

Original Research Article

***In vitro* Anti-inflammatory activity of leaves of *Jeffreyia zeylanica* Using the Egg albumin denaturation method and Human Red Blood Cell stabilization method.**

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ABSTRACT

AimsTo investigate the *in vitro* anti-inflammatory activity of aqueous, methanol, dichloromethane(DCM), and hexane extracts of *Jeffreyia zeylanica* leaves extracts. *In vitro* protein(Egg albumin) denaturation method and *in vitro* Human Red Blood Cell (HRBC) membrane stabilization methods were used to evaluate the anti-inflammatory activity of the leaf extracts.

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Methodology Plant leaves were collected, washed, air dried, and obtained crude plant material. Cold maceration was used to extract the plant materials. Aqueous, methanol, DCM, and hexane were used as solvents for maceration. Diclofenac sodium was used as the positive control for the evaluation.

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ResultsIn the egg albumin denaturation method, the positive control diclofenac sodium indicated an IC 50 value of 179.2 µg/ml and R² of 0.9979. Similarly, hexane leaf extract suggested an IC 50 value of 154.9 µg/ml and R² of 0.9114. P values for all the extracts were P<0.05 indicating that there's a strong correlation between plant extract concentration and % inhibition of egg albumin denaturation. According to the IC 50 values and R² value hexane extract indicated the highest potential anti-inflammatory activity. In the HRBC membrane stabilization method, the positive control diclofenac sodium indicated an IC 50 value of 77.05µg/ml and R² of 0.9929 similarly DCM leaf extract indicated an IC 50 value of 154.0 µg/ml and R² of 0.9787. P values for all the extracts were P<0.05 indicating that there's a strong correlation between plant extracts concentration and % protection of RBC membrane. According to the IC 50 values and R² value DCM extract indicated the highest potential anti-inflammatory activity.

ConclusionHexane leaf extract indicated the highest potential anti-inflammatory activity for the egg albumin denaturation method meanwhile DCM leaf extract indicated the highest potential anti-inflammatory activity for the HRBC membrane stabilization method, evidencing that nonpolar or less polar secondary metabolites of the plant leaves can contribute more to the plant's anti-inflammatory activity.

Keywords-*Jeffreyia zeylanica*, *Vernonia Zeylanica*, *Anti-inflammatory activity*, *Endemic plant*, *Sri Lanka*, *Protein denaturation method*, *Human Red Blood Cell Stabilization method*.

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1. INTRODUCTION

The immune system's reaction to potentially dangerous stimuli including pathogens, damaged cells, toxic substances, or radiation is called inflammation[1]. The inflammatory reaction is indicated by redness, swelling, heat production, loss of tissue function, and pain. Simply inflammation is the body's immune system's response to an irritant [2]. This inflammation can be acute or chronic. As a result of the inflammation, inflammatory signaling pathways get activated, and the above-mentioned inflammatory reactions occur in the body. Inflammation can sometimes be harmful. When the inflammatory response's control systems are defective or the body's capacity to remove damaged tissue and foreign objects is compromised, tissue loss may result [3]. Also, the denaturation of proteins which leads to the formation of antigens associated with type III hypersensitivity can create inflammation. In some situations, an ineffective immune response may result in a prolonged and harmful inflammatory response. responses caused by allergies or hypersensitivity are examples[4].

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Anti-inflammation is the reaction that occurs to minimize inflammation. Anti-inflammatory drugs, Non-Steroidal Anti-inflammatory Drugs (NSAIDs) are used to treat pain and other inflammatory responses[5]. These NSAIDs are known as anti-inflammatory agents, an anti-inflammatory agent is a chemical compound along with a medication that can decrease inflammation[6]. The simple mechanism of an anti-inflammatory drug can be explained below, Wide class of therapeutic agents with analgesic and anti-inflammatory properties that inhibit the two recognized isoenzymes of prostaglandin G/H synthase (cyclo-oxygenase (COX)) known as, COX 1 and COX 2. Since the therapeutic action of these drugs is highly mediated by the inhibition of COX 2, their gastrointestinal adverse reactions are largely due to COX 1 inhibition. Cyclooxygenase is required to convert arachidonic acid into thromboxane, prostaglandins, and prostacyclin so this is simply the inhibition of prostaglandin production [7]. This is the main mechanism of action of NSAIDs. Some non-selective NSAIDs are ibuprofen, naproxen, and diclofenac. And COX-2 selective NSAIDs are, glecoxib, rofecoxib, valdecoxib. Although these are effective, these medications are relatively pricey, especially in developing nations[8]. They also have significant and harmful side effects, such as gastric ulcers, gastric pain, cardiovascular, hepatic, and renal dysfunctions, increased risk of bleeding, fluid retention, hypertension, headache and dizziness, and skin rashes some of which may be life-threatening. Other than that, these NSAIDs may interact with other medications and cause unwanted side effects[9]. NSAIDs should always be used cautiously for as short a time as possible and at the lowest effective dose. NSAIDs have accounted for the prevention of protein denaturation [10]. As a result of these complications, people tend to find alternatives for NSAIDs. Therefore, plant resources are the best option for creating novel NSAIDs[11].

