg/ml

Sample ID: 45µg/ml			Gate: G_2	
Gated Events: 9379			Total Events:10000	
Marker	Events	% Gated	% Total	Mean
All	9379	100.00	93.79	223.45
Sub G_0-G_1	187	2.02	1.87	143.54
G_0-G_1	8123	86.76	81.23	198.78
S	324	3.57	3.24	332.23
G_2-M	745	7.65	7.45	416.78

Table 5. Percentage of cell Apoptosis at different cell cycle distribution of MCF-7 cell line

Sample ID: Control			Gate: G_2	
Gated Events: 8939			Total Events:10000	
Marker	Events	% Gated	% Total	Mean
All	8939	100.00	89.39	206.30
Sub G_0-G_1	411	5.71	4.11	152.16
G_0-G_1	7787	88.10	77.87	188.20
S	474	5.70	4.74	306.15
G_2-M	165	0.49	1.65	415.18

Table 6. Effect of EPHE on percentage of cell apoptosis in MCF-7 at 31.25µg/ml

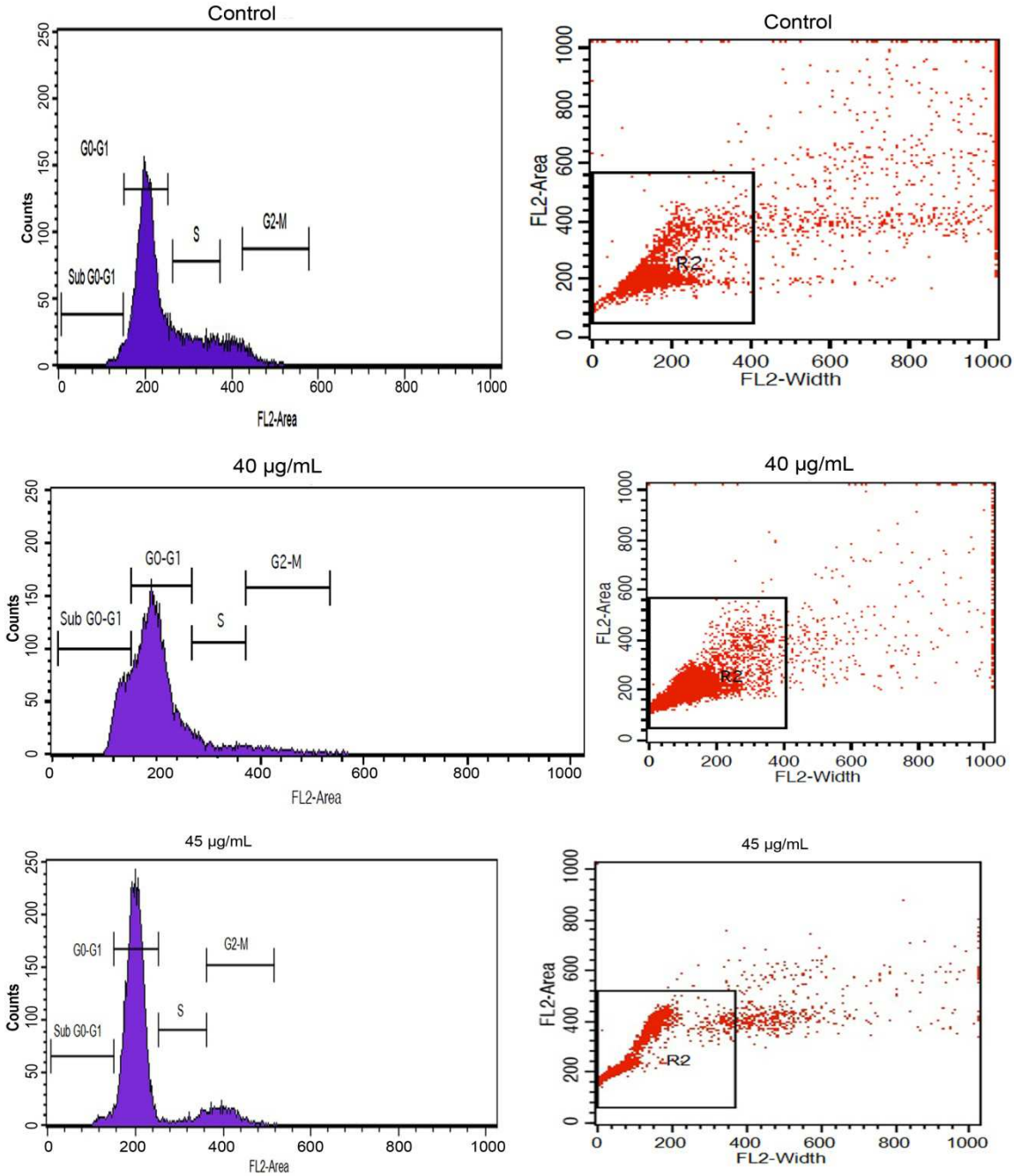
Sample ID: 31.25µg/ml			Gate: G_2	
Gated Events: 9782			Total Events:10000	
Marker	Events	% Gated	% Total	Mean
All	9782	100.00	97.82	224.49
Sub G_0-G_1	3	0.10	0.03	156.67
G_0-G_1	7552	77.47	75.52	214.12
S	1657	16.51	16.57	332.02
G_2-M	577	5.95	5.77	416.28

