In vitro Evaluation of Antiproliferative, Lipoxygenase and Xanthine Oxidase Inhibitory Activities of *Artemisia nilagirica* (C.B.Clarke) Pamp. Leaf Extracts

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Bhat *et al.*: Cytotoxicity and Inhibition of Proinflammatory Enzymes by *Artemisia nilagirica*

The current study was performed to screen the extracts of *Artemisia nilagirica* (C.B. Clarke) Pamp. for antiproliferative and antiinflammatory properties. Antiproliferative activity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Antiinflammatory activity was evaluated in terms of lipoxygenase and xanthine oxidase inhibitory activities. Results indicated that out of the 3 extracts tested, methanol extract was found to inhibit Y79 (human retinoblastoma) and MCF-7 (human breast adenocarcinoma) cell lines. The same extract was also found to inhibit lipoxygenase and xanthine oxidase enzymes in a concentration-dependent manner with an IC_{50} of 128.20±3.39 and 144.23±2.04 µg/ml for lipoxygenase and xanthine oxidase, respectively. The present study highlighted the antiproliferative potential of the plant against cancer cell lines. The extracts inhibited lipoxygenase and xanthine oxidase enzymes. Therefore, this plant could be a valuable source for anticancer and antiinflammatory molecules.

Key words: *Artemisia*, inflammation, lipoxygenase, xanthine oxidase, cytotoxic

Plants are well known as a treasure house of medicinally important secondary metabolites. Since ancient periods and across a number of civilizations, mankind has used this to their advantage to treat a variety of diseases and for maintaining proper health. The purpose of synthesis of secondary metabolites by plants is actually the coordination of biological interactions and defense against infections, predation and herbivory. But, many of these possess a remarkable therapeutic value and serve as preliminary material for the development of agents of modern medicine[1,2]. Natural products obtained from plants are well-accepted sources for a number of drugs currently used against several human ailments like cancer. Several plant-based molecules that include vinblastine, vincristine, taxol and camptothecin derivatives are used clinically to cure patients of various types of cancers[3]. The pursuit of new anticancer agents from plants is still a necessity to widen the range of availability of more effective and tumor-specific drugs. Biosphere harbors a great plant diversity with estimates of about 2 to 4.5 lakh species of flowering plants present in it. Approaches for selecting any plant for new bioactivity includes either random selection or taking a clue from ethnopharmacology or related traditional knowledge of the concerned plant[4,5].

Lipoxygenase (LOX) enzyme transforms linoleic, arachidonic and other polyunsaturated fatty acids into biologically active metabolites implicated in the inflammation-related and immunological reactions like hydroperoxy fatty acids and leukotrienes[6]. The products of this transformation are involved in the pathogenesis of pain, fever and sepsis[7]. Likewise, xanthine oxidase (XO) is a complex iron-sulfur flavoprotein that catalyses hydroxylation of hypoxanthine into xanthine and finally xanthine to uric acid[8]. Excess production of uric acid results in hyperuricemia and subsequently in its deposition in joints ultimately leading to inflammation with pain[9]. XO inhibitors that obstruct the production of uric acid within the body have been used for treating hyperuricemia. However, the currently used XO inhibitors suffer from certain side effects and limitations such as allergic reactions and nephropathy[10,11]. In this scenario, the utilization of compounds accessed from medicinal plants signifies an essential source of newer and clinically relevant drugs to cure of disorders arising due to hyperactivity of these enzymes[12].

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Artemisia nilagirica has been known to be used in traditional and folk medicine in the form of decoctions and infusions prepared from almost all parts of the plant to treat nervous disorders, gastrointestinal problems, epilepsy, urinary problems, skin disorders and inflammatory problems[13,14]. In addition to this, there have been reports of other biological properties from this plant like antioxidant[15], antibacterial[16], cytoprotective[17], anthelminthic, antifungal and antiulcer[13]. Essential oil obtained from this plant is also reported to have many medicinal applications[18]. The aim of the present study was to screen various extracts of *A. nilagirica* for antiproliferative activity on human cancer cell lines and inhibition of the enzymes, LOX and XO.

