

Full Length Research Paper

## Ethyl acetate extract of *Hemigraphis colorata* leaves shows anti-inflammatory and wound healing properties and inhibits 5-lipoxygenase and cyclooxygenase-1 and 2 enzymes

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***Hemigraphis colorata*, a prostrate herb, is used in traditional medicine for the treatment of wounds. Hexane, ethyl acetate and methanol extracts of leaves were analyzed for anti-oxidant and free radical scavenging abilities, of which ethyl acetate extract showed maximum activity. In addition, ethyl acetate extract also displayed high amounts of phenolic and flavonoid contents. 5-LOX, COX-1 and COX-2, enzymes involved in inflammatory processes, were also inhibited by the ethyl acetate extract. Lipopolysaccharide induced expression of pro-inflammatory genes (TNF- $\alpha$ , COX-2 and IL-1 $\beta$ ) was inhibited in a dose dependent manner in human keratinocyte cell line (HaCaT cells) treated with the ethyl acetate extract. Further, the ethyl acetate extract induced the proliferation of HaCaT cells at lower concentrations with no alteration of morphology. Scratch wound healing model confirmed the wound healing capabilities of the ethyl acetate extract. This study highlights the potential of the ethyl acetate extract of *H. colorata* leaves as an anti-inflammatory and wound healing agent.**

**Key words:** *Hemigraphis colorata*, anti-inflammatory, wound healing, HaCaT cells.

### INTRODUCTION

Inflammatory process is the collective response of cytokines and immune cells to injury and infection. Cytokines and immune cells create a micro environment for either pro or anti-tumor progression (Hanahan et al., 2011). Evidence points to a connection between inflammation and a predisposition of cancer. Chronic inflammation without resolution can lead to the development of cancer (Kuper et al., 2000). Oxidative stress either due to infections, pollutants, diet and age related cellular senescence may be critical in non resolution of chronic inflammation leading to dysplasia. Cyclooxygenases (COXs) COX-1 and COX-2 isoforms catalyze critical step in the oxidation of arachidonic acid (AA) to the prostanoids. COX-1 is a constitutively

expressed enzyme and COX-2 is induced in the presence of inflammatory stimulus. Pharmacological inhibition of COX provides relief from the symptoms of inflammation and pain and hence inhibition of COX activity is targeted for anti-inflammatory activity in several diseases (Warner et al., 2004). Lipoxygenases (LOXs) are non-heme iron containing dioxygenases that initiate the synthesis of oxylipins to produce hydroperoxy fatty acids which in turn are converted to different compounds. Lipoxygenases are implicated in the pathogenesis of hypersensitivity, asthma, psoriasis, atherosclerosis and cancer. Leukotrienes (LTs) play a major part in the inflammatory process (Morham et al., 1995). They are synthesized via the 5-LOX pathway. 5-LOX is the key

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enzyme in leukotriene biosynthesis (Ford-Hutchinson et al., 1994). Non-steroidal anti-inflammatory drugs (NSAIDs) are effective as anti-inflammatory agents but they fail to address the oxidative stress component of inflammation. Hence there is a need for the development of anti-oxidant based anti-inflammatory agents.

The conventional NSAIDs have gastric side effects while (COX-2 inhibitors) COXIBs have cardiovascular side effects. Many of these side effects of COXIBs have been attributed to the drift of AA towards the LOX pathway and hence there is a search for 5-LOX and COX-2 dual inhibitors (CLOXIBs) and many CLOXIBs are at different stages of clinical trials. Chebulagic acid, a COX/LOX dual inhibitor was recently isolated from *Terminalia chebula* and demonstrated potent anti-inflammatory and anti-cancer effects (Reddy et al., 2009). A number of anti-oxidants like C-phycoerythrin (Reddy et al., 2000; Roy et al., 2007) are particularly effective as anti-inflammatory and anti-cancer agents. The present study made an attempt to identify 5-LOX and COX inhibitors from leaves of *Hemigraphis colorata*.

Skin acts as a physical barrier and protects the body from exposure to environmental insults like chemicals, allergens, irritants, pollutants and ultra violet light. Physical injury to skin can compromise the barrier function, making it susceptible for infections. Infections can prolong the wound healing process by increasing the inflammatory phase of the healing process (Werdin et al., 2009; Robson, 1997).

The prolonged inflammation reaction releases inflammatory cytokines and increase oxidative stress leading to tissue damage. Traditional plant extracts or natural products which have wound healing property along with anti-oxidant and anti-inflammatory properties might serve as good candidates for treatment of wounds with increased inflammatory reaction. In traditional folk medicine, leaf paste of *H. colorata* is used for wound healing.

*H. colorata*, belonging to family Acanthaceae, is used as a traditional medicine for wound healing in southern part of India, with the local name "murikoodi" and is cultivated in Manila, Philippines with the local Chinese name "dahong pula". Among the mullu kuruma tribes of Wayanad district of Kerala state of India, this is extensively used for wound healing (Silja et al., 2008). Leaf paste of *H. colorata* was shown to have anti-inflammatory effect on the carrageenan induced paw edema model (Subramoniam et al., 2001). Leaf extracts were also shown to have anti-bacterial properties (Anitha et al., 2012). *H. colorata* is being used for generations in traditional medicine for its wound healing properties but its extracts have not been explored for anti-inflammatory property or as inhibitors of 5-LOX and COX-1 and 2 enzymes. Wound healing property of the extracts was also not explored. So, we hypothesize that the extracts of this plant could be potential source of active principles and therefore this plant was selected and leaves were

used as a source for identification of bioactive extracts with anti-inflammatory and wound healing properties.