Table7. Effect of EPHE on percentage of cell apoptosis in MCF-7 at 45µg/ml

Sample ID: 45µg/ml			Gate: G_2	
Gated Events: 9778			Total Events:10000	
Marker	Events	% Gated	% Total	Mean
All	9778	100.00	97.78	214.67
Sub G_0-G_1	1767	19.37	17.67	134.78
G_0-G_1	4564	48.88	45.64	198.64
S	1675	17.22	16.75	287.89
G_2-M	1543	14.43	15.43	415.89

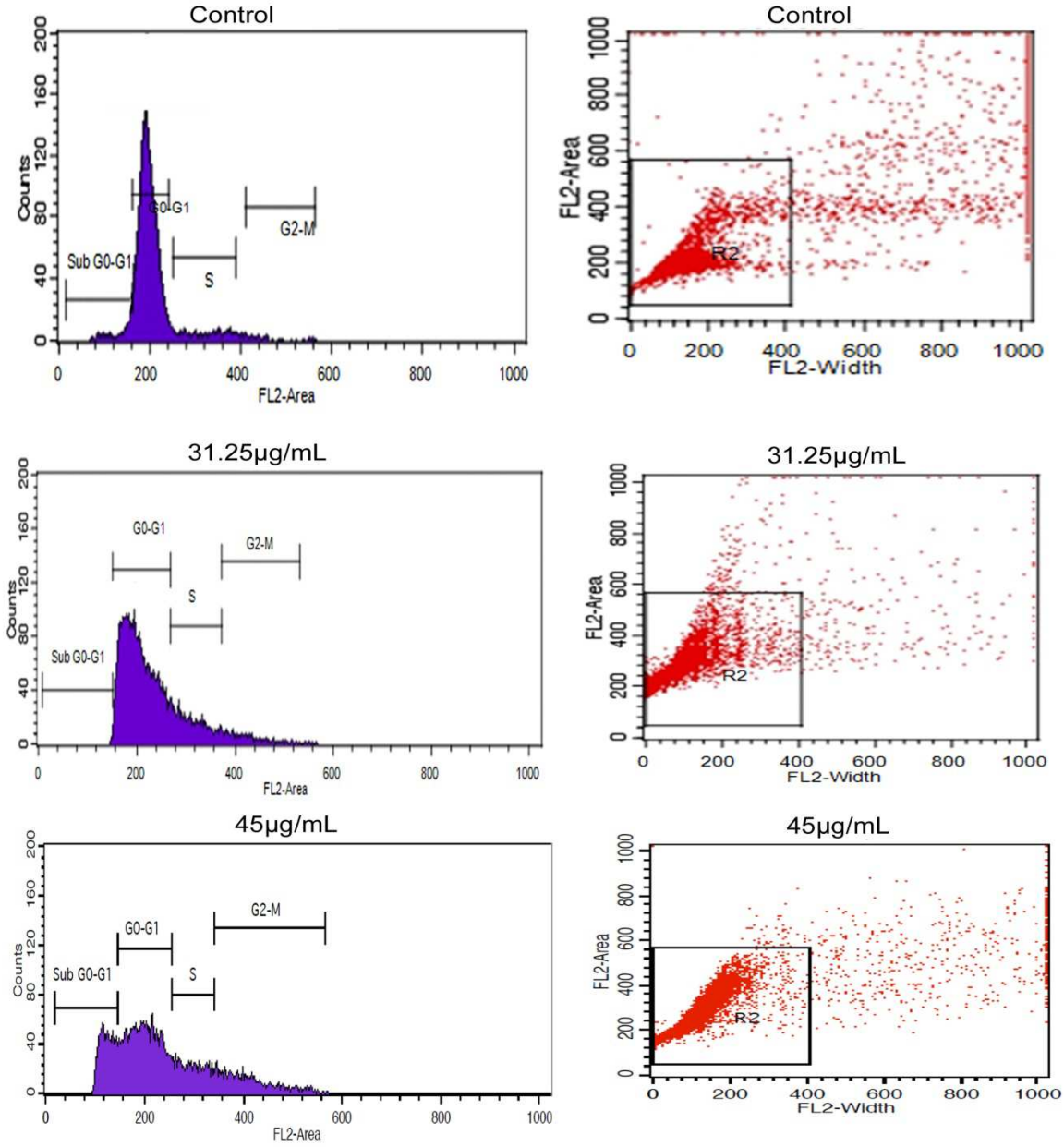
The effect of EPHE on $HepG_2$ and MCF7 cell cycle was investigated by flow cytometry. Results showed that $HepG_2$ cells (Fig.1) treated with IC_{50} concentration of EPHE displayed a dose-dependent accumulation of the G_0-G_1 region (i.e. loss of fragmented DNA), an increase of dose from 40µg to 45µg/ml for 72.64%, 81.23% similarly an increase from 31.25µg to 45µg/ml for 16.57%, 16.75% of the S region in MCF7cells respectively (Fig.2). After 24 h of treatment, IC_{50} concentration of EPHE induced cell cycle arrest in theG_0-G_1, S phase and resulted in a decline in the percentage of cells in the G_2/M phase (Table 2-7). These findings provided preliminary evidence that EPHE may activate the programmed cell death machine.

