

Anti-inflammatory profile of alnus nepalensis leaves



**ASSIGN
BUSTER**

Anti-inflammatory profile of *Alnus nepalensis* leaves extracts: *In-vitro* and *in-vivo* study

Archana Saxena ¹ , Deepti Yadav ² , Anant kumar ¹ , Madan M. Gupta ² ,
Dnyaneshwar U. Bawankule ¹

ABSTRACT

Ethnopharmacological relevance: *Alnus nepalensis* has been used in the traditional medicine to treat the inflammatory conditions in India, Korea and China but it lacks proper pharmacological intervention.

Aim of the study: To evaluate the *in-vitro* and *in-vivo* anti-inflammatory profile of *A. nepalensis* leaves extracts to provide experimental evidence for its traditional use.

Materials and methods: Methnolic extract (*ANM*), hexane fraction (*ANH*), chloroform fraction (*ANC*) and butanol fraction (*ANB*) were obtained from air dried leaves of *A. nepalensis* . These were tested *in-vitro* against lipopolysaccharide (LPS)-induced inflammation in macophage cells by quantifying the production of pro-inflammatory cytokine. *ANB* , a most active fraction was further validated for *in-vivo* anti-inflammatory study. The quality and the reproducibility of *ANB* was ensured by high-performance liquid chromatography fingerprint.

Results: *In-vitro* results revealed that *ANB* exhibited significant anti-inflammatory effect. *In-vivo* study further confirmed its anti-inflammatory property by significant (P <0. 05) inhibition of carrageenan-induced paw

edema in dose dependant manner. The HPLC analysis of *ANB* showed the presence of diarylheptanoids.

Conclusion: These results revealed that *ANB*, a butanol fraction of *A. nepalensis* possess the significant anti-inflammatory activity which supporting the traditional usage of the plant to treat inflammatory diseases.

Key words: *Alnus nepalensis*, Diarylheptanoids, Inflammation, Macrophages, Rats

1. Introduction

Alnus nepalensis belongs to the family Betulaceae, an alder species and open-pollinated forest tree found in the hilly regions (Chauhan and Misra, 2002). Almost all plants of genus *Alnus* have been used in the traditional medicine in India (Changkija 1999), Korea (Lee, 1996) and China (Vo, 1997) to treat inflammatory diseases, diarrhea, dysentery, and fever.

Platyphyllenone, alusenone, hirustenone, and hirsutanonol are the important pharmacological active diarylheptanoids isolated from *Alnus* species (Lee et al., 2000). Hirustenone and hirsutanonol show anti-influenzal (Tung et al., 2010), anti-oxidant (Kuroyanagi et al., 2005), cytotoxic (Choi et al., 2008), hepatoprotective (Park et al., 2010), anti-inflammatory activity (Kim et al., 2005). Furthermore, hirustenone has been reported to prevent cytokine and chemokine-mediated immune cell function and inflammatory reaction and was found to be an attractive starting point for the development of a topical drug for T cell-based anti-atopic dermatitis due to its calcineurin inhibitory effects (Joo et al., 2009). Alusenone and platyphyllenone showed significant antioxidant and hepatoprotective effects (Tung et al., 2010). Recently, our <https://assignbuster.com/anti-inflammatory-profile-of-alnus-nepalensis-leaves/>

group has reported that diarylheptanoids isolated from *A. nepalensis* leaves exhibiting *in-vitro* and *in-vivo* Antifilarial activity (Yadav et al., 2013).

Inflammation is the first response of the immune system to infection, injury or irritation, and macrophages play a crucial role during the inflammatory process (Lalenti et al., 1993). Chronic inflammation is a prolonged, dysregulated and maladaptive response that involves active inflammation, tissue destruction and attempts at tissue repair. Such persistent inflammation is associated with many chronic human diseases, including allergy, atherosclerosis, gastro-enteritis, arthritis, autoimmune diseases etc . To overcome the challenges of inflammatory disorders, several classes of anti-inflammatory drugs have been used. Despite considerable progress in the treatment of various inflammatory diseases and disorders using modern therapeutic agents, search for newer drugs continues because the existing synthetic drugs have several side effects. The current trend in research is the investigation of medicines of plant origin because of their affordability and accessibility with minimal side effects (Calixto, 2005). Considering the traditional use of *A. nepalensis*, this study was undertaken to evaluate its *in-vitro* and *in-vivo* anti-inflammatory profile for its ethnopharmacological validation.