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Plants can be used to generate and isolate a large variety of phytochemical substances, as secondary metabolites. At the present time, several anti-inflammatory compounds originating from plants are being used as medicines and have no adverse side effects[12,13,14]. In Sri Lanka, medicinal plants have been used for centuries to treat a variety of inflammatory conditions. The plant *J. zeylanica*, which is native to Sri Lanka, is also known as Pupula, formerly known as *Vernonia Zeylanica* is frequently used in Sri Lankan traditional medicine for a number of inflammation-related disorders, including fractures because it helps the bones fuse, boils, eczema, and asthma. Plant *J. zeylanica* is a member of the family ASTERACEAE. It is a small under shrub 1 - 2.5 m tall with many strangling, divaricate, cylindrical branches that are finely tomentose when young. In folk medicine, the whole plant, leaves, stem, and aerial parts are used for the treatments[15]. The plant consists of phytochemicals, saponins, steroids, terpenoids, alkaloids, flavonoids, lupane, and phenols are some of them. These phytochemicals or secondary metabolites can be extracted using polar, nonpolar, or in-between polar and nonpolar solvents. Literature suggests that previous studies have been conducted to evaluate the plant's anti-inflammatory activity but there's only a few research have been conducted[16]. Additionally, the egg albumin denaturation method and HRBC membrane stabilization method have not been used previously to investigate the anti-inflammatory activity of *J. zeylanica*.

Comment [h8]: zeylanica

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2. METHODS

2.1. Collection and Authentication of the plant

Matured well grown *Jeffreyia zeylanica* leaves were collected during the morning, in the flowering season of the plant, about 4 kg of the leaves were collected, at Aparakka, Matara district in the southern province of Sri Lanka. (Latitude: 6° 00' 0.00" N Longitude: 80° 36' 59.99" E). The plant was identified and authenticated at the Bandaranaike Memorial Ayurvedic Research Institute, Nawinna, Maharagama, Sri Lanka.

Comment [h12]: Rewrite as during the flowering season in the month of, at the morning time. Please also include the flowering month.

2.2. Preparation of aqueous, methanol, dichloromethane, and hexane extract of leaves of *J. zeylanica*.

The collected leaves were thoroughly washed and air-dried, when the dry weight reached a constant level, the leaves were ground to obtain a fine powder[17]. Aqueous, methanol, DCM, and hexane extracts were made using cold maceration. For 50 g of the powder 500ml of the solvent was added, 1:10 ratios[18]. Then allowed for cold macerating for about 1 week with frequent agitation (to preserve the heat labile phytochemicals). Then the micelle was filtered using a muslin cloth, obtained filtrate

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was concentrated using the Rotary vacuum evaporator [19]. Extracts were collected into sterile airtight containers and stored in the freezing compartment of the refrigerator until use[20].

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2.3. Evaluation of *in vitro* anti-inflammatory activity using the Egg albumin denaturation method.

2.3.1. Principle of egg albumin denaturation method

Protein denaturation has a correlation with inflammation[21]. The ability of a substance to inhibit the denaturation of protein manifests the possible anti-inflammatory activity. Anti-inflammatory activity is screened by the *in-vitro* egg albumin denaturation method using a fresh hen's egg. Egg white contains the proteins ovalbumin, clusterin, ovoinhibitor, ovotransferin, and prostaglandin D2 synthase. In this method, the ability to inhibit the egg protein (albumin) denaturation is measured using an NSAID (Diclofenac sodium). Also, the Plant extract is assumed as an NSAID and measures its anti-inflammatory potency. The reduction of the prostaglandin activity is screened using protein denaturation by heating. This is indicated by the % inhibition of egg albumin denaturation[3]. The higher the % inhibition of denaturation, the higher the anti-inflammatory ability[22].

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2.3.2. Preparation of the Standard concentrations and screening of the Egg albumin denaturation method.