## MATERIALS AND METHODS

### Plant

Leaves of the herb *H. colorata* (Acanthaceae) were collected from Kerala, India. The specimen was identified by Prof. K. Seshagirao, Department of Plant Sciences University of Hyderabad, Hyderabad, 500046, India.

### Chemicals

Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL, USA. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide], protease inhibitor cocktail were from Sigma-Aldrich, India. All the other chemicals and reagents were purchased from local companies and are of molecular biology grade.

### Preparation of plant extracts

Compounds were extracted from the leaves of shade dried and powdered *H. colorata* by a soxhlation method into hexane, ethyl acetate and methanol solvents, in increasing order of polarity. 100 g of dried leaf powder was used for extraction. The fractions were concentrated by rotary evaporation. These fractions were dissolved in dimethyl sulfoxide (DMSO) before treatments. Details of percentage yield of each extract are given in Table 1.

### Estimation of total phenolic content

Total phenolic content in leaf extracts was determined using Folin-Ciocalteu reagent. Briefly, 10  $\mu$ l of each extract was added to 100  $\mu$ l of reagent and mixed thoroughly. 300  $\mu$ l of 20% Na<sub>2</sub>CO<sub>3</sub> solution was added after 8 min and the volume made up to 1 ml using distilled water. The reaction mixture was left in dark for 2 h followed by measurement of absorbance at 765 nm. Gallic acid was used as the standard. The total phenolic content was expressed as milligram (mg) of Gallic acid equivalent (GAE)/gram (g) of dry weight of leaf extract.

### Estimation of total flavonoid content

Total flavonoid content was determined by the method using aluminium chloride (AlCl<sub>3</sub>) (Rao et al., 2010). Briefly, 0.03 ml of 5% sodium nitrite (NaNO<sub>2</sub>) was added after the addition of 0.1 ml of plant extract to 0.3 ml distilled water. After 5 min of incubation at 25°C, 0.03 ml of 10% AlCl<sub>3</sub> and 2 ml of 1 mM sodium hydroxide (NaOH) were added. The final volume of reaction mixture was made up to 1 ml with distilled water and the absorbance was measured at 510 nm. Quercetin was used as a standard and the flavonoid content was expressed as mg of quercetin equivalent (QE)/gram (g) dry weight of leaf extract.

### Estimation of anti-oxidant capacity

The total anti-oxidant capacity was determined using the method of Rao et al. (2010). Briefly, various concentrations of leaf extracts were mixed with 1 ml of ammonium molybdate reagent solution

which contains 0.6 M H<sub>2</sub>SO<sub>4</sub>, 28 mM sodium phosphate and 4 mM ammonium molybdate. The reaction mixture was incubated for 90 min in a water bath at 95°C. After cooling to room temperature, the absorbance was measured at 695 nm. The total anti-oxidant capacity of extract was expressed in gram equivalent of ascorbic acid.

#### Estimation of total reducing power

Total reducing power was estimated by taking 1 ml of various concentrations of leaf extracts (100 to 500 µg/ml) as described previously (Oyaizu, 1986). These extracts were mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide [K<sub>3</sub>Fe(CN)<sub>6</sub>]. The mixture was incubated at 50°C for 20 min. 2.5 ml of 10% trichloroacetic acid was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. 2.5 ml from the upper layer was mixed with 2.5 ml distilled water and 0.5 ml of 0.1% ferric chloride (FeCl<sub>3</sub>) and the absorbance was measured at 700 nm.

#### Free radical scavenging assay

α,α-Diphenyl-β-picryl-hydrazyl (DPPH) assay was used for determining free radical scavenging ability of various leaf extracts. This method is based on the reduction of DPPH to DPPH-H (non radical form) in methanol in the presence of a hydrogen donating anti-oxidant. Reduction of free radical results in a color change which is measured spectrophotometrically. The reaction mixture (3.0 ml) consisted of 1.0 ml of DPPH in methanol (0.3 mM), 1.0 ml of extract and 1.0 ml of methanol. Reaction mixtures were incubated for 10 min in dark before absorbance was measured at 517 nm. Ascorbic acid was used as the standard.

#### 5-LOX assay

5-LOX was isolated from potato tubers as described earlier (Reddanna et al., 1990). The enzyme activity was measured with Clark's oxygen electrode on a Gilson model 5/6 oxygraph using a polarographic method. Briefly, the reaction mixture contained 2 ml of assay buffer, 940 µl of distilled water, 50 µl of enzyme and 10 µl of AA. The reaction was performed at 25°C. The rate of decrease in oxygen (O<sub>2</sub>) concentration in the reaction mixture was measured to calculate the enzyme activity. The enzyme activity was calculated as µM of O<sub>2</sub> incorporated/min per ml of enzyme and was expressed as units/ml. The effect of leaf extracts on enzyme activity was assessed by adding 30 µl of extract (dissolved in DMSO) to the reaction mixture and measuring the difference in the O<sub>2</sub> concentration after incubating for 5 min. The percentage inhibition of 5-LOX enzyme by extracts was calculated by comparison with the control (30 µl DMSO). The experiment was repeated by using different concentrations (1, 10 and 100 µg/ml) of each of the extract.