2. Materials and Methods

2. 1. Plant material

Fresh leaves of *A. nepalensis* were collected from Kausani, Uttrakhand, India. The specimen was identified by Dr. S. C. Singh (Taxonomist) and was

deposited at the herbarium of Central Institute of Medicinal and Aromatic Plants Lucknow, India under voucher number 13644.

2. 1. 1. Extract preparation

The air-dried powdered leaves of *A. nepalensis* (600 g) were macerated with metanol (2 L) overnight and evaporated in vacuo to yield crude extract (200 g). This crude extract was suspended in water (600 ml) and extracted successively with hexane (3×500 ml), chloroform (3×500 ml) and butanol (3×500 ml). Vacuum concentration yielded hexane fraction (32 g), chloroform fraction (20 g) and butanol extract (90 g). Plant extract and fractions were coded as methnolic extract(*ANM*), hexane fraction(*ANH*), chloroform fraction(*ANC*), butanol fraction(*ANB*) for conducting the blind pharmacological study.

2. 2. Cells and cell culture

Primary cells were isolated and cultured as described (Singh et al., 2012). In brief, the macrophage cells were collected from the peritoneal cavities of mice after an intra peritoneal injection of 1. 0 ml of 1% peptone (BD Biosciences, USA) 3 days before harvesting. Mice were euthanized by cervical dislocation under ether anesthesia and peritoneal macrophage cells were obtained by intra-peritoneal (i. p.) injection of Phosphate Buffer Saline (PBS), pH-7. 4. The viability of the cells was determined by trypan blue exclusion and the viable macrophage cells at the concentration of 0.5×10^6 live cells/ml were used for the experimentation. The cells were suspended in RPMI 1640 medium (Sigma-Aldrich, USA) containing 10% heat-inactivated

fetal calf serum (Gibco, USA), 100 U/ml of penicillin and 100 µg/ml of streptomycin and incubated in a culture plate (Nunc, Germany) at 37 °C in 5% CO₂ in an incubator. Cells were pretreated with 10, 30 and 100 µg/ml of extracts and standard anti-inflammatory drug, Dexamethasone (Sigma Aldrich, USA) at 5 µg/mL for 30 min. The cells were stimulated with LPS (1 µg/mL). After incubation with LPS for 24h, supernatants were collected and immediately frozen at -80 °C. Harvested supernatants were tested for quantification of pro-inflammatory cytokines (TNF-α and IL-6) by ELISA according to the manufacturer's instructions (BD Biosciences, USA).

2. 3. Cytotoxicity study

Murine peritoneal macrophage cells were treated with potent extract (10, 30 and 100 µg/ml) for 24 h. The MTT (stock solution 5 mg/kg) was added to a final concentration of 0.5 mg/ml and cells were incubated for an additional 4h. The medium was removed and formazon precipitate was solubilized in 100µl DMSO for 10 min and absorbance was measured at 570 nm (molecular device, USA). The amount of color produced is directly proportional to the number of viable cells. Cell cytotoxicity was calculated as the percentage of MTT absorption as follows: Percentage (%) of survival = (mean experimental absorbance/mean control absorbance × 100).

2. 4. Experimental animals

Male Swiss albino mice and Wistar rats were used for the *in-vitro* and *in-vivo* inflammation study. The animals were bred and maintained in-house under standard environmental conditions (25±2 °C; 12/12 h light/dark cycle). All

studies were conducted after obtaining prior approval for animal studies from CPCSEA, Government of India through the Institutional Animal Ethics Committee.

2. 4. 1. Carrageenan-induced rat paw edema in rats

Rats were injected a 100 μ l saline containing 1% w/v carrageenan as a phlogistic agent into the sub plantar region of the left hind footpad to initiate an acute inflammation. Vehicle treated group of rats received the same volume of vehicle at the same time. Before injecting carrageenan, the volume of each left footpad was measured using an animal plethysmometer (IITC lifescience, USA) and the reading of the footpad swelling after injection was carried out at 3 h. To test the effect of ANB, the rats were orally fed (30, 100, 300 mg/kg) one hour prior to the injection of carrageenan. Rats treated with dexamethasone (3 mg/kg) serve as positive control. Increase in paw volume was calculated as the difference between the paw volume reading before and 3h after the injection of carrageenan and the percentage inhibition of edema was calculated as follow:

Percent inhibition of The percentage edema= $(V_c - V_t)/V_c \times 100$

Where, V_c = Increased in Paw Volume (ml) in vehicle treated rats

V_t = Increased in Paw Volume (ml) in extracts treated rats

2. 6. HPLC fingerprinting

The HPLC fingerprinting was performed using a Shimadzu modular HPLC system Shimadzu, Japan) consisting of an analytical column (Waters

