

**COMPARATIVE STUDY ON *IN-VIVO* ANTI-INFLAMMATORY POTENTIAL FROM LEAVES OF METHANOL AND CHLOROFORM EXTRACTS OF *AZADIRACHTA INDICA* A. JUSS AND *JUSTICIA ADHATODA* IN NEPAL**

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**ABSTRACT**

The present study was carried out to evaluate and compare anti-inflammatory potential in different extract from leaves of plants along with analysis of phytochemicals responsible for anti-inflammatory activity. Phytochemical analysis revealed presence of various phytoconstituents such as- Phenol, flavonoids, glycosides, carbohydrates, proteins and steroids. Total Phenolic Content (TPC) was estimated using gallic acid as standard. Total phenolic content was  $207.39 \pm 8.77$  mg GAE/g and  $58.08 \pm 4.41$  mg GAE/g for methanol and chloroform extract of *A. indica* respectively while  $110.00 \pm 6.87$  mg GAE/g and  $75.80 \pm 5.55$  mg GAE/g for methanol and chloroform extract of *J. adhatoda* respectively. A well established method:

carrageenan induced rat paw edema was adopted for the study of anti-inflammatory activity where adult Wistar albino rats (110-160)gm of either sex were selected for study. Adult rats were divided into 10 groups, carrageenan was injected in hind paw and treated with extract at the standard dose of 200 mg/kg and 400mg/kg. The paw volume was measured at different time intervals i.e. 0,2,4 and 12 hours. Percentage inhibition of inflammation was proportional with the total phenolic content. The result indicates that methanol extract of *A. indica* has highest anti-inflammatory activity than both extract of *J. adhatoda* while its chloroform extract has least activity among all extracts.

**KEYWORDS:** Phytochemical screening, anti-inflammatory activity, total phenolic content, *Azadirachta indica*, *Justicia adhatoda*.

## INTRODUCTION

Medicinal plants, the richest bio-resource of drugs, owing to their versatile pharmacological actions are used in wide range of fields comprising of traditional system of medicine, modern medicine, nutraceuticals, pharmaceutical intermediates and chemical entities for synthetic drugs.<sup>[1]</sup> Although, Nepal is a small country, it is enriched with diverse plants possessing medicinal and aromatic values due to geographical diversity. Most of the plants are being used in traditional medicine; however, some are not explored scientifically for their medicinal values yet.<sup>[2]</sup> Preliminary screening of phytochemicals is a valuable step for the detection of various bioactive principles present in plant which paved the way for drug discovery.<sup>[3]</sup> Almost every parts of *A. indica* are being used in traditional medicine for treating variety of human ailments as it possess diverse biological activities such as- antiallergenic, antidermatic, antifeedant, antifungal, anti-inflammatory, antioxidant, antipyorrhoeic, antiscabic, diuretic etc.<sup>[4]</sup> Similarly, *J. adhatoda* has been used in the indigenous system of medicine world widely as herbal remedy for treating cold, cough, chronic bronchitis, asthma, rheumatic inflammation. It can also be used as sedative, expectorant, antioxidant, hepatoprotective, antispasmodic and antihelmintic.<sup>[5,6]</sup> In general, *A. indica* contains secondary metabolites such as- steroids, triterpinoids, reducing sugars, alkaloids, phenolic compounds, flavonoids and tannins while *J. adhatoda* constitute- alkaloids, tannins, flavonoids, terpenes, sugars, and glycosides.<sup>[7,8]</sup> The experimental method for determining anti-inflammatory effect of extracts was studied by inflammatory response produced in rat by injecting carrageenan in the hind paw. Carrageenan is a phlogistic agent which upon administration produces edema.<sup>[9]</sup> Inflammation is a pathophysiological response and a non-specific internal system of defense towards- microbes, physical agents, chemicals agents and other harmful stimuli which is associated with pain. It involves occurrences such as: increased vascular permeability, increase in protein denaturation and membrane alteration.<sup>[10]</sup> During the course of inflammation, free radicals dependent system are involved in the formation of cytokines, inflammatory mediators, and cellular functions. However, reactive oxygen species (ROS) are involved in wound healing and tissue repair mechanisms.<sup>[11]</sup> Synthetic anti-inflammatory agents such as- NSAIDs and corticosteroids have various adverse effects so there is a need for investigation of efficacy of plants used in traditional medicine that posses anti-inflammatory activity.<sup>[12]</sup> Due to the similarities in

phytoconstituents and wide spread ethnomedicinal use by local communities, they are selected for comparative study as many people are not aware on selection of particular plant based on their effectiveness for particular therapeutic purposes.

