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**SYNERGISTIC IN VITRO ANTI INFLAMMATORY AND  
ANTIOXIDANT ACTIVITY OF THE METHANOLIC EXTRACT OF  
*SHOREA ROBUSTA* AND *ACORUS CALAMUS***

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**ABSTRACT**

The objective of this study was to assess the *in vitro* anti-inflammatory and the antioxidant properties of *Shorea robusta* and *Acorus calamus*. The anti-inflammatory properties of the methanolic extracts *Shorea robusta* and *Acorus calamus* were assessed through two *in vitro* tests namely hemolysis inhibition and protein denaturation inhibition. The results showed that both plant extracts inhibited protein denaturation in a concentration-dependent manner. At a concentration of 1000g/ml, the value of inhibition of protein denaturation of methanol extract of *Acorus calamus* and *Shorea robusta* was 83.99 µg/ml. When used in combination, the two plant extracts significantly increased the prevention of hemolysis and denaturation of proteins. Antioxidants are essential for reducing oxidative stress, which is a primary cause of many chronic illnesses. In this investigation, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) tests were used to measure the antioxidant activity. Both the extracts scavenged the DPPH free radicals in a concentration-dependent manner. Interestingly, the percentage of the combination of both plant extracts was higher than ascorbic acid. The present study of methanolic extracts *Acorus calamus* and *Shorea robusta* exhibited potent anti-inflammatory and antioxidant properties and can be considered as a synergistic drugs during their use in traditional herbal medicine.

**Keywords:** *Shorea robusta*, *Acorus calamus*, anti-inflammatory, antioxidant activity

**INTRODUCTION:**

For thousands of years, plants have been an essential source of medication. During ancient civilizations, humans have relied mostly on traditional therapies using herbs that have been employed in popular folk medicine [1-2]. According to Ayurvedic principles, a healthy human has a robust immune system capable of resisting the illness [3]. *Acorus calamus* is a beneficial medicinal herb that has been used in a variety of Ayurvedic medicines. *Acorus calamus* enhances human intelligence and self-expression [4]. This plant's rhizome is used to treat a variety of illnesses, including epilepsy, mental disorders, sporadic fevers, kidney, and liver problems [5]. The *Acorus calamus* has an ability to increase and maintain hunger [6]. The dried rhizome of *Acorus calamus* contains yellow fragrant volatile oil with beta asarone as a primary ingredient, as well as a tiny amount of terpenes and alcohols [7-8]. *Acorus calamus* methanolic extract show antifungal action including a variety of inducible defensive mechanisms against a wide range of bacterial and fungal infections [10]. *Shorea robusta* is a plant that has been used in Indian traditional medicine. Many secondary metabolites from terpenoids, flavonoids, polysaccharides, lignin, phenols, and sterols have been found in phytochemical analysis of *Shorea robusta* [7].

*Shorea robusta* (Sal) species is native to the Western Ghats and South Asia, spanning south of the Himalaya from Myanmar in the east to India, Bangladesh, Bhutan, and Nepal [11]. *Shorea robusta* has antioxidant, analgesic, anti-inflammatory, anti-pyretic; wound healing, anti-microbial, anti-obesity, anti-diabetic and immunomodulatory properties [12-13]. Pharmacological effects of the *Shorea robusta* plant are mainly due to phytochemicals. Numerous degenerative human diseases, including cancer, inflammation, and cardiovascular diseases, have been identified as outcomes of damage caused by free radicals. Free radical reactions take place within the human body. These free radicals, specifically reactive oxygen and nitrogen species, are naturally present and important for normal bodily functions. However, an excessive production of these reactive species can occur when there's an imbalance between the body's antioxidant defense system and the formation of free radicals. This condition is known as oxidative stress. These reactive species have the potential to interact with biomolecules, which can result in damage and even death of cells. This process can contribute to the development of chronic illnesses like cancer, as well as diseases affecting the heart and blood vessels. Inflammation is a component of the intricate

biological reaction of vascular tissues to harmful stimuli. It is often associated with pain and encompasses various biological processes, such as heightened vascular permeability, protein denaturation, and changes in cell membranes. The present study deals with the use of *Acorus calamus* rhizome and *Shorea robusta* resin for *in vitro* anti-inflammatory and antioxidant activity.

