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Original Article

In vitro Study on the Antioxidant and Anti-Inflammatory Capabilities of *Borassus flabellifer* Seedcoat

Madhubala Natarajan*, Arul Amutha Elizabeth

Department of Pharmacology, Sree Balaji Medical College and Hospital, Chennai, Tamil Nadu, INDIA.

ABSTRACT

Aims: *Borassus flabellifer*, commonly known as Palmyra palm, Ice apple, Nungu (Tamil) is prevalent in South Asia. This study is focused on determining the anti-oxidant and anti-inflammatory activity of ethanolic extract isolated from the seedcoat of *Borassus flabellifer*. **Materials and Methods:** The ethanolic extract of *Borassus flabellifer* seedcoat was prepared by cold extraction technique using sterile filter paper. The *in vitro* antioxidant activity was assessed using 2,2-Diphenylpicrylhydrazyl [DPPH] assay, Ferric Reducing Antioxidant Power [FRAP] assay, Hydrogen peroxide assay and Catalase assay. The *in vitro* anti-inflammatory activity was determined using the Membrane stabilization method, Protein denaturation method and inhibition of albumin denaturation method. **Results:** DPPH assay revealed stronger anti-oxidant activity than the standard, Butylated Hydroxy Toluene [BHT]. FRAP assay exhibited limited anti-oxidant activity but less than the standard, Ferrous sulfate [$FeSO_4$]. Hydrogen peroxide assay demonstrated good anti-oxidant activity but not more than the standard, Ascorbic acid [Vitamin C]. Catalase assay proved the presence of significant antioxidant activity. There was limited anti-inflammatory activity observed in all three methods employed. Diclofenac was used as standard. **Conclusion:** This study concludes that the seedcoat of *Borassus flabellifer* has strong anti-oxidant activity and limited anti-inflammatory activity. Traditional herbal medicine has been used since ancient times. Hence, it can be used for treating a variety of human ailments since it yields less serious side effects as well as cheaper.

Keywords: Anti-oxidant, Anti-inflammatory, *Borassus flabellifer*, Ethanolic extract, Ice apple, Seed coat.

Correspondence:
Dr. Madhubala Natarajan
Department of Pharmacology, Sree Balaji Medical College and Hospital, No 7, CLC Works Road, Chromepet, Chennai-600044, Tamil Nadu, INDIA.
Email: drmadhubala2013@gmail.com

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INTRODUCTION

Free radicals are inherently unstable molecules that are produced by our bodies as well as taken in from the environment. Although the body naturally produces free radicals, certain daily practices such as smoking, alcohol consumption and regular junk food intake increase the amount of these molecules, which eventually can cause neurological disorders, cancer and cardiovascular disorders.[1]

Reactive oxygen species have positive impacts on immunological system performance and cellular responses at low to moderate concentrations. As concentrations increase, they cause oxidative stress, a potentially lethal process that is crucial in the emergence of degenerative and chronic illnesses like cancer, autoimmune diseases and aging.[2] Antioxidants defend cells from the damaging effects of excess free radicals by neutralizing their effects and eventually help in preventing disease.

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Antioxidant supplements have also been shown to be detrimental in some research studies, especially if taken more than the daily recommended dose.[3] So, there arises a necessity for novel drugs that are herbal in origin as they might possess less serious side effects and can be utilized as they are easily available.

Inflammation is a normal defensive response of our body elicited by harmful stimuli like pathogens, toxic compounds and damaged cells.[4] Redness, swelling, pain sensation, warmth and loss of tissue function are the hallmarks of Inflammation. It involves a complex process involving tissue breakdown, the release of chemical mediators, arteriolar dilatation (Hyperaemia), increased capillary permeability (Swelling), extravasation of fluid, cell migration and repair.[5]

Anti-inflammatory drugs are used for treating several diseases like Rheumatoid arthritis, Diabetes, Psoriatic arthritis, Inflammatory bowel disease, COPD, Bronchial asthma, SLE, Multiple sclerosis.,[6] Use of anti-inflammatory drugs such as Aspirin[7] and NSAIDs[8] poses serious side effects like GI hemorrhage, hepatotoxicity and nephrotoxicity which mandates the search for alternatives from traditional herbal medicine. So, plants and their products can be

used as sources for developing novel drugs with minimal side effects and easy feasibility.

