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Evaluation of Anti-Inflammatory Activity of Aerial Part Extract of Nerium Indicum in Acute and Chronic *in vivo* Models in Mice

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Abstract
Medicines from natural sources have become a mainstay in drug discovery. Biocompatibility and lower side effects of natural drugs from plants, animals, microbes and their derivatives, are the main horizons to screen such drugs. In this study aerial parts of Nerium indicum were extracted together in ethanol and distilled water. Both extract were screened for anti-inflammatory potential using three models viz., Carrageenan induced Paw edema, Cotton pellet induced Granuloma and Carrageenan induced Peritonitis. The maximum inhibition was observed for at the dose of 400 mg/kg (49.39%) and minimum inhibition at the dose of 100mg/kg (1.25%) after 4h of drug treatment in Carrageenan induced paw edema, whereas diclofenac sodium (standard drug) produced 59.03% inhibition. In the chronic model (cotton pellet induced granuloma), ethanolic extract of N.indicum (at doses of 100, 200 and 400 mg/Kg), Diclofenac as standard drug showed decreased formation of granuloma tissue by 32.84%, 39.18%, 42.07% and 67.98% respectively. In addition, exudative model of inflammation was mimicked using carrageenan induced Peritonitis. Percentage of Leukocyte and Neutrophil inhibition was calculated. Dose related inhibition was observed. At 400 mg/kg N.indicum inhibited 42.69 % leukocytes and 44.05 % Neutrophils. Thus, the results indicate that ethanolic extract of N.indicum aerial parts have potent anti-inflammatory effects on both acute and chronic type of inflammation in mice.

Keywords
N.indicum, Carrageenan induced paw edema, Peritonitis, Cotton Pellet granuloma.

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INTRODUCTION
Plants are among the most widely sought source of therapeutics. The battle of existence is similar for plants and any other form life on earth. Mankind has always been curious about unearthing the mysteries behind this similarity. Exploring and exploiting the essence of healthy living from nature has achieved many milestones till date. Advanced understanding of air, water, earth, matter and life has enriched the ways of development and discovery. Every range of product for health and wellbeing constitute natural elements for e.g. morphine, quinine, colchicine, atropine, pilocarpine, or theophylline. Paclitaxel, Vinca alkaloids, theophylline, Colchicine, capsaicin curcumin, resveratrol, epigallocatechin-3-gallate (EGCG), and quercetin are other important plant

products. Examples for plant-derived compounds that served as lead structures and/or were chemically modified are salicylic acid (acetylsalicylic acid), morphine (scores of derivatives), camptothecin (topotecan and irinotecan), artemisinin (artemether), and dicoumarol (warfarin) [1]

Supplementation of core therapeutics with Adjuvant is improving the survival ratio of people with serious debilitating diseases like cancer, leprosy, Aids, flu, arthritis diabetes and infections etc. a huge list of medicinal plant and their products are in market, under clinical and preclinical trials [2]. Thorough congregation of information, knowledge and skills from various literary compilations, folkloric and traditional practitioners and internet tools has led the development of a new trend known as 'Reverse Pharmacology' [3].

Inflammation is a sign of active defense machinery. Sometimes a cascade of inflammatory mediators leads to damage and abrupt changes in the body [4]. Conventional anti-inflammatory drugs include NSAIDs, corticosteroids, immunosuppressant and other biological. All of these produce serious and numerous toxicities [5]. Demand for anti-inflammatory agents depends on the source of inflammation and this generates the interest among scientific community to search for newer agents. Likewise, antimicrobials are always an important research due to the increased rate of resistance to existing antimicrobials. Discovery and development of safe and effective antimicrobials carry an immense therapeutic relevance.

Transcription factor NFκB considered for anti-inflammatory activity.