All solvents and chemicals used in the study were purchased from HiMedia and Merck (India). Standard drugs and chemicals were procured from Sigma Aldrich (St. Louis, USA). Roswell Park memorial institute (RPMI-1640), Dulbecco modified Eagle medium (DMEM), antibiotics and fetal bovine serum were obtained from Gibco (Invitrogen, USA). The solvents used in this study were of analytical grade.

The plant material was procured from Central Research Institute of Unani Medicine, Hyderabad, India. The material was properly identified by a taxonomist and a voucher specimen of this plant (UoH/VS/AN-3) has been well-preserved for future reference.

Fresh leaves of *A. nilagirica* were shade-dried completely at room temperature. The dried leaves (200 g) were ground to a coarse powder and was extracted with 2 l of n-hexane by maceration for 48 h with constant shaking. The extraction was repeated two more times each time using 2 l of fresh *n*-hexane. The extract was filtered through Whatman no.1 paper and concentrated on a rotary evaporator to dryness. The marc was further extracted with absolute methanol (>98 %) and 70 % alcohol in a similar manner. The extracts were dried, lyophilized and were labelled, hexane extract of *A. nilagirica* (ANH), methanol extract of *A. nilagirica* (ANM) and 70 % alcoholic extract of *A. nilagirica*

Accepted 20 February 2019
Revised 29 September 2018
Received 09 August 2018
Indian J Pharm Sci 2019;81(2):389-395

(ANA), respectively. Stock solutions of 50 mg/ml were prepared in DMSO for experiments.

The yield of plant extracts was calculated as: yield (%) = $(W_1/W_2)\times100$, where W_1 is the weight of the extract after complete removal of the solvent and W_2 is the weight of the dry plant powder. The yield of the extracts was calculated using new powder each time (n=3).

The cell lines, MCF-7 (human breast adenocarcinoma) and Y79 (human retinoblastoma) were acquired from the National Centre for Cell Sciences, Pune, India. MCF-7 cells were cultured in DMEM and Y79 in RPMI 1640 medium complemented with 10 % fetal bovine serum. Culture media contained penicillin (100 units/ml) and streptomycin (100 µg/ml). These cells were cultured at 37 ° in a humidified milieu in a 5 % CO_2 incubator.

The extracts of *A. nilagirica* were examined for antiproliferative activity using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay[19]. Cells were equally dispensed in triplicate in a 96-well plate at optimized concentrations of 1×10^5 cells/ml. The culture medium was removed from the wells after 24 h of incubation. Subsequently, 200 µl of fresh medium containing the extracts at increasing concentration (5-250 µg/ml for MCF-7 and Y79) was added to each well and further incubated for 48 h. After incubation, medium in each well was taken out carefully without disturbing the cells and replaced with fresh media along with 20 µl of 5 mg/ml MTT solution (in sterile filtered phosphate-buffered saline, pH 7.4). The cells were incubated at 37° for 4 h, and thereafter the medium from each well was removed and replaced with dimethyl sulfoxide (DMSO, 100 µl) to solubilize the formazan crystals. A vehicle control of 0.1 % DMSO was also used with all the cell lines. Curcumin was used as a positive control for Y79 and doxorubicin for MCF-7 cells. Finally, the absorbance of the wells was measured at λ 540 nm in a microplate reader after shaking for 30 s and the percent cell inhibition was calculated using the formula: % cell inhibition = 1− (OD of sample/OD of control)×100.

