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## Assessment of the *in vitro* anti-inflammatory effects of *Cardiospermum halicacabum* extract

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### ABSTRACT

**Background:** Inflammation is the body's natural response to harmful stimuli. Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used to alleviate inflammation, but they often come with significant side effects. Consequently, there is a growing need for new, effective anti-inflammatory agents with minimal adverse effects, preferably derived from plants. **Aims and Objectives:** This study aimed to prepare aqueous and ethanol extracts of the entire *Cardiospermum halicacabum* plant using the cold maceration extraction method, evaluate their *in vitro* anti-inflammatory properties, and determine the IC<sub>50</sub> values through a concentration-response curve. **Methodology:** Whole *C. halicacabum* plants were collected, and extracts were prepared using cold maceration with ethanol and water. The anti-inflammatory properties of these extracts were assessed *in vitro* using the heat-induced ovalbumin denaturation method, with diclofenac sodium serving as the reference drug. **Results:** The ethanol and aqueous extracts yielded 15.7% and 10.4% w/w, respectively. Compared to diclofenac sodium (IC<sub>50</sub> at 1922 µg/mL), the extracts demonstrated mild-to-moderate, concentration-dependent anti-inflammatory activity, with IC<sub>50</sub> values of 5157 µg/mL for the ethanol extract and 8121 µg/mL for the aqueous extract. A statistically significant positive correlation was observed between the concentration and percentage inhibition of the extracts, with a p-value of less than 0.05 and an R-square value close to one. **Conclusion:** The extracts of *C. halicacabum* exhibit mild-to-moderate anti-inflammatory activity compared to standard diclofenac sodium.

**Keywords:** *Cardiospermum halicacabum*, Anti-inflammatory activity, Non-Steroidal Anti-Inflammatory Drugs, Cold maceration technique, Ovalbumin denaturation assay.

### INTRODUCTION

Inflammation is a complex biological response to harmful stimuli, characterized by symptoms such as pain, redness, swelling, warmth, and loss of function. This process involves both vascular and cellular responses, triggered by chemical mediators released from damaged cells [1]. These mediators, including arachidonic acid metabolites and cytokines, enhance vascular permeability and cause vasodilation, leading to fluid exudation, accumulation, redness, and warmth. The resulting fluid stasis facilitates leukocyte recruitment and activation [2]. Inflammation can be classified as either acute or chronic based on its duration. Acute inflammation represents the body's initial, short-term response to harmful stimuli, while chronic inflammation develops gradually and persists for extended periods, ranging from days to years [1].

Chemical mediators play a crucial role in initiating and sustaining acute inflammation by binding to specific receptors or through direct enzymatic or toxic activity [3]. Key substances released during inflammation include vasoactive amines (e.g., serotonin, histamine), eicosanoids (e.g., thromboxane, prostaglandins), and peptides (e.g., bradykinin), which induce complex reactions within the body [4]. Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used to manage inflammation [5]. These drugs function by inhibiting processes at various checkpoints, such as cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2), which are essential for prostaglandin synthesis [6].

However, prolonged use of NSAIDs can lead to several adverse reactions, including cardiovascular diseases, gastrointestinal mucosal impairment, hormonal imbalances, headaches, and allergic reactions. These side effects are attributed to the non-selective inhibition of both constitutive (COX-1) and inducible (COX-2) isoforms of cyclooxygenase [7]. Various drug classes exhibit anti-inflammatory activity, including NSAIDs like Ibuprofen and Diclofenac, corticosteroids such as Prednisolone and Methylprednisolone, and cytokine receptor inhibitors like Basiliximab [8].

Plants are significant sources of novel chemical substances with therapeutic potential and fewer side effects. Plant-based products have demonstrated various beneficial properties, including low toxicity,

synergistic effects, high purity, and minimal side effects. Natural products have significantly contributed to the advancement of modern medicine [9]. Consequently, the search for new medicines with fewer serious adverse effects and economic favorability has become crucial. Researchers worldwide have increasingly studied natural sources and phytochemicals with anti-inflammatory activity [10].

