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Chronic anti-inflammatory effect of methanolic extracts of leaf, bark and fruit of *Averrhoa bilimbi* on cotton pellet-induced chronic inflammation in rats

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Abstract

Inflammation is a complex biological response to harmful stimuli such as pathogens, damaged cells, or irritants. While acute inflammation is a protective mechanism, chronic inflammation is implicated in a variety of diseases. Medicinal plants have been used for centuries in managing inflammation. The present study aimed to evaluate anti-inflammatory potential of crude methanolic extract of leaf, bark and fruit of *Averrhoa bilimbi* Linn (Oxalidiaceae). The dried and powder leaves, stem barks and fruits of *A. bilimbi* Linn were extracted with methanol and the crude methanolic extracts were investigated for the evaluation of anti-inflammatory potential by cotton pellet-induced chronic inflammatory model in rats. The methanolic extracts of *A. bilimbi* leaves, stem barks and fruits were diminished inflammation as shown by lowered cotton pellet weight in cotton pellet-induced granuloma in rats. In this study, the results of leaves methanolic extract exhibited most significant anti-inflammatory action and results were comparable to diclofenac sodium, a standard NSAID. This research demonstrates that *A. bilimbi* has significant anti-inflammatory effects, indicating its potential as a source for isolating active compounds for treatment of inflammation.

Keywords: Anti-inflammatory activity, *Averrhoa bilimbi*, cotton pellet, diclofenac sodium, methanolic extracts

Introduction

Inflammation is a disorder including localized rises in white blood cell count and a variety of mediators involving prostaglandins ^[1]. Diseases of inflammation are considered one of the major life threatening difficulties worldwide and are a main reason of mortality of the labour force across the globe. These are named the "King of Human Miseries" ^[2]. Disorders of inflammation like rheumatoid arthritis, bronchial asthma, colitis and hepatitis are amid the lead reasons of mortality and disablement in the earth ^[3-5]. Plants have been applied for treating different inflammatory disorders in human beings and animals from past to the present. Acetyl salicylic acid, also known as aspirin, is the most well-known and widely used drug in the world. It is derived from the plant genera *Salix* and *Populus* and is related to salicin ^[6]. About 30% of today's anti-inflammatory medications come from natural sources, either directly or indirectly, and the most potent of them all is aspirin, or acetyl salicylic acid, which is derived from plants. Over 70% are synthetic anti-inflammatory medications ^[7].

Many NSAIDs and steroidal medications are currently used in clinical settings to treat inflammatory diseases. However, they have serious adverse effects include nephrotoxicity, hepatotoxicity, and peptic ulcers or other gastrointestinal disorders. Many natural anti-inflammatory chemicals have been found, however there are still a lot of new, safer natural anti-inflammatory molecules to be found. There is a high need for novel and cutting-edge medications with anti-inflammatory properties. According to current research, chemical variety, and ethnomedical data, plants are a great source of novel bioactive chemicals. Therefore, the goal of the current study is to quantify the anti-inflammatory properties of particular medicinal plants and to identify novel anti-inflammatory components from a powerful *Averrhoa bilimbi* Linn plant extract.

The medicinal plant *Averrhoa bilimbi* Linn., belonging to the Oxalidaceae family, is also referred to as "Billimbi" or "Cucumber tree."

Due to its possible application in treating inflammatory conditions, the herb is utilized in folk medicine. Various parts of the plant are used for the treatment of inflammatory diseases, cough, cold, syphilis, hypertension, diabetes; piles and scurvy; Whitlows; hepatitis, diarrhoea, pyrexia, beriberi, biliousness, rectal bleeding, internal hemorrhoids, control obesity, itching, pimples, swellings of mumps, rheumatism, eruptions of skin, bites of toxic organism, rectal inflammations, cough and thrush [8-15]. Malaysia and Indonesia's Moluccas are the native land of the plant. All around the world, it is grown in tropical fields. It can be found all over Bangladesh; however, it is most common in Dhaka, Brahmanbaria, Cumilla, and Chattogram. Ascorbic acid, oxalic acid, tannins, and minerals abound in bilimbi fruits. Amino acids, citric acid, cyanidin-3-O-h-D-glucoside, phenolics, potassium ion, sugars, and vitamin A are among the chemical components of *A. bilimbi* Linn [16]. The plant has been reported to possess analgesic, antioxidant, antimicrobial, anticoagulant, anti-fertility, antimalarial, anthelmintics, cytotoxic, hepatoprotective, hypoglycaemic, hypolipidemic, nephrotoxic, pediculicidal, thrombolytic and wound healing properties [17-39].