Acute and chronic inflammations are induced by chemical mediators such as prostaglandins, leukotrienes and platelet-activating factor. Anti-inflammatory agents show their activities through several activity mechanisms [6]. Non-steroid anti-inflammatory drugs (NSAIDs) are the most prescribed drugs for treatment of inflammatory diseases. The NSAIDs provide the patients with symptomatic relief; however, they do not modify the pathogenesis of inflammation [7]. Furthermore, prolonged use should be avoided due to severe side effects particularly on gastric mucosa. Therefore, searching for new drug candidates in the treatment of chronic inflammation has great importance. The scientific research on the biological effects of the medicinal plants led to the discovery and development of novel bioactive drug molecules. Indeed, natural products have proved to be a rich source of therapeutic agents. Due to the side effects caused mostly by synthetic drugs, research into natural products has advanced tremendously in academia and pharmaceutical companies. The consumer interest in such plant-based remedies is due to the lower cost of phytotherapy and the fact that many plant-based remedies are successfully replacing allopathic medicines in relieving malady symptoms.

For centuries, in rural areas, the efficacious plants have been used in simple formulations to treat several diseases. Inflammatory diseases are among the most common health problems treated with traditional remedies. In vivo and in vitro anti-inflammatory, antinociceptive and antipyretic evaluations of medicinal plants provide scientific evidence for the ethnomedicinal features. In the past few years, many studies have investigated the healing potential of higher plants with ethnobotanical histories. Many such plant-derived molecules have been isolated, identified and successfully introduced into international markets by pharmaceutical industries [8]. Particularly phenolic plant constituents such as flavonoids, phenolic acids and proanthocyanins were reported to modulate arachidonic acid metabolism. Similarly, a number of phenolic compounds were shown to be the active anti-inflammatory and antinociceptive compounds of folk remedies through bioassay-guided isolation procedures [9].

In an in vitro study, inhibitory activity against PGE1- and PGE2-induced contractions [10].Anti-inflammatory and antinociceptive activities of isolated compounds [11].

Importantly, the anti-inflammatory potency of secondary metabolites like, flavonoids was found to be [12], Alkaloids [13], Phenolics [14], Tannins, Glycosides [15]. In vitro inhibitory activity against both thromboxane and leukotriene synthesis [16]. In-vitro inhibitory activity against tumor necrosis factor α (TNF-α) production [17].

It is obvious that folk medicines are the most favorable sources for novel drug discovery. By using in vivo bioassay- or in vitro activity-guided fractionation and isolation techniques, hundreds of active constituents have been identified. Hence, it would be reasonable to follow an ethnopharmacological approach using traditional knowledge to increase success in drug discovery and development.

If unchecked, inflammation leads to development of rheumatoid arthritis, diabetes, cancer, Alzheimer's disease, and atherosclerosis along with pulmonary, autoimmune and cardiovascular diseases. Inflammation involves a complex network of many

mediators, a variety of cells, and execution of multiple pathways. e activated immune system, including activated immune cells and the biomolecules. Furthermore, chronic inflammation is also linked with various steps of tumorigenesis and recognized as risk factor for the occurrence of different types of cancers [18]. The chronic use of NSAIDs is connected with cardiovascular, gastrointestinal, and renal toxicities [19-20]. Similarly, the use of corticosteroids leads to hypertension, hyperglycemia, osteoporosis, and growth arrest [21]. Natural products are safe, efficacious, biocompatible, and cost-effective alternatives to treat inflammatory diseases [22]. The anti-inflammatory effects of phytoconstituents are exerted through their action on key regulatory molecules, including cyclooxygenase (COX), inducible nitric oxide synthase (iNOS), and cytokines.

Nerium indicum is one of the Apocynaceae family plants. There are varieties of Nerium in India alone. It is known for its traditional and conventional therapy of [23-28]. The plant is rich in toxic secondary metabolites which is a characteristic of Apocynacaea family [my all]. Presence of cardiac glycosides, alkaloids, terpenoids, antioxidants, tannins etc [29-30]. Has made the plant very sourceful to treat the aforementioned ailments. The main chemical constituents are, Anvirzel, noeflx a, olendrin, odoroside, are used in the [31-32].

"To date the best source of anticancer agents have come from toxic plants". Based on traditional claims, Arrow poisons are anticancer [33].