LOX assay was carried out according to the method of Chung *et al.* with a slight modification in 96 well plate[7]. An aliquot of 50 µl LOX (final concentration, 100 ng/ml) in 50 mM Tris HCl buffer (pH 7.4) was pre-incubated with 20 µl of the extracts or indomethacin (10-300 µg/ml) for 5 min at 25°. In this experiment, 50 µl of LOX solution and 20 µl of buffer with 0.5 % v/v DMSO were added to wells as a negative control. The reaction was triggered by adding 50 µl of 140 µM linoleic acid prepared in the same buffer. This reaction mixture was further incubated in dark for 20 min at 25°. The reaction was terminated by the addition of 100 µl freshly prepared ferric oxidation of xylenol orange (FOX) reagent. The composition of this reagent was sulfuric acid (30 mM), xylenol orange (100 µM), iron (II) sulfate (100 µM), methanol/water (9:1). Thereafter, the Fe^{3+}-dye complex was incubated for another 30 min at 25° for color development before being measured at λ 560 nm in a microplate reader (Infinite Pro, Tecan, Switzerland). Blanks contained the enzyme during incubation, but the substrate (linoleic acid) was added only after the FOX reagent. LOX inhibition in the presence of the extracts and standard was calculated according to the formula: % inhibition of LOX = $(A_c–A_b)–(A_s–A_b)/(A_c–A_b)$, where A_b; A_s and A_c are the absorbances of blank, sample and controls, respectively.

The generation of uric acid from xanthine was utilized to determine XO activity by the method of Spanou *et al.* with minor changes[20]. The total reaction mixture of 200 µl in the 96-well plate contained sodium phosphate buffer (pH 7.5), xanthine (4.8 µM) and EDTA (0.1 mM). The extract was added in increasing concentration of 10-400 µg/ml. An addition of XO (17.2 mU in deionized water) was made to initiate the reaction. The optical density was measured at λ 295 nm for 4 min at room temperature. Allopurinol, a known XO inhibitor, was used as positive control at a concentration of 50-400 µg/ml. A negative control was prepared with the assay mixture without the extract.

The results were presented as mean±standard deviation (SD) values derived from independent experiments done in triplicate. IC_{50} values were determined as the concentrations of the extracts or standards needed to inhibit enzyme activity by 50 %. Data calculations and statistical analysis were performed using GraphPad Prism software (version 7.0). Statistical differences among the samples were deduced by proper statistical tests (One-way ANOVA, Tukey's and Student's t-tests). *p* values ≤0.05 were set as statistically significant.

A wide range of the yields among extracts was observed showing considerable variations depending on the nature of extraction solvent. Maximum yield was for ANM at 9.09±1.08 % followed ANH at 5.23± 0.92 %, while the yield of ANA was only 4.13± 0.61 %. Physical appearance of the extracts was

somewhat semisolid paste type. The colour of ANH, ANM and ANA was yellowish-green, brownish-green and brown, respectively.

Out of the three extracts ANH, ANM and ANA, only ANH and ANM showed antiproliferative activity in a concentration-dependent manner from 10-250 µg/ml in most of the cancer cells used in this study. ANA, on the other hand, was least effective in killing the cancer cells and inhibited a negligible percentage of cancer cells even when applied at a higher concentration of 250 µg/ml. MCF-7 cells showed a concentration-dependent cell death with ANH and ANM with 87.12±6.39 and 96.84±2.21 % cell death at 250 µg/ml, respectively. Y-79 cell line was also affected to a considerable extent by ANM showing 65.79± 6.29 % cell death at 250 µg/ml with an IC_{50} value of 187.93±6.74 µg/ml. The cytotoxicity due to ANH was lower (29.14±4.23 %) even at the highest concentration used in the experiment (fig. 1). The percent inhibition and IC_{50} of extracts and positive controls are described in Table 1.

The extracts exhibited considerable variation in inhibiting LOX activity. ANM was most effective among the extracts and inhibited LOX activity up to 88.24±0.46 % at the highest concentration of 300 µg/ml with an IC_{50} value of 128.20±3.39 µg/ml. ANH also inhibited the activity of LOX although it was lower than that of ANM and its IC_{50} value was 196.07± 2.19 µg/ml. ANA was found to be the least effective and the highest concentration tested produced only 21.09±2.01 % inhibition. Indomethacin used as a standard inhibited LOX with an IC_{50} value of 60.97±2.31 µg/ml. The overall inhibitory of activity of the extracts is depicted in fig. 2A.

