

Phytochemical, Anti-inflammatory and *in vitro* anticancer activities of *Caesalpinia bonduc* stem bark

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ABSTRACT

Caesalpinia bonduc possess anti-inflammatory, anthelmintic, digestive, stomachic properties. The present study investigated anti-inflammatory and *in vitro* anticancer studies of stem bark of *C.bonduc*. The *in vitro* anti-inflammatory study of different extracts were done by Protein denaturation method. The total ethanolic extract of stem bark of *C.bonduc* was investigated for *in vivo* anti-inflammatory activity (carrageenan induced rat paw oedema) at the doses 200 and 400mg/kg body weight in male Wister albino rats. The *in vitro* cytotoxicity study was done by Trypan blue dye exclusion technique in Daltons Ascites Lymphoma (DLA) cells at 200, 100, 50, 20, 10 µg/ml concentrations. Estimation studies by Folin Cio-calteau method and Aluminium chloride colorimetric method showed that phenolics and flavonoids are abundant in the stem bark. The *in vitro* and *in vivo* anti-inflammatory studies shows that TEE exhibits more anti-inflammatory effect which increases in a dose dependent manner. TEE exhibits 100% cytotoxicity even at 100 µg/ml concentrations. The present study revealed that presence high quantities of phenolics and flavonoids in the stem bark may be responsible for its anti-inflammatory anticancer properties.

KEYWORDS: *Caesalpinia bonduc*, Anti-inflammatory activity, Protein denaturation, Trypan blue dye exclusion, Folin Cio-calteau, Aluminium chloride colorimetry.

INTRODUCTION

Medicinal plants constitute the main source of new pharmaceuticals and healthcare products. The Traditional system of medicines such as Ayurveda, Unani, Homeopathy and Siddha lean heavily on natural products (1).

Cancer is one of the thrust area for which effective drugs at affordable prices are not available as yet probably due to lack in understanding the cancer pathophysiology. Cancer is one of the major causes of death in developed nations. For such a dreadful disease, anticancer drugs have been developed from a variety of sources ranging from natural products (plants and microbes) to synthetic molecules (2). The widely used drugs that are cancer chemotherapeutic agents suffer from drawback of high toxicity such as bone marrow suppression, alopecia, nausea & vomiting and are not within the reach of common man. Therefore the challenging task at this moment is to identify the quick and novel methods than can identify and develop molecules, which can be of therapeutic value in human cancers. This urgently necessitates screening of a large number of compounds. For this purpose both, the *in vitro* and *in vivo* models are employed for systematic screening of an anticancer drug (3).

Cell viability may be judged by morphological changes or by changes in membrane permeability and/or physiological state inferred from the exclusion of certain dyes or the uptake and retention of others. Any compound that is cytotoxic to cells inhibits the cell proliferation and kills the cells. Trypan blue is a dye, which is capable of penetrating the dead cells; therefore, the dead cells take up the blue stain whereas the viable cells do not (4).

Inflammation is a universal host defense process involving a complex network of cell-cell, cell-mediator, and tissue interactions. It occurs in response to a variety of stimuli viz. physical, chemical, traumatic, antigen challenge and infectious agents (3). Synthetic drugs both steroidal and nonsteroidal being used for acute and chronic inflammation cause a number of side effects. Also they are not able to cure inflammation and arthritis completely. Hence, the traditional medicine practitioners and scientists are turning towards medicinal plants for curing these ailments (1).

Inflammation as a risk factor for most cancers Lung cancer, Gastric cancer, Pancreatic cancer, Melanoma, Hepatocellular carcinoma. Inflammatory mediators in cancer are TNF- α , interleukins (IL-1, IL-6, IL-8, and IL-18), COX-2, 5-LOX, and NF-kB (5).

Flavonoids possess several biological activities, including antimutagenic and anticancer properties (6). Polyphenols were found to be strong topoisomerase inhibitors, similar to some chemotherapeutic anticancer drugs including Etoposide and Doxorubicin (7).