#### COX-1 and COX-2 assay

COX-1 was isolated from ram seminal vesicles as described by Hemler et al. (1976). Enzymatic activities of COX-1 and COX-2 were measured as given by Copeland et al. (1994), with slight modifications using a chromogenic assay based on the oxidation of N,N,N,N-tetra-methyl-p-phenyl diamine, (TMPD) during the reduction of prostaglandin G<sub>2</sub> (PGG<sub>2</sub>) to prostaglandin H<sub>2</sub> (PGH<sub>2</sub>) (Egan et al., 1976; Pagels et al., 1983). The assay mixture contains 975 µl assay buffer (100 mM Tris-HCl pH 8.0, 16 µM Hematin, 3 µM ethylene-di-amine-tetraacetic acid (EDTA)), 10 µl of leaf extract, 5 µl

of partially purified enzyme, 5 µl TMPD and 5 µl of 20 mM AA. COX synthesizes PGG<sub>2</sub> and PGH<sub>2</sub> from AA, through cyclooxygenase and peroxidase activities, respectively. During the conversion of PGG<sub>2</sub> to PGH<sub>2</sub>, one molecule of O<sub>2</sub> is released and it oxidizes TMPD, whose absorbance is measured at 610 nm. The enzyme activity was measured by estimation of the initial velocity of TMPD oxidation.

#### Cell culture

Human keratinocytes (HaCaT) were grown in DMEM supplemented with 10% (v/v) heat-inactivated FBS, 100 IU/ml penicillin and 100 mg/ml streptomycin. Cultures were maintained in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C. The cultured cells were passed every 72 h. The confluent culture was subcultured using 0.25% of trypsin for 90 s. Trypsin is inactivated using serum containing medium. The cells were again seeded at a density of approximately 2 × 10<sup>3</sup> cells/ml. Cell viability was determined by the Trypan blue dye exclusion method before seeding for each experiment.

#### Cell viability assay

The effect of the ethyl acetate extract on HaCaT cell viability was assessed by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HaCaT cells were seeded at a density of 5000 cells/well in a 96 well plate and incubated at 5% CO<sub>2</sub> at 37°C. Ethyl acetate extract at concentrations of 0.01, 0.05, 0.1, 0.5, 1.0, 10, 50, and 100 µg/ml were added to each well. DMSO was used as the control. After 24 h incubation, 20 µl of 5 mg/ml MTT was added to each well and incubated in dark for 3 h. Crystals were dissolved in 100 µl of DMSO and absorbance was read at 570 nm using a multi well plate reader. Percentage of viable cells at particular concentrations of extract was calculated by using the following formula:

$$\text{Viability (\%)} = (A_T / A_C) \times 100$$

Where the A<sub>T</sub> and A<sub>C</sub> are the absorbencies of treated and control cultures, respectively at 570 nm.

#### Activity of ethyl acetate extracts on morphology of HaCaT cells

HaCaT cells were seeded in a 60 mm petri dish and allowed to grow to 70% confluent in a DMEM base medium. The cells were treated with 0.1 µg/ml concentration of ethyl acetate extract. The cell morphology in comparison to untreated cells was observed under phase contrast microscope (Leica, Germany) and photographs were taken at 40x magnification after 24 h incubation.

#### Expression analysis of pro-inflammatory genes using real time PCR

The anti-inflammatory efficiency of ethyl acetate extract was tested at various concentrations (0.01, 0.1, 1.0, 10.0 µg/ml) on lipopolysaccharide (LPS) (10 µg/ml) stimulated HaCaT cells. Cells are incubated simultaneously with extract and LPS for 24 h. Here, untreated cells were used as the control. Total RNA was isolated from HaCaT cells using TRI-reagent (Sigma, St. Louis, MO, USA). The concentration was assessed by NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies, Delaware, USA) and the quality was checked by formaldehyde agarose gel electrophoresis. 5 µg of total RNA was transcribed to cDNA using superscript III following the manufacturer's instructions (Invitrogen, Carlsbad, CA,

**Table 1.** Yield of various leaf extracts of *Hemigraphis colorata* (per 100 g dried leaf extract).

Plant	Solvent	Extract after evaporation/lyophilization (g)
<i>Hemigraphis colorata</i> Blume	Hexane	1.2
	Ethyl acetate	4.4
	Methanol	10.4

USA) using oligo(dT) primers. Gene specific forward and reverse primers used in each respective polymerase chain reaction (PCR) are as follows: TNF $\alpha$  Fw: AGCCCATGTTGTAGCAAACC, TNF $\alpha$  Rv: CCAAAGTAGACCTGCCAGA; COX-2 Fw: AACAGGAGCATCCTGAATGG, COX-2 Rv: GGTCATGGAAGCCTGTGATG; IL-1 $\beta$  Fw: AGCTGATGGCCCTAAACAGA, IL-1 $\beta$  Rv: TCTTCAACACGCAGGACAG; 18S Fw: GCTACCACATCCAAGGAAGGCAGC, 18S Rv: CGGCTGCTGGCACCAGACTTG. Real-time studies were performed on an ABI Prism H7500 fast thermal cycler (Applied Biosystems, CA, USA). Reaction mixtures of 25  $\mu$ l contained 1  $\mu$ l of template (1:10 dilution), 10 pmol of each primer and 12  $\mu$ l of Power SYBR Green PCR master mix (Applied Biosystems). The real-time PCR results were presented as change in expression relative to control, using target gene  $C_t$  values normalized to that of 18S gene  $C_t$  values, based on the comparative  $C_t$  method (Schmittgen et al., 2008).

#### Activity of ethyl acetate extract on scratch wound model of HaCaT cells

Ethyl acetate extracts were tested for their ability to effect wound closure in a scratch wound model. HaCaT cells were seeded in 90 mm culture dishes marked with two horizontal lines to trace the point of observation and allowed to grow 100% confluent. A sharp scratch was created on the confluent culture tangentially to the horizontal lines. Scratched cells were removed from the plate and fresh medium was added. The cells were now treated with 0.1  $\mu$ g/ml concentration of ethyl acetate extract. 10% FBS served as positive control for wound healing activity. Untreated cells served as negative control. The cells were observed to estimate the ability of ethyl acetate extract to stimulate wound closure using phase contrast microscope and photographs were taken at 10 $\times$  magnification.

