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Potential investigation of anti-inflammatory and anti-oxidative property of ethanolic extract of *Ixora nigricans* leaves

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Abstract

Our present work was meant to unleash pharmacological activity like cytotoxic, anti-oxidant and anti-inflammatory activity of *Ixora nigricans* (Family: Rubeacea). In ethnomedicine root extract of *I. nigricans* is taken for the treatment of diarrhea and leaf extract is for the treatment of dysentery. The ethanolic extract of *Ixora nigricans* leaves was studied using 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging activity and quantitative analysis of anti-oxidative compound like flavonoids were made to estimate or to identify its total anti-oxidant property by using a spectrophotometric method. In vitro anti-inflammatory activity was evaluated using membrane stability and protein (albumin) denaturation assay at different concentration. The DPPH scavenging result was 131.43±2.03µg/ml and anti-oxidative compounds most importantly flavonoid content was 84.03±0.78 and total phenolic content was found 20.80±1.44. The anti-inflammatory action by human RBC membrane stabilizing methods resulted 81.74±2.54% at 1000µg concentration where as the comparability standard (Aspirin) results 90.23±1.48% and in protein denaturation method, it resulted 78.07±2.4% which was also close to Aspirin (86.28±1.48%). From these results it can be concluded that this has quite promising result and that may be due presence of flavonoids and some phenolic content.

Keywords: *Ixora nigricans,* anti-inflammatory, anti-oxidant, protein denaturation, phytochemical analysis

1. Introduction

From the very start of the civilization there is an extreme relationship between human organisms and plants. In ancient period the people used to utilize several parts of plants in different treatment purposes. A single piece of plant may consist of numerous medicinal values, but it has been shown that direct intake of crude plant is not in effect; as it contains both substantive and non-essential elements. The non-essential one may not be needed by the body in healing purposes or in other conditions, the non-essential components may in fact be toxic to the body under some cases. Even the intake of the essential components via the crude extract may lead to an improper dosage.[1]

Phytochemicals like alkaloids, tannins, flavonoid and phenolic compounds are extensively found in different layers in various medicinal plants and applied in herbal medicine to treat various ailments such as cough, malaria, wounds, toothache and rheumatic diseases, inflammation, disease related to reactive oxygen species (ROS) formation and many others. The most common ROS is superoxide anion ($O2^{-}$), hydroxyl radicals (OH-) and hydrogen peroxide (H_2O_2) which has been known as a major cause of critical human diseases such as ageing[2], cancer, coronary heart disease, Alzheimer's disease[3][4], neurodegenerative disorders, coronary artery disease, cataracts and inflammation[5]. As ROS are free radicals, can easily react with and oxidize most bio-molecules like carbohydrates, proteins, lipids and DNA. The ability of phenolic compounds to function as anti-oxidants have been picked out by donating a hydrogen molecule.[6] Inflammation is a normal protective response to the injured region of the body or tissue, specifically, and it implies a complex array of enzyme activation, mediator release, fluid extravasations, cell migration, tissue breakdown and repair. It is a complex procedure, which is often linked with pain and involves occurrences such as an increase in vascular permeability, increase of protein denaturation and membrane alterations. Currently available all drugs for inflammation or pain such as nonsteroidal anti-inflammatory drugs (NSAIDs) are generally synthetic and all of them have common side effects like

gastric irritation[7]. Prostaglandin is an autacoid that release extracellularly and initiate pain, most of NSAIDs inhibits the pathway of prostaglandin synthesis. Anti-inflammatory agents either block this attached synthesis by inhibiting the COX enzyme or protecting the lysosomal membrane from breakdown. Therefore, search for other alternatives seems necessary and essential for health benefits.

2. Materials and Method

2.1 Plant Material

The leaves of our experimental plant were collected from Chittagong hill tracts and were authenticated by Dr. Sheikh Bakhtiar Uddin, Botany, University of Chittagong, Bangladesh. A voucher specimen (accession no. 40256) is deposited at the Department of Pharmacy, International Islamic University Chittagong, Bangladesh.