<https://assignbuster.com/anti-inflammatory-profile-of-alnus-nepalensis-leaves/>

Spherisorb ODS-2, 250 x 4.6 mm, 10 µm), pumps (LC-10AT), autoinjector (SIL-10AD) and PDA (SPD-M10A). Separation of butanol extract and artificial mixture of compounds was carried out by using an isocratic solvent system with a flow rate of 1 ml/min. Methanol and water (40:60 (v/v) ratios were used as mobile phase and eluents were monitored at 275 nm. A 10 µl aliquot of the sample solution was injected on the column.

2.7. Statistical analysis

Results were presented as the means \pm SEM and analyzed using GraphPad Prism 4. Student's paired t test was performed to compare the results of vehicle and treatments. P value <0.05 was considered statistically significant.

3. Results and Discussion

We have evaluated the *in-vitro* anti-inflammatory status of extract and fractions isolated from the leaves of *A. nepalensis* against the production of pro-inflammatory cytokines (TNF- α and IL-6) using ELISA technique in LPS-induced inflammation in macrophage cells at the concentration 10, 30 and 100 µg/ml. Production of pro-inflammatory cytokines was significantly ($P < 0.05$) increased in LPS-stimulated cells when compared with normal unstimulated cells. Among the extract and fractions, *ANB* possessed most promising anti-inflammatory agent which was able to significantly inhibit the production of pro-inflammatory cytokines (TNF- α and IL-6) in dose dependent manner (Table 1). Cytotoxicity study of *ANB* using MTT assay demonstrated that it is not toxic to the normal cells (supplementary fig.). To substantiate the physiological function of most potent fraction, we have further evaluated <https://assignbuster.com/anti-inflammatory-profile-of-alnus-nepalensis-leaves/>

its therapeutic efficacy in *in-vivo* system using carrageenan-induced paw edema in rats. The oral administration of *ANB* (30, 100 and 300 mg/kg respectively) showed significant inhibition of paw edema (39.73, 47.02 and 55.62 % respectively) after 3 h (Fig. 1.). The HPLC analysis showed the presence of hirsutanonol 5-O- β -D-glucopyranoside (Tr 7.3 min) oregonin (9.2 min) and Platyphylloside (Tr 17.8min) in the *ANB*. The compounds were identified in comparison of their retention time and UV spectra with external standards (Fig. 1).

In a previous study, it was established that the *Alnus* species have been found to be a good source of active diarylheptanoids (Yadav et al., 2013). Naturally occurring diarylheptanoids had demonstrated to possess anti-inflammatory activities (Jin et al., 2007) in laboratory animals (Mukhopadhyay et al., 1982) as well as human (Kohli et al., 2005) due to significant inhibition of inflammatory mediators such as cyclooxygenase (Lee et al., 2000), TNF- α , NO production in LPS-stimulated macrophage cells via suppressing activities of NF-kB (Lee et al., 2005). The production of pro-inflammatory cytokines in LPS-stimulated macrophage cells isolated from mice and also able to reduced the carragenan-induced paw edema in rats by *ANB* treatment revealed that it may be due to presence of the active diarylheptanoids in *ANB*, a butanol fraction of *A. nepalensis* leaves. These findings further confirm the use of *A. nepalensis* as an anti-inflammatory agent.

4. Conclusion

The result of this study revealed the further validation of traditional use of *A. nepalensis* as an anti-inflammatory agent. Study confirms its suitability as a candidate for further studies to obtain a prototype for anti-inflammatory drugs.

Acknowledgements

We acknowledge the Council of Scientific and Industrial Research (CSIR), New Delhi, India for financial support through XII FYP networking project BSC 0203 and DST, Govt of India (SR/ FT/LS-85/2011). We are thankful to Director, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, India for rendering essential research facilities and support.

References

- Calixto, J. B., 2005. Twenty-five years of research on medicinal plants in Latin America: a personal view. *Journal of Ethnopharmacology* 100, 131-134.
- Changkija, S., 1999. Folk medicinal plants of the Nagas in India. *Asian Folklore Studies*, pp. 205-230.
- Chauhan, V. S., Misra, A. K., 2002. Development of molecular markers for screening of *Alnus nepalensis* (D. Don) genotypes for the nitrogenase activity of actinorhizal root nodules. *Molecular Genetics and Genomics* 267, 303-312.
- Choi, S. E., Kim, K. H., Kwon, J. H., Kim, S. B., Kim, H. W., Lee, W. M., 2008. Cytotoxic activities of diarylheptanoids from *Alnus japonica*. *Archives of Pharmacal Research* 31, 1287-1289.