#### Statistical analysis

All the experiments were repeated thrice and the results were expressed as mean  $\pm$  standard error of mean (SEM) of three replicates for each sample. Statistical significance between control and treated groups were assessed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls' post hoc test. Statistical analysis was performed using Sigma Plot 11.0 software (Systat Software Inc., USA). A probability of  $P < 0.05$  was considered statistically significant.

## RESULTS

#### Estimation of total phenolic and flavonoid contents, anti-oxidant and free radical scavenging capacity of extracts

Percentage yield of the extracts into hexane, ethyl

acetate and methanol is given in Table 1. The total phenolic content in ethyl acetate and methanolic extracts was 23 and 41 mg, respectively as calculated using gallic acid as reference. The flavanoid content was highest in ethyl acetate extract (238 mg) followed by hexane extract (108 mg) in comparison to quercetin. Ethyl acetate extract also showed good anti-oxidant capacity of 660 mg followed by hexane extract with 120 mg comparable to ascorbic acid (Table 2). Total free radical scavenging power of the extracts was assessed by comparison with ascorbic acid, where ethyl acetate extract had the highest free radical scavenging capability with an  $IC_{50}$  of  $> 108$   $\mu$ g/ml (Table 2) and was concentration dependent (Figure 1A). All the extracts exhibited equal reducing power (100 to 500  $\mu$ g/ml of extracts equivalent to 10 to 50  $\mu$ g/ml of ascorbic acid) (Figure 1B). As ethyl acetate extract showed good anti-oxidant and free radical scavenging abilities, further experiments were performed with the ethyl acetate extract.

#### Effect of ethyl acetate extract on 5-LOX, COX-1 and COX-2 activities

The ethyl acetate extract from leaves of *H. colorata* obtained from soxhlation was checked for the ability to inhibit 5-LOX, COX-1 and COX-2 enzyme activity. Percentage inhibition of these enzymes at different concentrations of ethyl acetate extract is given in Table 3. Ethyl acetate extract displayed high inhibition of COX-1 ( $IC_{50}$  10  $\mu$ g/ml), whereas inhibition of 5-LOX and COX-2 was relatively low ( $IC_{50}$ : 90 and 48  $\mu$ g/ml, respectively) (Table 3).

#### Expression analysis of pro-inflammatory genes in the presence of ethyl acetate extract

As the ethyl acetate extract demonstrates significant anti-oxidant and free radical scavenging activities, anti-inflammatory activity of ethyl acetate extract was tested using human keratinocyte cell line (HaCaT cells) treated with lipopolysaccharide (LPS). These cells induced the expression of COX-2, TNF $\alpha$  and IL-1 $\beta$  upon treatment with LPS. Ethyl acetate extract decreased the LPS induced expression of pro-inflammatory genes COX-2, TNF $\alpha$  and IL-1 $\beta$  in HaCaT cells in a dose dependent manner (Figure 2) signifying anti-inflammatory activity of the extract. The levels of these cytokines in the cells treated with extract at 1 and 10  $\mu$ g/ml

**Table 2.** Estimation of total phenolic content (equivalent to gallic acid), flavonoid content (equivalent to quercetin), anti-oxidant capacity (equivalent to ascorbic acid) and IC<sub>50</sub> value of free radical scavenging capacity by DPPH assay in leaf extracts of *H. colorata*.

Extract	Amount of extract (g)	Phenolic content Eq. to gallic acid (mg)	Flavanoid content Eq. to quercetin (mg)	Anti-oxidant capacity Eq. to ascorbic acid (mg)	Free radical scavenging capacity IC <sub>50</sub> value (in µg/ml)
Hexane	1	--	108	120	>400
Ethyl acetate	1	23	238	660	>108
Methanol	1	41	5.03	90	>400

**Table 3.** Percentage inhibition and IC<sub>50</sub> values of 5-LOX, COX-1 and COX-2 activities of Ethyl acetate extract of *Hemigraphis colorata*.

Extract	Concentration (µg/ml)	Inhibition of 5-LOX activity (%)	IC <sub>50</sub> value (µg/ml)	Inhibition of COX -1 activity (%)	IC <sub>50</sub> value (µg/ml)	Inhibition of COX -2 activity (%)	IC <sub>50</sub> value (µg/ml)
Ethyl acetate	1	13.32	90	21.8	10	11.3	48
	10	23.38		49.0		27.9	
	100	53.21		84.2		64.1	

concentration were lower than the no LPS control.

#### Cell viability assay on HaCaT cells

Cell viability assay was performed using HaCaT cells by treating them with different concentrations of ethyl acetate extract. In cells treated with ethyl acetate extract, an increase in 30% more proliferation at 0.1 µg/ml was compared to untreated control. At higher concentrations, the extract showed cytotoxicity (Figure 3A). Morphology of cells treated with ethyl acetate extract (Figure 3C) showed no change compared to control (Figure 3B).

#### Wound healing potential of ethyl acetate extract in HaCaT cells

Scratch wound healing assay suggests role of

plant extracts in wound healing. HaCaT cells treated with ethyl acetate extract showed considerable wound healing capabilities compared to control cells (Figure 4). FBS treated cells showed complete wound closure (Figure 4C). These results indicate the wound healing potential of ethyl acetate extract of *H. colorata*.