*Cardiospermum halicacabum*, a member of the Sapindaceae family, is widely distributed in tropical and subtropical regions [11]. This plant exhibits antimicrobial, antifungal, antidiarrheal, antiparasitic, anxiolytic, and antipyretic effects [12]. It is also effective in managing arthritic inflammatory conditions and is extensively used in traditional medical systems such as folk medicine, Ayurveda, and Unani [13].



Figure 1: *Cardiospermum halicacabum* plant

## MATERIAL AND METHODS

### Collection and Authentication of Plant Material

1.5 kg of mature, whole *C. halicacabum* plants were collected during the day from Ingiriya (6.7404° N, 80.1625° E), Kalutara District, Western Province, Sri Lanka. The dried and pressed specimen was sent to the Division of Pharmaceutical Botany at the Ayurveda Research Institution, Navinna, Sri Lanka for authentication. Identification was confirmed using a voucher specimen (Acc. no 2090) deposited at the herbarium.

### Preparation of Crude Extracts of Plant Material

The whole plants were cleaned with running tap water and air-dried at  $28 \pm 2^\circ\text{C}$  until a constant weight was achieved. Then the dried plants were powdered using a domestic grinder (INNOVEX, Model: IMG 010) to obtain a coarse powder used for the extraction procedure.



Figure 2A: Air-dried *C. halicacabum* plant



Figure 2B: Coarse powder of *C. halicacabum*

### Ethanol and aqueous extraction procedures

One hundred grams (100 g) of powdered *C. halicacabum* was placed in a closed vessel and macerated with 500 mL of ethanol, shaking continuously for 24 h at 130 rpm at room temperature using a laboratory shaker incubator (Model No: IN-666, S/N: 112783). The resulting extract was suction-filtered through filter paper and evaporated using a rotary evaporator (HAHNVAPOR, Model No: HS-2005S, S/N: S-00352) to remove the solvent. The crude dry powder obtained was then freeze-dried for 7 days using a freeze dryer (Shandong, Model No: BK-FD10PT). Similarly, 100 g of powdered *C. halicacabum* was macerated with 500 mL of distilled water under the same conditions. The resulting extract was suction-filtered and freeze-dried for 17 days using the same freeze-dryer to obtain the sludge.

### Evaluation of Physicochemical Characteristics of the *C. halicacabum* Extracts

#### Nature, yield, and solubility of the freeze-dried *C. halicacabum* crude sample and UV-visible Spectrum

Each freeze-dried crude sample was observed visually for organoleptic properties and physical appearance. The yield of each extract was calculated by using the weight of each freeze-dried crude extract and the weight of the dry coarse powder of the *C. halicacabum* plant used for each extraction process. One milligram of ethanol and aqueous freeze-dried crude extracts were tested for solubility in distilled water. Each extract was accurately weighed (1 mg), placed in a test tube, and dissolved in distilled water using a micropipette. Samples were agitated for one minute with a laboratory vortex after adding 100  $\mu\text{L}$  of distilled water. The UV-visible spectrum of the ethanol and aqueous extracts was obtained using a spectrophotometer. The extracts were examined under visible and UV light at wavelengths ranging from 200–800 nm.

### Evaluation of *In vitro* Anti-Inflammatory Activity – Concentration Dependent Activity of Ethanol and Aqueous Extract of *C. halicacabum* and Diclofenac Sodium (DC) on Heat-Induced Ovalbumin Protein Denaturation

#### Preparation of Test and Standard Solutions

Stock solutions and control solutions were prepared as follows;

640 mg of each plant extract (ethanol and aqueous) was dissolved in 40 mL of distilled water. Serial dilutions were made to obtain concentrations of 16 mg/mL, 8 mg/mL, 4 mg/mL, 2 mg/mL, 1 mg/mL, and 0.5 mg/mL. 60 mg of DC (98% w/w) was dissolved in 30 mL of distilled water. Serial dilutions were made to obtain concentrations of 1.6 mg/mL, 0.8 mg/mL, 0.4 mg/mL, 0.2 mg/mL, 0.1 mg/mL, and 0.05 mg/mL. Positive Control was prepared with 0.2 mL ovalbumin, 2.8 mL PBS, and 2 mL distilled water. Negative Control 5 mL of distilled water.