The secondary plant metabolites presence in *A. bilimbi* include alkaloids, flavonoids, glycosides, steroids, saponins and tannins [40-43]. Considering the conventional uses and shown potential medicinal characteristics of *A. bilimbi*, current research was undertaken to investigate its anti-inflammatory potential in cotton pellet-induced granuloma in rats.

Materials and Methods

Collection and Identification of Sample

In March 2017, the plant was taken from the campuses of the Institute of Health Technology (IHT), located in Mohakhali, Dhaka, and the Central Medical Stores Depot (CMSD), located in Tajgaon, Dhaka. The taxonomist of the Bangladesh National Herbarium, located in Mirpur, Dhaka, Bangladesh, determined the identity of the medicinal plant to be *A. bilimbi* Linn.

Preparation of Plant Material

Collected leaves, fruits and stem barks were washed and dried. The dried plant materials were then ground into a coarse powder using a grinder. The plant components in powder form were stored in an airtight container with appropriate labeling.

Extraction of Plant Material

The powdered leaves, fruits, and stem barks (500 mg of each portion) of *A. bilimbi* Linn were each separately soaked in 3L of 95% methanol for seven days before being filtered. A concentrated crude extract was prepared by evaporating the filtrate at a low temperature of 40 ± 2 °C and reducing pressure using a Buchii Rotavapour. Every concentrated extract was preserved for use in future research.

Animals for Experiment

For the experiment, 120-150g Swiss albino rats of either sex were obtained from the Pharmacy Department's animal house at Jahangirnagar University in Savar, Dhaka, Bangladesh. Standard conditions for the experimental animals included a 12-hour light/dark cycle, a temperature of 27.0 ± 1.0 °, and a relative humidity of 55–65%. They received an optimal food and unlimited supplies of water.

One week before to the exams, they were acclimated to the lab setting.

Acute Toxicity Test

Acute toxicity is the term used to describe the harmful effects of a chemical that might occur from one dose or several doses within a brief period of time, usually no more than 24 hours. The experimental specimens' LD50 values were determined using an acute toxicity test. The experimental specimens were administered orally to the experimental animals at different doses (0.1, 0.25, 0.5, 1.0, 2.0, 3.0, and 4.0 grams per kilogram body weight). Fatality or toxicity sign or any toxicity test parameter such as general appearance, activity, movement, stimulus response (sound, touch, light), lacrimation, salivation, piloerection, irritability, sedation, paw licking, convulsion etc. were observed for 1 hr, after administration of the test samples. The experimental animals were then watched every hour for the next five to six hours. Again the experimental animals were observed for one week [44].

Design for Experiment

Eight categories, each with five animals, were created from the experimental animals (Table 1). For the administration of all dosages, the oral route is selected.

Table 1: Category Ordination and Chemical administered with dose.

Category	Chemical Administered and Dose
Category 1	Control (1% Tween 80, 10ml per kg body wt.)
Category 2	Standard (Model specific, mg per kg body wt.)
Category 3	ALM (250 mg per kg body wt.)
Category 4	ALM (500mg per kg body wt.)
Category 5	ABM (250 mg per kg body wt.)
Category 6	ABM (500 mg per kg body wt.)
Category 7	AFM (250 mg per kg body wt.)
Category 8	AFM (500mg per kg body wt.)

Animals Identification during Experiment

The test animals were divided into eight groups, each with five animals, making it challenging to recognize and observe one at a time. During the test, it is therefore crucial to identify a specific species within a category. The test animals were identified by their tail numbers, which were I, II, III, IIII, and none (for number five).

