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TARGETING OXIDATIVE STRESS AND INFLAMMATION: PRECLINICAL EVALUATION OF CASSIA ABSUS FOR ARTHRITIS MANAGEMENT IN RAT MODELS

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Abstract

Background: *Cassia absus seeds contain different phytochemicals which are used for the management of inflammation and arthritis. This study was carried out to evaluate the anti-inflammatory, anti-arthritic effect and antioxidant activity of aqueous methanolic extract of Cassia absus seeds.*

Methodology: *Aqueous methanolic extract was prepared by maceration. High performance liquid chromatography (HPLC) was used for to detect phytochemicals. Extract doses of 250 mg/kg, 500 mg/kg, 750mg/kg and standard Naproxen 30mg/kg were used in all the models. Carrageenan, Histamine and serotonin induced acute edema models were used to observe the effect of extract seeds on acute inflammation. These models showed the inhibition of edema formation in dose dependent manner. Formaldehyde and CFA model were used to study the anti-arthritic effect. Injections of CFA and formalin were injected in the paws of rats for induction of arthritis.*

Results: *In formaldehyde model, a significant difference was seen on the 7th day on the doses of 250 (P=0.001) and 500mg/kg (P=0.0054). In CFA model a significant difference of anti-arthritic effect was seen on 18th, 21th, 25th and 28th day in the paw of rats. The weight was improved only in groups of standard control and those treated at 750mg/kg. In-vitro anti-oxidant activity was determined by DPPH, phosphomolybdenum and reducing power assay by comparing the log IC_{50} value of extract with the Log IC_{50} value of standard. In in-vitro testing, aqueous methanolic extract showed strong anti-oxidant activities. To check the bone erosion and extent of inflammation radiographic evaluations were conducted on 28th day of CFA model. Histopathology of paw's cell was also conducted to evaluate necrosis and inflammation. In hematological findings, extract down regulated the C-reactive protein, alanine transaminase, aspartate aminotransferase and urea level. Effect of extract on the gene expression (IL-1, IL-4, IL-6, IL-10, COX-2, TNF-α) in the blood was determined by q-RT-PCR. The extract up-regulated the effect of mRNA expression of IL-4 and IL-10 while down-regulated the mRNA expression of IL-1, IL-4, COX-2 and TNF-α.*

Conclusion: *This study provides the evidence that seeds of Cassia absus have anti-inflammatory and anti-arthritic effect.*

INTRODUCTION

Rheumatoid Arthritis is the most prevalent chronic autoimmune disease characterized by the Inflammation of joints and affecting pulmonary, ocular, cardiovascular, and cutaneous systems[1] Tenderness, swelling of joints, morning stiffness, fatigue, elevated CRP (C - reactive protein) and increased ESR (Erythrocyte Sedimentation Rate) levels with consistent underlying synovitis contribute to the initial clinical diagnosis of rheumatoid arthritis. Numerous types of arthritis have been classified into non-inflammatory and inflammatory arthritis. Inflammatory arthritis can be caused by crystal deposition, bacteria, virus and autoimmune processes.[2]

Medicinal plants possessing anti-inflammatory activity seem to have lesser side effects and provide a way for natural drug therapy for rheumatoid arthritis[3]. *Aloe barbadensis* shows anti-inflammatory properties due to the presence of anthraquinone compound present in it[4]. The alkaloids and steroidal lactones present in *Withenia somnifera* root powder have shown anti-arthritic effect in adjuvant induced arthritic rats[5]. In *Zingiber officinale* the main constituents are sesquiterpenoids, with zingiberene. The anti-inflammatory activity in it is due to the presence of Sesquiterpene Lactones (SLs). A study revealed anti-arthritic activity similar to that of Aspirin in adjuvant induced arthritic rats by the leaves of the plant *Justicia gendarussa*.[6] this research is also focused on the use of medicinal plants for the treatment of rheumatoid Arthritis due to toxic and teratogenic side effects of anti-arthritic drugs. The objective of this study is to evaluate the anti-arthritic and anti-inflammatory effect of *Cassia absus* seeds by pharmacological models and anti-oxidant assays like DPPH assay, phosphomolybedenum assay and reducing power assay.

Cassia absus belongs to the family Fabaceae and its common names are Chaksu (Hindi), Jasmeejaz (English) and ShimiKul (Ayurveda). It is present in tropical regions of the world and mainly found in India and Sri Lanka. In Pakistan, it is found in western regions. Its leaves and seeds have been used for therapeutic purposes; roots and seeds have also been studied for medicinal purposes[7]. The seeds comprise of alkaloids, fatty acids and saponins in large quantity whereas, tannins and terpenoids are present in trace amounts. The flavonoids present in it have anti-bacterial and antifungal effect. Condensed tannins and derivative of gentisic acids 5-O-β-D-glucopyranoside is also present. Amino acids, gums and resins are also present in the seeds.[8]Some isolated constituents of the seeds are linoleic acid, leutolin, chaksine, isochaksine, raffinose, β-sitosterol, and ketooctadec-cis-15-enoiec acid.[9]

Material and Methods

Ethical Considerations: Punjab university College Of Pharmacy institutional committee approved the project.

Chemicals: Methanol, distilled water, conc. sulfuric acid, sodium hydroxide, carrageenan 0.1g, 37% formalin, histamine, 0.1g, Benedict's reagent, Fehling's A and B, Ninhydrin reagents, CFA cell suspension, conc. Hydrochloric acid, formaldehyde, chloroform, ferric chloride and glacial acetic acid.

Drug: Naproxen 30mg/kg, used as an oral dosage form.

Animals: Albino wistar rats of both sexes were acclimated for 14 days. Rats were sheltered in stainless steel cages at 25°C±2°C and humidity of 45-56%. Access to diet, water and human care were provided according to the requirements of National Institute of Health (NIH) Center.

Plant material and Extraction: The seeds of the plant *Cassia absus* were collected from Papar Mandi, Lahore, Pakistan in the month of February. Plant was identified from the Government College University, Lahore, Pakistan with identification number 3879 by Prof. Dr. Zahir-ud-din Babar (Botany department). Around 2kg of *Cassia absus* seeds were weighed and grinded into a powder. Then extraction was carried out through the

maceration process by soaking the powder in aqueous methanol for 7 days with regular shaking. The ratio of methanol and water was 70:30. After 7 days, the soaked powder was filtered through Whatman's filter paper. Powder was soaked again for 7 days in aqueous methanol and the procedure was repeated. The filtrate then was set on rotary at 67°C to evaporate the solvent (Methanol). It was further dried in Petri dishes under a fan. Extract obtained after solvent evaporation was 50g.[10]

Solubility testing: The extract was dissolved into different solvents and its solubility was analyzed. Water bath and vortex mixture were used as required. Solvents used were distilled water, normal saline, ethanol, methanol, Ringers' lactate, DSMO (Di-methyl sulfoxide), Tween 20, Tween 80.[10]

Phytochemical Analysis: Several phytochemical tests were performed for the screenings of chemical constituents.

Qualitative Tests:

Tests for Saponins: 5mL of distilled water and 5mL of aqueous extract were shaken and warmed. The test was positive which was indicated by stable foam formation.

Test for Alkaloids: In 2mL of aqueous extract, a few drops of Wagner's reagent were added. Reddish brown precipitates were formed indicating the presence of alkaloids.

Test for Tannins: In a test tube, 2mL of distilled water was added to the extract. To identify tannins few drops of ferric chloride were added to the test tube. Green colored precipitates were formed indicating the presence of tannins.

Test for Terpenoids: 2mL of extract and 2mL of chloroform were heated in a test tube. After that, 2mL of sulfuric acid was added and heated for about 2 min. Grayish color formation indicated the presence of terpenoids.[11]

Test for Glycosides: Keller Kiliani test was performed to test glycosides. 1 drop of 2% ferric chloride was added to 4mL glacial acetic acid. This mixture was added to 10mL aqueous extract and 1mL sulfuric acid. Brown ring was formed indicating presence of glycosides.

