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ATTENUATION OF INFLAMMATION BY ETHANOLIC EXTRACT OF *CAESALPINIA BONDUCELLA* USING *IN-VITRO* INFLAMMATORY MODELS

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Keywords:

Caesalpinia bonducella, Anti-inflammatory, Ethanolic extract, HRBC, Membrane stabilization, proteinase inhibitory

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ABSTRACT: Background: *Caesalpinia bonducella* F. (Leguminosae) has long been used in traditional medicinal treating methods. It has high therapeutic values in India Ayurveda. They contain tannin, saponins, flavonoids, and alkaloids. The seeds of *Caesalpinia bonducella* are claimed to be styptic, purgative, anthelmintic and cures inflammations. Today the interest in traditional medicine and demand for more plant-based drugs has increased. Here the study is focused on anti-inflammatory efficiency and analysis of phytochemical compounds from the *Caesalpinia bonducella* seed kernel extract. As part of the immune response, inflammation plays a significant role in defending or protecting the body against pathogens like viruses, bacteria, fungi, and other parasites. Inflammation leads to a complete detriment at the sites of inflammation and promotes the renewal or regeneration of normal tissues. During inflammation, Chemotaxis is the event with cells like Fibroblasts, leukocytes, Macrophages, and Tumor cells are involved in the migration of cells towards a chemical signal in the cell's surrounding environment. Our research focuses on unravelling the anti-inflammatory efficacy of ethanolic fraction, which comprises the active leads necessary for fighting against inflammation. The main part of this research project is to carry out an ethanolic extract of *Caesalpinia bonducella* seed kernel and the in-vitro assessment of the anti-inflammatory effects of plant extract.

INTRODUCTION: Inflammatory responses stimulated by the immune system acts as a defense mechanism against any foreign agent it encounters, such as microorganisms, dust particles, and damaged tissues/cells. Because of modifications or remodeling of the membrane, which causes an increase in protein denaturation along with an increase in vascular permeability, this body mechanism is responsible for the redness, discomfort, and swelling in the infected region [1].

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The primary goal of inflammation is to restore cellular homeostasis in response to any compromised condition. As a result, the fundamental mechanism of initiation of inflammation is inextricably linked to the physiological state of homeostasis. Cellular homeostasis restoration in response to any damaging condition is the goal of inflammation.

It's seen that inflammation usually begins within seconds in a host with a functional innate immune system. In inflammatory response, immune cells like lymphocytes, macrophages, neutrophils and mast cells play a crucial role. Non-immune cells like fibroblasts, epithelial cells, endothelial cells, and immune cells contribute to the inflammatory process. Inflammatory pathways and the tissues

they target are vastly different. Inflammatory pathways and their targeted tissues differ significantly [2]. Inflammatory mediators like inflammatory chemokines and cytokines are induced by the activation of the production of pathogen-specific receptors. The progress of inflammation through modification of vascular endothelial permeability, attracting of neutrophils, and these inflammatory mediators quickly stimulate excess plasm into the side of injury. At the same time, immune cells attack and kill the pathogens that have been captured.

Pathogenic wound invasion was the fundamental reason for the evolution of innate inflammation. These signals are triggered by the release of necrotic cells and damaged tissue necrotic molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). Activation of resident immune cells such as mast cells, Langerhans and T cells, and macrophages is triggered by the binding of pattern recognition receptors to these PAMPs and DAMPs, activating downstream inflammatory pathways [2].

NF-B has been shown to play an important role in the pathogenesis of organ injury caused by sepsis, according to the findings of a large number of studies. The Toll-like receptor 4-NF-kB pathway was a well-known signaling pathway that was activated when a pathogen-associated molecular pattern (PAMP) or a danger-associated molecular pattern (DAMP) was detected. As a result, a plethora of cytokines, chemokines, and peptides are produced [3, 4].