2.2 Plant Part Extraction

The collected plant was dried for a few days in a natural way and then in hot air oven (37 ± 2^0C) for 3 hours. It was macerated to powder form and about 250gm powder was dissolved in 500ml ethanol (95%) following cold extraction [8][9]. It takes a couple of days for proper dissolution, then filtered through a Buchner funnel and again dried at water bath (40^0C) for evaporation of methanol and extract preserved at $<4^0$C for next use.

2.3 Phytochemical Evaluation

Ethanolic extract of *Ixora nigricans* was studied for its phytochemical compounds such as alkaloid, steroid, tannin, glycoside and flavonoid.[10]

2.4 Assay for anti-oxidant activity

2.4.1 1, 1-Diphenyl, 2-Picrylhydrazyl scavenging activity

The scavenging ability of the extract was evaluated using DPPH free radicals[11]. 0.5ml of ethanolic extract of *I. nigricans* at different concentration was mixed with 0.5ml of 100mM ethanolic solution of DPPH and the mixer was allowed to stand idle for 30min in darkness at room temperature. The resultant absorbance was recorded at 517nm. The percentage of inhibition was calculated by the following equation-

$$\frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

IC_{50} values were calculated as the average of three readings.

2.4.2 Reducing Power Capacity

The reducing power capacity was determined by the method of Oyaizu[12] with simple modification. A substance which has reduction potency, reacts with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}) which then react with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700nm. Different concentration of ethanolic extract of experimental plant was mixed with 1ml distilled water with phosphate buffer (2.5ml) and potassium ferricyanide (2.5ml) and incubated at 50^0C for 15min. 2.5ml of trichloroacetic acid was added to the mix and was centrifuged for 10min at 3000rpm. Upper layer of the freshly prepared solution was withdrawn carefully and mixed with ferric chloride solution (0.5ml) and diluted with distilled water (2.5ml). A blank solution was made without using extract.

2.5 Quantitative Analysis

Phenolic content was estimated by following method described by Hammerscmidt[13]. 0.2ml of test solution was mixed with 1ml Folin-Ciocalteu solution (10%) and 0.8ml sodium carbonate solution (7.5%). This mixture was incubated for 1 hour at room temperature and absorbance was measured at 760nm. The result was converted to phenolic content in accordance to the calibration curve of Gallic acid.

Total flavonoid content was estimated colorometrically as described by Zhishen et al[14]. 0.1ml extract (10mg/ml) was added to distilled water to make the volume 5ml and 0.3ml of 5% $NaNO_2$ was added also. 5min later 3ml of 10% $AlCl_3$ was added to the mixture. After 6 minutes, 2ml sodium hydroxide (1M) was added and absorbance was measured at 510nm. Flavonoid content was calculated from calibration curve of Rutin.

2.6 Anti-inflammatory Activity Assessment

2.6.1 *In vitro* anti-inflammatory activity by Protein denaturation

The mixture was consisting of test extract at different concentration and 1% aqueous solution of bovine albumin fraction. Tiny amount 1N HCl was added to the mixture to adjust the pH of the reaction. The samples were incubated at 37^0C for 20 min, in addition 20 min more heated at 57^0C. The solution was cooled and turbidity was measured spectrophotometrically at 660nm for evaluation of a percent of inhibition. The calculation was done by following equation[15]:

$$\frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

2.6.2 Anti-inflammatory activity by membrane stability method

The lysosomal enzyme released during inflammation produces a variety of disorders. The extracellular activity of these enzymes is said to be related to acute or chronic inflammation. The non-steroidal drugs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane. Since HRBC (Human Red Blood Cell) membrane is similar to lysosomal membrane, the study was undertaken to check the stability of the HRBC membrane by the extracts to predict the anti-inflammatory activity *In-vitro*. The various extracts at the concentration of 125, 250, 500 and 1000µg/ml were incubated separately with HRBC solution[16].