Test for Proteins: 2mg of extract and 2 mL of Millon's reagent were mixed thoroughly and heated. The white precipitates turned brick red after boiling which indicated the presence of proteins.

Quantitative Test: HPLC (High Performance Liquid Chromatography):

HPLC is used identification, quantification and purification of individual photochemical present within a mixture. Shim-Pack CLC-ODS (C118), 25cm x 4.6mm, 5µm column was used for separation of reverse phase on gradient HPLC. The UV-visible detector with λ max 280nm was used. Column oven CTO-10VP, pump LC-10AT, SPD10AV detector was implied in the process. Flow rate was kept at 1mL/min, 10 samples per second. Mobile phase gradient used were water and acetic acid in 94:6 ratio with a pH of 2.27 and acetonitrile 100%.The procedure was performed at room temperature.

In-Vivo Anti-Inflammatory Models:

Carrageenan Induced Edema: To induce edema, 0.1mL carrageenan (1%w/v) was injected subcutaneously in sub-plantar region of hind paw of rats, 1 hour before the treatment in curative model. This was done to each group except for the normal controlled group. Rats were given the treatment drug orally. Paw size was measured at 0hr, 1hr, 2hr, 3hr, and 4hr interval through digital vernier caliper.[12] Rats were divided in 6 groups.

Histamine Model: Edema was induced in the right hind paw of rats of each group except normal control groups by injecting 0.1mL histamine (1%w/v) subcutaneously 1hour before the treatment in the curative model. Treatment

drug (Naproxen 30mg/kg) was given orally to the rats. Paw size was measured at 0hr, 1hr, 2hr, 3hr and 4hr interval through digital vernier caliper. [13]

The groups of rats were as under:

Serotonin Model: To induce edema, 0.1mL serotonin (1%w/v) was injected in sub-plantar region of hind paw of rats, subcutaneously, 1 hour before the treatment in curative model. This was done to each group except for the normal controlled group. Rats were given the treatment drug orally. Paw size was measured at 0hr, 1hr, 2hr, 3hr, and 4hr interval through digital vernier caliper. Rats were divided in 6 groups.[14] Each group contained 5 rats each.

Group 1: Distilled water 10mg/kg
Group 2: (Carageeanan/ Histamine/ Serotonin) and distilled water 10mg/mg
Group 3: (Carageeanan/ Histamine/ Serotonin) and 30mg/kg naproxen
Group 4: (Carageeanan/ Histamine/ Serotonin) and 250mg/kg extract
Group 5: (Carageeanan/ Histamine/ Serotonin) and 500mg/kg extract
Group 6: (Carageeanan/ Histamine/ Serotonin) and 750mg/kg extract

In-Vivo Anti-Arthritic Models

Formaldehyde Model: The right hind paw of rats of each group except for normal control group were injected subcutaneously with 0.1mL formaldehyde (2% w/v) to induce arthritis, 1 hour before administration of treatment drug in curative model. Drug treatment was given for 9 more days after day 0. 0.1mL formaldehyde (2%w/v) was injected into the same paw on day3.[15]Paw size was measured by vernier caliper. The rats were divided in groups with 5 animals each:

CFA Model: 0.1mL of CFA was administered subcutaneously into the sub-plantar region of the left hind paw on 1st and 7th day to all rats of all groups except for control group. Naproxen 30mg/kg was also orally administered once daily from day 12 to day 28. Physical parameters like body weight, paw diameter was determined on days 11, 14, 18, 21, 25 and 28.[16]

The rats were grouped in all acute and chronic inflammation models as follows:

Group 1: Distilled water 10mg/kg
Group 2: Formaldehyde/ CFA and distilled water 10mg/kg
Group 3: Formaldehyde/ CFA and 30mg/kg naproxen
Group 4: Formaldehyde/ CFA and 250mg/kg extract
Group 5: Formaldehyde/ CFA and 500mg/kg extract
Group 6: Formaldehyde/ CFA and 750mg/kg extract

Percentage inhibition was calculated by formula:

$$\textbf{Percentage inhibition} = \frac{\textit{Increase in paw edema (control)} - \textit{Increase in paw edema (Test)} \times 100}{\textit{Increase in paw edema (Control)}}$$

Hematology Findings: According to the groups, all rats were anaesthetized by diethyl ether and blood was obtained through cardiac puncture and collected into blood collection tubes. The hematological parameters analyzed were LFT (Liver Function Test) and RFT (Renal Function Test). C-reactive protein was analyzed from the serum. The blood samples were then sent to Al-Hameed LAB and Research Center, Lahore, Punjab for the hematological findings.

Polymerase Chain Reaction (PCR)

RNA isolation: RNeasy kit (Qiagen) was used to separate the total RNA from blood samples. The quantity and quality of RNA was checked on Nano-drop.

cDNA synthesis: Super Script III first strand cDNA synthesis kit was used to perform the synthesis of first strand of cDNA. 5.0µg of total RNA was used for cDNA synthesis. 40µl of cDNA synthesis was achieved with poly-A tail primed oligo dT.

PCR Reaction: 2µL cDNA templates were used with gene specific primers in a tube to perform PCR reaction.

Thermal Cycle:

Steps of thermal cycle in PCR reactions

Step	Temperature	Duration
Denaturation 1st step	95°C	10min
Denaturation	95°C	15min
Annealing and extension	60°C	1min

Primer Sets

List of Primers used in PCR

Marker	Forward/ Reverse Primer	Primer Sequence
IL-1	Forward Reverse	5'-GAA ATG CCA CCT TTT GAC AGT G -3' 5'-TGG ATG CTC TCA TCA GGA CAG -3'
IL-4	Forward Reverse	5'-GTA CCG GGA ACG GTA TCC AC-3' 5'-TGG TGT TCC TTG TTG CCG TA-3'
IL-10	Forward Reverse	5'-TTG AAC CAC CCG GCA TCT AC-3' 5'-CCA AGG AGT TGC TCC CGT TA-3'
IL-6	Forward Reverse	5'-CCC ACC AGG AAC GAA AGT CA-3' 5'-ACT GGC TGG AAG TCT CTT GC-3'
TNF-α	Forward Reverse	5'-ATG GGC TCC CTC TCA TCA GT-3' 5'-GCT TGG TGG TTT GCT ACG TT-3'
COX-2	Forward Reverse	5'-ATG CTA CCA TCT GGC TTC GG-3' 5'-TGG AAC AGT CGC TCG TCA TC-3'

ELISA kit analysis:

C - Reactive Protein (CRP) ELISA: CRP is an acute phase reactant protein found in the blood serum. It is a pentameric protein synthesized by the liver even in non-pathological state. Its level rises in response to inflammation in the body. So, it is mainly used an inflammation marker.

Materials: Microwells coated with streptavidin 96, CRP standard, CRP conjugate reagent, TMB substrate, 6 vials 0.25mL, and Stop solution 12mL, sample diluents 50mL and wash buffer 25mL.

Procedure: 5µL of sample is added to 495uL of sample diluents in the ratio 1:100. Then 10µL samples and standards are added into appropriate wells. 100µL of conjugate reagent is added to all wells. They are then incubated for an hour at

room temperature. After removing the liquid from the wells, it is washed with 300µL of wash buffer. After that, the wells are incubated for 15 minutes at room temperature after adding 100µL of TMB substrate. Then, 50µL of stop solution is added and absorbance is measured within 15min of adding the stop solution.

Standard curve: A linear graph is used to plot a standard curve having concentration and respective absorbance values on both axes. Concentration of samples is obtained by linear regression. The values obtained are multiplied by dilution factor 100 to obtain CRP values in mg/mL.