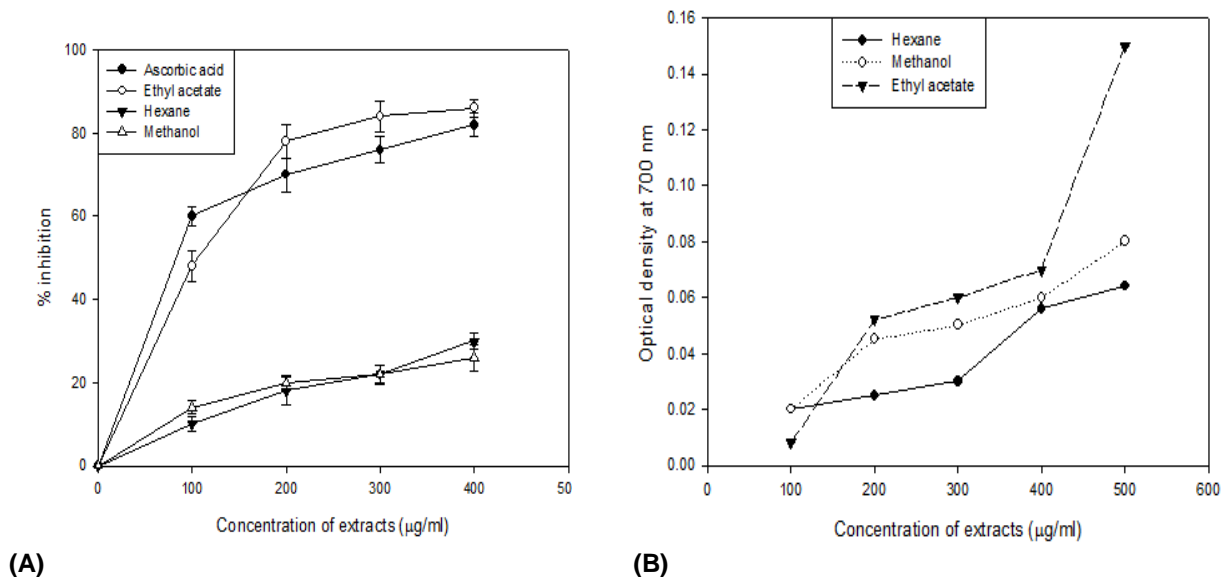
#### DISCUSSION

Plants have the ability to synthesize secondary metabolites with varied structures for their self defense. These secondary metabolites were being explored for treating human diseases. Natural products derived from plants have greatly varied structure and have been shown to act as anti-oxidants, free radical scavengers, enzyme inhibitors and regulators of gene expression (Reddy et al., 2003). Plant derived natural products still form a major source of treatment in

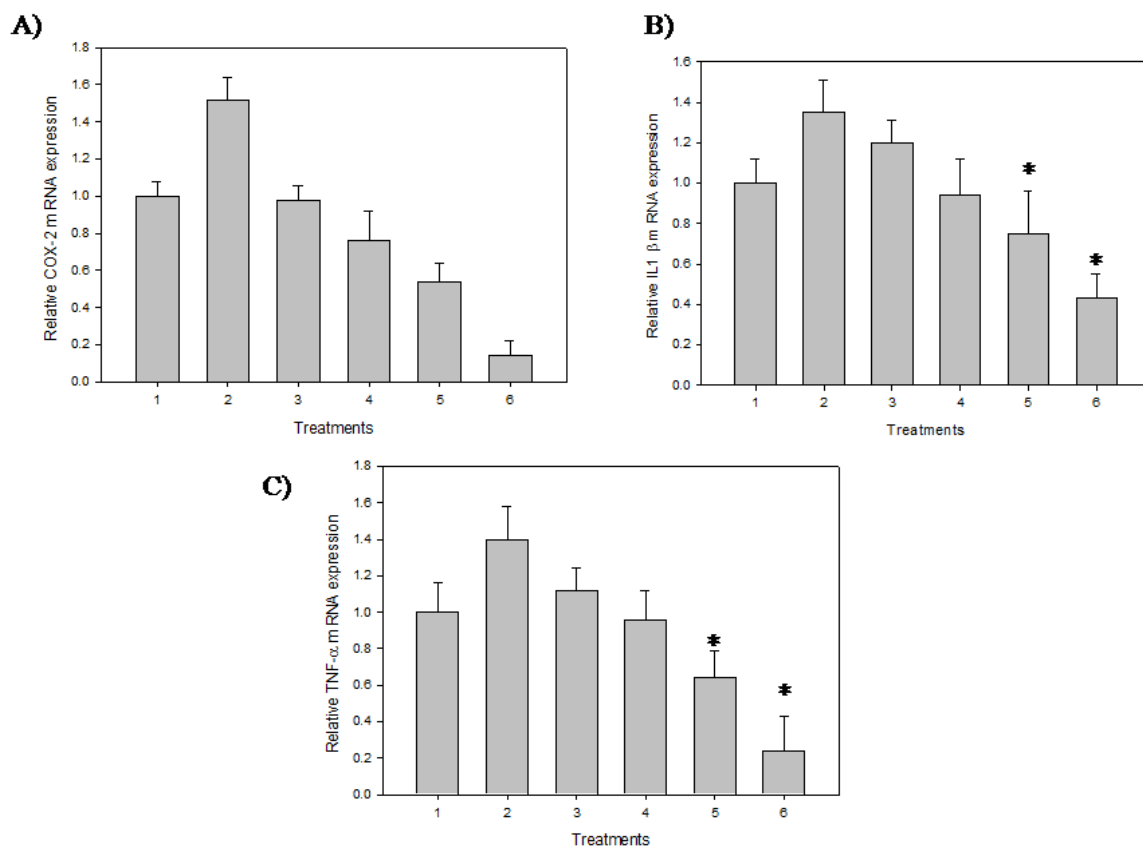
many communities worldwide. This traditional knowledge combined with the modern molecular methods can help us understand the active principles along with elucidation of mechanisms of action. The present study was undertaken to understand active principles and modes of action of *H. colorata* leaves which are traditionally used in southern parts of India for wound healing.