The extracts of *A. nilagirica* also showed considerable variation in inhibiting XO activity. In a manner similar to that observed in the LOX assay. ANH and ANM inhibited XO activity in a concentration-dependent manner. ANM showed the highest activity with an IC_{50} value of 144.23±2.04 µg/ml. ANH and ANM inhibited XO activity by 20.41±0.37 and 80.60±1.43 % at 400 µg/ml respectively, at the highest concentration tested. ANA was again least effective among the extracts tested and produced only 16.02±0.79 % inhibition of XO at 400 µg/ml. Allopurinol, an inhibitor of XO gave an IC_{50} value of 108.74±3.20 µg/ml. The results of this experiment are depicted in fig. 2B.

As per WHO, traditional medicine based on plants is popular in every corner of the world and after realizing the benefits, its uses are rapidly spreading even in developed countries[21]. In the contemporary era, plants not only have been a source of drugs for various life-threatening and other disorders, but several standardized crude extracts obtained from medicinal plants have in many cases been used to cure various ailments and much more showed promising biological activity[22,23]. Although several anticancer drugs currently in market trace their basic origin to plants, a good deal of research continues to identify more number of plant species that demonstrate anticancer activity with a huge focus on those being used as herbal remedies by people in developing countries. Most of the plant-based

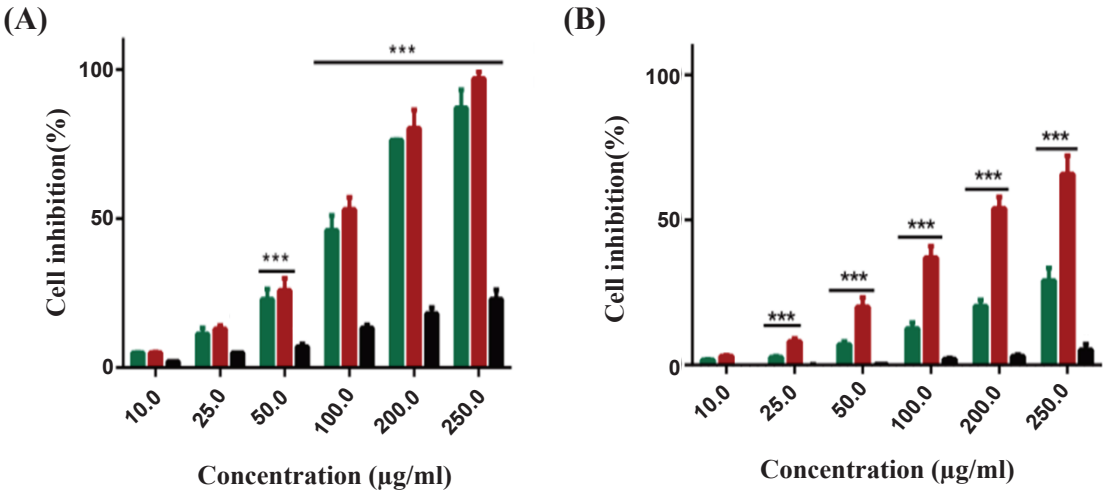

Fig. 1: Cytotoxic activity of *A. nilagirica* extracts against MCF-7 and Y79 cells
MTT assay was employed to study cytotoxicity of extracts (■) ANH, (■) ANM and (■) ANA against A. MCF-7 and B. Y79 cell lines. Cytotoxicity was estimated after treating the cells with various concentrations of extracts and incubation for 48 h. Values are mean±SD (n=3). ***p<0.001 as compared with control