## MATERIALS AND METHODS

### Study species

*Azadirachta indica* A. Juss is a versatile plant belonging to the family Meliaceae which is inhabitant to tropical and subtropical parts of the world.<sup>[13]</sup> Mainly two species of *Azadirachta* have been reported, *Azadirachta indica* A. Juss - native to Indian subcontinent and *Azadirachta excelsa* Kock - confined to Philippines and Indonesia.<sup>[14]</sup> The leaves of *A. indica* are imparipinnate, alternate, exstipulate; leaflets are alternate or opposite, very shortly stalked, ovate-lanceolate, attenuate at the apex, unequal at the base and are medium to dark green in colour.<sup>[15]</sup>

*Justicia adhatoda* Linn. is an indigenous herb belonging to family Acanthaceae. It is a perennial evergreen and highly branched shrub (1.0 m to 2.5 m. height) having bitter taste and opposite ascending branches with white, pink or purple flowers. It is widespread throughout the tropical regions of Southeast Asia.<sup>[16,17]</sup>

### Plants and chemicals

The leaves of *A. indica* and *J. adhatoda* were collected from Paklihawa, Siddharthanagar Municipality, Rupandehi district of Nepal in the month of August, 2015. The leaves were identified from Department of environmental sciences, Institute of Agriculture and Animal Sciences, Tribhuvan University. All the chemicals used in the experiment were of analytical grade and were purchased from S.d. Fine-chem Ltd; Himedia Laboratories Pvt. Ltd. and Qualigens fine chemicals.

### Preparation of extracts

The leaves of plant were washed with distilled water, dried at room temperature in the laboratory for 3 weeks to obtain consistent weight and were powdered using mechanical grinder. About 200 g of the crushed leaves were extracted by maceration using pure methanol as solvent and other 200 g crushed leaves via chloroform for 7 days with frequent agitation. The extracts were filtered using Buckner Funnel and Whatmann No. 1 filter paper. Each filtrate was concentrated to dryness under reduced pressure by Rotary vacuum evaporator below 40°C. Extracts were stored at 4°C in air tight container with proper labeling.

### **Phytochemical screening**

Phytochemical analysis for alkaloid, glycoside, saponin, steroid, phenol, flavonoid, tannin, protein and amino acids was carried out following the procedure as mentioned by the authors.<sup>[18,19]</sup> Mayer's reagent and Hager's reagent were used to detect the presence of alkaloids; Molish's reagent and Fehling's reagent for carbohydrate; Legal's test for glycosides; Froth test and foam test for saponin; Salkowski's test for steroid; Ferric chloride test for phenol; Alkaline reagent test and Lead acetate test for flavonoid; Ferric chloride test and Bromine water test for tannins and Xanthoproteic test for the proteins and amino acids.

### **Total phenolic content**

#### ***Preparation of standard***

The total phenolic content in plant extracts were determined by using spectrophotometric method based on oxidation-reduction reaction with some modifications.<sup>[20]</sup> Various concentrations of gallic acid solutions in methanol (0.5, 0.4, 0.3, 0.2, 0.1, 0.05, 0.025, 0.0125 mg/ml) were prepared. In a 20 ml test tube, 1 ml gallic acid of each concentration was added, 5 ml of Folin-Ciocalteu reagent (10%) and 4 ml of 7.0% Na<sub>2</sub>CO<sub>3</sub> were added to get a total volume of 10 ml. The blue coloured mixture was shaken well and incubated for 40 minutes at 40°C in a water bath. Afterward, the absorbance was measured at 760 nm against blank. All the experiments were carried out in triplicate. The average absorbance values obtained at different concentrations of gallic acid were used to plot the calibration curve.