Caesalpinia bonduc is a large straggling, very thorny shrub distributed widely in the tropics and subtropics; it is also found in the deltaic regions of western, eastern and southern India (8). This plant is reported to have multiple therapeutic properties. The seeds are anti-inflammatory, antipyretic and analgesic, leaves are Antioxidant, hypoglycemic, antimicrobial, cytotoxic and root bark is emmenagogue, febrifuge, expectorant, anthelmintic and stomachic (8,9,10).

Methanol extract of *Caesalpinia bonducella* leaves have been reported to have antitumor activity against Ehrlich ascites carcinoma (EAC)-bearing Swiss albino mice (11). Previous bioactivity studies have been revealed that ethanolic extract of *Caesalpinia bonducella* seed kernel possesses potent antipyretic and analgesic activities (12).

Many compounds have isolated from *C. bonduc* like caesalpinolide-C, caesalpinolide-D, caesalpinolide-E (13), neocaesalpin C and D (14), neocaesalpin P, neocaesalpin H, cordylane A, caesalpinin B, bonducellpin E, caesalpinolide A, 17-methylvouacapane-8(14)-9(11)-diene (15)

The anticancer activity of stem bark has not yet been explored scientifically. In the present study, we examined the phytochemical, anti-inflammatory and *in vitro* anticancer activities of *C. bonduc* stem bark.

MATERIALS AND METHODS

Plant material and preparation of extract- *C. bonduc* stem bark used in the present study were collected from Athani, Kerala in December 2010 and authenticated by the botanist, Mr. Joby paul, Department of Environmental science, M.G University, Athirampuzha, Kottayam, Kerala. A voucher specimen has been deposited in the herbarium of University College of Pharmacy under number (SES, M.G UTY No. 1505)

Fresh stem bark were collected from the tree and dried at room temperature to remove moisture, and size reduced. The dried powdered stem bark of *C. bonduc* was kept for cold maceration in a 1000 ml round bottom flask for 3 d. Total 475g of dried powder was soaked in 2.1L ethanol (95%) for 3 d for cold maceration. Extraction of the stem bark of *C. bonduc* was done using hot continuous percolation in a Soxhlet apparatus using ethanol (95%) as solvent for 5 h. Then the TEE was fractionated using increasing polarity, petroleum ether, chloroform, ethyl acetate and water in increasing order of their polarity.

Phytochemical evaluation

The three extracts (TEE, ethyl acetate, aqueous) were subjected for qualitative chemical analysis for the identification of various phytoconstituents. Viz. Phenolics, Flavonoids, Carbohydrates, Proteins and aminoacids, Terpenoids, Sterols, and Saponins.

Total phenolic estimation (16, 17, 18)

The total phenolic content in the extracts was found out by Folin cio-calteau method. The absorbance was measured at 765nm. The standard graph was plotted using the various concentration of gallic acid. Then the total phenolic content in the extract was determined and it is expressed as gallic acid equivalent/100g of extract.

Total flavonoid estimation (16, 17)

Aluminium chloride colorimetric method is used for the estimation of total flavonoid content. The absorbance was measured at 510nm. The standard graph was plotted using the various concentration of Quercetin. Flavanoid content is expressed in equivalence of quercetin/100g of extract.

Experimental Animals: Male Wister albino rats (about weight of 150-250g) were used for the present study. They were maintained under standard environmental conditions.

Acute toxicity study (19): Acute toxicity studies of the drug is conducted according to OECD guidelines 423. The test is administered in a single dose of 2000 mg/kg body weight by gavage using a stomach tube or a suitable intubation cannula. Animals were observed for toxicity and mortality for 14 d.