This research article reports Invitro antioxidant, antimicrobial, antiproliferative and antiinflammatory activity in mice. Reporting above activities on the aerial parts extract of pink variety of Nerium indicum Mill found in Jhansi region of Bundelkhand is not done previously.

MATERIAL AND METHODS

Collection and authentication of plant

The plant growing wildly at Jhansi region of Bundelkhand was collected in the month of April. The Plant were identified and authenticated by Dr. Mudailiya, taxonomist NVARI, Jhansi, UP India. Herbarium specimen no. 24381 for future reference.

Plant extract preparation

The parts of plants (leaves, flowers, fruit follicles and stem) washed thoroughly and dried. Pulverized and sieved drug was extracted exhaustively in Soxhlet apparatus using water, 60% ethanol and ethanol as solvent. The extracts were concentrated using vacuum rotary evaporator and Lyophilizer and dried in incubator. Percentage yield calculated and stored in deep freezer (-4^{0}C) for future use.

Phytochemical study

Qualitative analysis of Phytoconstituents performed according to standard methods [34-35].

Antiinflammatory Study

Animals

Animal study protocol was approved by the Institutional Animal Ethics Committee (IAEC), Bundelkhand University [Project no. BU/Pharm/IAEC/a/16/15]. Adult healthy female and male Swiss Albino mice, of age and body weight (6-8 week, 25-35g) were used in the study. Animals were housed in the Institutional Animal house facility. Guidelines of CPCSEA (Regd. No. 716/02/a/CPCSEA) and IAEC were strictly followed for the care and use of animals. All animals were provided sanitized paddy husk bedding and fed with nutrient rich diet and water ad libitum. Mice were kept in polypropylene lab cages in a room conditioned with a12h light: dark cycle, temperature at 20 ± 2°C and relative humidity 54% ± 12%. The animals were tamed and caressed for one or two weeks before use.

Acute toxicity Study [36-38]:

The limit test for acute toxicity was carried out at 2000 mg/kg oral dose of Nerium extract as per OECD 423 guidelines (OECD 423/425, 2003). Swiss albino female mice (20-30g) were fasted for 6 hrs prior to dosing. Each single dose (1ml) was administered in three mice by oral gavage feeding needles for mice. The mice were observed for any change in the behavior, neurological profile and autonomic profile keenly watched at 24, 48 and 72 hrs for any co-morbidity or morbidty.

Drugs/chemicals

Diclofenac, carrageenan, and dextran were purchased from Sigma Aldrich (USA) and formalin was purchased from CDH Mumbai. All other reagents were of analytical grade

Paw edema induced by Carrageenan [39-40]

Carrageenan-induced acute inflammation in the mice paw is used as a classical model of edema formation and hyperalgesia, for the study of non-steroidal anti-inflammatory drugs and selective cyclooxygenase-1 (COX-1) and COX-2 inhibitors.

Five groups of 6 mice each were treated with Diclofenac sodium (10 mg/kg, p.o), or vehicle (distilled water, p.o). One hour after receiving the drug(s), each animal received a subcutaneous injection of 0.02 ml of 1%w/v λ-carrageenan (dissolved in 0.9% NaCl) in the right hind paw of each mouse under the subplantar aponeurosis. The paw volume was measured by dipping the foot in the mercury bath of a plethysmometer up to the

anatomical hairline on lateral malleolus and compared with control animals at 0, 1, 2, 3 and 4 h interval. The difference between the final and initial volumes used to calculate Percent inhibition according to the following formula:

$$\text{Percentage inhibition} = (Ct - C_0)_{control} - (Ct - C_0)_{treated} \;/\; (Ct - C_0)_{control} \times 100$$

Where, Ct=mean paw volume for each group at time t, and C_0=mean paw volume for each group before carrageenan injection.

Cotton Pellet Induced Granuloma [41-42]

This study was carried out following the protocol of Ray SD, 2015 and Ismail TS, 1997 [26] with fewer modification. Sterile cotton pellets (10 ± 0.5 mg) were implanted subcutaneously on the dorsa region of mice. The five groups (n = 6/group) of mice were treated with Normal saline, Extract (100, 200 mg/kg and 400 mg/kg) and Diclofenac (10 mg/kg) orally, once daily over 7 consecutive days. On day 8, the mice were anaesthetized, and the implanted cotton pellets were taken out carefully. The pellets coated with granulomatous tissue were dried overnight at 60 °C and weighed. The mean difference between the weight of control and treated animal in each group was calculated. And precent inhibition calculated as above.