TNF-α ELISA

Materials: Standard solution (32U/mL), standard diluents, streptavidin HRP, pre-coated plate, stop solution, substrate solution A and B, wash buffer, biotinylated human TOS antibody.

Procedure: 50µL standard and 40µL are added to appropriate wells. 10µL TOS antibody and 50µL streptavidin antibody is added to sample wells. They are then incubated for 60 minutes at 37°C. The plate is then washed with a wash buffer 5 times for at least 30 seconds each. Later, 50µl of substrate A and 50µL of substrate B are added to each well and incubated at 37°C for 10 minutes. 50µL stop solution is then added to terminate the reaction and then optical density is measured at 450nm.

Standard curve: A standard curve is constructed on linear graph by taking concentration and respective absorbance values on both axes. Concentration of samples is obtained by linear regression. The values obtained are then multiplied by dilution factor 100 which gives CRP values in mg/mL.

Histopathological Examination: After the end of CFA protocol, paws of all rats were dissected and placed in 10% formalin solution for fixation. The slides were made after staining with hematoxylin and eosin stains.

Cutting Technique: The tissues are placed in paraffin wax and sections are cut at 5 microns with tungsten carbide plate.17

Staining procedure: The paraffin wax is removed from the paraffinized sections and dipped in 3 changes of xylene for 3 minutes. These are then hydrated by 100% and 95% ethyl alcohol giving 10 dips each. They are then washed with distilled water and stained with hematoxylin for 1to5 minutes. Then they are washed in three changes of water. The slides are then dipped in lithium carbonate, and aqueous tap water. They are washed in three changes of tap water, rinsed with distilled water and the excess is drained from the slides. Then they are given 10 dips in 70% alcohol. Counter staining is done in eosin Y working solution for 30 seconds. Dehydration is done by dipping them in two changes of 95% alcohol for 1 min and two changes of 100% ethyl alcohol, ten dips each. Then they are cleared in 3 changes of xylene in 10 dips. Cover slip is put with mounting medium.

The identification of slides is done by camera microscope. Slides were scored on a scale of 0-3 depending upon pannus formation, bone erosion and chronic inflammation.

X-ray radiographic evaluation: At the 21st day of CFA model, the animals were given anesthesia by chloroform and the paw of each rat was observed under a digital X-ray machine. The focal film distance was 40cm at 125kVp and 500mA.[16] each paw was scored on a scale of 0-4 on the basis of joint space reduction.

In vitro Antioxidant Activity

DPPH Radical Scavenging Assay

Procedure: 0.1mM solution of DPPH and methanol is prepared (Solution A). Along with this extract is prepared in different concentrations of methanol (1.25-150µg/mL) (Solution B). 2.4mL of solution A and 1.6mL of Solution B are mixed and vortexes thoroughly and placed in dark at RT for 30 mins. The absorbance is measured with spectrophotometer at 517nm. BHT is used as reference. The percentage of free radical scavenging ability of the extract with DPPH is calculated by following formula:

Percentage Inhibition= $(A_0 – A_1)/A_1 \times 100$

Where,

A_0= Absorbance of the control

A_1= Absorbance of standard

A graph is plotted between percentage inhibition and the respective concentration. IC_{50} is calculated from the graph. The experiment is repeated thrice at each concentration.

Phosphomolybdenum Assay:

Procedure: A solution of 10mg of plant extract and 1mL of DSMO is prepared. Out of this solution, 100µL is taken and 1mL of phosphomolybdenum assay reagent (0.588ml sulphuric acid, 0.049g ammonium molybdate, 0.063g sodium phosphate and 10mL distilled water) is added to it. It is then incubated in a hot water bath at 95°C for 90 mins. After normalizing the solution at room temperature for 20 minutes, the absorbance is checked at 695nm against ascorbic acid as a standard. The phosphomolybdenum reduction potential (PRP) is then calculated as a percentage.

Reducing Power Assay:

Procedure: 0.75mL of extract is mixed with 0.75mL of phosphate buffer (pH6.6, 0.2M) and 1%w/v of 0.75mL potassium hexacyanoferrate. The mixture is then incubated at 50°C in a hot water bath for 20 mins. 0.75mL trichloroacetic acid (TCA) is added to the mixture to stop the reaction. It is then centrifuged at 800g for 10 mins. After this, 1.5mL of the obtained supernatant is mixed for 10 minutes with equal quantity of distilled water and 0.1mL of ferric chloride solution (0.1% w/v). The absorbance of the mixture is read at 700nm. Higher the absorbance value, higher the reducing power.

Acute Toxicity Model

Acute toxicity is the undesired effect that can occur immediately or at short intervals right after a toxic substance is administered within 24 hours. The criterion to measure acute dose is lethal dose assessment (The dose at which 50% of the test population gets killed). LD_{50}, therapeutic index and degree of safety of a pharmacological agent are assessed by acute toxicity. Before doing the toxicology assessment, a drug cannot be marketed. To estimate the LD_{50} of *Cassia absus*, Lorke's method was implied. It has two phases.

Phase 1: Nine animals were placed into three groups and each group was administered different doses of the test substance i.e., 10, 100, 1000 mg/kg). The animals were kept under observation for 24 hours. The behavior and mortality were observed.

Phase 2: One animal from each group was taken and each was administered higher doses (3000, 4000, 5000 mg/kg) of test substance and then observed for 24 hours. The LD_{50} is calculated by the following formula:

$LD_{50}=\sqrt{(D_0 \times D_{100})}$

D_0= the highest dose that gave no mortality

D_{100}= the highest dose that caused mortality

Results

Table 1. Solubility Analysis of Cassia absus

Solvent	Results
Distilled water	Not soluble
Normal Saline	Not soluble
Ringers Lactate	Not soluble
Tween 20	Soluble
Tween 80	Soluble
Methanol	Soluble
Ethanol	Soluble
DSMO	Soluble

Phytochemical Screening:

Table 2. Phytochemical Analysis

Test	Observation	Result
Saponins	Foam formation	Present
Alkaloids	Reddish brown precipitates	Present
Tannins	Green colored precipitates	Present
Terpenoids	Grey color	Present
Glycosides	Brown ring formation	Present
Proteins	Brick red color precipitates	Present

Quantitative analayis

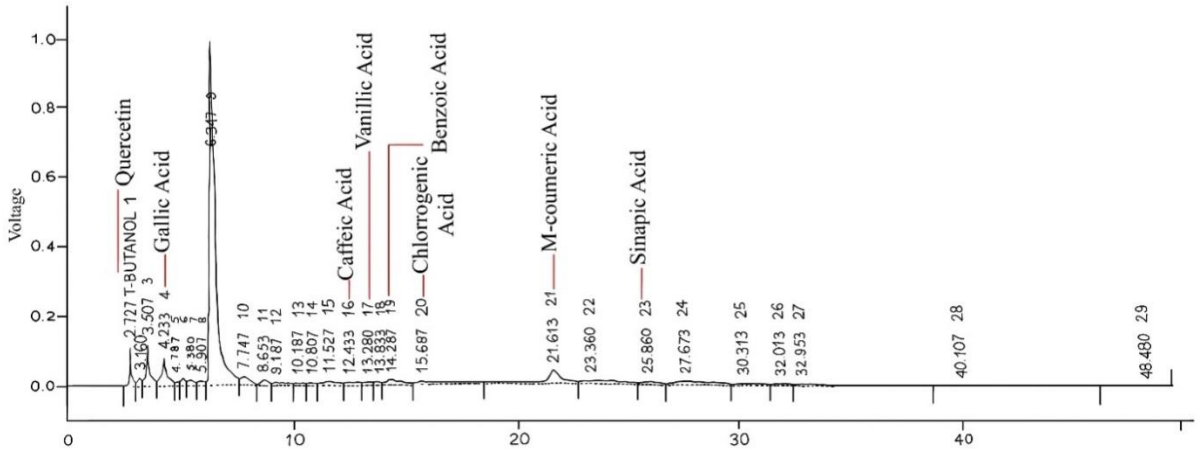

Figure 1. HPLC Chromatogram

Table 3. HPLC Analyzed Results of Phytochemicals

Compound Name	Retention time	Area [mV.s]	Area Percentage	Amount [ppm]
Quercetin	2.727	793.697	2.4	42.06
Gallic acid	4.233	1476.378	4.5	53.14
Caffeic acid	12.433	301.528	0.9	13.87
Vanillic acid	13.280	199.385	0.6	12.36
Benzoic acid	14.287	638.074	2.0	67.63
Chlorogenic acid	15.687	865.162	2.6	67.48
M-coumeric acid	21.613	2279.872	7.0	27.35
Sinapic acid	25.960	493.218	1.5	6.41