Among Mullu kuruma tribes of Wayanad district of Kerala state of India, the leaves of this plant are extensively used for wound healing (Silja et al., 2008). Leaf paste of *H. colorata* has been shown to have anti-inflammatory, anti-bacterial and wound healing properties (Subramoniam et al., 2001; Anitha et al., 2008).

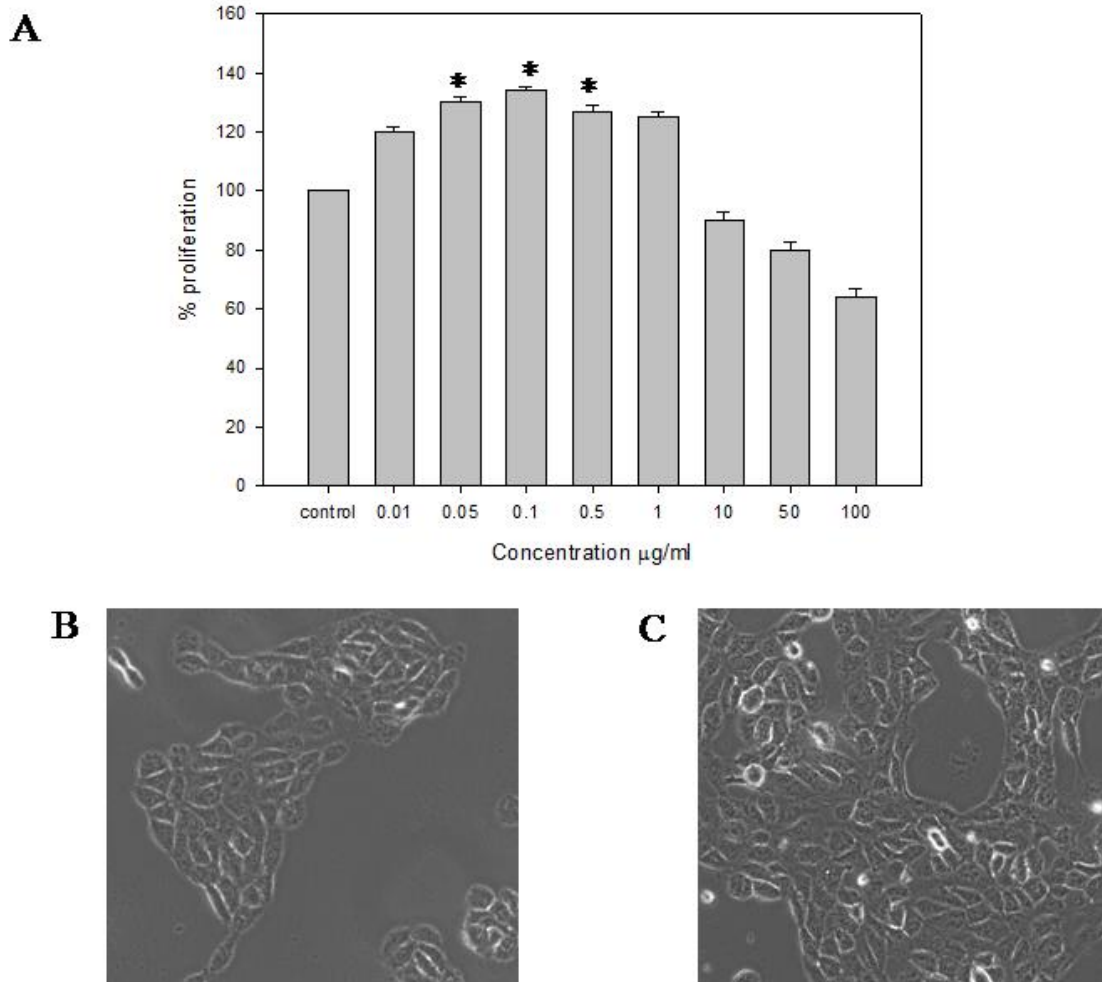
The lipid molecules, eicosanoids, derived from AA are the biologically active mediators involved in inflammation, cancer and other neurological and auto immune diseases. These eicosanoids include leukotrienes, thromboxanes and prostaglandins AA as substrate. 5-LOX