Vascular cell adhesion molecule 1 (VCAM1), e-select in (SELE) on endothelial cells, and intercellular adhesion molecule 1(ICAM1), which mediate neutrophil adherence to blood vessel walls, are the adhesion molecules whose expression is stimulated by Chemokines [5]. The cytoskeleton of neutrophils is changed when the endothelial cells are attached, and chemokines are exposed. Neutrophils remain for a few days if the wounds don't get infected and perform phagocytosis to remove debris. Tumor necrosis factor (TNF)-, interleukin (IL)-1, and IL-6 are the cytokines for which neutrophils are responsible for production. These cytokines amplify the inflammatory response.

All over the world, complementary medicine and herbal medicine are used. Herbs were the main source of the drug in ancient times. Plants' medicinal properties have long been recognized. There are numerous plant species found throughout the world. *Caesalpinia bonducella* L. is an Ayurvedic herb used in traditional Indian medicine. The name "Bonducella" means "little ball," referring to the seed's round shape.

It has been studied early that those parts of*the Caesalpinia bonducella* plant have therapeutic properties. Saponins, terpenoids, alkaloids, flavonoids, steroids, and tannins are among the phytochemicals in the *Caesalpinia bonducella* seed. The color of the seed is grey and looks like eyeballs. The seed is styptic, purgative, anthelmintic and anti-inflammatory, making it useful for the treatment of severe pain in the abdomen caused by wind or obstruction in the intestines and leprosy [6].

When applied to a wound, drugs derived from the seed kernel of *Caesalpinia bonducella* can be used to destroy parasitic wounds and stop bleeding. The seed kernel has wound healing properties, anti-cancer, and anti-diabetic [7]. This study aims to see if *Caesalpinia bonducella* ethanolic extract has anti-inflammatory activity *in-vitro.*

MATERIALS AND METHODS:

Chemicals and Drugs: Dulbecco's modified essential medium (DMSO), dimethyl sulfoxide, fetal bovine serum (FBS), L-nitro-arginine methyl ester (L-NAME) and -(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), RIPA Buffer (10X), sulphuric acid, chloroform, liquid ammonium hydroxide was purchased from HiMedia Laboratories (Mumbai, Maharashtra, India). Sigma-Aldrich was the vendor for the acquisition of sulfanilamide and naphthyl ethylenediamine. *Caesalpinia bonducella* seed was brought from a botanical garden in Chennai, India.

Cell Culture Studies: L929 Fibroblast was grown in DMEM supplemented with 10% FBS and 5% antibiotic, i.e., 100X Liquid in 10,000 U Penicillin and 10mg Streptomycin per ml in 0.9% normal saline, was acquired from HiMedia (Mumbai, Maharashtra, India) in an atmosphere containing 5% CO_2.

Preparation of Extract: The seed's shell was broken, and the kernel was extracted. The seed kernel was broken and dried in a hot-air oven set to 70° C. Using a household mixture, the broken dried seed was ground into a fine powder. The Soxhlet apparatus extracted 50g of dried seed kernel powder with 500ml of 95 percent ethanol. The apparatus was switched on at 9:00 am. It was monitored regularly for proper condensation by INLET and OUTLET water supply. The column was made sure that it is not dried, and Ethanol was added little by little to avoid dryness. The crude extract that was obtained was filtered using watchman paper, and the solvent was dried by vacuum rotary evaporator under reduced pressure at a temperature of 50 °C.

Yield of Ethanol Extract: The extracted material was dried and weighed under reduced pressure. The yield was determined.

The formula below is used to calculate the yield extract of ethanol extract:

: (Weight of the sample extract obtained (g)) / (Weight of the powdered sample used (g) × 100

***In-vitro* Anti-inflammatory Assays:**

Inhibition of Albumin Denaturation: The inhibition of the albumin denaturation technique was used to investigate *Caesalpinia bonducella's* anti-inflammatory activity. The reaction mixture was supplemented with a 1% aqueous solution of Bovine Albumin and test extracts of varying concentrations. The concentrations at the end were 31.25g/ml, 62.5g/ml, 125g/ml, 250g/ml, 500g/ml, and 1000g/ml. For 20 minutes, the sample extract was incubated at 37°C. It was heated for 20 minutes at 51°C after incubation. It was then allowed to cool. A spectrophotometer was used to measure the turbidity at 660nm after the sample had been cooled. The reference drug was diclofenac sodium or aspirin at the same final concentration as the extract. The experiment was repeated three times.