In-Vivo Anti-Inflammatory Model:

Carrageenan Induced Paw Edema: Paw size was measured with vernier caliper, used to determine the percentage inhibition for each hour. Percentage inhibition values are shown in the Table 4 represented as mean±SEM and the data was analyzed by two-way ANOVA. The comparison was done by applying Tukey's multiple comparison tests.

From hour 4 to hour 6, a significant decrease in paw size and a significant increase (P<0.0001) in percentage inhibition was observed for standard group and groups of 250, 500 and 750mg/kg as compared to the disease group. The graphical representation is shown in figure 2.

Table 4. Percentage Inhibition Values Of All Groups In Carrageenan Model

Time	Disease	Standard	250mg/kg	500mg/kg	750mg/kg
1 hour	0±0	28.8±0.29	6.1±0.17	11.03±0.38	17.2±0.4
2 hours	0±0	34.83±0.21	15±0.26	22.9±0.26	27.9±0.48
3 hours	0±0	46.7±0.36	21.4±0.41	35.2±0.29	41.2±0.35
4 hours	0±0	55.36±0.34	28±0.33	41.1±0.46	49.76±0.6
5 hours	0±0	68.8±0.54	35±0.38	52.6±0.35	56.8±0.5
6 hours	0±0	82.2±0.68	42.2±0.57	59.5±0.38	68.5±0.9

There was a significant statistical difference in anti-inflammatory effect in all groups (P<0.0001) in the course of study period from 0 hour to 6 hour (n=6).

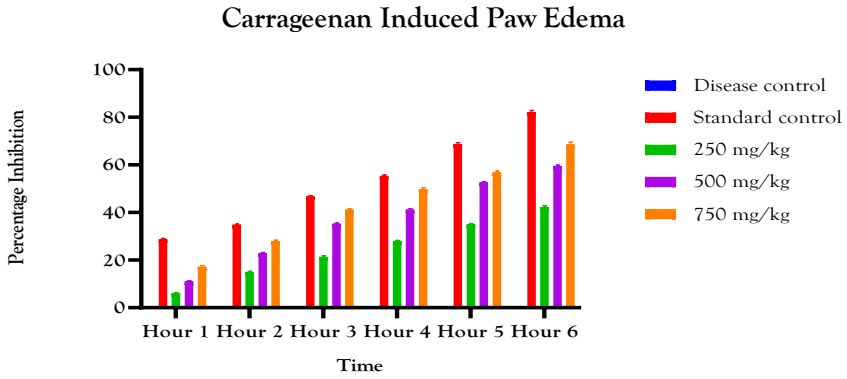

Figure 2. The Effect Of HME Of Cassia Absus Seeds On Paws In All Groups (250, 500 And 750 Mg/Kg) In Carrageenan Model

All the doses of extract; 250mg/kg (4.14±0.38), 500mg/kg (4.49±0.51) and 750mg/kg (4.61±0.61) have shown considerable anti-inflammatory effect as compared to disease group (5.34±1.3).

Histamine Induced Paw Edema: Paw size was gradually decreased in all the treatment groups when compared to the disease group. The percentage inhibition was calculated for all groups and shown in Table 5 and represented as Mean ±SEM and the data was analyzed by 2-way ANOVA. The graphical representation is shown in Figure 3.

Table 5. Percentage Inhibition Values Of All Groups In Histamine Model

Time	Disease	Standard	250mg/kg	500mg/kg	750mg/kg
1 hour	0±0	0.83±0.02	0.41±0.06	0.96±0.12	1.76±0.18
2 hours	0±0	51.16±0.43	8.7±0.18	19.4±0.44	21.5±0.48
3 hours	0±0	71.63±0.44	20.9±0.3	33±0.4	39.5±0.4
4 hours	0±0	87.61±0.63	33.8±0.63	47.4±0.49	59.06±0.47

There was a significant statistical difference in anti-inflammatory effect in all groups ($P<0.0001$) in the course of study period from 0 hour to 4 hour (n=6).

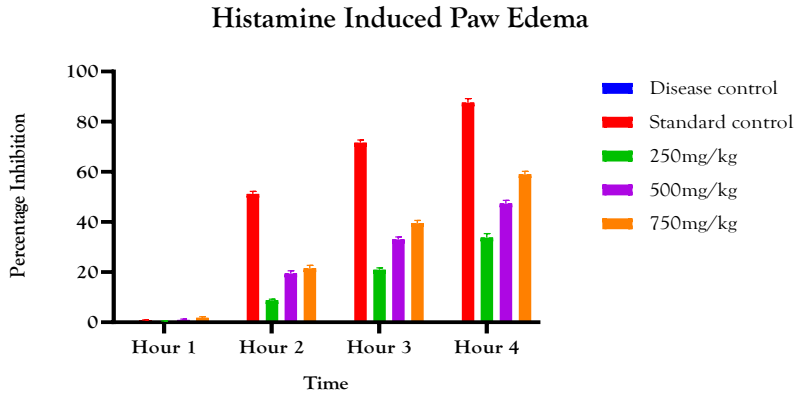

Figure 3. The Effect Of HME Of Cassia Absus Seeds On Paws In All Groups (250, 500 And 750 Mg/Kg) In Histamine Model

All the doses; 250mg/kg (4.14±0.40), 500mg/kg (4.46±0.53), 750mg/kg (4.68±0.62) have shown greater anti-inflammatory effect as compared to disease group (5.49±1.305).

Serotonin Induced Paw Edema: Paw size was gradually decreased in all the treatment groups as compared to the disease group. The percentage inhibition was calculated for all groups and shown in Table 6 and represented as Mean ±SEM. The data was analyzed by 2-way ANOVA. The graphical representation is shown in Figure 4.

Table 6. Percentage Inhibition Values of All Groups In Serotonin Model

Time	Disease	Standard	250mg/kg	500mg/kg	750mg/kg
1 hour	0±0	36.2±0.83	18±0.47	21.8±0.53	24.6±0.69
2 hours	0±0	48.1±1.4	24.2±0.46	30.7±0.45	37.2±0.78
3 hours	0±0	65.4±0.7	34.5±0.53	42.9±0.65	50.5±0.55
4 hours	0±0	84±1.06	42.38±0.93	53.2±0.9	65.1±0.5

There was a significant statistical difference in anti-inflammatory effect in all groups ($P<0.0001$) in the course of study period from 0 hour to 4 hour (n=6).