The botanical name is *Borassus flabellifer* (Palmyra palm), belonging to the family Arecaceae. Ice apple, Nungu (Tamil), Taati ningu (Telugu), Tale hannu (Kannada) and Tadgola (Hindi) are some of the common names used in India. Palmyra palm is indigenous to Southeast Asia and they are more prevalent in South India, Malaysia, Cambodia and Sri Lanka. The health-promoting perspective of several parts of the palmyra palm is attributed to its rich phytochemical components like Tannins, Flavonoids and Saponins.[9] There are works of literature portraying that different part of *Borassus flabellifer* possess different medicinal properties. The roots and male flowers were reported to possess anti-inflammatory properties.[10] The fruit of palmyra palm was observed to have antioxidant potential.[11]

Hence, this present study is undertaken to provide more concrete evidence on the antioxidant and anti-inflammatory activity of *Borassus Flabellifer* seedcoat which is usually discarded (Figure 1a, 1b).

MATERIALS AND METHODS

Plant material collection

The plant material used for this study is the seedcoat found within the fibrous fruit of *Borassus flabellifer*. In the month of May, the seeds of the same were collected from Madurai, South Tamil Nadu. Using a sterile knife, the washed seeds were peeled off and then dried in the shade for a week (Figure 1c). They were smooth-grounded and kept in air-sealed jars at room temperature for further use.

Preparation of Plant Extract

Cold maceration extraction technique[12] was employed for extracting the prepared seedcoat of *Borassus flabellifer* using ethanol as solvent. With 100 mL of ethanol, the grounded seedcoat powder was made to stand for 72 hr. The diluted extract after filtering was incubated at room temperature to concentrate the extract by evaporation. The eventual extract was collected in sterile jars and maintained at 4°C for later use.

Evaluation of Antioxidant Property

The antioxidant activity of the *Borassus flabellifer* seedcoat was evaluated using the following different methods.

DPPH [2,2′-diphenyl-1-picrylhydrazyl radical] Radical scavenging assay

The free radical scavenging ability of *Borassus flabellifer* seedcoat extract was assessed using DPPH assay following the technique adopted by Tailor *et al.*,[13] Using the Dilution method, different concentrations of *Borassus flabellifer* seedcoat extract were prepared. The seedcoat extract in different concentrations was made to react with the DPPH solution to determine the percentage of antioxidant activity. The mixture was mixed sufficiently and it was let to stand at 28°C for 30 min. Later, a spectrophotometer was used to determine the absorbance at 517 nm. The standard, BHT [Butylated Hydroxy Toluene] was used to compare the potential of seedcoat extract's antioxidant capacity. The scavenging activity percentage (%) was found using the formula below,

$$\text{Percent inhibition (\%)} = [X-Y]/X \times 100\%$$

X-Absorbance of control,

Y-Absorbance of extract/Standard.

Figure 1a: Fruit and Seeds of *Borassus flabellifer*.

Figure 1b: *Borassus flabellifer* without/with seedcoat.

Figure 1c: Shade-dried seedcoat peels of *Borassus flabellifer*.

Ferric Reducing Antioxidant Power [FRAP] Assay

The antioxidant potential of *Borassus flabellifer* seedcoat extract was observed employing the method followed by Gohari *et al*.,[14]

Reagents used

By integrating 1, 2 and 3 in a 10:1:1 ratio, the FRAP reagent was freshly made and maintained warm.

Acetate buffer: 300 mM, pH 3.6.

TPTZ(2,4,6-tri(2-pyridyl)-s-triazine), 10 mM in 40 mM HCl.

Ferric chloride (FeCl3.6H2O), 20 mM.

Ferrous sulfate ($FeSO_4.7H_2O$), 1 mM.