The Percentage inhibition of protein inhibition = (Absorbance of control) / (absorbance of sample)-1) × 100

Membrane Stabilization:

Preparation of RBCs (Red Blood Cells) Suspension: In EDTA-containing tubes, 10mL of human blood was collected. Blood cells were washed three times in saline after plasma was aspirated. The cells were filled with ice-cold distilled water and left overnight at 0°C. Centrifugation at 10000 rpm for 20 minutes at 4°C separated the hemolysate. Pellets were thoroughly washed with distilled water twice. After centrifuging it for 10 minutes, it was suspended in 50ml of Tris-HCl buffer.

Heat-induced Hemolysis-HRBC Membrane Stabilization Assay: 1 ml of RBC suspension was combined with 1 ml of *Caesalpinia bonducella* ethanolic extracts. In the control tube, 1ml of RBC suspension in normal saline was placed. Diclofenac sodium or aspirin was prescribed as standard medication. All reaction mixture-containing centrifuge tubes were wrapped in aluminum foil and incubated at 56°C for 30 minutes. It was then cooled with running tap water. The reaction mixture was spun at 2500rpm for 5 minutes. At a wavelength of 560nm, the optical density of the supernatant was determined. The experiment was done three times.

Membrane stabilization activity = (absorbance of test) / (absorbance of control) × 100

Proteinase Inhibitory Action: The reaction mixture (2ml) contained 0.06mg trypsin, 1ml of 20mM Tris HCl buffer at a pH of 7.6 of and 1ml of a test sample of different concentrations (100-500µg/ml). The mixture was incubated at 37°C for 5mins, and then 1ml of 0.8% (w/v) casein was added. The mixture was incubated at room temperature for about 20min. The reaction was terminated by adding 2ml of 70% perchloric acid. The mixture was vortexed, and the absorbance of the supernatant was measured at 210nm again; the set buffer was blank. The experiment was performed in triplicates. Percentage inhibition of proteinase inhibitory was calculated using:

% Proteinase inhibitory = (Absorbance o test) / (Absorbance of control)-1) × 100.

***In-vitro* Assays:**

Cell Viability Assessment of Ethanolic Extract of *Caesalpinia Bonducella* in L929 Fibroblast Cell Line: Reduction of MTT (3- [4, 5-dimethylthiazol- 2-yl]-2, 5-diphenyltetrazolium bromide) was used in checking the cell viability in L929 Fibroblast. Cell seeding was done on a 96-

well plate and after the 24 h of incubation it was then treated with different compounds that were incubated again for another 24 h. After 4hr of incubation, 100μl of MTT solution was added to every well. Later, the medium was aspirated to dissolve the insoluble purple formazan crystals with the solubilizing agent (DMSO) [7]. The absorbance was measured at 570nm. The percentage of cell viability was determined by using the formula given below

% Cell viability = (O.D of Test) / (O.D of Control) × 100

LPS Induced Nitrite Production in L929 Fibroblast Cell Line: 1 μg/mL of LPS stimulation was used to study the inhibitory effect of phloretin NO production using the L929 Fibroblast cell line. A 96-well plate was taken, and in the plate, L929 Fibroblast cells were plated. After achieving 80% confluency, 1μg/ml of LPS i.e., Lip polysaccharide, was added to each of the wells except the control. Before the previous step, 10μL of Ethanolic extract of *Caesalpinia bonducella* and NO synthase inhibitor L-NAME (200μM) was added as a positive control. For 1 hour, the plate was incubated. After incubating, 1μg/ml of LPS was added to each of the wells except the control. For 24hours, the cells were incubated at 37℃. Following incubation, the supernatant was used to estimate nitrite using the Griess nitrite assay [8].

Griess Nitrite Assay: An equal amount of supernatant and Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylenediamine in 5% phosphoric acid) was mixed thoroughly and incubated at room temperature for 10 minutes. At 540nm, the absorbance was measured8. As a control, sodium nitrite was used.