Anti-inflammatory activity:

Protein denaturation method (20, 21):

1. The Test solution (0.5ml) consist of 0.45ml of Bovine serum albumin (5%W/V aqueous solution) and 0.05ml of test solution (250 µg /ml).
2. Test control solution (0.5ml) consist of 0.45ml of bovine serum albumin (5%W/V aqueous solution) and 0.05ml of distilled water.
3. Product control (0.5ml) consists of 0.45ml of distilled water and 0.05 ml of test solution (250 µg /ml).
4. Standard solution (0.5ml) consists of 0.45ml of Bovine serum albumin (5%w/v aqueous solution) and 0.05ml of Diclofenac sodium (250 µg /ml).

All the above solutions were adjusted to pH 6.3 using 1N HCl. The samples were incubated at 37°C for 20 minutes and the temperature was increased to keep the samples at 57°C for 3 minutes. After cooling, add 2.5 ml of phosphate buffer to the above solutions. The absorbance was measured using Shimadzu 1800 UV-Visible spectrophotometer at 416 nm.

The percentage inhibition of protein denaturation can be calculated as,

$$\text{Percentage inhibition} = [100 - (\text{optical density of test solution} - \text{optical density of product control}) \div (\text{optical density of test control})] \times 100.$$

The control represents 100% protein denaturation. The results were compared with Diclofenac sodium (250 µg/ml).

Carrageenan induced rat paw oedema method (22, 23, 24, 25)

Rats were divided into four groups containing six animals in each group, wherein group I served as control and received isosaline only whereas group II and group III served as test drug dose TEE-2 (200 mg/kg p.o) and TEE-4 (400 mg/kg p.o) and group IV served as standard and received Indomethacin (10 mg/kg body weight) respectively. The extracts were administered in the form of suspension in saline through oral route. Male Wistar albino rats were induced paw oedema by injecting 0.1 ml of 1% carrageenan in isosaline into the sub plantar tissues of left hind paw.

The paw volume was measured at 1, 2, 3, 4 h after carrageenan administration by mercury displacement method using plethysmograph, immediately before and after 3 hr of injection. The change in the paw volume was observed and it is recorded.

Statistical analysis

The values are mean ± SEM of 6 animals in each group. Statistical differences were evaluated using one-way ANOVA followed by Dunnett's test. Results were considered to be statistically significant at $p < 0.001$.

Trypan blue dye exclusion method (26, 27)

Experimental design-10 mg of the sample is dissolved in 1 ml DMSO. Preparation of higher concentration was done by mixing PBS with above prepared sample solution so as to obtain 200, 100, 50 µg/ml solutions. From these, lower concentrations such as 20, 10, 5 µg/ml solutions were prepared.

The tumour cells (DLA cells) were aspirated from the peritoneal cavity of tumour bearing mice were washed thrice with normal saline and checked for viability using Trypan blue exclusion method. The cell suspension (1×10^6 cells in 0.1 ml) was added to tubes containing various concentrations of the test compounds and the volume was made up to 1 ml using phosphate buffered saline (PBS). Control tube contained only cell suspension. These assay mixtures were incubated for 3 hr at 37°C and percent of dead cells were evaluated by Trypan blue exclusion method. The cells were counted using a haemocytometer.

Percentage of cytotoxicity was calculated by the following formula:

$$\% \text{ dead cells} = (\text{no. of dead cells} / \text{sum of dead cells and living cells}) \times 100.$$

RESULTS AND DISCUSSION

Inflammation has long been known as a localized protective reaction of tissue to irritation, injury, or infection, characterized by pain, redness, swelling, and sometimes loss of function. But there has been a new realization about its role in a wide variety of diseases, including cancer. Chronic inflammation has been linked to various steps involved in tumorigenesis, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis⁵. Inflammation is a risk factor for cancers like Lung cancer, Gastric cancer, Hepatocellular carcinoma, Pancreatic cancer (5).

