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Original Research Article

## Suppression of inflammatory mediators by aqueous leaf extract of *Crotalaria verrucosa*: *in vivo* and *in vitro* analysis

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### ABSTRACT

**Background:** *Crotalaria verrucosa* is a traditional plant frequently prescribed by the tribes for its medicinal value against inflammation. The present study was designed to investigate the scientific basis for medicinal value in inflammation by *in vivo* and *in vitro* analysis.

**Methods:** Anti-inflammatory activity of the plant's leaf was evaluated by two *in vivo* methods - carrageenan induced rat paw edema and xylene induced mice ear edema. Moreover, *in vitro* analysis was performed through heat induced hemolysis and heat induced protein denaturation methods.

**Results:** The inflammation produced by carrageenan and xylene were effectively suppressed by the aqueous leaf extract of *C. verrucosa* (CVAQ) at 600 mg/kg body weight which was comparable to the standards. In heat induced hemolysis test the extract was able to inhibit the lysis up to 70% at 500 µg/ml whereas in heat induced protein denaturation test it reduces the percentage till 69% at the same concentration.

**Conclusions:** The findings suggested that CVAQ possess moderate to high anti-inflammatory activity when applied in low to high concentrated doses. However, the study can only conclude from this basic evaluation that the extract needs to be further investigated for identifying the potential compound which contributed to such medicinal value of the plant.

**Keywords:** Anti-inflammatory activity, *Crotalaria verrucosa*, Paw edema, Ear edema, Protein denaturation, Haemolysis

### INTRODUCTION

The use of medicinal plants in the treatment of diseases is common from the ancient time till date. According to World health organization 80% of the populations in developing countries rely on the traditional medicines for their primary treatments.<sup>1</sup> The discovery of many essential phytocompounds and their traditional use

together found to attract the bio-model researchers and triggered for pharmacological investigations. As part of this, thousands of medicinal plants being traditionally used in Bangladesh are under investigation for their scientific basis of efficacy. The principle objective of these studies is to find responsible compounds or fractions to suggest the best preparation of traditional medicine, to get the optimum effect as well as to take approach to extract or synthesize such compound for

allopathic preparation through research and development.<sup>2</sup>

*Crotalaria verrucosa*, commonly known as blue rattlesnake, is a member of Leguminosae (family: Fabaceae).<sup>3</sup> So far it has been reported as the only perennial shrub solely possessing medicinal value among *Crotalaria* species. They are found in unploughed land of Chittagong, Rajshahi, Mymensingh, Khulna, Sylhet districts of Bangladesh.<sup>4</sup> The plant is a branched herbaceous shrub, grows up to 80-100 cm high which rises to triangular branches with 5-15 cm ovate deltoid leaves. Its leaf extract which contains tropane alkaloids, is considered efficacious in decreasing salivation and also used in impetigo and scabies.<sup>5</sup> It also contains crotaverine, anacrotine and O12-acetyl-crotaverine. Researchers have found its effectiveness in dyspepsia, cough, biliousness, cardiac abnormalities and other oral disease. Tribal people use it as traditional medicine in skin allergies in different countries.<sup>4</sup> Moreover, its hepatoprotective, antifertility activity and efficacy against estrogenic implantation has also been reported.<sup>6</sup> The present study was designed to investigate its anti-inflammatory response with aqueous leaf extract by established methods. Such investigation has not been reported in any literature.

## METHODS

### *Collection of plant leaf*

The leaves of *Crotalaria verrucosa* were collected from the hilly areas of Mymensingh district of Bangladesh on April when the leaves were in abundance. A sample specimen was sent to Bangladesh National Herbarium (BNH), Mirpur, Dhaka and authenticated with Accession number: DACB 42010.