RESULTS AND DISCUSSION:

Extraction of Plant Material: The ethanolic extracts were obtained using the Soxhlet apparatus.

Yield of Ethanolic Extract:

Percentageof yield = (weight of the sample extract obtained (g) / (weight of the powdered sample used (g) × 100

Weight of the sample extract obtained = 3.25g

Wight of the product sample used = 50g

% yield = x 100

% yield = 0.065 x 100

% yield = 6.5%

Thus, a 6.5% yield of ethanolic extract was obtained.

Inhibition of Albumin Denaturation: Protein denaturation is the loss of tertiary and secondary structures due to external stress or compounds, such as a strong acid or base, a concentrated inorganic salt, an organic solvent, or heat. When biological proteins are denatured, their biological function is lost. Protein denaturation is well known to cause inflammation. The ability of plant extract to inhibit protein denaturation was investigated as part of the investigation into the mechanism of anti-inflammatory activity. It effectively prevented heat-induced albumin denaturation. At 1000g/ml, the maximum inhibition of 82% was observed **Fig. 1.**

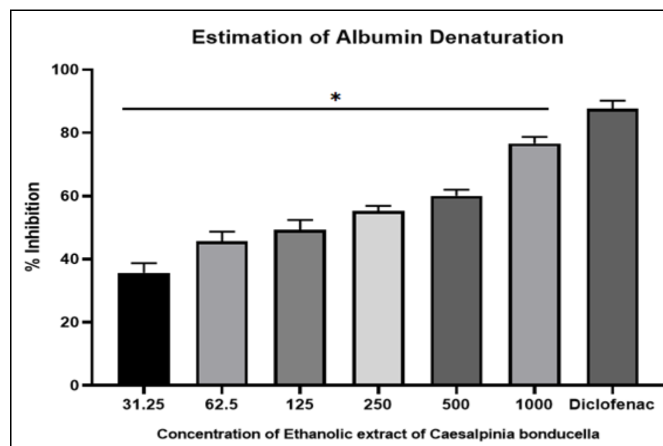

FIG. 1: ASSESSMENT OF % OF INHIBITION OF ALBUMIN DENATURATION IN ETHANOLIC EXTRACT OF *CAESALPINIA BONDUCELLA*. * Denotes $p < 0.05$.

Membrane Stabilization: The stabilization of erythrocytes indicates that the extract may stabilize lysosomal membranes in the *in-vitro* anti-inflammatory activity, as lysosomal membranes and erythrocytes are alike; this has been investigated by HRBC membrane stabilization. Lysosomal stabilization is critical for limiting the inflammatory response because they prevent the release of activated neutrophil lysosomal constituents like bacterial enzymes and proteases, which cause extracellular tissue inflammation and damage. Lysosomal enzymes, which are released during inflammation, can cause a number of issues.

The extracellular activity of these enzymes is thought to be linked to either acute or chronic

inflammation. Lysosomal enzymes are inhibited or stabilized by nonsteroidal medications.

Test extract 31.25µg/ml, 62.5µg/ml, 125µg/ml, 250µg/ml, 500µg/ml and 1000µg/ml were showed a possible membrane stabilization potency of RBCs. At 1000g/ml, the maximum stabilization of ethanolic extract was 72 percent. The standard drug, aspirin, showed maximum inhibition of 92 % at 1000g/ml as shown in **Fig. 2.**

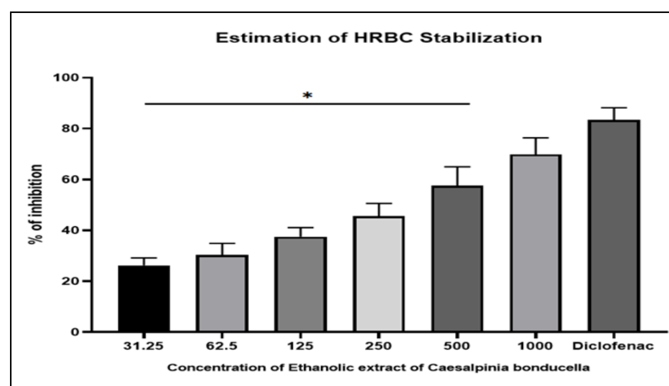

FIG. 2: ASSESSMENT OF % OF STABILIZATION OF HRBC MEMBRANE IN ETHANOLIC EXTRACT OF *CAESALPINIA BONDUCELLA.* * Denotes $p < 0.05$.