Standard concentration series were prepared, and they are, 2000, 1000, 500, 250, 125, 62.5, 31.25, and 15.625 µg/mL. Diclofenac sodium was used as a positive control, and all the diclofenac sodium concentration series were made similarly. Phosphate buffer saline (PBS, pH 6.4) was made using 28.80 g of the disodium salt of hydrogen phosphate and 11.45 g of dipotassium salt of hydrogen phosphate dissolved in 1000 mL of distilled water [5]. For the preparation of determined test samples 2.8 ml of PBS, 0.2 ml egg albumin, and 0.2 ml plant extract was added. For the reference drug diclofenac sodium test samples were prepared using 2.8 ml of PBS, 0.2 ml egg albumin, and 0.2 ml diclofenac sodium. A control sample was prepared using 2.8 ml of PBS, 2 ml of distilled water, and 0.2 ml of egg albumin. All prepared samples were incubated at 37 °C in the water bath for 15-20 minutes using the laboratory shaking water bath, followed by heating at 70 °C, for five minutes. Allowed the samples to cool for 10 minutes at room temperature. Absorbances were taken at 660 nm by using the vehicle as a blank, using the Double beam UV spectrophotometer. The whole test procedure was done in triplicates. % inhibition of egg albumin denaturation was measured using the below equation [5].

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$$\% \text{ inhibition of egg albumin denaturation} = \left[\frac{\text{Absorbance of the control} - \text{Absorbance of the Standard}}{\text{Absorbance of the control}} \right] \times 100$$

2.4. Evaluation of anti-inflammatory activity using Human Red Blood Cell membrane stabilization method.

2.4.1. Principle of the HRBC method

Human red blood cells (RBCs) are the most abundant blood cells. RBCs are round biconcave and very flexible cells, containing a two-dimensional structure. According to studies, substances that can maintain the integrity of the lysosomal membrane can stop the determined effects of the release of phospholipase A₂ that results from the breakdown of arachidonic acid metabolites[20]. The breakdown of the arachidonic acids leads to the production of thromboxane, prostaglandins, and prostacyclin. The production of prostaglandins plays a major role in inflammation. Any substance that can minimize the production of prostaglandin has anti-inflammatory activity. Indicating that the lysosomal membrane has this anti-inflammatory ability. Furthermore, it has been suggested that the erythrocyte membrane shares structural similarities with the lysosomal membrane. It is possible to generalize the use of

Comment [h20]: In the Abstract and Introduction section, author mentioned it as HRBCs.....and here RBCs. Please be consistent in writing.

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erythrocyte membrane instead of lysosomal membrane to evaluate the stabilization of *in vitro* anti-inflammatory activity [23].

2.4.2. Preparation of the Standard concentrations and screening of Human Red Blood Cell membrane stabilization method.

Concentration series were made similar to the egg albumin method. Prepared concentrations were 2000, 1000, 500, 250, 125, 62.5, 31.25, and 15.625 µg/mL. Diclofenac sodium was used as the positive control, and all the diclofenac sodium concentration series were made similarly. Phosphate buffer saline (PBS, pH 7.4) was made by dissolving 0.19 g of potassium dihydrogen phosphate, 2.38 g of disodium hydrogen phosphate, and 8 g of sodium chloride in 100 ml of distilled water. Hypotonic saline was prepared by dissolving 0.36 g of sodium chloride in 100 mL of distilled water. Isotonic saline was prepared by dissolving 0.85 g sodium chloride in 100 mL of distilled water. 10% w/v red blood cell solution was made using the blood samples (confirmed that NSAIDs were not taken for two weeks prior to the test). The blood samples were mixed with the same amount of Alsevers solution, (containing, 0.8 g Sodium citrate, 2 g of dextrose, 0.005 g citric acid, and 0.42 g of sodium chloride, dissolved in 100 mL of distilled water). The blood solution was centrifuged at 3000 rpm for 10 minutes. Then the supernatant was discarded and washed again two times with isotonic saline [20,24].

Test samples were prepared using 1 ml phosphate buffer, 2 ml hypotonic saline, 0.5 ml of the plant extract, and 0.5 ml of prepared red blood solution. Test control was prepared with 1 ml phosphate buffer, 2 ml distilled water, and 0.5 ml of 10% RBC suspension. Diclofenac standard control samples were made using 1 ml phosphate buffer, 2 ml hypotonic saline, 0.5 ml of the plant extract, and 0.5 ml of prepared 10% RBC suspension. Prepared assay mixtures were incubated at 37°C for 30 minutes. After incubation, the assay mixtures were centrifuged at 3000 rpm for 10 minutes, and assay mixtures were carefully handled without disturbing the pellet. Then absorbances of the supernatant were measured at 560 nm wavelength. The whole test procedure was done in triplicates. % Protection (stabilization) of the RBC membrane was measured [24].