Peritonitis induced by carrageenan [43-44]

This model predicts neutrophil migration to the peritoneal cavity and leukocyte rolling. Mice were treated Diclofenac (10 mg/kg, po) or vehicle (distilled water, po). One hour after treatment, each animal received an intraperitoneal injection of 0.25 mL 1% carrageenan to induce the inflammatory process and, 4 h later, the animals were sacrificed by cervical dislocation after CO_2 euthanasia. The peritoneal cavity was washed with 2 mL phosphate-buffered saline (PBS) containing heparin, and the exudates/peritoneal cavity cells were collected for analysis. The volume recovered was similar in all experimental groups and equated to approximately 95% of the injected volume. An exudates sample of 20 µL was taken, and a 0.4-mL Turk's solution was added to it. The total number of neutrophils per cavity was then counted in a Neubauer chamber using light microscope. Data are reported as the mean (x $10^3/mm^3$) number of leukocytes and neutrophils.

Statistical analysis of Data

Statistical Calculations were performed using GraphPad Instat 3.0. Data are reported as means ± SEM for 6 animals per group. Statistical analyses were carried out using one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls post hoc test for multiple comparisons. P values < 0.05 were considered to be statistically significant.

RESULT AND DISCUSSION

The results obtained are provided in Table 1-4 and Graphs 1-3. Phytocontituents analysis (Table 1) showed the presence alkaloids, glycosides, terpenoids, phytosterols, tannins etc.

Table 1: Preliminary Phytoconstituents analysis

S.NO.	Test	Nerium indicum		
Tests	**Extracts**	**N1**	**N2**	**N3**
Glycosides-cardiac/anthraquinone	Borntrager's test	++	++	+/-
Saponins	Foam test	-	-	++
Oils and fat	Spot test	+++	++	+
Phlobatannins/ Chalcones	HCl test/spot test	-	-	-
Flavonoids	$AlCl_3$ test/ alkaline reagent test/lead acetate test	+++	+++	+++
Tannins/Phenolic compound	$FeCl_3$ test/ Lead acetate test	+++	+++	+++
Alkaloids	wagner's test/mayer's test/hagers/dragendorffs	++++	++++	++++
Protein/Amino acids	ninhydrin/ xanthoproteic	++	++	++
Steroids	salkowski's test/ Liebermann buchard	-	-	-
Phytosterols	sulphuric acid test	+	-	-
Carbohydrates/ sugar	molish's test/ Benedict's test	+	-	+
Coumarins	fluorescens test	+	-	-

Table 2: Carrageenan induced rat paw oedema: (n=6)

Group/ Treatment	Dose	Percentage inhibition							
		1 hour		2 hours		3 hours		4 hours	
		Volume (ml)	% Inhibition	Volume (ml)	% Inhibition	Volume (ml)	% Inhibition	Volume (ml)	% Inhibition
Group-I (Control)	5 ml/ kg	0.87±0.03	…….	0.89±0.04	……..	0.84±0.03	……..	0.83±0.03	……..
Group-II (Diclofenac)	10 mg/kg	0.36±0.03***	58.62%	0.39±0.03***	56.17%	0.32±0.02***	61.90%	0.34±0.01***	59.03%
Group-III	100 mg/kg	0.83±0.05$$$	4.58%	0.85±0.05$$$	4.49%	0.80±0.05$$$	4.76%	0.82±0.05$$$	01.25%
Group-IV	200 mg/kg	0.83±0.06$$$	4.58%	0.84±0.05$$$	5.61%	0.81±0.06$$$	3.75%	0.79±0.07$$$	4.81%
Group-V	400 mg/kg	0.45±0.07***$$$	48.27%	0.46±0.07***$$$	48.31%	0.43±0.08***$$$	48.80%	0.42±0.06***$$$	49.39%