## MATERIALS AND METHODS:

### Collection of plant material:

*Shorea robusta* plant specimens were gathered from the Bastar district Forest (19.1071oN, 81.9535oE), which is located in the southern region of Chhattisgarh and has an area of 4029.98 km [14]. *Acorus calamus* was obtained from Pathare farms in Ratnagiri, Maharashtra. The plants' resin and rhizome were gathered using a conventional procedure according to the C.C.R.A.S standards [15] for the collection of the rhizome and resin [16]. Botanists identified and validated both plant specimens, which were stored in the Blatter herbarium at St. Xavier's College in Mumbai, India. For this investigation, fresh plant material was used.

The raw rhizomes and resins were properly cleaned. For drying, a known amount of rhizome and resin (30g each) were stored at room temperature. Rhizomes and resins were ground in a mixer grinder after being dried. The powdered components were kept

in sealed polythene bags until they were used.

### Preparation of extracts:

The extract was made by dissolving 30 grams of powdered plant components in 300 ml of methanol. This combination was shaken at 50 rpm on an orbital shaker for four days. After 4 days, the mixture was filtered using No. 1 Whatman filter paper. The filtrate was allowed to evaporate on a rotary evaporator, and extractive values in percentages were determined. The extract was kept in the refrigerator for later use. The stock solution of the extract was prepared by taking 100 milligrams of extract per ml of Dimethyl sulfoxide (DMSO). The stock solution was stored at 4<sup>0</sup>C [17].

### *In vitro* anti-inflammatory activity:

#### Proteinase inhibitory assay-

The proteinase inhibitory assay was performed using methanolic extracts of *Shorea robusta* and *Acorus calamus* according to method modified by Oyedepo and Femurewa [18-19]. The reaction mixture (1.2 ml) contained 0.06 mg trypsin, 0.4 ml of 20 mM Tris-HCl buffer (pH 7.5) and 0.4 ml of methanolic extract's solution (100-1000 µg/ml). The reaction mixture was incubated at 37 °C for 5 min and then 0.4 ml of 0.8% (w/v) casein was added. The reaction mixture was incubated for an additional 20 minutes. Reaction was stopped by addition of Perchloric acid (2 ml, 70%). The turbid solution was centrifuged at

3000 rpm for 20 minutes, and the absorbance of supernatant was measured at 430 nm using buffer as a control. The following equation was used to determine the % inhibition of proteinase inhibitory activity [20].

Percentage inhibition =  $(\text{Abs control} - \text{Abs sample}) \times 100 / \text{Abs control}$

#### **HRBC membrane stabilization assay-**

In this study, an erythrocyte suspension was prepared by using the following method.

- a) Blood Collection: Whole human blood was collected from a healthy subject.
- b) Centrifugation: The collected blood was placed in heparinized centrifuge tubes and then centrifuged at 3000 rpm for 5 minutes.
- c) Washing: After centrifugation, the blood components separated, and the erythrocytes (red blood cells) were isolated. The erythrocytes were washed three times with an equal volume of normal saline solution (0.9% NaCl). The volume of the isolated erythrocytes was measured after washing.
- d) Reconstitution: The washed erythrocytes were reconstituted to form a 10% (v/v) suspension. To achieve this, an isotonic buffer solution with a pH of 7.4 was used. The composition of the buffer solution (per liter) was as follows: NaH<sub>2</sub>PO<sub>4</sub> (0.2 g), Na<sub>2</sub>HPO<sub>4</sub> (1.15 g), and NaCl (9.0 g). A reaction mixture of 3 ml was prepared, consisting of 1 ml of a 10% red blood cell suspension, 1 ml of hyposaline, 1 ml of methanolic plant extract at various

concentrations. All of the test mixtures underwent a 30 minute incubation period at 37°C and a 10 minute centrifugation at 3000 rpm. Using a spectrophotometer set at 560 nm, the supernatant liquid was decanted to determine how much hemoglobin was present.