Cotton Pellet Induced Granuloma Model for Chronic Inflammation

Cotton pellet induced granuloma model is a technique for chronic inflammation⁴⁵. Two sterile pellets of cotton (each weight 10 ± 1 mg) were aseptically implanted in subcutaneous route in the interscapular distance of the dorsal region of rats which were anaesthetized by Ketamine. Various categories of rats were treated with above-mentioned experimental design chemical and doses (Table 1). From the day of pellet implantation the experimental chemical was given orally once daily for consecutive 7 days. Here, standard category was taken Diclofenac (100 mg/kg, p.o.) and control category was taken vehicle (aqua, 10 ml/kg, p.o.). The experimental animals were immolated on the eighth day and the pellets of cotton were withdrawn and dried-up at 60 °C for a period of 24 hours. Weight was taken from both moist and dry cotton pellets. The difference between weight of dry cotton and cotton prior insertion was

thought as granuloma weight.

The percentage (%) inhibition of granuloma formation was estimated as follows:

$$\% \text{ Inhibition} = \left(1 - \frac{\text{Granuloma Weight (Drug/ Standard)}}{\text{Granuloma Weight}} \right) \times 100$$

Phytochemical screening of crude extract

The methanolic extracts of *A. bilimbi* Linn leaf, bark, and fruit were screened for different phytochemical constituents using standard screening assays following traditional phytochemical screening protocols like Alkaloids by Mayer's, Hager's and Dragendorff's reagent; Flavonoid (Shinoda test); Steroids (Salkowski's Test); Tannin (Ferric

chloride test) [46-47].

Statistical Analysis

Statistical analysis for animal tests was completed by Independent-Sample T Test. The achieved results were compared with the vehicle control category and expressed as mean $\pm$ SEM. *p* values < 0.05, 0.01 and 0.001 were thought to be statistically important.

Results

Phytochemical Screening

The different extracts of *A. bilimbi* were tested for preliminary plant constituents screening to find out the presence of different plant constituents are mentioned in table 2.

Table 2: Result of phytochemical screening test of *A. bilimbi* extracts.

Extract	Alkaloid			Steroids (Salkowski's Test)	Tannin (Ferric chloride test)	Flavonoids (Shinoda test)	Saponin (Frothing test)
	Mayer's Test	Hager's Test	Dragendorff's Test				
ALM	+	+	+	+	+	+	+
ABM	+	+	+	+	+	+	+
AFM	-	-	-	-	+	+	+

Here, ALM= *A. bilimbi* leaf methanolic extract, ABM=*A. bilimbi* bark methanolic extract, AFM=*A. bilimbi* fruit methanolic extract
[(+) = Presence, (-) = absence]

Acute Toxicity Test

From this toxicity test no lethality or toxic sign was found until 4000 mg/kg dose of all extracts during the period of the study (observation 7 days). In addition, no weight-loss, change of pellet consumption or visceral change (macroscopic) of treated animals were detected. The acute toxicity results were same for all extracts and doses. The methanolic extracts of *A. bilimbi* were thought to be safe with wide therapeutic range because median lethal dose (LD₅₀) was not found in this study. So, 250 mg/kg and 500 mg/kg dose of extracts were chosen for each *in-vivo* test models.

Cotton Pellet Induced Granuloma Formation

The methanolic extracts of the *A. bilimbi* were tested to estimate chronic antiinflammatory activity using Diclofenac as reference standard. The methanolic extracts of *A. bilimbi* inhibited granuloma formation which dependent on dose of extracts. It is notable from table 3, that methanolic extract of *A. bilimbi* leaf (250 mg/kg and 500 mg/kg) and bark (500 mg/kg) and diclofenac sodium presented significant (*p*<0.001) action in inhibiting granuloma formation. The *A. bilimbi* leaf methanolic extract (ALM) given at 500 mg/kg orally had a greater anti-granuloma (39.95%) action but lower than diclofenac sodium (74.73%). Granuloma inhibition activity decreased in the following order: Diclofenac sodium>ALM> ABM>AFM (Table 3).

Table 3: Results of various extracts of *A. bilimbi* in cotton pellet induced granuloma test.