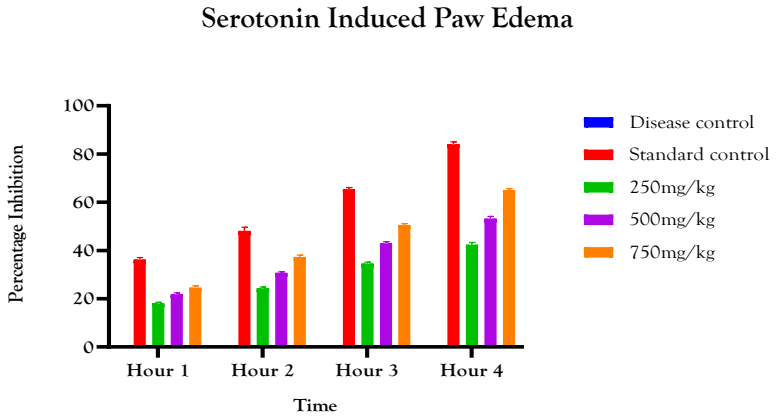

Figure 4.The Effect Of HME Of Cassia Absus Seeds On Paws In All Groups (250, 500 And 750 Mg/Kg) In Serotonin Model.

All they doses; 50mg/kg (4.16±0.38), 500mg/kg (4.62±0.71), 750msg/kg (4.73±0.82) have shown greater anti-inflammatory effect as compared to disease group (5.48±1.24).

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In-Vivo Anti-Arthritis Model:

Formaldehyde Induced Arthritis: The percentage inhibition was calculated for all groups in Formaldehyde induced arthritic model and shown in Table 7 and represented as Mean ±SEM.

Table 7. Percentage Inhibition Values Of All The Groups In Formaldehyde Model

Days	Disease	Standard	250mg/kg	500mg/kg	750mg/kg
1st day	0±0	16.9±0.7	12.5±0.6	18.8±0.14	21.9±0.59
4th day	0±0	37.6±0.63	22.2±1.10	27±0.7	29.9±0.7
7th day	0±0	57.09±0.8	49.5±0.68	57.11±0.7	56.3±0.7
10th day	0±0	86.9±1.02	65.19±0.26	68.7±0.2	74.2±0.2

There was a significant statistical difference in anti-inflammatory effect in all groups ($P<0.0001$) in the course of study period from 1st day to 10th day (n=6).

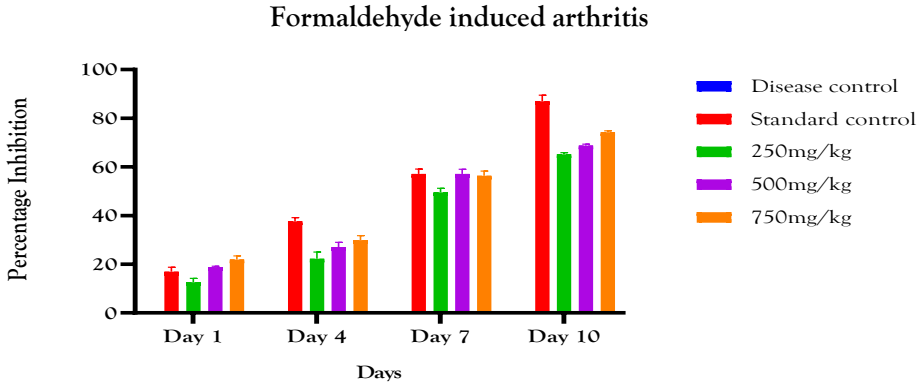


Figure 5. The Effect Of HME Of Cassia Absus Seeds On Paws In All Groups (250, 500 And 750 Mg/Kg) In Formaldehyde Model.

All the doses; 250mg/kg (4.31±0.66), 500mg/kg (4.47±0.60), 750mg/kg (4.60±0.70) have shown considerable anti-inflammatory effect as compared to disease group (5.93±1.04).

CFA induced arthritis:

Percentage inhibition:

In CFA induced arthritis, all the groups showed anti-arthritic activity including the naproxen standard group as compared to the disease group. The percentage inhibition values are represented as Mean ±SEM in Table 8. The data was analyzed by graph pad prism 9 using Two-way ANOVA with Tukey's multiple comparison test. The graphical representation is shown in Figure 6.

Table 8. Percentage Inhibition Values Of All The Groups In CFA Model

Days	Disease	Standard	250mg/kg	500mg/kg	750mg/kg
1st day	0±0	4.89±0.16	2.74±0.13	3.51±0.08	3.97±0.25
11th day	0±0	34.12±0.45	8.97±0.2	11.26±0.19	15.9±0.23
14th day	0±0	57.3±0.3	19.28±0.2	27.5±0.42	29.3±0.19
18th day	0±0	61.8±0.73	30.3±0.2	36.6±0.2	43.4±0.26
21st day	0±0	73.16±0.5	35.4±0.27	51.07±0.32	55.3±0.22
25th day	0±0	77.06±0.21	42±0.53	53.1±0.13	61±0.4
28th day	0±0	83.9±0.69	48.8±0.13	57.9±0.4	63.5±0.26

There was a significant statistical difference in anti-inflammatory effect in all groups ($P<0.0001$) in the course of study period from 1 day to 28th day (n=6).

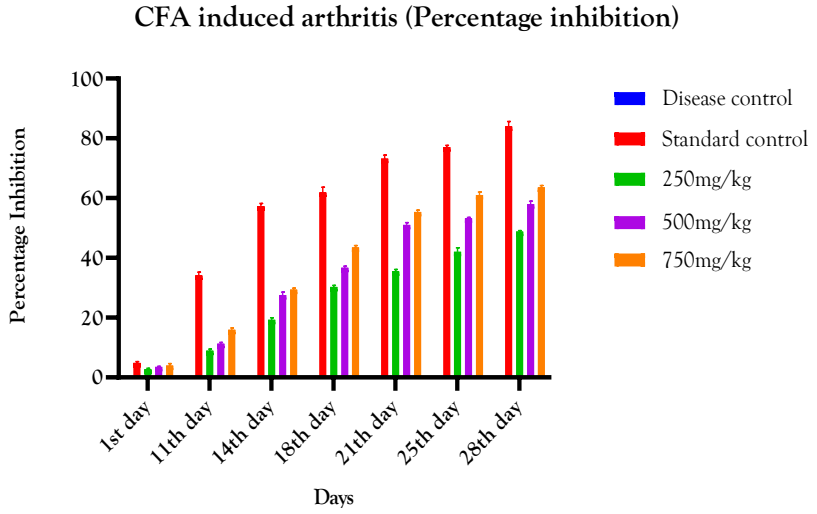


Figure 6. The Effect Of HME Of Cassia Absus Seeds On Paws In All Groups (250, 500 And 750 mg/Kg) In CFA Model.

All the doses; 250mg/kg (4.96±0.07), 500mg/kg (4.77±1.03), 750mg/kg (5.007±0.95) have shown greater anti-inflammatory effect as compared to disease group (5.93±1.15).

Weight Variation in CFA induced model

The weight variations of all the groups were studied in comparison to the disease group in CFA model. The weight variation values are represented as Mean ±SEM in Table 9. The data was analyzed by graph pad prism 9 using Two-way ANOVA with Tukey's multiple comparison test. The graphical representation is shown in Figure 7.

Table 9. Weight Variation Of All Groups In CFA Model

Days	Normal	Disease	Standard	250mg/kg	500mg/kg	750mg/kg
1st day	120±18.54	147±18.01	152.83±13.86	154.33±9.83	136±12.71	142±14.58
11th day	129±14.35	144.5±15.97	144.33±10.36	152±11.22	134.66±12.24	137.16±14.20
14th day	133±10.23	137.16±21.41	152.5±12.32	149.88±11.10	131.33±12.94	147.6±10.94
18th day	137.5±10.69	131.33±21.01	160±15.65	146.88±12.15	130.33±12.61	172.33±34.98
21st day	141.33±11.6	133±17.36	163±10.88	144.88±14.16	127.5±15.07	161.1±12.49
25th day	137.66±14.74	131.83±17.33	174±10.33**	142.50±14.08	124.83±13.89	164.6±18.79
28th day	139.5±11.86	132.66±16.04	180.8±10.08**	141.50±14.99	122.5±14.36	171.6±16.39*

There was no significant statistical difference in weight of all the groups (P<0.0001) in the course of study period from 1 day to 28th day except for the weight of standard group (P=0.0075) on 25thday. On 28th day, weight of standard (P=0.0019) and 750mg/kg group (P=0.017) showed significant difference as compared to the disease group (n=6).