The percentage hemolysis was calculated using the below mentioned formula:

Percentage membrane stabilization =  $(\text{Abs control} - \text{Abs sample}) \times 100 / \text{Abs control}$

#### ***In vitro* antioxidant activity:**

##### **DPPH free radical scavenging assay-**

0.1 mM solution of DPPH (2, 2-diphenyl-1-picrylhydrazyl) in 90% ethanol was prepared. Subsequently, 3 ml of this DPPH solution was vigorously mixed with 1 ml of various concentrations (ranging from 100 to 1000 g/ml in ethanol) of each extract. Ascorbic acid was used as control. After incubating the mixture for 30 minutes in the dark, the absorbance (A) was measured at 518 nm [21]. The percentage of radical scavenging activity was then calculated using the following equation:

DPPH Scavenged (%) =  $[(A \text{ control} - A \text{ test}) / A \text{ control}] \times 100$

Where A control is the absorbance of the control reaction and A test is the absorbance in the presence of the sample of the plant extracts.

##### **Ferric reducing antioxidant power assay-**

The FRAP (Ferric Reducing Antioxidant Power) assay was conducted to estimate the

antioxidant activity of the *Acorus calamus* and *Shorea robusta* plant extracts [22]. In this assay, a FRAP solution consisting of 2.5 ml of 0.2 M sodium phosphate buffer, 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution, and 20 mM FeCl<sub>3</sub> solution was prepared. To this solution, 0.15 ml of different concentrations of the plant extracts (50, 100, 150, 200, 250, 500, 750, and 1000 µg /ml) was added. The mixture of FRAP solution and plant extracts was then incubated at 37°C for 30 minutes in the dark. After the incubation, the absorbance of the colored product was measured at 593 nm. Percentage of FRAP effect = (Abs control – Abs sample) × 100/Abs control.

## RESULT AND DISCUSSION:

### Determination of *In-vitro* Anti-inflammatory activity:

#### Proteinase inhibitory assay-

The *in vitro* anti-inflammatory studies on the effect of methanol extract of *Acorus calamus* and *Shorea robusta* are summarized in **Table 1**. The experimental data obtained shows that there is a concentration dependent inhibition of protein denaturation by methanol extract of *Acorus calamus* and *Shorea robusta* throughout the concentration range of 100-1000µg/ml. Diclofenac the standard drug also showed inhibition of protein denaturation in a concentration dependent manner. But the effect of the standard drug diclofenac sodium when compared to the

methanol extract of *Acorus calamus* and *Shorea robusta* was found to be almost equivalent when both plant extracts were added together. The value of methanol extract of *Acorus calamus* and *Shorea robusta* was found to be 83.99 µg/ml whereas that of diclofenac was 85.91 µg/ml. Certain plant extracts have been observed to prevent the denaturation of proteins. While the exact way these extracts stabilize cell membranes is not fully understood, however it's suggested that they might hinder the discharge of lysosomal contents from neutrophils at the site of inflammation. These lysosomal contents found within neutrophils encompass enzymes that can kill bacteria and proteinases. When released outside the cells, these enzymes and proteinases can lead to more inflammation and harm to surrounding tissues.

#### HRBC membrane stabilization assay-

Methanol extracts of *Acorus calamus* and *Shorea robusta* were able to inhibit hemolysis in a concentration-dependent manner. Inhibition percentage of hemolysis from these extracts was in the range from 33.19 % to 85.59 %, at the concentrations of 100 µg/ml to 1000 µg/ml, when added together. The order of the inhibition percentage of extracts was *Shorea robusta* > *Acorus calamus* > *Acorus calamus* and *Shorea robusta*. Since human red blood cell membranes are similar to lysosomal membrane components, the prevention of

hypotonicity induced HRBC membrane lysis can be taken as a measure of anti-inflammatory activity of drugs. The results obtained demonstrated that combination of *S. robusta* and *A. calamus* methanol extract can significantly and dose-dependently inhibit HRBC hemolysis [23]. The results are given in Table 2.