## Ovalbumin Fraction Separation and Heat-Induced Ovalbumin Denaturation Assay

The egg white was separated from the yolk using a paper knife and mixed well with a glass rod.

Following [14] method, the reaction mixture included 0.2 mL ovalbumin fraction, 2.8 mL PBS (pH 6.4), and 2 mL of various concentrations of *C. halicacabum* extracts or standard drug. Mixtures were incubated at 37°C for 15 minutes, then heated to 70°C for 5 minutes with continuous stirring. After cooling, turbidity was measured at 660 nm using a spectrophotometer. Results were obtained in triplicate, and protein denaturation inhibition was calculated as:

$$\text{Percentage Inhibition} = \frac{(\text{Absorbance of the control} - \text{Absorbance of the sample})}{\text{Absorbance of the control}} \times 100$$

## Determination of IC<sub>50</sub> Values

IC<sub>50</sub> values for each extract and standard were determined using nonlinear regression (sigmoidal, 4PL, X = log (Concentration)) in GraphPad Prism 9 (version 9.0.0).

## Statistical Analysis

Results are presented as mean ± SEM (Standard Error of Mean) and ± SD (Standard Deviation). GraphPad Prism 9 (version 9.0.0) was used to calculate concentration dependencies and IC<sub>50</sub> values via nonlinear regression. Significance was set at p < 0.05.

## RESULTS

### The Physicochemical Characteristics of the *C. halicacabum* Extracts

#### Nature, yield and solubility of the ethanol and aqueous extracts

The ethanol extract was a dry powder, which was green in color (Figure 3A) and with a fragrant odor. Aqueous extract was a brownish color sludge (Figure 3B) and had no odor.



Figure 3A: Dry powder of ethanol extract



Figure 3B: Sludge of aqueous extract

The percentage yields of ethanol and aqueous extracts of whole plants of *C. halicacabum* were 15.7% (w/w) and 10.4% (w/w) respectively.

The data revealed that the ethanol extract of *C. halicacabum* has higher water solubility compared to the aqueous extract of *C. halicacabum*. The solubility of both extracts is present in Table 1.

#### UV-visible spectrum of *C. halicacabum* extracts

Figure 4 represents the UV-visible spectrum of ethanol and aqueous extracts.

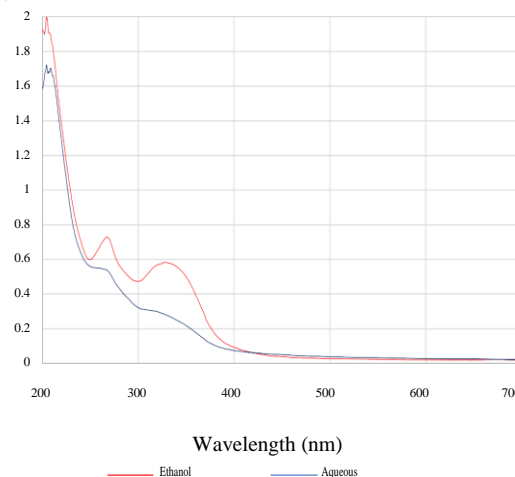


Figure 4: UV absorption spectrum of ethanol and aqueous extracts of *C. halicacabum* in distilled water

### The Results of *In vitro* Anti-Inflammatory Assay

**Diclofenac Sodium (98% w/w) Anti-inflammatory Activity:** The percentage inhibition of Diclofenac Sodium at various concentrations was calculated using the methodology equation. The mean percentage inhibition results are shown in Table II and represented as mean ± SEM. According to Table 2, the percentage inhibition of ovalbumin denaturation by Diclofenac Sodium ranged from 26.41 ± 0.49% to 79.60 ± 0.19% at concentrations of 50-1600 µg/mL.