Proteinase Inhibitory Action: Neutrophils, which are found in lysosomes, are known to be a rich source of serine proteinase. It has previously been reported that leukocyte proteinase plays an important role in the development of tissue damage during inflammatory responses and proteinase inhibitors provide a significant level of protection. At various concentrations, Ethanolic extract of *Caesalpinia bonducella* exhibited significant antiprotease activity. At 1000g/ml, it showed maximum inhibition of 63% as shown in **Fig. 3.**

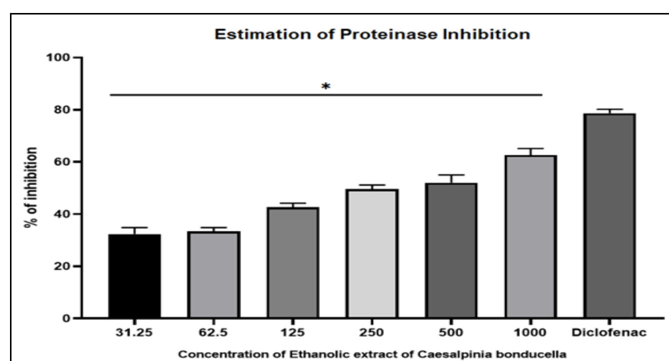

FIG. 3: ASSESSMENT OF % OF INHIBITION OF PROTEINASE ACTION IN ETHANOLIC EXTRACT OF *CAESALPINIA BONDUCELLA.* * Denotes $P < 0.05$.

Cell Viability Ethanolic Extract of *Caesalpinia bonducella* in L929 Fibroblast: During the tissue formation period of the healing process, fibroblast proliferation plays a role in the reconstruction of structure and function. A fibroblast's collagen secretion affects the maturation of the extracellular matrix into the mature dermis [7]. Because fibroblasts are the primary targets in the development of therapeutic drugs, bioactive compounds that can induce the proliferation of fibroblasts may be able to speed up the healing process, as was the case in the study that we are reporting here [11].

The MTT assay that was used in this investigation serves multiple purposes. One of the aims was to identify the active extracts and determine the optimal concentrations of phloretin so that we could both measure the rate of cell proliferation and influence the metabolic activity of the cells 12. It was only possible for metabolically active cells to reduce MTT by mitochondrial dehydrogenases to determine the viability of the cells.

The cytotoxic effect of the concentration of ethanolic extract of *Caesalpinia bonducella* in L929 Fibroblast was evaluated with the help of an MTT reduction assay. In this illustration, the viability of the cell is shown while ethanolic extract of *Caesalpinia bonducella* is present. It showed in **Fig. 4**. That at 1ng to 10µg concentration, the result showed that the ethanolic extract of *Caesalpinia bonducella* did not affect the viability of the cells, and these results were used for further research.

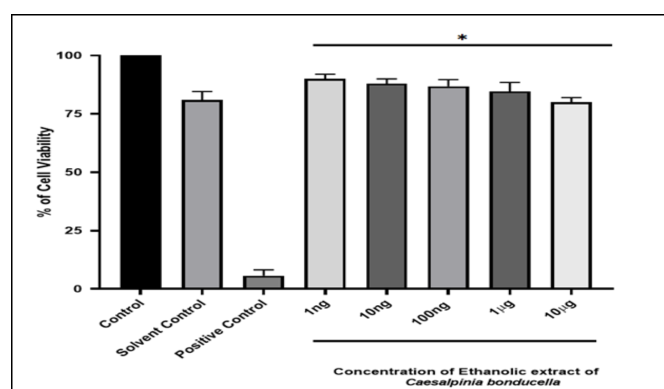

FIG. 4: MTT ASSAY OF ETHANOLIC EXTRACT OF CAESALPINIA BONDUCELLA IN L929 FIBROBLAST. Data represents mean ± SD from three individual experiments. * Denotes $p < 0.05$, which is significantly different from the positive control.