### *Preparation of extract*

Leaves were thoroughly washed after collection and dried under ceiling fan. The dried leaves were then grinded into powder (approx. 500 gm) by a grinding machine and soaked in water (2L) for 3 days with rigorous shaking. The mixture was filtrated with filter paper and the filtrate was inspissated using a rotary evaporator to obtain the viscous aqueous extract (approx. 5 gm) of Aqueous extract of *C. verrucosa* (CVAQ). Obtained extract was finally stored in air tight container till its use.

### *Drugs and chemicals*

Prednisolone, indomethacin, xylene, carrageenan, acetyl salicylic acid used in experiments were collected from Merk, Germany.

### *Acute toxicity study*

Separate acute toxicity tests were performed for Wister rat and Swiss Albino mice. Five groups (n=5) of each

type of animal were given high dose of the plant extract (500 mg/kg, 1000 mg/kg, 1500 mg/kg and 2000 mg/kg) and compared with a control (water) group. Animals were observed for next 72 hours for mortality, disorders and abnormal behavior.

### *In vivo anti-inflammatory tests*

#### *Approval of institutional ethical committee*

The standard guideline for treating and handling animals adopted by Institutional ethical committee (Ref. no. IPSDRLAB/AHCP/01/18) was pursued for *in vivo* tests.

#### *Xylene induced ear edema*

The experiment xylene induced ear edema was undertaken as described by Tang et.al with slight modification.<sup>7</sup> Swiss albino mice, weighing 22-28 gm of both sexes were taken. They were divided into six groups (N=5); control (water), test drugs (CVAQ 200 mg/kg, CVAQ 400 mg/kg, CVAQ 600 mg/kg body weight) and standards (indomethacin 10 mg/kg and prednisolone 5 mg/kg body weight) were administered orally to the animals prior the bilateral application of xylene (inflammatory substance) to the right ear of each mouse. After 15 minutes, animals were sacrificed by dislocating their spinal cord from the neck and both ears were removed from test, standard and control animal. A 0.3 cm diameter circular area was cut from each ear of the animal. The weights of control (left) and inflamed ear (right) were taken and difference was calculated.

#### *Carrageenan induced rat paw edema*

The method was performed as described by Winter et. al with slight modifications.<sup>8,9</sup> Both male and female Wister rat weighing 130-150 mg were taken and grouped as the experiment above (N=5). Test drug (CVAQ 200 mg/kg, CVAQ 400 mg/kg, CVAQ 600 mg/kg body weight) and standard drugs (Indomethacin 10 mg/kg and prednisolone 5 mg/kg body weight) were administered orally to the animals 1 hour prior to injection of carrageenan (inflammation inducer) to the right paw while its left paw was left untreated to serve as its own control. Paw thickness was measured by vernier calipers measurement scale before the carrageenan injection as a base value. Thickness of the paw were again measured after treatment in intervals (1, 2, 3, 4 and 24 hours after injection).

### *In vitro anti-inflammatory tests*

#### *Heat induced hemolysis*

Fresh whole blood was collected from a healthy volunteer and washed it with 0.9% normal saline. It was then centrifuged at 3000 rpm for 10 minutes. A 10% v/v HRBC suspension was obtained using isotonic phosphate buffer (154 mM NaCl in 10 mM sodium phosphate buffer

at pH 7.4). 1 ml phosphate buffer [pH 7.4, 0.15 M], 2 ml hyposaline [0.36%], 0.4 ml HRBC suspension with 0.6 ml of aqueous extracts of different concentrations (100, 200, 300, 400, 500 µg/ml) or same concentrations of acetyl salicylic acid for a standard or distilled water for control constituted the final reaction mixture (4 ml). The mixture was then incubated for 30 minutes at 37° C. After incubation the mixture was centrifuged at 3000 rpm for 20 minutes. The supernatant was separated and its absorbance was taken at 560 nm using UV spectrophotometer.<sup>10</sup>

#### Heat induced protein denaturation

The method used for extraction of egg albumin was described by S.J. Carter.<sup>11</sup> A reaction mixture was prepared for the investigation of the inhibition capacity of albumin denaturation. The mixture contained 0.2 ml of egg albumin, 32.8 ml of phosphate buffer saline (pH 6.4) and 2 ml of CVAQ extract at various concentration (100, 200, 300, 400, 500 µg/ml). Aspirin at the same doses and distilled water served as the standard and control respectively. The mixture was filled in tubes to incubate at 37° C for 10 minutes. After incubation the mixture containing tubes were heated at 70°C for 5 minutes. The mixture was then left to be cooled. The UV spectrophotometer was used to measure its absorbance at 660 nm.