Phytochemical studies

Numerous studies have been conducted to prove flavonoids efficacy as antimycotic, antibacterial, antiviral, anti-inflammatory, antioxidant, immune modulator, enzyme inhibitor, mutagenic and toxic agents (28). Flavonoids were found to be strong topoisomerase inhibitors and induce DNA mutations in the MLL gene, which are common findings in neonatal acute leukemia (29,30). The DNA changes were increased by treatment with flavonoids in cultured blood stem cells (31). Plant phenolics possess various biological properties like antioxidant, anti-inflammatory and estrogenic activities (32). Phenolic compounds in herbs act as antioxidants due to their redox properties, allowing them to act as reducing agents, hydrogen donors, free radical quenchers and metal chelators (33). The estimation studies shows that phenolics and flavonoids more in EAE and TEE. Comparison of phenolics and flavonoids showed that phenolics are abundant in the stem bark than flavonoids.

Phenolic and flavonoid content of different extracts were compared in figure 1.

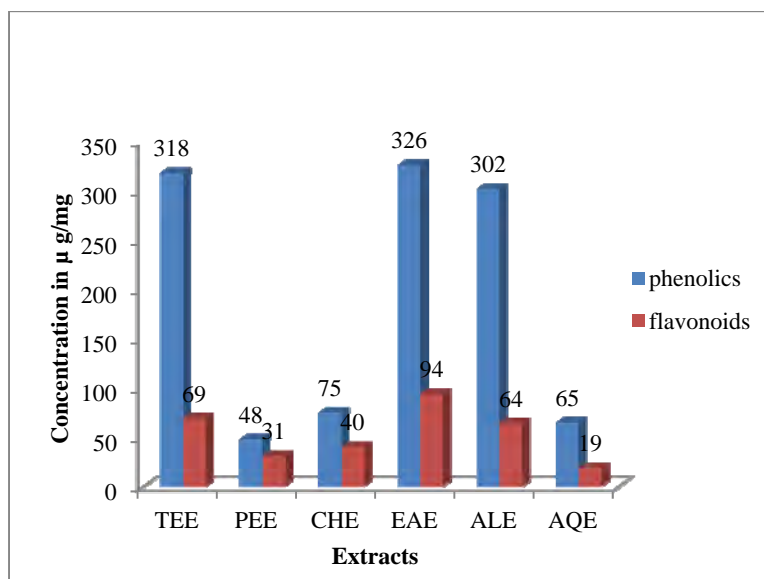


Fig I: Comparison of total phenolic and flavonoid content

Where, TEE- total ethanolic extract, PEE- pet ether extract, CHE- chloroform extract, ALE-alcoholic extract, AQE- aqueous extract

Anti-inflammatory study

Protein denaturation method

Denaturation of proteins is a well documented cause of inflammation. Protein denaturation has been considered as an *in vitro* screening method for anti-phlogistic agents by Mizushima & his coworkers (34, 35). In the present study TEE (total ethanolic extract) exhibits maximum activity by inhibiting protein denaturation than other extracts.

Table 4: Effect of *Caesalpinia bonduc* on Protein denaturation

Extract (250µg/ml)	Abs of test control (416nm)	Abs of product control (416nm)	% inhibition
PEE	0.045	0.014	11.43
EAE	0.046	0.022	31.43
TEE	0.038	0.016	37.15
AQE	0.048	0.078	14.29
CHE	0.045	0.016	17.14
ALE	0.055	0.018	5.71

Carrageenan induced rat paw oedema method

Carrageenan is a well known phlogistic agent, which can induce inflammation in animals at very low concentration. The percentage protection of the drug from the oedema is measured as the anti-inflammatory activity. The development of oedema in the paw of the rats after the injection of carrageenan has been described by Vinergar et al as a biphasic event. The initial phase seen at +1 h is attributed to the release of histamine and serotonin. The oedema maintained during the plateau phase is presumed to be due to kinin-like substances (36,37,38) The second accelerating phase of swelling is due to the release of prostaglandin like substances(12). It has been reported that the second phase of oedema is sensitive to both clinically useful steroidal and non-steroidal anti- inflammatory agents (36,39,40). In our study the TEE exerts maximum activity at a dose 400mg/kg. This indicates the specificity of the test drug towards selective anti-inflammatory mediators. The exact mechanism may be revealed after detailed phytochemical analysis.