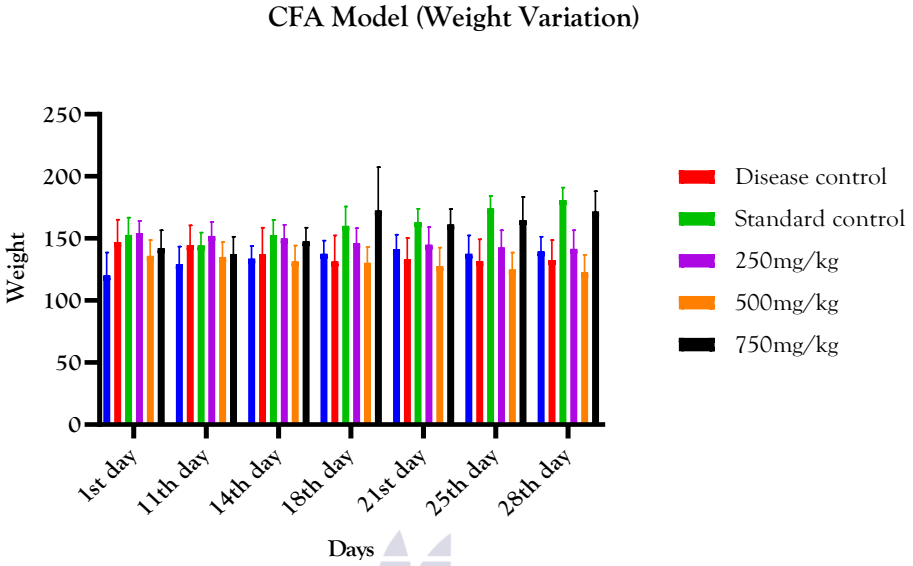


Figure 7. The effect of HME of Cassia absus seeds on weight variation in all groups (250, 500 and 750 mg/kg) in CFA model.

All the doses; 250mg/kg (147±4.86), 500mg/kg (129.6±4.96), 750mg/kg (156.7±14.32) have shown greater significant effect of weight as compared to disease group (136.8±6.46).

In-Vitro Pharmacological Testing

Hematological Findings: The value of all parameters is represented as Mean ±SEM in Table 10. One way-ANOVA with Tukey's multiple comparison was applied on the data to obtain the results. All the groups; 250mg/kg, 500mg/kg and 750mg/kg have shown significantly difference in biomarker values as compare to disease group. The graphical representation of these values is shown in figure 8.

Table 10. Hematological Findings

Parameters Mean±SD					
Groups	**C-Reactive Protein**	**ALT**	**AST**	**Urea**	**Creatinine**
Normal	3.90±1.25	74.0±1.75	115±1.75	39±1.75	0.7±0.03
Disease	14.60±1.25	386.0±1.75	298±1.75	77±0.90	1.0±0.03
Standard	5.90±1.25	64.0±1.75	49±1.75	56±1.75	0.67±0.03
250mg/kg	5.20±1.25	24.83±1.75	28.6±1.75	49±1.75	0.90±.03
500mg/kg	5.40±1.25	53.0±1.75	78±1.75	69±1.75	1.10±0.03
750mg/kg	5.20±1.25	59.0±1.75	52±1.75	40±1.75	0.90±0.03

The value of all parameters is represented as Mean ±SEM in Table 10. One way-ANOVA with Tukey's multiple comparison was applied on the data to obtain the results. All the groups; 250mg/kg, 500mg/kg and 750mg/kg have shown significantly difference in values as compare to disease group. The graphical representation of these values is shown in figure 8.

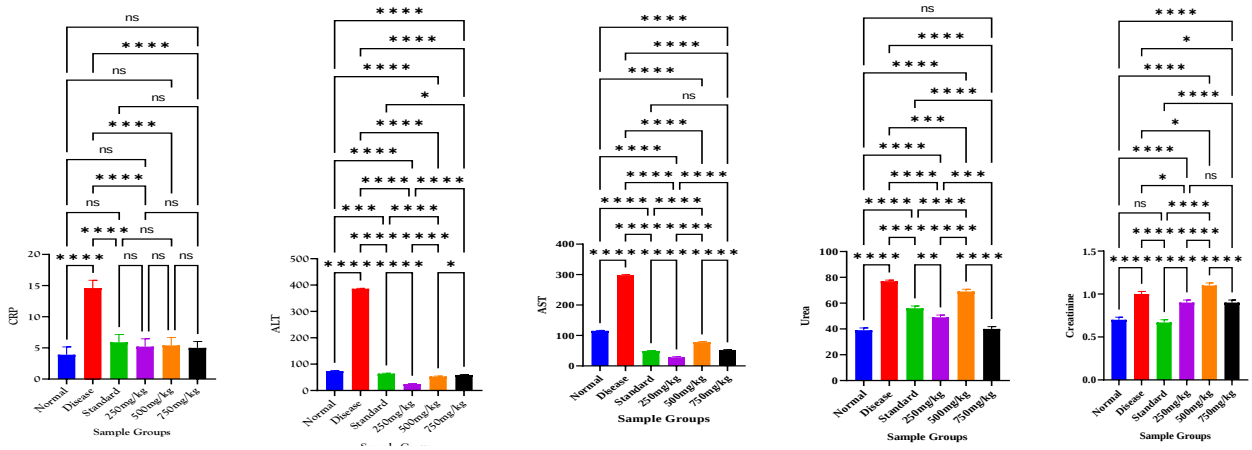


Figure 8. The Effect Of HME Of Cassia absus On Hematological Parameters Of All Groups (250, 500 And 750 mg/Kg) In Rats

There was a significant statistical difference in values of all groups (P<0.0001) in the course of study period from 1 to 28 days (n=6).

PCR Analysis:

The blood samples of all the animals were collected to prepare serum on the 28th day of CFA model. The mRNA expression by real time Polymerase reaction was quantified for IL-1, IL-4, IL-6, IL-10, COX-2 and TNF-α. The values of all parameters are represented as Mean ±SEM in Table 11. One way-ANOVA with Tukey's multiple comparison was applied on the data to obtain the results. The graphical representation of all values is shown in figure 9.

Table 11. PCR Findings

Groups	Parameters Mean±SD					
	IL-1	IL-2	IL-6	IL-10	COX-2	TNF-α
Normal	0.11±0.03	0.07±0.03	0.59±0.03	0.23±0.03	1.02±1.23	1.45±0.03
Disease	5.75±0.30	0.26±0.03	8.97±0.03	0.81±0.03	3.70±0.03	9.43±0.03
Standard	0.30±0.30	3.20±0.03	4.08±0.03	6.76±0.03	2.44±0.03	1.23±0.03
250mg/kg	3.58±0.03	0.18±0.03	2.5±0.03	0.61±0.03	2.44±0.03	6.37±0.03
500mg/kg	0.26±0.03	0.26±0.03	0.65±0.03	0.81±0.03	0.19±0.03	1.03±0.03
750mg/kg	0.084±0.03	0.49±0.03	0.60±0.08	2.37±0.03	0.46±0.03	0.53±0.03