Group	Dose (mg/kg)	Granuloma wt. (mg/mg cotton)	Inhibition (%)
Control	1% Tween 80 in water (10 mL/kg)	4.63 $\pm$ 0.12	-
Diclofenac Na	100	1.17 $\pm$ 0.13**	74.73
ALM	250	3.26 $\pm$ 0.12**	29.58
	500	2.78 $\pm$ 0.07**	39.95
ABM	250	4.17 $\pm$ 0.11	9.94
	500	3.56 $\pm$ 0.12**	23.11
AFM	250	4.26 $\pm$ 0.11	7.99
	500	4.09 $\pm$ 0.17*	11.66

Results are submitted as mean $\pm$ SEM (*n* = 5). One way ANOVA succeeded by Dunnett's multiple comparisons was carried out to analyze this dataset. **p*<0.05, ***p*<0.001 were deemed statistically important when compared against control.

Discussion

The crude methanolic extracts of *A. bilimbi* Linn leaf, bark, and fruit were subjected to different qualitative tests to identify the appearance of plant constituents and *in-vivo* pharmacologic research to assess their toxicity and anti-inflammatory actions.

Various extracts of *A. bilimbi* have many phytochemical compounds such as alkaloid, glycosides, steroids, tannins and saponin. No alkaloid and steroid were detected in methanolic fruits extract. The phytochemical compounds appeared in the extracts may responsible for their diverse pharmacologic actions demonstrated in other *in-vivo* and *in-vitro* investigation [47].

Cotton pellet-induced granuloma is commonly used to evaluate the transudative and proliferative element of chronic inflammation [45]. So, Cotton pellet-induced granuloma model was used to measure the anti-inflammatory action in chronic inflammation. Chronic

inflammatory response mediated by subcutaneous implantation of cotton pellet are divided into 3 phases: 1) transudative phase, happens during first 3 hr; 2) exudative phase, happening within 3 to 72 hr following implantation of pellets of cotton; 3) proliferative phase, estimated as the rise in dry granuloma weight that occurs within 3 to 6 days following implanting pellet [48]. Furthermore, inflammatory response is induced by implanted cotton pellet and inflammatory mediator release is also regulated by implanted materials that eventually generate tissue proliferation and granular production [49-50]. The inhibition of proliferative phase of chronic inflammation could result in reduce in the weight of granuloma formation. It is noted from current reports that NSAIDs *viz* acetyl salicylic acid reveal a little suppression contrariwise steroidal anti-inflammation drugs have a powerful suppression on both transudative and proliferative phase of inflammation [51]. The methanolic extracts of *A. bilimbi* showed a dose dependent suppression of granuloma production. Granuloma formation was most significantly reduced ($p < 0.001$) by 250 mg/kg and 500 mg/kg oral doses of *A. bilimbi* leaf methanolic extract (ALM). The *A. bilimbi* bark methanolic extract (ABM) also exhibited most significant ($p < 0.001$) result only at high dose 500 mg/kg orally. Fruit extract demonstrated significant result ($p < 0.05$) at dose 500 mg/kg orally. Diclofenac, a nonsteroidal anti-inflammatory drug, exhibited high significant ($p < 0.001$) inhibition (74.73%). From the aforementioned investigation it is clear that methanolic extracts of *A. bilimbi* have anti proliferative action but lower than that of the standard drug.

Conclusion

In the current study, methanolic extracts exhibited significant anti-inflammatory potential representing that the plant extracts are biologically active. Methanolic crude extracts of various parts of the *A. bilimbi* were used to cotton pellet-induced granuloma formation test for probable anti-inflammatory action. In this experiment, leaf and bark methanolic extracts exhibited most significant anti-inflammatory actions 39.95% and 23.11% respectively at the dose of 500 mg/kg while standard anti-inflammatory drug Diclofenac sodium showed 74.73% inhibition of edema with a dose of 100 mg/kg. On the other hand, fruits methanolic extract showed 11.66% reduction of weight of cotton pellet granuloma at the dose of 500 mg/kg. The current research finding supports the conventional claims and gives a scientific basis for anti-inflammatory action of *A. bilimbi* in inflammatory disorders.

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