Fig.1. Effect of EPHE on the cell cycle distribution profile of the $HepG_2$ cells by flow cytometry



The cells were treated with EPHE for 24h and concentrations 40 and 45µg/ml, then stained with propidiumiodied (PI). The cycle distribution profile of the cells were determined by flow cytometry and graphs show the cell counts in Sub G_0-G_1,G_0-G_1,S and G_2/M phases.

Fig.2. Effect of EPHE on the cell cycle distribution profile of the MCF7 cells by flow cytometry



The cells were treated with EPHE for 24h and concentrations 31.25 and 45µg/ml, then stained with propidiumiodied (PI). The cycle distribution profile of the cells were determined by flow cytometry and graphs show the cell counts in Sub G_0-G_1,G_0-G_1,S and G_2/M phases.

Discussion

EPHE at concentrations of 200–1000 µg/ml protected the human erythrocyte membrane against lysis induced by hypotonic solution. In the inflammation phase, the lysosomal lysis releases their component enzymes that produce different degrees of inflammation. Non-steroidal anti-inflammatory drugs (NSAIDs) exert their beneficial effects by either inhibiting the release of lysosomal enzymes or by stabilizing the lysosomal membranes(16). Exposure of red blood cells (RBCs) to hypotonic medium, results in the lysis of the membranes, i.e., haemolysis accompanied by oxidation of hemoglobin (17). Since human red blood cell (HRBC) membranes are similar to lysosomal membrane components, the inhibition of hypotonicity induced red blood cell membrane lysis was taken as a measure of the mechanism of anti-inflammatory activity of EPHE. The hemolytic effect of hypotonic solution is related to excessive accumulation of fluid within the cell resulting

in the rupturing of its membrane. Injury to red cell membrane will render the cell more susceptible to secondary damage through free radical induced lipid peroxidation (18). Membrane stabilization leads to the prevention of leakage of serum protein and fluids into the tissues during a period of increased permeability caused by inflammatory mediators (19). EPHE perhaps stabilized the red blood cell membrane by preventing the release of lytic enzymes and active mediators of inflammation.

In this study, EPHE inhibited cell proliferation and induced cell apoptosis. Results ascertained that treatment with EPHE led to the G_0-$G_1$and S phase arrest and subsequently caused a decline in the G_2/M phase. The EPHE-induced apoptosis was confirmed by the cell cycle distribution assays.

Conclusion

The results of HRBC membrane stability assay of EPHE proved the potent anti-inflammatory properties by reducing the inflammatory injury and tissue damage. Also a significant dose-dependent increase in the percentage of apoptotic cells was noted in the EPHE-treated groups on the surface of cells and an accumulation of sub-G1 peak. Further study is required to investigate the underlying molecular mechanisms.

Acknowledgement

The authors wish to thank Dr. Ishari. K. Ganesh, Chancellor, Vels University, Chennai, Tamil Nadu, India for providing the facilities necessary to carry out the research work.

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