#### Determination of *In-vitro* Antioxidant activity:

##### DPPH free radical scavenging assay

It was observed that the DPPH radical scavenging activity was positively correlated to the concentration of the extract [24]. All extracts were able to reduce the stable, purple-colored radical DPPH into yellow-colored DPPH. In this assay, it can be observed that the antioxidant activity of methanol extract of *A. calamus* and *S.*

*robusta* individually showed lower DPPH radical scavenging activity at 1000µg/ml concentration than standard. However when both plant extracts were used together in combination they were effective, indicating higher antioxidant activity than standard drug (Table 3).

##### Ferric reducing antioxidant power assay (FRAP) -

Antioxidant capacity of the methanolic extract of *A. calamus* and *S. robusta* showed the maximum reducing power. In addition, the reducing power of methanolic extract was comparable to that of ascorbic acid. The results obtained demonstrated that combination of *S. robusta* and *A. calamus* methanolic extract can significantly and dose-dependently reduce power. The results are given in Table 4.

Table 1: In-vitro Anti-inflammatory activity of methanol extract of *A. calamus* and *S. robusta* by Proteinase inhibitory assay

Conc.(µg/ml)	100 (µg/ml)	250 (µg/ml)	500 (µg/ml)	750 (µg/ml)	1000 (µg/ml)
Diclofenac (Std)	36.87	49.33	55.88	63.7	85.91
<i>Acorus calamus</i>	30.8	38.78	47.73	58.75	66.74
<i>Shorea robusta</i>	28.72	35.75	40.85	50.44	63.71
<i>S. robusta</i> + <i>A. calamus</i>	34.47	48.37	56.2	61.63	83.99

Table 2: In-vitro Anti-inflammatory activity of methanol extract of *A. calamus* and *S. robusta* by HRBC membrane stabilization assay

Conc.(µg/ml)	100(µg/ml)	250 (µg/ml)	500 (µg/ml)	750 (µg/ml)	1000(µg/ml)
Diclofenac (Standard)	32.71	58.43	68.66	75.84	89.42
<i>Acorus calamus</i>	28.88	40.7	55.56	65.62	75.68
<i>Shorea robusta</i>	23.61	32.87	45.65	56.83	68.97
<i>S. robusta</i> + <i>A. calamus</i>	33.19	52.68	65.46	72.49	85.59

Table 3: In-vitro Antioxidant activity of methanol extract of *A. calamus* and *S. robusta* by DPPH free radical scavenging assay

Conc.(µg/ml)	100 (µg/ml)	250 (µg/ml)	500 (µg/ml)	750 (µg/ml)	1000 (µg/ml)
Ascorbic acid	16.34	34.76	50.55	67.66	95.29
<i>Acorus calamus</i>	9.76	29.5	50.55	65.02	90.02
<i>Shorea robusta</i>	5.81	20.29	38.71	58.45	79.5
<i>S. robusta</i> + <i>A. calamus</i>	12.39	33.45	54.5	74.24	97.92

Table 4: In-vitro Antioxidant activity of methanol extract of *A. calamus* and *S. robusta* by ferric reducing antioxidant power (FRAP) assay

Conc.(µg/ml)	50 (µg/ml)	100 (µg/ml)	150 (µg/ml)	200 (µg/ml)	250 (µg/ml)	500 (µg/ml)	750 (µg/ml)	1000 (µg/ml)
Ascorbic acid	20.73	24.37	27.17	41.17	53.78	68.49	77.87	89.63
<i>Shorea robusta</i>	4.76	15.96	21.43	35.01	48.32	60.92	65.68	78.29
<i>Acorus calamus</i>	13.16	20.31	25.07	38.09	51.68	62.32	72.13	81.09
<i>S. robusta</i> + <i>A. calamus</i>	21.57	27.87	31.37	44.68	60.08	74.09	80.39	91.03

**CONCLUSION:**

This study revealed the *in vitro* anti-inflammatory and antioxidant activity of *A. calamus* and *S. robusta*.

The activities of the two plants were synergistic in action. The characterization of the anti-inflammatory and antioxidant activity of the two plants could lead to more useful source in the traditional plant based medicine.

**CONFLICT OF INTEREST:**

The authors have no conflicts of interest regarding this investigation.

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