#### Statistical analysis

Data obtained were analyzed via statistical analysis of variance (ANOVA) method using SPSS 24 for Windows and assessed for the significance of the result at 95%, 99% and 99.99% confidence level and denoted respectively as p values <0.05, 0.01 and 0.001. All results were compared with the negative control.

## RESULTS

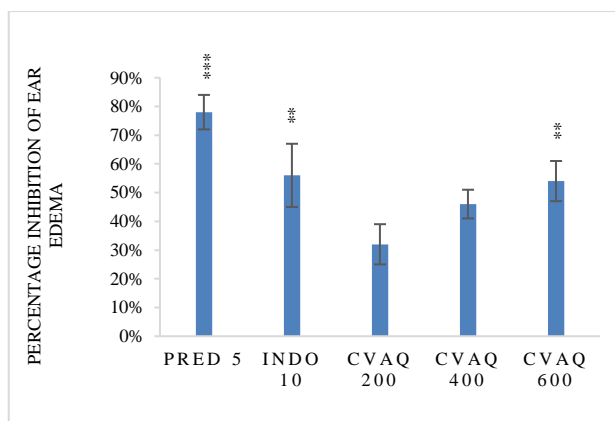
#### Acute toxicity study

The acute toxicity study resulted with no morbidity or abnormal condition for the treated groups of animals. The study suggested a safe dose line for further experiments.

#### In vivo anti-inflammatory tests

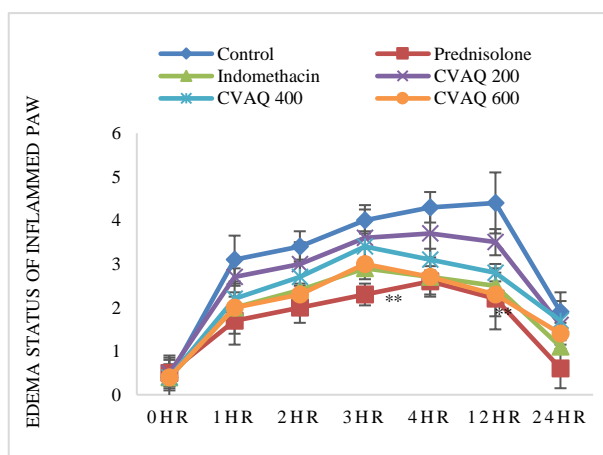
##### Xylene induced ear edema

Xylene increased the edema by producing inflammation in control group. In response to the treatment, prednisolone and indomethacin decreased the inflammation which increased their percentage of inhibition as 78% and 56% respectively. The aqueous extract at 600 mg/kg body weight was able to exhibit maximum potential to suppress (54%) edematous condition which was comparable to indomethacin at 10 mg/kg body weight. However, CVAQ at 200 and 400 mg/kg doses could not produce significant activity.



**Figure 1: Percentage inhibition of ear edema in xylene induced ear edema test.**

Figure represented the percentage inhibition of ear edema induced by xylene in respective groups of Swiss Albino mice. CVAQ = Aqueous extract of *C. verrucosa*, PRED 5= Prednisolone 5 mg/kg body weight, INDO 10 = Indomethacin 10 mg/kg body weight. Data represented as mean+SEM and analyzed by one-way ANOVA followed by Dunnett t test where \*\*, \*\*\* denoted p<0.01 and p<0.001 respectively and as statistically significant. All groups (n=6) were compared to control.