#### ***Preparation of sample***

Two different concentrations of the extracts (1, 0.1 mg/ml) were prepared. Following the procedure described for standard, absorbance for each concentration of extract was recorded. Total phenolics content of plant extracts were expressed as mg gallic acid equivalents (GAE) per gram of sample in dry weight (mg/g). The TPC in all samples were calculated using the formula  $C = c \times V/m$  where, C = total phenolic content mg GAE/g dry extract, c = concentration of gallic acid obtained from calibration curve in mg/ml, V = volume of extract in ml, m = mass of extract in gram.

### **Anti-inflammatory activity**

It was carried out by carragennan induced rat paw edema inhibition method. Wistar albino rats were divided into 10 groups each containing three rats. Control group received no drug, only normal saline was given.

**Table 1: Phytochemical screening of methanol and chloroform extract of plants.**

S/N	Phytochemical Tests	Reagents used / Test performed	Result			
			<i>J. adhatoda</i>		<i>A. indica</i>	
			Methanol	Chloroform	Methanol	Chloroform
1.	Alkaloid Test	Mayer's Reagent	+	+	+	-
		Hager's Reagent	+	+	+	+
2.	Carbohydrate Test	Molish's Reagent	+	-	+	+
		Fehling's Reagent	+	+	-	+
3.	Glycoside Test	Legal's Test	-	+	+	-
4.	Saponin Test	Froth Test	-	-	-	-
		Foam Test	+	-	+	-
5.	Steroid Test	Salkowski's Test	+	+	+	+
6.	Phenol Test	Ferric chloride Test	+	+	+	+
7.	Flavonoid Test	Alkaline reagent Test	+	+	+	-
		Lead acetate Test	+	+	+	+
8.	Tannin Test	Ferric chloride Test	-	-	+	+
		Bromine water Test	-	-	-	+
9.	Protein and Amino acid	Xanthoproteic Test	+	+	+	-

+ → Positive - → Negative

Standard control received indomethacin in the dose of 20 mg/kg. Remaining rats received methanol and chloroform extract of *A. indica* and *J. adhatoda* in the dose of 200 mg/kg and 400 mg/kg. Inflammation was produced by injecting 0.1 ml of carrageenan suspension in normal saline to right hind paw. All the drugs and extracts were given orally by feeding tube one hour prior to carrageenan injection. A mark was made on leg at malleolus to facilitate uniform dipping at subsequent readings. The paw edema volume was measured with the help of plethysmograph by mercury displacement method at zero hour (immediately after injecting carrageenan). The same procedure was repeated at different (2, 4 and 12) hour after injection. The percentage inhibition of edema in various treated groups was then calculated by using the formula.

$$\% \text{ edema inhibition} = (V_c - V_t) / V_c \times 100$$

Where,

V<sub>t</sub>- mean edema volume in the drug treated group.

V<sub>c</sub>- mean edema volume in control group.<sup>[21]</sup>



**Figure 1: Carrageenan induced rat paw edema.**

### Statistical analysis

All the data were expressed as mean value  $\pm$  standard error of mean (SEM) of the number of experiments (n=3). Microsoft EXCEL program 2010, and Statistical Package for Social Sciences, Version 16.0 (SPSS V.16.0) were used for data analysis.

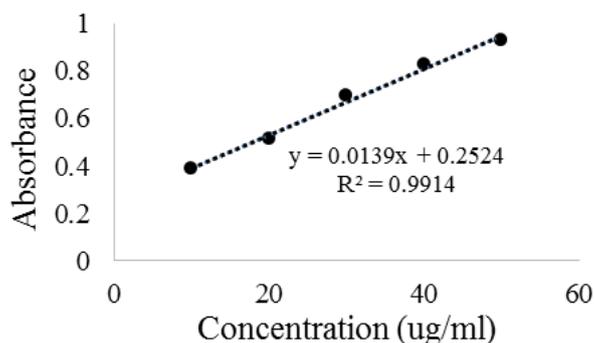
## RESULTS

### Phytochemical screening

The preliminary phytochemical screening of methanol and chloroform extract of plants showed presence of different Phytochemicals which are presented in Table 1. The two extract of plants showed presence of similar phytochemicals such as- alkaloids, glycosides, carbohydrate, phenol, flavonoid, steroids, protein and amino acids.