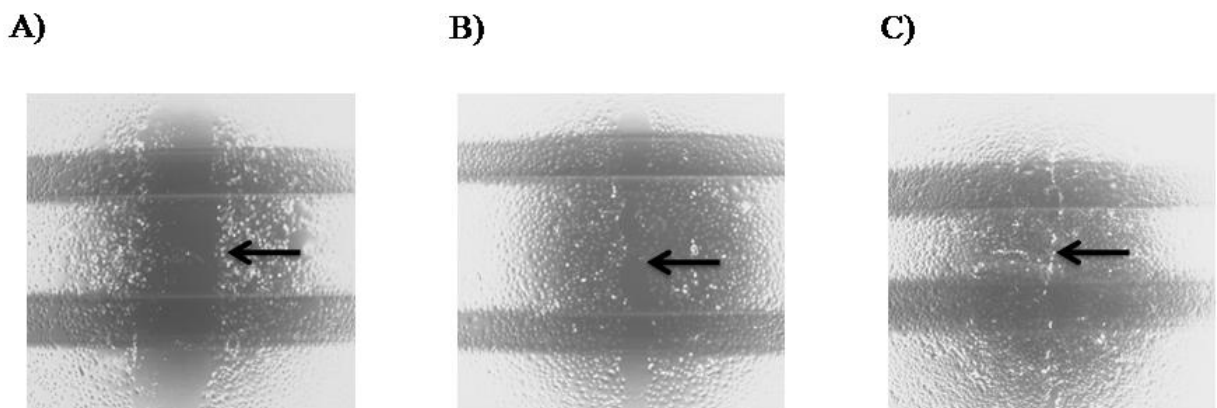
**Figure 1.** (A) Free radical scavenging capacity of ethyl acetate, hexane, methanol extract compared to standard (A) ascorbic acid. IC<sub>50</sub> values of ethyl acetate extract for DPPH radical scavenging was > 108 µg/ml. (B) Estimation of total reducing power of different *H. colorata* leaf extracts at different concentrations by utilizing potassium ferricyanide.



**Figure 2.** Effect of ethyl acetate extract on expression of pro-inflammatory genes. Real time PCR quantitative analysis of (A) COX-2; (B) IL-1β mRNA and (C) TNF-α expression in HaCaT cells relative to 18S rRNA expression at different concentrations of ethyl acetate extract given along with or without LPS (1- Control, 2- LPS (1 µg/ml), 3- LPS + 0.01 µg/ml, 4- LPS + 0.1 µg/ml, 5- LPS + 1 µg/ml, 6- LPS +10 µg/ml) by qRT-PCR is reported as fold change relative to untreated control calculated using  $2^{-\Delta\Delta CT}$ . X-axis represents different treatment conditions. Values are mean ± SEM, n = 3. \*Indicates significance at  $p < 0.05$ .



**Figure 3.** Cell viability assay on HaCaT cells using ethyl acetate extract (A) and phase contrast micrographs showing the morphological features of HaCaT cells treated with ethyl acetate extract (C) compared to untreated cells (B). Cells treated with ethyl acetate extract showed proliferation at lower concentrations and cytotoxicity at higher concentrations. Photographs were taken at 40x magnification.



**Figure 4.** Effect of ethyl acetate extract on wound healing. Scratch wound model shows 100% confluent HaCaT cells treated differently. (A) Control cells (B) Ethyl acetate extract treated cells (C) FBS treated cells. Arrow shows the point of wound.

synthesizes 5-HETE, leukotrienes and lipoxins that play an important role in acute inflammation. 5-LOX and COX-2 are co-expressed and up-regulated in inflammation and in many forms of human cancers, including colon, prostate, breast and lung cancers and hence these can be better targets for development of anti-inflammatory and anti-cancer drugs (Pommery et al., 2004). Due to these advantages, focus on developing compounds acting as COX-LOX dual inhibitors has increased.

In the present study, among different leaf extracts isolated from *H. colorata*, ethyl acetate extract exhibited considerable anti-oxidant, free radical scavenging and reducing capabilities. Ethyl acetate extract also have high quantities of phenolic and flavanoid compounds. Due to the high anti-oxidant and free radical scavenging properties, the ethyl acetate extract was selected for further analyses. Ethyl acetate showed inhibition of 5-LOX, COX-1 and COX-2 enzymes. Ethyl acetate extract may help in the resolution of inflammatory process by inhibiting these enzymes. The wound healing process depends on the resolution of the inflammatory phase (Werdin et al., 2009). As the ethyl acetate extract inhibited enzymes involved in inflammation, its ability to inhibit LPS induced pro inflammatory cytokines was assessed. Ethyl acetate extract decreased the LPS induced expression of pro-inflammatory genes like, TNF $\alpha$ , COX-2 and IL-1 $\beta$  in the HaCaT cells. Levels of these cytokines fell below the levels of no LPS control in the cells treated with ethyl acetate extract at 1 and 10  $\mu$ g/ml. This might be due the complete basal level inhibition of transcription of these genes by the extract. The potential of active principles of ethyl acetate extract to resolve inflammation in cases of prolonged infections can be further explored.

Proliferation of HaCaT cells was observed with ethyl acetate extract treatment at lower concentrations. At higher concentration of around 50 to 100  $\mu$ g/ml, this extract showed cytotoxicity. This effect can be explored for anti-proliferative potential of this extract after thorough investigation. Growth factors which induce cell proliferation also show wound healing abilities (Bielefeld et al., 2013). Ethyl acetate extract being pro proliferative induced wound healing in scratch wound model of HaCaT cells. Earlier studies showed leaf paste of *H. colorata* induced wound healing in mice model (Anitha et al., 2012). Anti-inflammatory properties were shown with leaf paste of *H. colorata* on the carrageenan induced paw edema model (Subramoniam et al., 2001). Leaf extracts were also shown to have anti-bacterial properties (Anitha et al., 2012).

Our studies showed that ethyl acetate extract of *H. colorata* has anti-oxidant and free radical scavenging activities. Ethyl acetate extract inhibited 5-LOX, COX-1 and COX-2 enzymes and LPS induced proinflammatory cytokines in human keratinocytes. Proliferative potential and wound healing capability was shown by ethyl acetate extract. Our studies showed for the first time the wound

healing properties of ethyl acetate extract of *H. colorata* leaves on *in vitro* wound healing model. Our findings showed for the first time that the leaf extracts of *H. colorata* inhibit enzymes involved in inflammation (5-LOX, COX-1 and COX-2) and LPS induced inflammatory cytokines. Further purification of ethyl acetate fraction for the identification of potent 5-LOX and COX inhibitors with anti-inflammatory and wound healing potential would increase the scope of the treatment regime of unresolved inflammation in wounds.