#### Statistical Analysis and Concentration-Response Curve

The ovalbumin denaturation assay results were analyzed using GraphPad Prism 9. The concentration-response curve (Figure vii) was plotted with percentage inhibition (Y-axis) against log concentration (X-axis) to determine the IC<sub>50</sub> value of Diclofenac Sodium. Figure 5 shows a positive correlation ( $r^2 = 0.9978$ ) between log concentrations and inhibition percentages, with an IC<sub>50</sub> value of 1922 µg/mL.

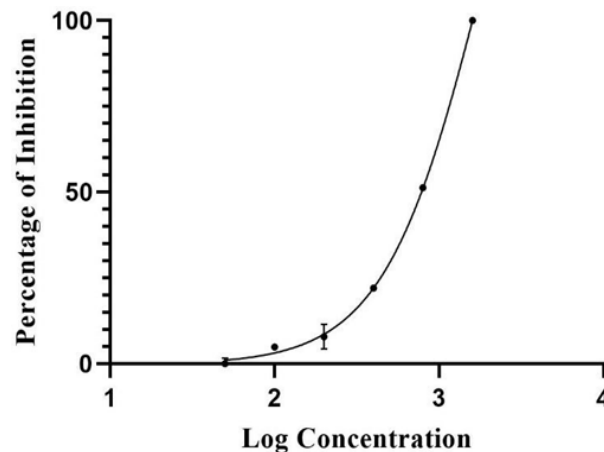


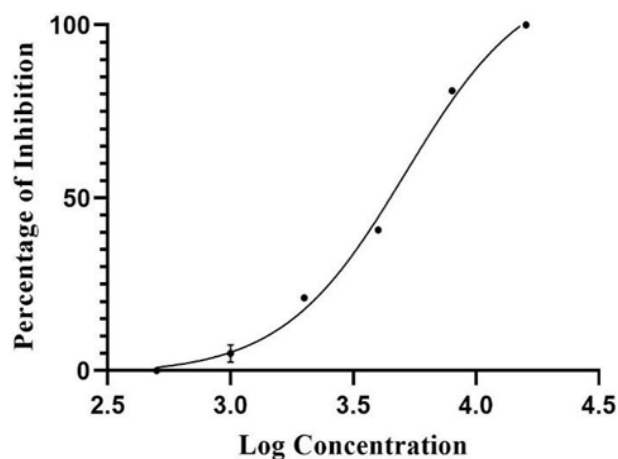
Figure 5: Concentration-response curve for anti-inflammatory activity of Diclofenac Sodium based on inhibition percentage

*Ethanol Extract of C. halicacabum Anti-inflammatory Activity*

The percentage inhibition of the ethanol extract of *C. halicacabum* at various concentrations was calculated using the methodology equation. The mean percentage inhibition results are shown in Table III and represented as mean  $\pm$  SEM. According to Table 3, the percentage inhibition of ovalbumin denaturation by the ethanol extract ranged from  $07.13 \pm 0.00\%$  to  $88.04 \pm 0.11\%$  at concentrations of 500-16000  $\mu\text{g/mL}$ .

*Statistical analysis and concentration-response curve of ethanol extract of C. halicacabum*

Using GraphPad Prism 9, a concentration-response curve was plotted with percentage inhibition (Y-axis) against log concentrations of ethanol extract of *C. halicacabum* (X-axis) (Figure 6). This curve determined the  $\text{IC}_{50}$  value of the extract. Figure viii shows that as log concentrations of the extract increased, percentage inhibition also increased. A strong positive correlation ( $r^2=0.9945$ ) was observed between log concentrations and inhibition percentages, with an  $\text{IC}_{50}$  value of 5157  $\mu\text{g/mL}$ .