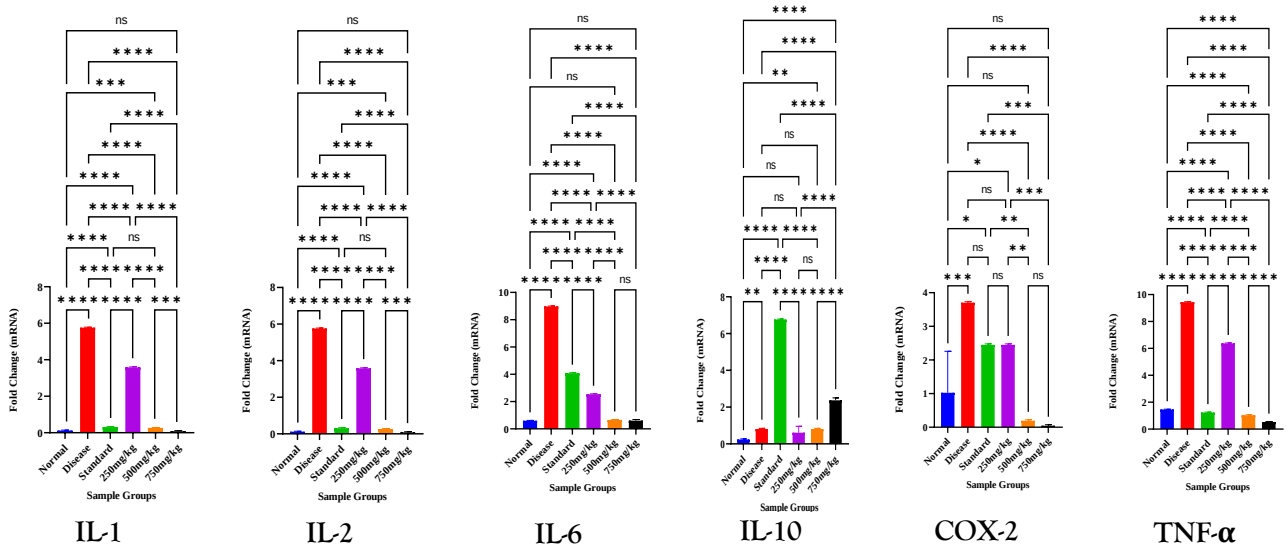


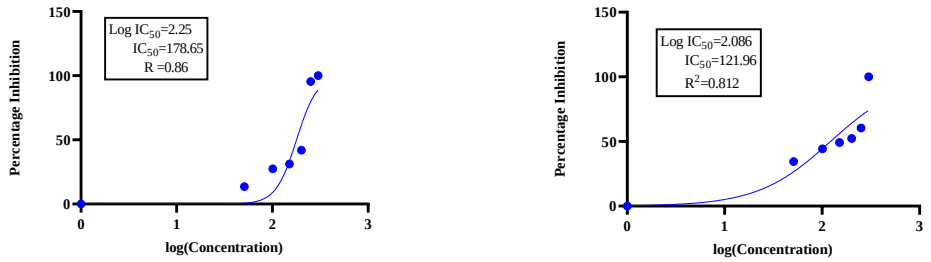
Figure 9. The Effect Of HME Of Cassia Absus On PCR Parameters Of All Groups (250, 500 And 750 mg/Kg) In Rats

There was a significant statistical difference in fold change value of all groups (P<0.0001) in the course of study period from 1 to 28 days. All the groups; 250mg/kg, 500mg/kg and 750mg/kg have shown difference in values when compared to disease group (n=6).

Antioxidant Activity

DPPH Assay:

The graphical representation of DPPH assay of *Cassia absus* and standard gallic acid is shown in the figure 10. The dilutions of hydro-methanolic extract of *Cassia absus* showed a log IC_{50} value of 2.086 with SEM 0.104. While the values for standard gallic acid were 2.25 with SEM=0.045. The Log IC_{50} represents the anti-oxidant activity. Lesser the Log IC_{50} value more is the anti-oxidant activity. The extract showed more anti-oxidant activity when compared to the standard dilutions.

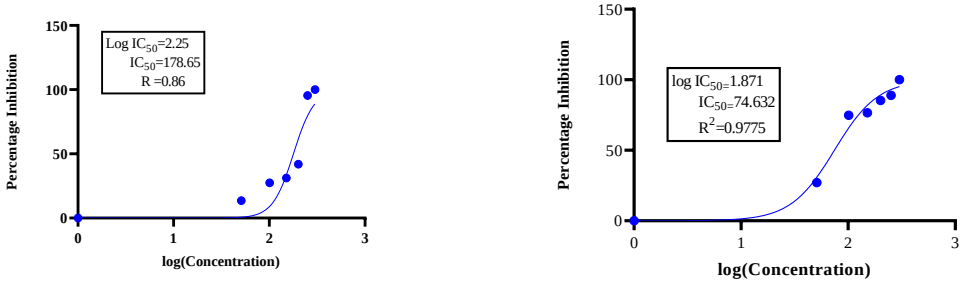


(a) (b)

Figure 10. Graphical Representation Of DPPH Assay Of HMEs Cassia Absus (b) when compared to gallic acid (a)

Phosphomolybdenum Assay:

The graphical representation of Phosphomolybdenum Assay of *Cassia absus* and standard gallic acid is shown in the figure 11. The dilutions of hydro-methanolic extracts of *Cassia absus* showed a log IC_{50} value of 1.871 with SEM 0.037 and logic IC_{50} value of standard was 2.25 with SEM=0.045. The Log IC_{50} represents the anti-oxidant activity. Lesser the Log IC_{50} value more is the anti-oxidant activity. The extract showed more anti-oxidant activity when compared to the standard gallic acid dilutions.

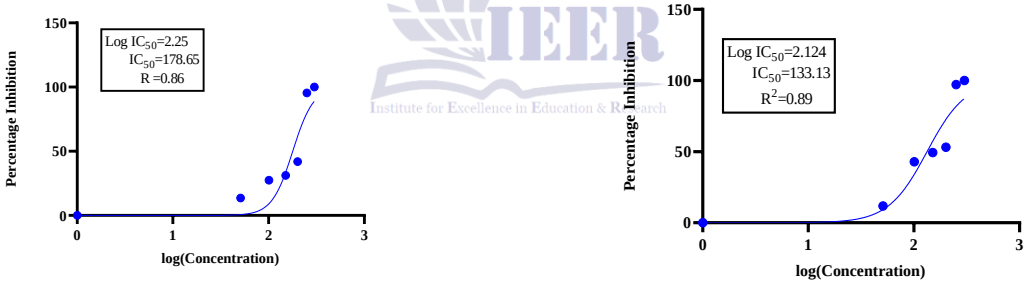


(a) (b)

Figure 11. Graphical Representation Of Phosphomolybdenum Assay Of HMEs Cassia Absus (b) when compared to gallic Acid (a)

Reducing Assay:

The graphical representation of reducing power assay of *Cassia absus* and standard gallic acid is shown in the figure 12. The dilutions of hydro-methanolic extract of *Cassia absus* showed a log IC_{50} value of 2.124 with SEM 0.060. While the values for standard gallic acid were 2.25 with SEM=0.045. The Log IC_{50} represents the anti-oxidant activity. Lesser the Log IC_{50} value more is the anti-oxidant activity. The extract showed more anti-oxidant activity when compared to the standard dilutions.



(a) (b)

Figure 12. Graphical Representation Of Reducing Assay Of HMEs Cassia Absus (b) when compared to gallic Acid (a)

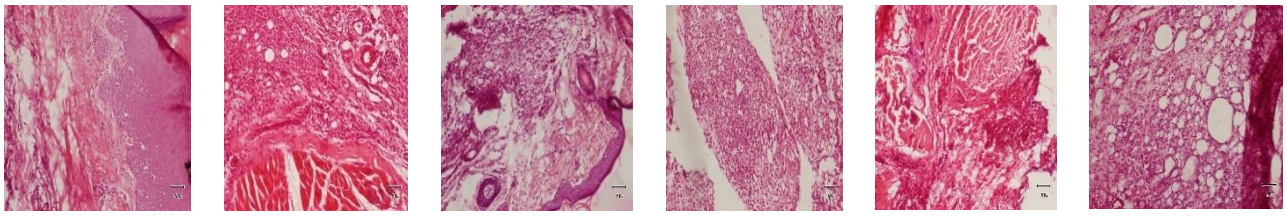
X-ray Evaluation:

Clinical Interpretation of bone X-rays also supported the beneficial effect of plant extract on rat paw when it was compared to the diseased group as shown from the X-rays in figure 13.