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## REFERENCES

- Anitha VT, Antonisamy JM, Jeeva S (2012). Anti-bacterial studies on *Hemigraphis colorata* (Blume) H.G. Hallier and *Elephantopus scaber* L. Asian Pac. J. Trop. Med. 5:52-57.
- Bielefeld KA, Amini-Nik S, Alman BA (2013). Cutaneous wound healing: recruiting developmental pathways for regeneration. Cell Mol. Life Sci. 70:2059-2081.
- Copeland RA, Williams JM, Giannaras J, Nurnberg S, Covington M, Pinto D, Pick S, Trzaskos JM (1994). Mechanism of selective inhibition of the inducible isoform of prostaglandin G/H synthase. Proc. Natl. Acad. Sci. U S A. 91:11202-11206.
- Dong YL, Fleming RY, Yan TZ, Herndon DN, Waymack JP (1993). Effect of ibuprofen on the inflammatory response to surgical wounds. J. Trauma. 35:340-343.
- Egan RW, Paxton J, Kuehl FA Jr (1976). Mechanism for irreversible self-deactivation of prostaglandin synthetase. J. Biol. Chem. 251:7329-7335.
- Ford-Hutchinson AW, Gresser M, Young RN (1994). 5-lipoxygenase. Annu. Rev. Biochem. 63:383-417.
- Hanahan D, Weinberg RA (2011). Hallmarks of cancer: the next generation. Cell 144:646-674.
- Hemler M, Lands WE (1976). Purification of the cyclooxygenase that forms prostaglandins. Demonstration of two forms of iron in the holoenzyme. J. Biol. Chem. 251:5575-5579.
- Kuper H, Adami HO, Trichopoulos D (2000). Infections as a major preventable cause of human cancer. J. Intern. Med. 248:171-183.
- Morham SG, Langenbach R, Loftin CD, Tian HF, Vouloumanos N, Jennette JC, Mahler JF, Kluckman KD, Ledford A, Lee CA, Smithies O (1995). Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. Cell 83:473-482.
- Oyaizu M (1986). Studies on product of browning reaction prepared from glucose amine. Jpn. J. Nutr. 44:307-315.
- Pagels WR, Sachs RJ, Marnett LJ, Dewitt DL, Day JS, Smith WL (1983). Immunochemical evidence for the involvement of prostaglandin H synthase in hydroperoxide-dependent oxidations by ram seminal vesicle microsomes. J. Biol. Chem. 258:6517-6523.
- Pommery N, Taverne T, Telliez A, Goossens L, Charlier C, Pommery J, Goossens JF, Houssin R, Durant F, Hénichart JP (2004). New COX-2/5-LOX inhibitors: apoptosis-inducing agents potentially useful in prostate cancer chemotherapy. J. Med. Chem. 47:6195-6206.
- Rao AS, Reddy SG, Babu PP, Reddy AR (2010). The antioxidant and antiproliferative activities of methanolic extracts from *Njavara* rice bran. BMC Complement Altern. Med. 10:4.
- Reddanna P, Whelan J, Maddipati KR, Reddy CC (1990). Purification of arachidonate 5-lipoxygenase from potato tubers. Methods Enzymol. 187:268-277.
- Reddy CM, Bhat VB, Kiranmai G, Reddy MN, Reddanna P, Madyastha KM (2000). Selective inhibition of cyclooxygenase-2 by C-



- phycocyanin, a biliprotein from *Spirulina platensis*. *Biochem. Biophys. Res. Commun.* 277:599-603.
- Reddy DB, Reddy TC, Jyotsna G, Sharan S, Priya N, Lakshmipathi V, Reddanna P (2009). Chebulagic acid, a COX-LOX dual inhibitor isolated from the fruits of *Terminalia chebula* Retz., induces apoptosis in COLO-205 cell line. *J. Ethnopharmacol.* 124:506-512.
- Reddy L, Odhav B, Bhoola KD (2003). Natural products for cancer prevention: a global perspective. *Pharmacol. Ther.* 99:1-13.
- Robson MC (1997). Wound infection. A failure of wound healing caused by an imbalance of bacteria. *Surg. Clin. North Am.* 77:637-650.
- Roy KR, Arunasree KM, Reddy NP, Dheeraj B, Reddy GV, Reddanna P (2007). Alteration of mitochondrial membrane potential by *Spirulina platensis* C-phycocyanin induces apoptosis in the doxorubicin resistant human hepatocellular-carcinoma cell line HepG2. *Biotechnol. Appl. Biochem.* 47:159-167.
- Schmittgen TD, Livak KJ (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc.* 3:1101-1108.
- Silja VP, Varma KS, Mohanan KV (2008). Ethanomedical plant knowledge of the Mulla kuruma tribe of Wayanad district, Kerala. *Indian J. Tradit. Know.* 7:604-612.
- Subramoniam A, Evans DA, Rajasekharan S, Sreekandan GN (2001). Effect of *Hemigraphis colorata* (blume) h. G. Hallier leaf on wound healing and inflammation in mice. *Indian J. Pharm.* 33:283-285.
- Warner TD, Mitchell JA (2004). Cyclooxygenases: new forms, new inhibitors, and lessons from the clinic. *FASEB J.* 18:790-804.
- Werdin F, Tennenhaus M, Schaller HE, Rennekampff HO (2009). Evidence-based management strategies for treatment of chronic wounds. *Eplasty* 9:e19.