### Total phenolic content

The content of total phenol (TPC) was determined by using Folin-Ciocalteu reagent in terms of gallic acid equivalent (standard curve equation:  $y=0.013x+0.252$ ,  $R^2 = 0.991$ ). Total phenolic content in extract of both plants expressed in terms of mg GAE/g of sample of dry weight. The values obtained are  $207.39 \pm 8.77$  mg GAE/g and  $58.08 \pm 4.41$  mg GAE/g for methanol and chloroform extract of *A.indica* respectively while methanol and chloroform extracts of *J. adhatoda* showed total phenolics to be  $110.00 \pm 6.87$  mg GAE/g and  $75.80 \pm 5.55$  mg GAE/g respectively.



**Figure 2: Calibration curve of gallic acid.**

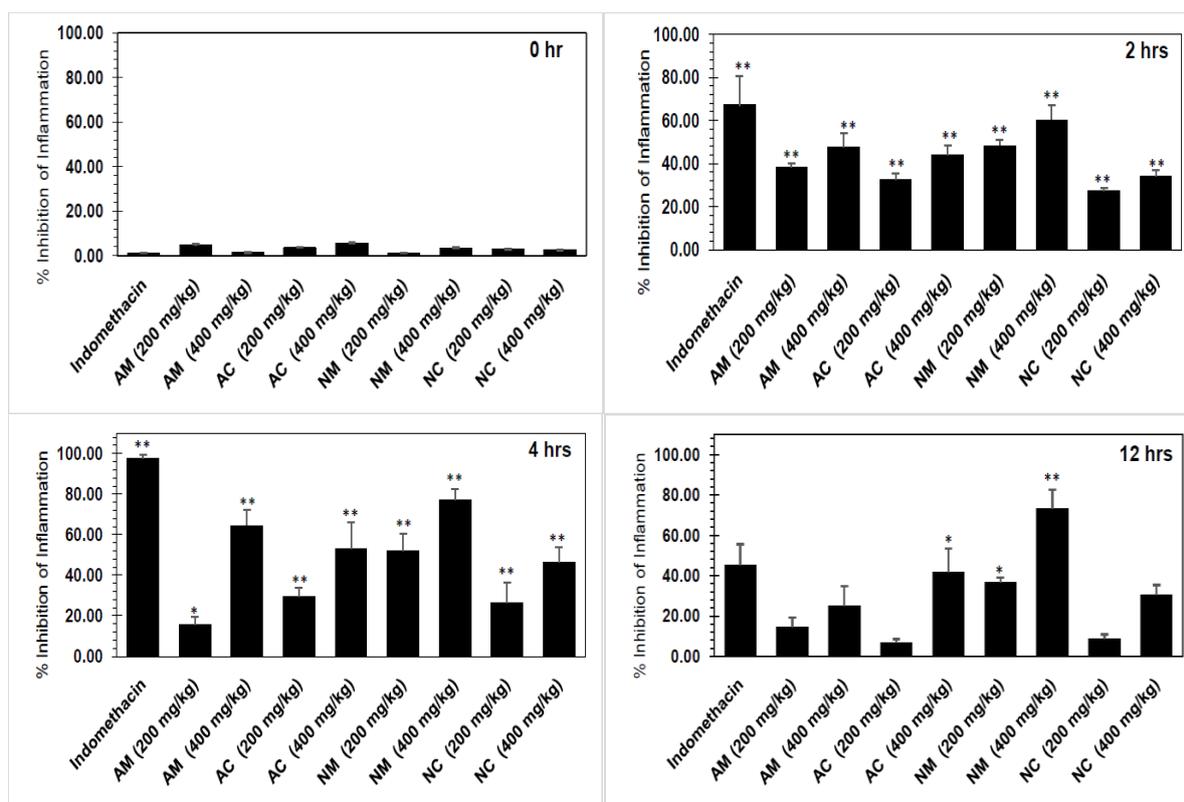
### Anti-inflammatory activity

Anti-inflammatory activity of *A.indica* and *J.adhatoda* was studied in wistar albino rat and paw volume was measured at different time intervals. Anti-inflammatory activity is expressed by percentage inhibition of inflammation as shown in Figure 3. Control, test and standard were given 1 hour prior to carrageenan injection. The paw volume was measured immediately after carrageenan injection at 0 hour; 2 hour, 4 hour and 12 hour. At zero hour, percentage inhibition of inflammation was very less nearly equal to zero. After 2 hours of injection, standard indomethacin showed highest percentage inhibition of inflammation 67.05% followed by *A.indica* extract in methanol, *J. adhatoda* extract in methanol, *J. adhatoda* extract in chloroform and *A. indica* extract in chloroform, all extract being given at a dose of 400mg/kg. Similar pattern of anti-inflammatory activity was observed for remaining extracts at dose 200 mg/kg. After 4 hours, all extract showed high percentage inhibition of inflammation with similar pattern as 2 hours. After 12 hour, the activity of all the extract and standard decreased, methanol extract of *A. indica* showed highest inhibition of inflammation which was 73.17 %.

### DISCUSSION

Plants are the richest source of drugs due to the chemical diversity of phytoconstituents present in plant. Discovery and efforts to include them in treatment as an anti-inflammatory agents are under consideration due to the side effects associated with Non Steroidal Anti-inflammatory Drugs (NSAID's).<sup>[22]</sup> Similar phytochemical constituents such as- phenol, flavonoid, alkaloid, glycoside, tannin etc are present in both methanol and chloroform extract of both plants which might be responsible for anti-inflammatory activity.<sup>[23]</sup> Carrageenan induced paw edema is one of the most reliable test to evaluate the anti-inflammatory properties of plant extracts because of its sensitivity to detect orally active anti-inflammatory