Figure 13. Dorsal X-ray of Rat paws (N: Normal, D: Diseased, S: Standard, 1: 250 mg/kg, 2: 500 mg/kg, 3: 750 mg/kg)

Histopatholoical Studies

Tissues of paw at a magnification of 10x10 of normal rat (a) shows intact dermis while diseased rat (b) showing necrosis and inflammation and standard rat (c) showing mild inflammation with no necrosis can be seen from figure 14. Whereas, Figure 14 (d, e and f respectively) shows tissue at a magnification of 10x10, of rats group treated at 250, 500 and 750 mg/kg are showing recovery from inflammation and necrosis with increased dose i.e. no inflammation and necrosis at 750 mg/kg dose.



(a) (b) (c) (d) (e) (f)

Figure 14. Histopathlogy of Rat paws (a: Normal, b: Diseased, c: Standard, d: 250 mg/kg, e: 500 mg/kg, f: 750 mg/kg)

Toxicological Studies

Table 11. Toxicological results:

Phase	Group Number	Dose Administered	Animals per group	Observation after 24hrs
1	1	10 mg/kg	3	No mortality
	2	100 mg/kg	3	No mortality
	3	1000 mg/kg	3	No mortality
2	1	3000 mg/kg	1	No mortality
	2	4000 mg/kg	1	No mortality
	3	5000 mg/kg	1	No mortality

LD_{50}>5000 mg/kg

Discussion

An in-vitro study reported the anti-inflammatory effect of *Cassia absus* seeds extract.[9] No detailed in- vivo work was previously done. So, this study was done to assess the anti-inflammatory and anti-arthritic effect of *Cassia absus* seeds in acute and chronic rat models. Hydro methanolic extract (HME) of *Cassia absus* seeds has shown the anti-inflammatory and anti-arthritic activity at doses i.e.,250mg/kg, 500mg/kg and 750mg/kg. The bio-active phytochemical constituents i.e. alkaloids, saponins and tannins were responsible for the anti- inflammmatory activity. Saponins resist vascular infiltration caused by inflammatory mediators and thus reduce the rate of swelling.[18] Saponins also have been reported to show anti-inflammatory activity by inhibiting lipopolysaccharide-induced iNOS and COX-2 which in-turn inhibit PGE-2, nitric oxide and tumor necrosis factor-α.[19]Alkaloids show the anti-inflammatory effect by inhibiting the cyclooxygenase enzyme.[20] Tannins show their anti-inflammatory activity by activating anti-oxidant enzymes.[21]

HPLC analysis showed the presence of compounds like quercetin, gallic acid, caffeic acid, vanillic acid, benzoic acid, chlorogenic acid, M-coumaric acid and sinapic acid. Flavonoids like quercetin have been reported to inhibit the initial process of inflammation.[22] Chlorogenic acid exhibits anti-arthritic activity by inhibition of NF-

κB activation.[23] Gallic acid inhibits PGE_2 production. Benzoic acid has been reported to inhibit matrix metalloproteinase which are responsible for tissue degradation in arthritis.[24] Acute inflammatory models were used which had a crucial role in drug development. The inflammatory response in these models was quantified by increased paw size (edema). The inflammatory cascade involved in carrageenan model has two phases. The first phase includes the release of serotonin, bradykinin and histamine. The 2nd phase involves with the release of prostaglandins.[25] The carageenan model has shown that the hydro methanolic extract of *Cassia absus* inhibits COX-2 and inflammatory cytokines like IL-1, IL-6, contributing to its anti-inflammatory and anti-arthritic effect. A similar inhibition of such inflammatory mediators has been exhibited in serotonin model by the hydro methanolic extract of *Cassia absus*. The extract also has shown the inhibition of inflammatory neuropeptides released in histamine model at all doses (250mg0kg, 500mg/kg, 750mg/kg).[26]

The chronic inflammation was observed through Formaldehyde and CFA induced arthritis models. The 1st phase of chronic inflammation involves the secretion of Substance P and bradykinin in neurogenic phase by formaldehyde. In the 2nd phase, inflammatory mediators like histamine, 5-HT and prostaglandins are released.[27]*Cassia. absus* extract decreased the paw inflammation caused by formaldehyde at all doses (250mg/kg, 500mg/kg, 750mg/kg). The CFA (Freund's adjuvant arthritis) model comprises of two phases. The first acute phase remains for 0 to 18th days and it occurs due to release of histamine and serotonin from immune cells. These mediators released from the immune cells are followed by the chronic phase which remains from 18th day to 28th day. Bone erosion, hypertrophy and synovitis are manifested by the action of pro-inflammatory mediators in the 2nd phase. Paw size decreases in the first phase and in 2nd phase it starts to increase again leading to chronic inflammation characterized by arthritis. The correlation between weight loss and inflammation was also observed. Less food intake, altered metabolism and stress during ailments may lead to weight loss or slow weight gain.[28] Weight loss occurred more in the diseased group when compared to standard group and also with treated group of extracts i.e. 250, 500 and 750mg/kg. The hydromethanolic extract of *Cassia. absus* restored the weight loss in all the groups due to presence of anti-arthritic activity.

Hematological parameters like CRP, ALT, AST, serum urea and creatinine were also analyzed. Elevated CRP level is an indicator for acute inflammatory phase of arthritis. CRP induces its pro-inflammatory effect by activating platelets, stimulating the production of IL-6, IL-1β and TNF-α and at the same time inhibiting nitric oxide.[29] The CRP values of the *Cassia. Absus* extract showed a significant decrease when compared to the diseased group. High ALT and AST levels indicate hepatocellular injury and the level of these enzymes were not elevated, indicating *Cassia. absus* extract has no hepatotoxicity. The PCR analyses conducted for IL-1, IL-4, IL-6, IL-10, COX-2 and TNF-α.IL-4 and IL-10 proved reduced production of inflammatory macrophages. The diseased group showed low levels of IL-4 and IL-10 proving these to be anti-inflammatory agents. IL-1 and IL-6 are pro-inflammatory cytokines leading to chronic inflammation.[30] The current study showed a decreased expression of IL-1 and IL-6 in the treated groups, indicating the anti-inflammatory and anti-arthritic effect of the extract of *Cassia. absus.* COX-2 and TNF-α are pro-inflammatory agents. COX-2 upon pathological stimulation activates TNF-α which causes its inflammatory effect by activating NF-κB pathway, protein kinases and caspase.[31] HME showed marked decrease in expression of COX-2 and TNF- α value for the standard and treated groups.

DPPH, phosphomolybdenum and reducing power assay were also carried out in the current study. DPPH assay determines potential oxidative damage.[3] The log IC_{50} value in DPPH determines the anti-oxidant activity. Low log IC_{50} value show high anti-oxidant activity. The extract showed comparable anti-oxidant activity. In phosphomolybdneum assay the log IC_{50} value of

extract was comparable to that of standard which shows that it has anti-oxidant activity. The log IC_{50}value of extract in reducing power assay was also comparable to that of standard (gallic acid) which shows that it has the anti-oxidant activity. X-ray scanning and histopathological evaluations also supported the anti-arthritic activity of hydro methanolic extract at all doses. Lastly, toxicological study showed that LD_{50} of plant extract is >5000 mg/kg.

Conclusion

The leave extract of *Cassia absus* is a promising and convincing option for the treatment of inflammation in future clinical practices. Further studies can be carried out to find the mechanisms and active constituents involved that are actually responsible for anti-arthritic and anti-inflammatory effect.

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