$$\% \text{ Protection of RBC membrane} = 100 - \left[\frac{\text{Optical density of the sample}}{\text{Optical density of the control}} \right] \times 100$$

3. RESULTS AND DISCUSSION

Plant extract dose-response data for aqueous, methanol, dichloromethane, and hexane leaves of *J. zeylanica* along with positive control using the egg albumin denaturation method are shown in Table 1. The dose-response curve for extracts and positive control is shown in Fig 1. Plant extract dose-response data for aqueous, methanol, dichloromethane, and hexane leaves of *J. zeylanica* along with positive control using the HRBC membrane stabilization method are shown in Table 2. The dose-response curve for extracts and positive control is shown in Fig 2. All the extracts show a strong positive statistically significant correlation ($P < 0.05$) between concentration, percentage inhibition and percentage protection. P values and R^2 values were obtained using GraphPad Prism 8 (version 8.2.1).

In the egg albumin denaturation method, diclofenac sodium indicated an IC 50 value of 179.2 µg/ml and R^2 value of 0.9979 suggesting a very reliable correlation with log concentration and percentage inhibition of egg albumin denaturation. Considering the IC 50 values of leaves extracts, potential anti-inflammatory activity indicated by plant extracts were, methanol (26.14 µg/ml, R^2 0.3812) > hexane (154.9 µg/ml, R^2 0.9114) > dichloromethane (568.1 µg/ml, R^2 0.9898) > aqueous (1297 µg/ml, R^2 0.9962). When comparing the R^2 values of four extracts and diclofenac sodium, the methanolic extract did not indicate a reliable coefficient determination indicating that data does not fit well with the regression model, considering that fact, hexane leaves extract has the best potential anti-inflammatory activity compared to the positive control, diclofenac sodium [25].

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In the HRBC membrane stabilization method, diclofenac sodium indicated an IC₅₀ value of 77.05 µg/ml and R² value of 0.9922 suggesting a very reliable correlation with log concentration and percentage protection of HRBC membrane. Considering the IC₅₀ values, potential anti-inflammatory activity indicated by plant extracts were, dichloromethane (154.0 µg/ml, R² 0.9787) > aqueous (199.5 µg/ml, R² 0.9873) > methanol (371.9 µg/ml, R² 0.9431). When comparing the IC₅₀ and R² values of four extracts and diclofenac sodium, DCM extract indicated the highest potential anti-inflammatory activity compared to the positive control, diclofenac sodium.

Table 1. Dose-response curve details of normalized % inhibition of egg albumin denaturation of diclofenac sodium, aqueous, methanol, DCM, and hexane leaves extracts of *J. zeylanica*.

Tabular results	Diclofenac	Aqueous	Methanol	DCM	Hexane
IC ₅₀ (µg/ml)	179.2	1297	26.14	568.1	2.190
P value	0.0098	0.0382	0.0054	0.0351	0.0075
R ²	0.9979	0.9962	0.3812	0.9898	0.9114

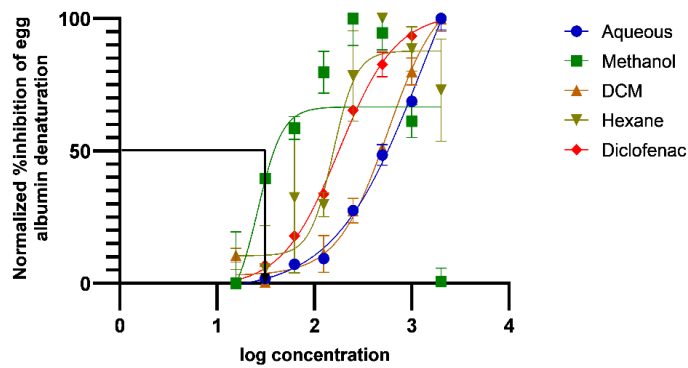


Fig 1. Dose-response curve of % inhibition of egg albumin denaturation of the reference drug diclofenac, aqueous extract, methanol extract, DCM extract, and hexane extract of *J. zeylanica* leaves.

These graphs were prepared using GraphPad Prism 8 (version 8.2.1), using a non-linear regression model according to the equation: $Span = Top - Bottom$, $Y = Bottom + (Top - Bottom) / (1 + 10^{-(Log IC_{50} - X) * HillSlope})$, where X is the log of dose-response or concentration, Y is a response.

Table 2. Dose-response curve details of % protection of HRBC membrane with reference drug diclofenac sodium, aqueous, methanol, and DCM leaves extract of *J. zeylanica*.