**Figure 2: Edema status of inflamed paw in carrageenan induced paw edema test.**

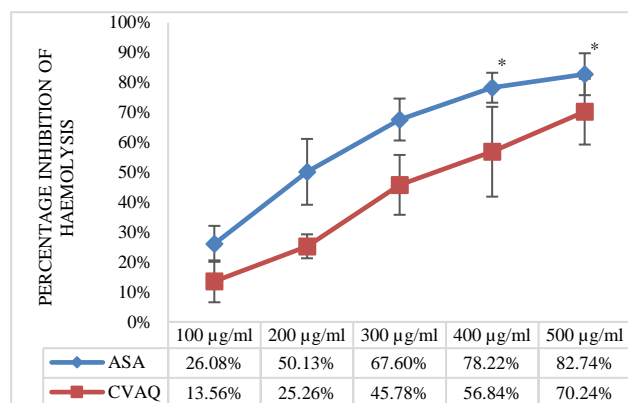
Figure represented the thickness of paw measured in cm inflamed by carrageenan in respective groups of Wister Rat. CVAQ = Aqueous extract of *C. verrucosa*. Data represented as mean+SEM and analyzed by one-way ANOVA followed by Dunnett t test where \*\* denoted p<0.01 as statistically significant. All groups (n=6) were compared to control.

Carrageenan showed inflammatory response from the first hour of the treatment and increased the edematous condition gradually in next hours. Prednisolone was found to be highly effective in reducing the edema in all intervals compared to control whereas indomethacin mimicked prednisolone closely in suppressing the inflammation (Figure 2). Like xylene test, CVAQ 600 showed to be effective as indomethacin from 0-12 hrs, though in low dose exhibited with mild response.

**In vitro anti-inflammatory tests**

**Heat induced hemolysis**

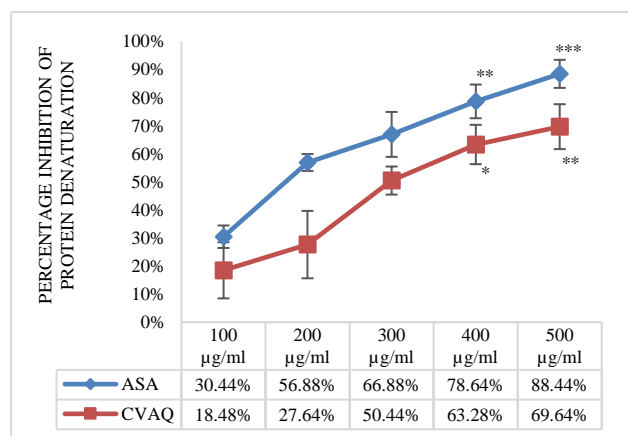
A standard curve was drawn with the applied concentration of acetyl salicylic acid to compare with that of the aqueous extract (Figure 3). The curves showed gradual increase in percentage of inhibition with increased concentration. The concentration 500 µg/ml was found to be most effective to inhibit hemolysis for both ASA (82%) and CVAQ (70%).



**Figure 3: Percentage inhibition of haemolysis in heat induced haemolysis test.**

Figure represented the percentage inhibition of haemolysis induced by heat. CVAQ = Aqueous extract of *C. verrucosa*. ASA = Acetyl salicylic acid. Data represented as mean+SEM and analyzed by one-way ANOVA followed by Dunnett t test where \* denoted  $p < 0.05$  as statistically significant. All groups ( $n=3$ ) were compared to control.

**Heat induced protein denaturation**



**Figure 4: Percentage inhibition of protein denaturation in heat induced protein denaturation test.**

Figure represented the percentage inhibition of protein denaturation induced by heat. CVAQ = Aqueous extract of *C. verrucosa*. ASA = Acetyl salicylic acid. Data represented as mean+SEM and analyzed by one-way ANOVA followed by Dunnett t test where \*, \*\* and \*\*\* denoted  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$  respectively.