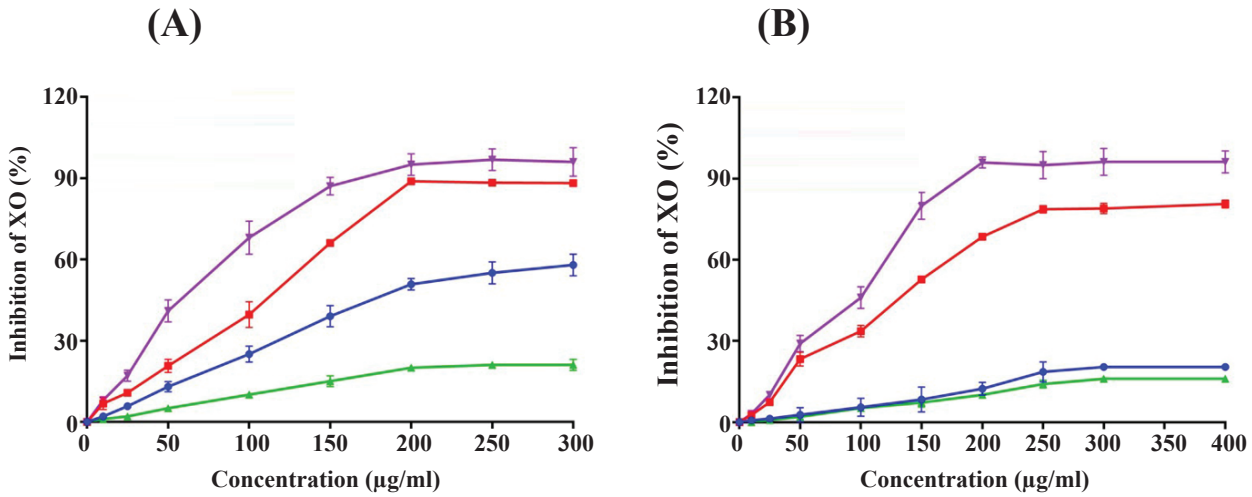

Fig. 2: Inhibitory effects of ANH, ANM and ANA on lipoxygenase and xanthine oxidase
Different concentrations of (—●—) ANH, (—■—) ANM, (—▲—) ANA were studied on the activity of (A) lipoxygenase and (B) xanthine oxidase enzymes in comparison to indomethacin (—▼—) and allopurinol (—▼—). Results were expressed as mean percent inhibition±SD, n=3

TABLE 1: IC_{50} VALUES FROM MTT ASSAY OF CELL LINES WITH EXTRACTS OF *A. NILAGIRICA*

Cell line	Extract/ standard extracts	IC_{50} value (µg/ml)	Inhibition at highest concentration (%)
MCF-7	ANH	104.16±4.11	87.12±6.39
	ANM	94.82±3.09	96.84±2.21
	ANA	ND	23.00±3.09
	Doxorubicin	0.76±0.08	96.28±3.49
Y79	ANH	ND	29.14±4.23
	ANM	187.93±6.74	65.79±6.29
	ANA	ND	5.28±1.92
	Curcumin	13.45±1.24	88.28±4.79

Values are expressed as mean±SD. ND stands for not determined. Highest concentration used for doxorubicin and curcumin is 1 and 20 µg/ml, respectively. Treatments were compared using one way ANOVA and probability values were calculated to less than or equal to 0.05 for these cell lines

chemical entities have an ideal property of being non-lethal to normal cells and more specifically cytotoxic to cancer cells and thus are ideal for drug development[3]. Chronic inflammatory disorders include; asthma, diabetes, rheumatoid arthritis, inflammatory bowel diseases, various neurodegenerative diseases and even cancer. The current treatment option is with antiinflammatory drugs, which often is not effective enough, but would result in unendurable side effects like gastrointestinal complications and immunosuppression[24]. A number of plants were reported to have been ethnopharmacologically used for management of inflammation-related ailments and injury healing. There is an increased interest in researchers worldwide to find new antiinflammatory remedies, which apart from possessing improved therapeutic index are also comparatively harmless[25]. It is generally believed that plant products can exhibit an antiinflammatory action by acting at various stages of inflammation. A significant number of molecules isolated from plants are already in clinical use and many more such as; curcumin, resveratrol and colchicine are in different stages of clinical trials[24]. *A. nilagirica* is an ethnopharmacologically important medicinal plant, which is a component of many Ayurvedic and Unani elixirs and the current study was an attempt to scientifically validate the medicinal claims.