Table 5: Measure of Paw oedema by using Plethysmograph

Treatment	Dose (mg/kg body weight)	Paw volume in ml			
		1 h	2 h	3 h	4 h
Negative control	Isosaline	0.64±0.002***	0.74±0.002***	0.88±0.003***	0.93±0.002***
TEE (Test-2)	200mg/kg	0.47±0.019***	0.48±0.023***	0.39±0.034***	0.33±0.030***
TEE (Test-4)	400mg/kg	0.44±0.031***	0.45±0.015***	0.35±0.034***	0.29±0.025***
Standard (indomethacin)	10mg/kg	0.37±0.006***	0.32±0.006***	0.23±0.006***	0.14±0.006***

Results are presented as mean± SEM. (n=6), ANOVA followed by Dunnett test. Comparisons were made between Group control Vs std, test-2, test-4 *** P- values: p<0.001.

Anti-inflammatory activity of two different concentrations of total ethanolic extracts were compared in figure 2.

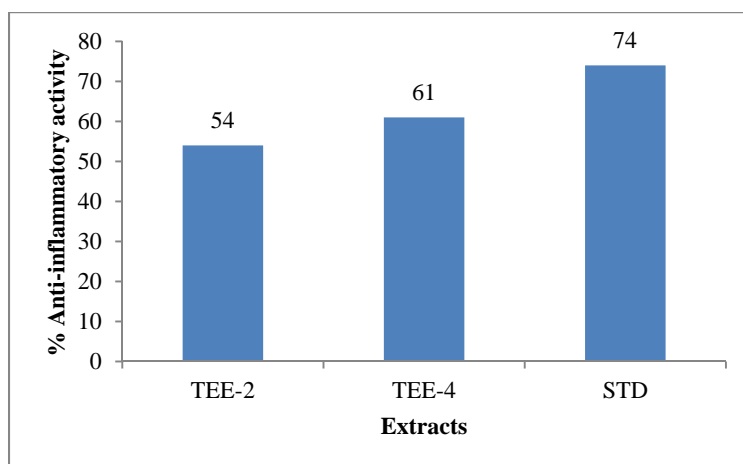


Fig II: Comparison of anti-inflammatory activity

Where, TEE-2 is total ethanolic extract (200mg/Kg); TEE-4 is total ethanolic extract (400mg/Kg) & STD is standard drug indomethacin (10mg/Kg).

Trypan blue dye exclusion method

A cytotoxic compound inhibits the cell proliferation & kills the cells. The dye exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, eosin, or propidium, whereas dead cells do not. The Trypan blue is capable of penetrating the dead cells & stain them. In our study the TEE exhibits 100% cytotoxicity even at 100 µg/ml concentrations compared to other extracts.

Table 6: Cytotoxicity of extracts by Trypan blue dye exclusion method.

Type of cancer cells(1X10 ⁶)	Conc. of extract(µg/ml)	% cytotoxicity			
		TEE	EAE	CHE	PEE
DLA cells	200	100	62	31	35
''	100	100	40	24	26
''	50	75	20	13	18
''	20	70	8	7	7
''	10	50	0	3	2

Literature review shows that homoisoflavonoids (41) (Caesalpinone & 6-o-methylCaesalpinone); Caesaldehyrin J & Pipataline (42) present in this plant have Glutathione S-transferase inhibiting activity an enzyme which causes resistances during treatment of cancer. Cassane-type furanoditerpenoids (43) have anti-inflammatory properties.

CONCLUSION

Presence of high quantities of phenolics and flavonoids were found in this plant may be responsible for the anti-inflammatory and anticancer properties of this plant. Future trends include the bioactivity guided fractionation to isolate the active principles of the plant. The results of the present study further support the traditional use of this plant in Ayurvedic medicine.

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