Blood was collected from healthy volunteers. The collected blood was mixed with equal volume of Alsever solution (Dextrose 2%, Sodium citrate 0.8%, Citric acid 0.05%, Sodium chloride 0.42% and Distilled water 100 ml) and centrifuged with isosaline. To 1 ml of HRBC suspension equal volume of test drug in three different concentrations was added. All the assay mixtures were incubated at 37ºC for 30 minutes and centrifuged. The hemoglobin content in the supernatant solution was estimated by using spectrophotometer at 560nm[17]. The percentage of haemolysis was calculated then, by the formula as given below:-

$$\text{Percent of hemolysis} = \frac{\text{OD of test}}{\text{OD of control}} \text{ X } 100$$

The percentage of protection can be hence calculated from the equation as presented under,

$$\text{Percentage of protection} = 100 - \frac{\text{OD of test}}{\text{OD of control}} \text{ X } 100$$

Here 'OD of test'= optical density or the test sample's absorbance and 'OD of control'= optical density or absorbance of the negative control.

Hither, the negative control used was the Alsever's solution with blood in it and it contained no Aspirin or methanolic extract of the plant material in it.

2.7 Statistical Analysis

The outcomes are represented as mean±SD and means were calculated from three consecutive values of each exam. The IC_{50} was calculated from linear regression of the graph.

3. Result and Discussion

3.1 Phytochemistry

The experimented plant part shows the presence of carbohydrate, tannin, phenolic content, flavonoids and glycoside (**Table 1)**.

Table 1: Phytochemicals and quantitative analysis of ethanolic extract of *I. nigricans*

Compounds	Ethanolic extract of *I. nigricans*
Phytochemicals	Steroid, Tannin, Glycoside, Carbohydrate, Alkaloid
Phenolic content	20.80±1.44
Total Flavonoid	84.03±0.78

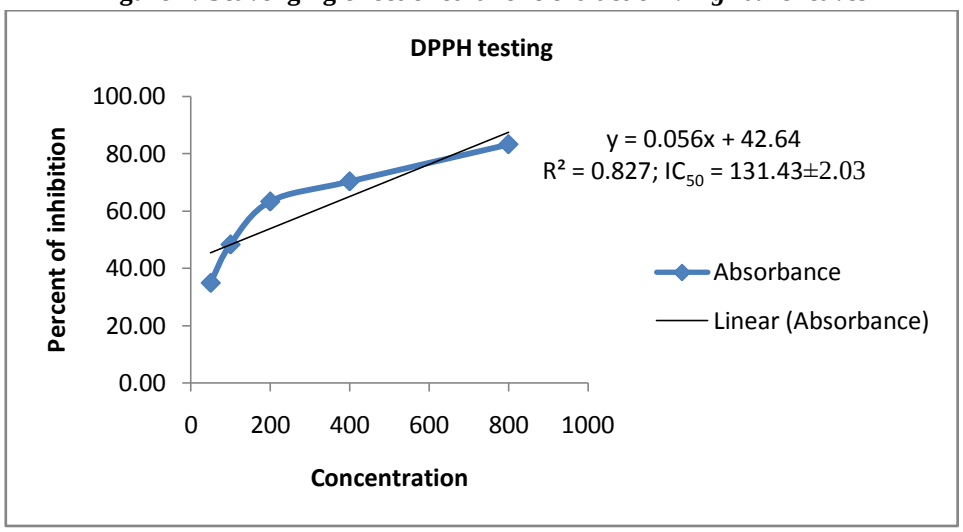
*phenolic content was calculated as (mg) of Gallic acid per gram of extract; value represents mean±SD
**total flavonoid was calculated as (mg) of Rutin per gram of extract; value represents mean±SD

3.2 DPPH scavenging activity

1, 1-Diphenyl-2-picrylhydrazyl radical scavenging assay is the most common method for screening anti-oxidant action, since it can accommodate many samples in a short period and detect active ingredient at low concentration[18][19].

The decrease in absorbance caused by antioxidant is due to scavenging of free radicals by hydrogen donation. It is also visually noticeable by color change (from violet to yellow). The fall in absorbance was reported in a manner of concentration dependent, the correlation (r) value was 0.827 which implies the values were related closely and the IC_{50} value found 131.43±2.03µg/ml.