**Figure 6:** Concentration-response curve for the anti-inflammatory activity of ethanol extract of *C. halicacabum* based on inhibition percentage

*Anti-inflammatory activity of aqueous extract of C. halicacabum*

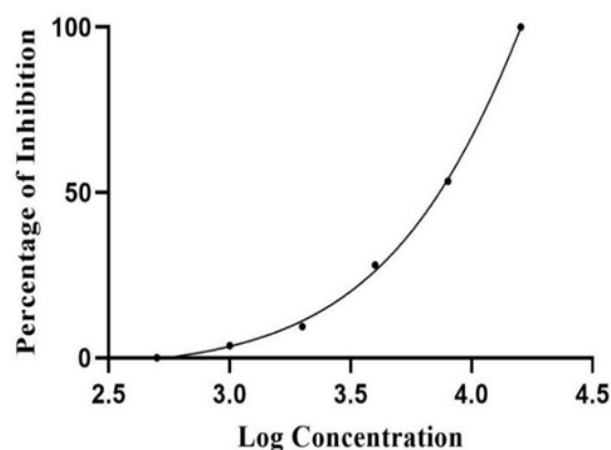
The percentage inhibition of the aqueous extract of *C. halicacabum* was calculated using the methodology's equation. The mean percentage inhibition results are shown in Table 4, represented as mean  $\pm$  SEM. The inhibition of ovalbumin denaturation ranged from  $2.51 \pm 0.11\%$  to  $79.73 \pm 0.06\%$  at concentrations of 500-16000  $\mu\text{g/mL}$ .

*Statistical analysis and concentration-response curve of aqueous extract of C. halicacabum*

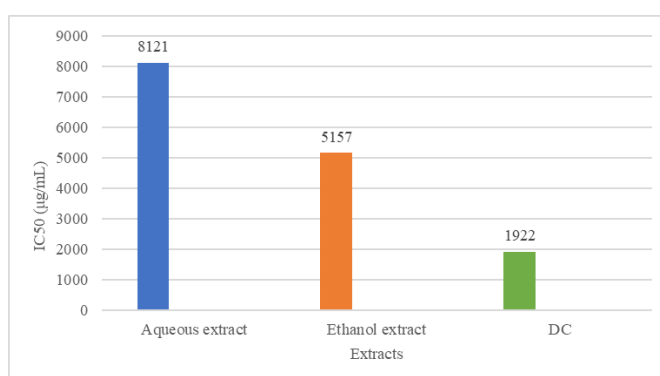
Using GraphPad Prism 9, a concentration-response curve was plotted with percentage inhibition (Y-axis) against log concentrations (X-axis) of the aqueous extract of *C. halicacabum* (Figure 7). The curve determined the  $\text{IC}_{50}$  value of the extract. Figure 9 shows that as log concentrations of the extract increased, percentage inhibitions also rose. A strong positive correlation ( $r^2 = 0.8121$ ) was observed between log concentrations and inhibition percentages, with an  $\text{IC}_{50}$  value of 8121  $\mu\text{g/mL}$ .

*Comparison of  $\text{IC}_{50}$  Values Among DC, Ethanol and Aqueous Extracts of C. halicacabum*

The  $\text{IC}_{50}$  values of DC, ethanol and aqueous extracts were compared and shown in Figure 8. The graph displayed the  $\text{IC}_{50}$  value of DC is smaller than the  $\text{IC}_{50}$  value of both extracts.



**Figure 7:** Concentration-response curve for the anti-inflammatory activity of aqueous extract of *C. halicacabum* based on inhibition percentage



**Figure 8:**  $\text{IC}_{50}$  values of DC, ethanol and aqueous extracts of *C. halicacabum*

**Table 1:** Solubility of *C. halicacabum* Extracts

Extract	Solubility (mg/mL)
Ethanol	5
Aqueous	2

**Table 2:** Mean Percentage Inhibition of Ovalbumin Denaturation with Diclofenac Sodium at Different Concentrations

Concentration ( $\mu\text{g/mL}$ )	Log Concentration (X-axis)	Mean % Inhibition $\pm$ SEM (Y-axis)
1600	3.204	$79.60 \pm 0.19$
800	2.903	$53.68 \pm 0.22$
400	2.602	$38.12 \pm 0.13$
200	2.301	$30.62 \pm 1.11$
100	2.000	$28.99 \pm 0.30$
50	1.698	$26.41 \pm 0.49$