Several *in vitro* screening methods like enzyme inhibition, cell-based assays have been widely employed for preliminary scientific validation of therapeutic claims of a plethora of medicinal plants used in traditional system of medicine, as food supplements and spices[26,27]. In this study with MTT assay, it was demonstrated that ANM was most potent in inhibiting the cell lines in a concentration-dependent manner followed by ANH. However, there is also a significant variation in the sensitivity of cells to different extracts. Y79 cell line was least affected by the extracts even by the most active ANM extract. These observations indicated that the antiproliferative potential with in the same plant depended upon which plant part was extracted and with which solvent[28,29]. Moreover, cancer cell lines were sensitive to different plant extracts to different extents. Such variations of cell lines to different extracts are very commonly encountered during cytotoxicity screening[30,31]. Previously, certain semi-purified fractions were reported to inhibit monocyte leukaemia cells and certain terpenoids and

flavonoids were identified in those fractions[32]. The cytotoxic components that have been identified in this plant included germacrene, caryophyllene, and borneol, all are components of the essential oil[30]. *A. nilagirica* is rich in phenolics, flavonoids, terpenoids and alkaloids which could contribute to the cytotoxic activity of the extracts as well as responsible for the variations observed in the antiproliferative activity of these extracts[14].

Inhibition of LOX activity could have potential therapeutic applications because leukotrienes play an important role in inflammatory disorders[33]. ANH and ANM extracts produced concentration-dependent inhibition of LOX activity and the activity was as good as that of indomethacin. Keeping in view the importance of this enzyme, continual attempts have been made from time to time to identify LOX inhibitors from medicinal plants[7,34]. *A. nilagirica* extracts have been reported to contain phenolics, flavonoids, terpenoids and other secondary metabolites, which could exhibit synergism while inhibiting LOX[14,35]. Hyperactive XO and oxidative stress would lead to increased levels of uric acid in tissue, which could result in rheumatic diseases such as acute inflammatory arthritis and gout[36]. ANM extract exhibited good XO inhibitory activity although at a higher concentration of 400 µg/ml. *A. nilagirica* plant has been traditionally known to cure inflammatory complications like arthritic gout and the LOX and XO inhibitory activities observed in this investigation provides a scientific explanation to support the plants use in traditional medicine[37,38].

To conclude, ANM exhibited cytotoxic activity against human breast cancer cells. The extracts also inhibited the enzymes LOX and XO in a concentration-dependent manner. The findings of this study warrant future research for isolation, identification and characterization of chemical constituents of this plant responsible for these activities which could serve as leads for developing better anticancer and antiinflammatory drugs.

Acknowledgements:

The authors MYB and MZG acknowledge the financial support in the form of Senior Research Fellowship from UGC and CSIR, India respectively. Authors would also like to thank DBT-CREBB, DST-FIST level I & II, DST-PURSE Phase I & II and UGC-SAP-CAS, UGC-XI plan seed money for supporting infrastructural facilities of Department of Plant Sciences and School of Life Sciences, University of Hyderabad. The authors also acknowledge the financial support provided by University of Hyderabad as UPE-II research grant to IAG lab (UH/UGC/UPE-II/Interface Studies/ Research Projects/R58). The authors also thank Dr. Insaf A. Qureshi, Department of Biotechnology and bioinformatics, School of life sciences, University of Hyderabad for his generous support during the research work.

Conflict of interest:

Authors have declared that they have no conflict of interest.

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