Tabular results	Diclofenac sodium	Aqueous	DCM	Methanol
IC ₅₀ (µg/ml)	77.05	199.5	154.0	371.9
P value	0.0037	0.0063	0.0104	0.0056
R ²	0.9929	0.9873	0.9787	0.9431

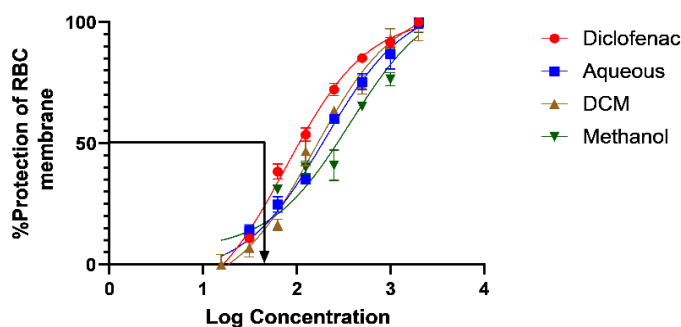


Figure 2. Dose-response curve for % protection of the Human RBC membrane with reference drug diclofenac sodium and aqueous, methanol, DCM extracts of *J. zeylanica* leaves.

These graphs were prepared using GraphPad Prism 8 (version 8.2.1), using a non-linear regression model according to the equation: $Span = Top - Bottom$, $Y = Bottom + (Top - Bottom) / (1 + 10^{-(Log\ IC50 - X) * HillSlope})$, where X is the log of dose-response or concentration, Y is a response.

Both methods indicated that *J. zeylanica* has an effective anti-inflammatory activity. The hexane plant extract expresses the highest potential activity in the egg albumin denaturation method. The DCM extract presented the highest potency in the HRBC membrane stabilization method. Evidence that less/nonpolar compounds of the leaves are more contribute to the plant's anti-inflammatory properties. When considering the reported anti-inflammatory activity of *J. zeylanica* through the study it is possible to reason out that nonpolar secondary metabolites of the leaves of *J. zeylanica* contributed more to the anti-inflammatory activity. The use of natural plant materials to manage various inflammatory conditions has been successful. According to evidence, phytochemicals may enhance the production of anti-inflammatory genes while suppressing the expression of pro-inflammatory genes[26]. It suggests that there's a high probability that phytochemicals can contribute to the anti-inflammatory properties of a medicinal plant. The medically important plants that have anti-inflammatory properties are rich in phytochemicals such as flavonoids, phenolic compounds, terpenoids, polyphenols, and so on[27, 28]. According to the previously investigated phytochemical properties of *J. zeylanica*, the plant consists of triterpenoids(which is a terpenoid), phenols, saponins, alkaloids, flavonoids, ethuliacoumarin (coumarin/ a phenolic compound), lupanol (type of a lupane). Another study suggests that *J. zeylanica* is rich in triterpenoid (which is a terpenoid), lupanol (type of a lupane)[29]. In this previous study, the extraction of the crude plant material was done by using nonpolar solvents [30]. It indicates that these compounds have a high solubility in nonpolar solvents, and they are important in anti-inflammatory activity, not only triterpenoids, phenols, and flavonoids are also constituents of the plant which can directly contribute to anti-inflammatory activity.

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When evaluating the egg albumin denaturation method, it is important to use a fresh hen's egg. Egg white thinning, which happens when egg white loses its viscosity and turns into a thin liquid, is a typical effect of storing eggs[21]. This phenomenon can lead to changes in egg proteins including ovalbumin, clusterin, ovoinhibitor, ovotransferin, and prostaglandin D_2 synthase, and can affect the assessment of the anti-inflammatory ability. *J. zeylanica* is an endemic plant in Sri Lanka, and it has seasonal differences as flowering seasons and fruiting seasons. The plant was collected for experimental study during the flowering season. These seasons also can contribute to its bioactivities. Also, the time of the collection can be a factor in the plant's activity. It is important to consider those factors about the plant, and no studies have been done regarding the effect of environmental factors.

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4. CONCLUSION

In conclusion, the study suggests the possible anti-inflammatory activity of *J. zeylanica* leaves. The study also indicated that less/nonpolar properties of the leaves are more favorable for the plant's anti-inflammatory activity. *Jeffreyia zeylanica* is an endemic plant in Sri Lanka, so it is important to

investigate its biological activity. The anti-inflammatory activity of the plant should be evaluated using a more efficient extraction method than cold maceration. Investigations should be carried out to determine the biological properties of other plant parts, including flowers, roots, and fruits. Since the plant has a flowering and fruiting season, evaluating the biological changes according to the time period should be done.

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