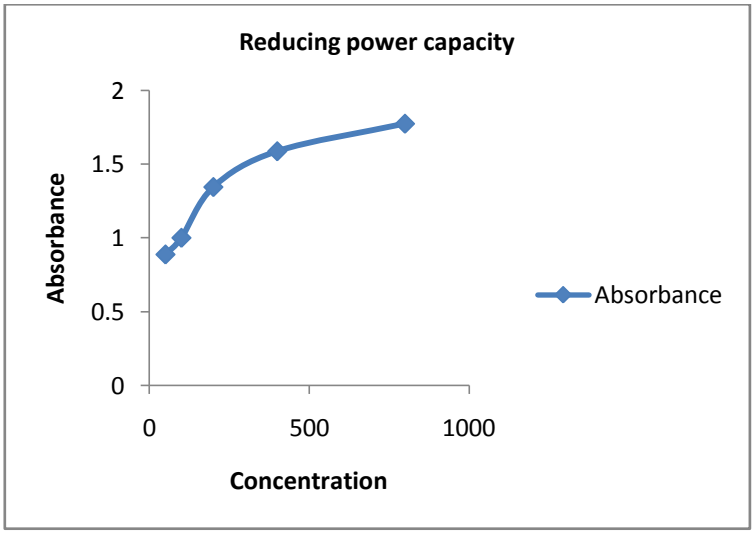
Figure 1: Scavenging effect of ethanolic extract of *I. nigricans* leaves



3.3 Reducing Power Assay

In the assay for reducing power capacity measurement the initial color (yellow) was changed to different grade of green and low, depending on the subduing ability of each immersion. The change of color was due to conversion of Fe^{3+} to Fe^{2+}. So the higher the absorbance at 700nm means the presence of Fe^{2+} at higher concentration, i.e. the reducing power is high at this stage. Our experimental plant part shows the concentration gradient result and from the final graphs (Figure 2), it's readable that it reduces free radical as its concentration rises.

Figure 2: Reducing Power capacity measurement of ethanolic extract of *I. nigricans*



3.4 Anti-inflammatory activity by Protein (albumin) denaturation

Protein denaturation is a well known and established cause of inflammation. Drugs like salicylic acid, phenylbutazone (anti-inflammatory drugs) have shown dose dependent response to thermally induced protein denaturation. As a part of our investigation in a search of anti-inflammatory activity, potency of extract of *I. nigricans* in inhibition of protein denaturation, it was found effective at different concentration **(Table 2).** It results 78.07±2.4% of inhibition at maximum concentration. Aspirin, an established anti-inflammatory drug showed 86.28±1.48% of inhibition, it was near to our studied plant part.

3.5 Anti-inflammatory activity by membrane stabilizing

Membrane stabilizing activity was studied to further ensure the anti-inflammatory property of ethanolic extract of *I. nigricans*. The selection is very much potent in inhibiting heat induced hemolysis. The actual mechanism of this infusion may be due to inhibit the discharge of lysosomal content of neutrophils at the site of excitation. These lysosomal constituents are involved in bactericidal action by containing bactericidal enzyme and protease, these when release extracellularly cause damage to tissue and inflammation[20]. Test extract inhibited hemolysis at different level according to the concentration (Table 2). It inhibited 81.74±2.54% at 1000µg concentration where as the comparability standard (Aspirin) results 90.23±1.48%.

Table 2: Effect of ethanolic extract of *I. nigricans* in inhibition of heat induced protein denaturation and hemolysis (membrane stability)

Group	Concentration (µg/ml)	Membrane Stability (% inhibition)	Protein Denaturation (% inhibition)
Ethanolic extract of *I. nigricans*	125	17.79±1.67	33.22±1.98
	250	36.46±1.28	46.01±2.22
	500	75.00±2.30	63.09±0.54
	1000	81.74±2.54	78.07±2.40
Standard	200	90.23±1.48	86.28±1.48

4. Conclusion

This study revealed that ethanolic extract of *I. nigricans* has effective anti-inflammatory potency and it contains some useful anti-oxidant compound that's important for human health. The actual mechanism of action of anti-inflammatory is yet not confirmed, but it is possible that the extract produced this event due to surface area/volume ration of cells, which could be brought around by an expansion of membrane or the shrinking of the cells and an interaction with membrane proteins[21]. Further investigation is taken in this selection to confirm the mechanism of activity.

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Ethical Consent

All authors read this article and approved this article and they also declared nothing done in this research that violates any ethical state.

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