LPS Induced Anti-inflammatory Assay L929 Fibroblast: Fibroblasts are important in tissue damage and wound healing by maintaining hemostasis and triggering inflammation. They

begin wound repair. These cells produce nitric oxide (NO), essential for growth and inflammation resolution. NO's ability to inhibit cytotoxicity and chemotactic action against bacteria, and tumor cells affect cell proliferation, differentiation, collagen deposition, and wound contraction. Extreme NO production harms normal cell function by targeting healthy tissue around wounds. Regulated reactive radical production draws fibroblasts to a wound to begin the proliferative phase of healing [13]. LPS interacts with TLR4 to produce NO, causing inflammation imbalances. iNOS gene-targeted anti-inflammatory drugs are important for treating diabetic wounds and Alzheimer's [14].

In this study, the concentration of ethanolic extract of *Caesalpinia bonducella* was evaluated for the inhibition of NO production in the LPS - stimulated L929 fibroblast cell line. The LPS treatment increased the accumulation of nitrite in the cells. To carry out this experiment, cells were treated with 1µg/ml LPS and the various concentrations of ethanolic extract of *Caesalpinia bonducella* simultaneously. In untreated cells, we get to see that we got 7µM of nitric production were cells that were treated with LPS stimulated the production of nitrite was 35µM but when the concentration of ethanolic extract of *Caesalpinia bonducella* is added i.e., 1ng, 10ng, 100ng, 1µg and 10µg, we got to see that at 10µM concentration nitric level significantly reduces *i.e.,* it comes to 11µM shown in **Fig. 5.**

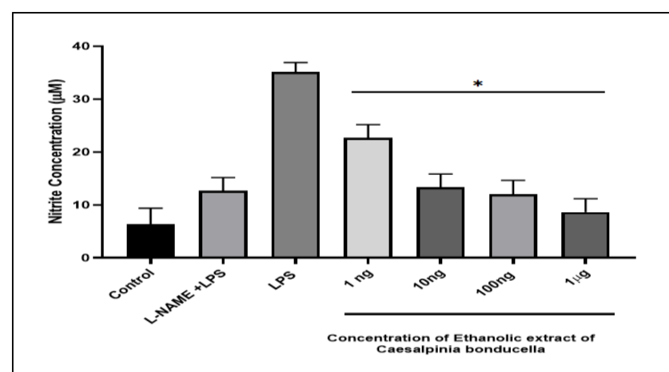

FIG. 5: INHIBITION OF NITRIC OXIDE PRODUCTION MEASURED AS NITRITE IN LPS INDUCED L929 FIBROBLAST. Data represents mean ± SD from three individual experiments, * denotes $p < 0.05$.

CONCLUSIONS: Many herbal remedies have been used to treat and manage a wide range of diseases in various medical systems. *Caesalpinia bonducella* has been used for the treatment of human diseases and ailments in various traditional medicine systems. Tea, grapes, berries, cocoa, and other plants contain phytochemicals, which are types of molecules. These have a wide range of pharmacological properties. The results presented in this study show that an ethanolic extract of *Caesalpinia bonducella* possesses anti-inflammatory properties. Polyphenolic compounds such as alkaloids, flavonoids, tannins, steroids, and phenols with high concentrations may be responsible for anti-inflammatory activity. The extract fractions act as free radical inhibitors or scavengers, or possibly as primary oxidants, inhibiting heat-induced albumin denaturation and stabilizing the Red Blood Cells Membrane. This study suggests that the plant Caesalpinia bonducella can be used as a lead compound to develop a potent anti-inflammatory drug that can be used to treat diseases such as cancer, neurological disorders, aging, and inflammation.

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CONFLICTS OF INTEREST: No potential conflict of interests.

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