The percent inhibition for each group was calculated by comparison with the control group. Values indicate mean±S.E.M (ANOVA test followed by Tukey's test). * Data differs significantly (p≤0.0001) when compared against control group.**

Table 3: Cotton pellet induced Granuloma: (n=6)

Group/ Treatment	Dose	Wt of cotton Mean ±S.E.M.	% Protection
Group-I Control (Saline)	5ml/kg	62.20±6.10	…….
Group-II (Diclofenac)	10mg/kg	19.93±2.05***	67.98%
Group-III	100mg/kg	41.77±3.75$$$	32.84%
Group-IV	200mg/kg	37.83±2.1$$$	39.18%
Group-V	400mg/kg	36.03±2.25***$$$	42.07%

*** **Data differs significantly (p≤0.0001) when compared against control group**

Table 3: Carrageenan Induced Peritonitis: (n=6)

Group/ Treatment	Dose	Parameters			
		Leucocytes (10^5 mm^{-3})	Leucocytes inhibition	Neutrophils (10^5 mm^{-3})	Neutrophils inhibition
Group-I Control (Saline)	5ml/kg	4.45±0.35	……..	2.02±0.03	……..
Group-II (Diclofenac)	10mg/kg	2.02±0.12***	54.60%	0.92±0.02***	54.45%
Group-III	100mg/kg	3.96±0.31$$$	11.01%	1.94±0.04$$$	3.96%
Group-IV	200mg/kg	3.85±0.25$$$	13.48%	1.95±0.02$$$	3.46%
Group-V	400mg/kg	2.55±0.19***$$$	42.69%	1.13±0.02***$$$	44.05%

*** **Data differs significantly (p≤0.0001) when compared against control group**

Figure 1: Carrageenan induced Paw edema

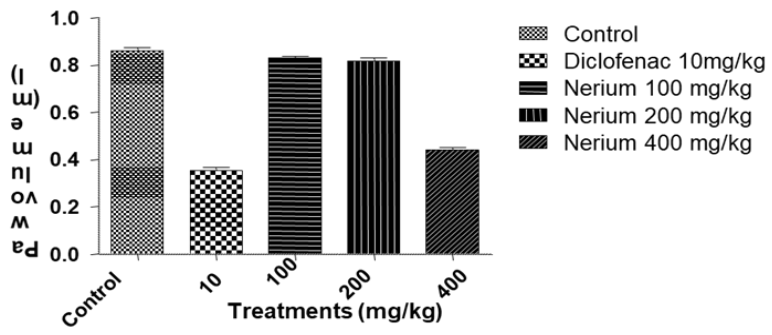


Figure 2: Cotton Pellet induced Granuloma

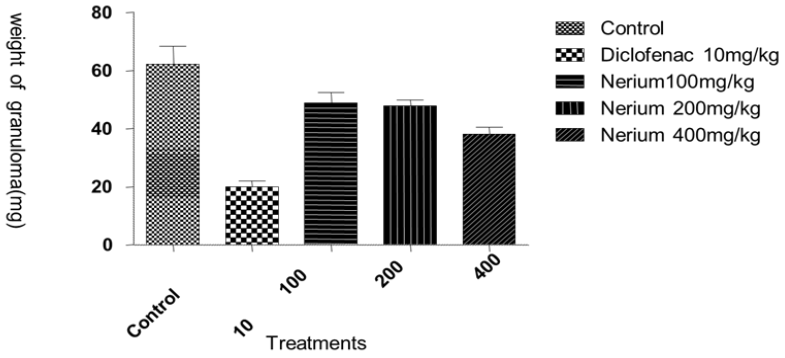
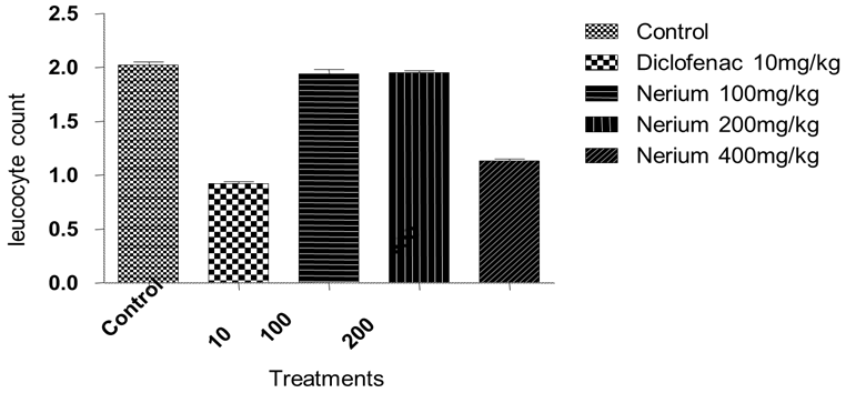