Jin, W., Cai, X. F., Na, M., Lee, J. J., Bae, K., 2007. Diarylheptanoids from *Alnus hirsuta*

inhibit the NF-kB activation and NO and TNF-alpha production Biological & Pharmaceutical Bulletin 30, 810-813.

Joo, S. S., Kim, S. G., Choi, S. E., Kim, Y. B., Park, H. Y., Seo, S. J., Choi, Y. W., Lee, M. W., Lee, D. I., 2009. Suppression of T cell activation by hirsutenone, isolated from the bark of *Alnus japonica*, and its therapeutic advantages for atopic dermatitis. European Journal of Pharmacology 614, 98-105.

Kim, H. J., Yeom, S. H., Kim, M. K., Shim, J. G., Paek, I. N., Lee, M. W., 2005. Nitric oxide and prostaglandin E2 synthesis inhibitory activities of diarylheptanoids from the barks of *Alnus japonica* Steudel. Archives of Pharmacal Research 28, 177-179.

Kohli, K., Ali, J., Ansari, M. J., Raheman, Z., 2005. Curcumin: a natural antiinflammatory agent. Indian Journal of Pharmacology 37, 141-147.

Kuroyanagi, M., Shimomae, M., Nagashima, Y., Muto, N., Okuda, T., Kawahara, N., Nakane, T., Sano, T., 2005. New diarylheptanoids from *Alnus japonica* and their antioxidative activity. Chemical and Pharmaceutical Bulletin 53, 1519-1523.

Lalenti, A., Moncada, S., DiRosa, M., 1993. Modulation of adjuvant arthritis by endogenous nitric oxide. British Journal of Pharmacology 110, 701-706.

Lee, M. W., Park, M. S., Jeong, D. W., Kim, K. H., Kim, H. H., Toh, S. H., 2000.

Diarylheptanoids from the leaves of *Alnus hirsuta* Turcz. Archives of Pharmacal Research 23, 50-53.

Lee, S. J., 1996. Korea Folk Medicine. Seoul National University Publishing Center Press, Seoul, Korea, pp. 40.

Lee, S. L., Huang, W. J., Lin, W. W., Lee, S. S., Chen, C. H., 2005. Preparation and antiinflammatory activities of diarylheptanoid and diarylheptylamine analogs; Bioorganic & Medicinal Chemistry 13: 75-81.

Mukhopadhyay, A., Basu, N., Ghatak, N., Gujral, P. K., 1982. Anti-inflammatory and irritant activities of curcumin analogues in rats. Agents Actions 12, 508-515.

Park, D., Kim, H. J., Jung, S. Y., Yook, C. S., Jin, C., Lee, Y. S., 2010. A new diarylheptanoid glycoside from the stem bark of *Alnus hirsuta* and protective effects of diarylheptanoid derivatives in human HepG2 Cells. Chemical and Pharmaceutical Bulletin 58, 238-241.

Singh, R., Trivedi, P., Bawankule, D. U., Ahmad, A., Shanker, K., 2012. HILIC quantification of oenotheralanosterol A and B from *Oenothera biennis* and their suppression of IL-6 and TNF- α expression in mouse macrophages. Journal of Ethnopharmacology 141, 357-62.

Tung, N. H., Kim, S. K., Ra, J. C., Zhao, Y. Z., Sohn, D. H., Kim, Y. H., 2010. Antioxidative and hepatoprotective diarylheptanoids from the bark of *Alnus japonica* . Planta Medica 76, 626-629.

Tung, N. H., Kwon, H. J., Kim, J. H., Ra, J. C., Ding, Y., Kim, J. A., Kim, Y. H., 2010. Antiinfluenza diarylheptanoids from the bark of *Alnus japonica* . Bioorganic and Medicinal Chemistry Letters 20, 1000-1003.

Vo, V. C., 1997. The Dictionary of Vietnamese Medicinal Plants. Publishing House Medicine, Ho Chi Minh City.

Yadav, D., Singh, S. C., Verma, R. K., Saxena, K., Verma, R., Murthy, P. K., Gupta, M. M., 2013. Antifilarial diarylheptanoids from *Alnus nepalensis* leaves growing in high altitude areas of Uttarakhand, India. Phytomedicine 20, 124-132.