agents particularly in the acute inflammatory model.<sup>[24]</sup> All the extracts used showed variable degree of anti-inflammatory activity up to 12 hours at different dose. Indomethacin showed highest degree of anti-inflammatory activity after 2 hours and 4 hours of carrageenan injection but not in the last 12 hour. This is probably due to elimination of indomethacin and decrease in plasma concentration as the half life is around 4.5 hour. Among all the extracts, methanol extract of *A. indica* showed highest degree percentage inhibition of inflammation at both dose 200 mg/kg and 400 mg/kg followed by methanol extract of *J. adhatoda*, chloroform extract of *J. adhatoda* and chloroform extract of *A. indica*. Dominant anti-inflammatory activity of methanol extract revealed high solubility of polar phytochemical constituents in methanol than in chloroform.<sup>[25]</sup> The anti-inflammatory activities exhibited by the extracts are proportional with the total phenolic content in extract, although a small degree of variation is observed in activity of methanol and chloroform extract of *J. adhatoda*. Similar studies conducted before showed potent anti-inflammatory activity of methanol and chloroform extract of both plants<sup>[26-28]</sup>.



**Figure 3:** Bar diagram showing anti-inflammatory activity of methanol and chloroform extracts of *A. indica* and *J. adhatoda* in carrageenan induced rat paw edema at different time intervals (0 hour, 2 hours, 4 hours and 12 hours respectively).

Data are expressed as Mean  $\pm$  SEM, Statistical analysis was carried out by ANOVA.\* Indicates  $P < 0.05$  (Significant) and \*\* Indicates  $P < 0.005$  (Highly significant) compared to control. **Key:** AM- Methanol extract of *J. adhatoda*, AC- Chloroform extract of *J. adhatoda*, NM- Methanol extract of *A. indica*, NC- Chloroform extract of *A. indica*.

## CONCLUSION

The result obtained in the study clearly demonstrates significant anti-inflammatory activity of all extracts, methanol extract being predominating over chloroform. Thus, based on the efficacy of plant extracts observed in the study, they can be designed to any pharmaceutical formulations as an anti-inflammatory agent for therapeutic purpose. Further studies are required to study synergistic effects of plant extracts, efficacy in terms of chronic inflammation and identification of chemical constituents for promoting scientific use of plants around the globe.

## CONFLICT OF INTERESTS

Author(s) have not declared any conflict of interest regarding the publication of this article.

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