**Table 3:** Mean Percentage Inhibition of Ovalbumin Denaturation with Ethanol Extract of *C. Halicacabum* at Different Concentrations

Concentration ( $\mu\text{g/mL}$ )	Log Concentration (X axis)	Mean % Inhibition $\pm$ SEM (Y axis)
16000	4.204	$88.04 \pm 0.11$
8000	3.903	$72.63 \pm 0.20$
4000	3.602	$40.07 \pm 0.15$
2000	3.301	$24.20 \pm 0.09$
1000	3.000	$13.15 \pm 3.48$
500	2.698	$07.13 \pm 0.00$

**Table 4:** Mean Percentage Inhibition of Ovalbumin Denaturation with Aqueous Extract of *C. Halicacabum* at Different Concentrations

Concentration (µg/mL)	Log Concentration (X-axis)	Mean % Inhibition ± SEM (Y-axis)
16000	4.204	79.73 ± 0.06
8000	3.903	43.77 ± 0.35
4000	3.602	24.18 ± 0.10
2000	3.301	09.82 ± 0.19
1000	3.000	05.35 ± 0.13
500	2.698	02.51 ± 0.11

## DISCUSSION

Inflammation is a healing process initiated by tissue injury and serves as a non-specific local defense mechanism. It can be caused by physical agents, chemical agents, and microbes. While inflammation is protective, excessive responses can be harmful [15]. NSAIDs and corticosteroids are commonly used to prevent excessive inflammation but have significant adverse effects, such as severe gastric irritation [5]. Therefore, there is a need for alternative, safe, and potent anti-inflammatory medicines. This study aimed to evaluate the *in vitro* anti-inflammatory activity of *Cardiospermum halicacabum* extract. Previous studies focused on the leaves aqueous and ethanolic extracts [16]. However, the *in vitro* anti-inflammatory activity of the whole plant (root, stem, leaves, fruits, and flowers) has not been evaluated.

The whole plant was dried without direct sunlight to prevent phytochemical degradation. Extracts were obtained by cold maceration using ethanol and purified water, the most suitable solvents for extracting chemical constituents from *C. halicacabum* [16]. The percentage yields of ethanol and aqueous extracts were 15.7% and 10.4%, respectively. Both extracts were water-soluble, with the ethanol extract showing higher solubility, facilitating the ovalbumin denaturation assay. UV-visible spectrum analysis revealed that the ethanol extract had higher potency than the aqueous extract. The ethanol extract contained gallic acid, indicated by a sharp peak at 267 nm [17]. The *in vitro* anti-inflammatory study was conducted avoiding the use of animal models. Inhibition of ovalbumin denaturation indicated the presence of anti-inflammatory phytoconstituents. Protein denaturation triggers several inflammatory responses, making its inhibition a viable method for testing anti-inflammatory activity [18].

The ethanol extract exhibited greater inhibition of protein denaturation compared to the aqueous extract. The anti-inflammatory activity of both extracts was concentration-dependent, with IC<sub>50</sub> values of 5157 µg/mL and 8121 µg/mL, respectively. Diclofenac Sodium used as a standard, showed a dose-dependent response with an IC<sub>50</sub> value of 1922 µg/mL. The anti-inflammatory effect of 1000 mg of ethanol and aqueous extracts was equivalent to 372.69 mg and 236.67 mg of Diclofenac Sodium, respectively. Thus, the ethanol and aqueous extracts were 2.68 and 4.23 times less potent than Diclofenac. The extracts showed mild-to-moderate anti-inflammatory activity, with *r*<sup>2</sup> values of 0.9945 (*p*<0.05) and 0.8575 (*p*<0.05) for ethanol and aqueous extracts, respectively. The reference drug had an *r*<sup>2</sup> value of 0.9978 (*p*<0.05), confirming the concentration-dependent effect as authentic and statistically significant.

## CONCLUSION

The results of this study conclusively showed that the ethanol and aqueous extracts of the plant *C. halicacabum* possess mild-to-moderate *in vitro* anti-inflammatory properties when compared to standard diclofenac sodium.

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## Conflict of interest

The authors declared no conflict of interest.

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