$p < 0.001$  respectively and as statistically significant. All groups ( $n=3$ ) were compared to control.

Like hemolysis test, ASA showed a gradual increase in inhibiting the percentage of protein denaturation which was statistically significant at 400 (78%) and 500 (88%) µg/ml concentrations (Figure 4). In comparison, CVAQ at the same concentrations exhibited moderate suppression as 63% and 69% respectively.

**DISCUSSION**

Inflammation belongs to release of mediators and activation of receptors at the target site. Thus, anti-inflammatory activity is obtained through a competitive binding of the antagonists to these receptors. In this study two *in vivo* methods were adopted having different inflammatory pathways and mediator’s involvement so as to test the sample in broader spectrum.<sup>6</sup> Carrageenan and xyline, two inflammatory substances which are still under investigation for their mechanism of triggering inflammation, were applied to living models. Standard anti-inflammatory agents were put in the frontline to understand the participation of mediators in each examination. Previous studies suggested carrageenan’s potential to trigger a two-phase biological response – initially by activating histamine, serotonin, kinin like mediators within first two hours and finally by releasing excessive prostaglandins, oxygen-derived free radicals and cyclooxygenase; where polymorphonuclear (PMN) leucocyte infiltration and a significant increase in the level of neutrophils were reported through observation of a large numbers of inflammatory cells in the paw tissues.<sup>12,13</sup> Unlike carrageenan, xyline is known to be partially associated with substance P, a neuromodulator which is extensively distributed throughout the central and peripheral nervous system. Xyline facilitates the release of substance P causing neurogenic inflammation through vasodilatation and plasma extravasations.<sup>14</sup> This event is caused by an innate immunity response produced by activated T-cells which results in migration of PMN leucocytes and augmentation of swelling and heaviness of the ear of mice.<sup>15</sup>

In both tests, indomethacin, a common non-steroidal anti-inflammatory (NSAID) agent found to have strong involvement in suppressing the mediators whereas the greater efficacy shown by prednisolone was largely due to its steroidal properties.<sup>16-18</sup> In comparison, the aqueous extract of *C. verrucosa* elucidated its potential for mediator suppression as similar to that of indomethacin, however the particular mediator involvement and the specific mechanism could not be predicted at this point of study without knowing the responsible components of the sample.

To support the claims, two *in vitro* study were performed to evaluate the protective ability of the sample towards protein denaturation and hemolysis.<sup>19</sup> A chronic inflammatory response has been reported to occur due to

protein denaturation, a biochemical reaction that could result in attenuation of tissue function. Pro-inflammatory markers are released by the lysis of lysosomal membranes during chronic inflammation which activates neutrophils, proteases and histamines at local site of tissue damage causing type III hypersensitivity reactions as well as the diseases like serum sickness.<sup>20,21</sup> As conventional NSAID, acetyl salicylic acid (aspirin) not only blocked COX enzyme and synthesis of endogenous PGE2 but also found to suppress the process of protein denaturation.<sup>22</sup> However, the leaf extract mimicked the NSAID to a moderate extent showing similar pattern in results. The other experiment suggested heat induced hemolysis is a result of erythrocyte membrane destabilization which promotes the production of free radicals, such as lipid peroxides and superoxides.<sup>23</sup> As the leaves have been reported to contain flavonoids, tannins and other phenolic content which act as effective scavengers of free radicals, the biological membrane stabilization properties shown by its extract can be attributed to these chemicals which prevented stress-induced destruction of the plasma membrane.<sup>24</sup>

## CONCLUSION

The findings of the study demonstrated that the aqueous leaf extract of *C. verrucosa* possess potential anti-inflammatory activity. However, the study was yet to indicate the biological compounds, specific mediators' involvement and mechanism of suppression relating to this anti-inflammatory effect. Further investigation was recommended on phytochemical screening and *in vitro* analysis to establish the scientific basis of its activity.

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*Conflict of interest: None declared*

*Ethical approval: The study was approved by the Institutional Ethics Committee*

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