Figure 3: Carrageenan induced peritonitis



The rationale behind this work was to scientifically authentify and confirm the traditional, folk and preliminary claims of Nerium indicum for its anti-inflammatory activity. Results of the present study indicate that the extract of N.indicum posses significant anti-inflammatory activity when used in doses of 100, 200 and 400 mg/kg, both in acute and chronic models of inflammation. The significant activity observed in the suppression of carrageenan induced acute model of inflammation extract as well as by diclofenac, may be due to inhibition of release of various mediators released during different phases of carrageenan induced inflammation [45]. Development of oedema in the rat paw after injection of carrageenan is a biphasic event. The early phase (1-2 h) is mainly mediated by histamine, serotonin and maintained during the plateau phase by kinin and kinin-like substances. The second accelerating phase of swelling is due to the increased synthesis and release of prostaglandins, protease and lysosome in damaged tissue surrounding [46].

Paw swelling, or footpad edema, is a convenient method for assessing inflammatory responses to antigenic challenges and irritants eg carrageenan, croton oil, Sheep Red Blood Cells etc. This model has long been used to assess the anti-inflammatory properties of agents such as nonsteroidal anti-inflammatory drugs (NSAIDs) that inhibit prostaglandin production.

The cotton pellet granuloma method has been widely employed to assess the transudative, exudative and proliferative component of chronic inflammation. It can be noted from the foreign body induced granuloma studies that the extract at the dose of 400 mg/kg showed 42.07% inhibition whereas diclofenac showed 67.98% inhibition in the mean dried weight of granuloma. These results also indicate that the extract possess dose dependent anti-transudative activity.

Nerium reduces the neutrophil migration in carrageenan-induced peritonitis in mice. Mice were pretreated subcutaneously (1 min) with saline (Control), ethanolic extract of N.indicum (100, 200 and 400 mg/kg) and received an intraperitoneal injection of carrageenan (0.2 ml/cavity). The neutrophil migration was evaluated 4 h later. The

values are means ± SEM of six mice per group. P < 0.05, compared with carrageenan group and P < 0.05 compared with saline group (analysis of variance followed by Bonferroni's t-test).

The role played by phytoconstituents on meristematic cell is similar to that played on human cells [47]. Secondary metabolites are generated as a result of homeostasis in plants or they could the by-products of various metabolic/ biochemical processes within the cell including primary metabolites [48]. Oleandrin, odoroside, indirubin, wrightial, carrindone, carrisol etc are very important leads from this family [49-56].

CONCLUSION

The plant N.indicum is a rich source of phytoconstituents like; cardiac glycoside, alkaloids, tannins, terpenoids, irridoids, secoirridoids and flavonoids . From the current study it is observed and hence conluded that Nerium indicum belonging to Apocynaceae family possessed anti-inflammatory effects in carrageenan-induced rat paw edema, Cotton pellet Granuloma and carrageenan induced peritonitis models, which are comparable to Diclofenac. The anti-inflammatory mechanism of the extracts may be due to the reduction of inflammatory mediators or inhibition of leukocyte rolling and neutrophil adhesion. Our Results provide new perspectives on the therapeutic use N.indicum extract and its isolated compounds in the management of inflammatory process in the body.

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CONFLICT OF INTEREST- none

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