Procedure

The fundamental process entails reducing the ferric Tripyridyl Triazine (Fe III TPTZ) complex to ferrous form at low pH, which can be observed by noting the shift in absorption at 593 nm. Different concentrations of the seedcoat extract were portioned, diluted to 1 mL along with distilled water, then combined with 3 mL of the freshly prepared FRAP reagent. Later, the mixture was made to incubate for 30 min at 37ºC and the absorbance was measured at 593 nm in a spectrophotometer. The standard used was Ferrous sulfate [$FeSO_4$]. The final output was expressed as the concentration of antioxidants having a ferric-reducing ability equivalent to that of 1 mmol/L $FeSO_4$.[15] The calibration curve was plotted employing different concentrations of ferrous sulfate as generated for the seedcoat extract.

Hydrogen peroxide [H_2O_2] assay

The Hydrogen peroxide assay was employed to assess the antioxidant capacity of *Borassus flabellifer* seedcoat extract following the technique described by Rangasamy *et al.*,[16]

Reagents used

H_2O_2, 50 mM

FOX reagent [9 mL of solution A+1 mL of solution B].

Solution A-4.4 mM BHT in methanol.

Solution B-1mM Xylenol orange.

2.56 mM Ammonium ferrous sulfate in 0.25 M H_2SO_4.

Procedure

A combination of seedcoat extract with different concentrations and an equivalent volume of 50 mM H_2O_2 was combined and incubated at 28ºC for about 30 min. Following incubation, 90 mL of the extract-H_2O_2 solution was mixed with 10 mL of methanol and 900 mL of FOX [Ferrous Oxidation-Xylenol Orange] reagent. After vortexing, the final mixture was kept at 37ºC for 30 min. The absorbance of the complex was quantified at 560 nm.

$$\text{Percent Inhibition (\%)} = [X-Y]/X \times 100\%$$

X-Absorbance of control,

Y-Absorbance of extract/Standard.

Catalase assay

The antioxidant potential of *Borassus flabellifer* seedcoat was analyzed using the technique employed by Sinha *et al.*,[17]

Reagents

Potassium dichromate

0.2 M H_2O_2

Phosphate buffer 0.01M (pH 7.8).

Procedure

About 50 mL of 5% potassium dichromate aqueous solution in distilled water was prepared and 150 mL of glacial acetic vinegar was instilled gradually into the dichromate solution. The assay mixture contained 1.5 mL of phosphate buffer, 1 mL of hydrogen peroxide and 0.1 mL of seedcoat extract. Then, 1 mL of prepared dichromate-acetic acid reagent was gradually instilled and the mixture was made to sit for about 10 min in a boiling water bath. Using a spectrophotometer, the absorbance was noted at 240 nm after cooling the mixture.

Evaluation of Anti-Inflammatory Activity

The *in vitro* anti-inflammatory activity of *Borassus flabellifer* seedcoat was evaluated using the following different methods.

Membrane stabilisation method

The anti-inflammatory activity of *Borassus flabellifer* seedcoat extract was estimated using the method followed by Dragan *et al.*,[18] with minimal alterations.

About 1 mL of blood was drawn from healthy volunteers who had a negative history of any NSAID intake for at least two weeks before blood extraction. The packed cells were separated by combining the blood collected with an equivalent amount of Alsever solution (0.8% sodium citrate, 2% dextrose, 0.42% NaCl, 0.05% citric acid and 100 mL of distilled water) and centrifuged at 3000 rpm. These cells were washed with an isosaline solution (NaCl 0.85%). Later, centrifuged at 3000 rpm for 5 min. About 1 mL of red blood cells and 9 mL of isosaline were added to obtain the HRBC suspension. Test tubes were filled with different concentrations of seedcoat extract, which were then diluted with distilled water to make up to 1 mL. A total of 0.5 mL of HRBC suspension, 2 mL of hyposaline (0.36% NaCl) and 1 mL of phosphate buffer were added to each test tube respectively. The mixture was again centrifuged at 3000 rpm for 20 min after being incubated at 37ºC for 30 min. Using a spectrophotometer at 560 nm, the supernatant layer was decanted to determine the amount of hemoglobin present. Diclofenac (1 mg/mL) was used as standard and a control was prepared without seedcoat extract.

The percentage (%) of HRBC protection was determined using the following formula,

$$\text{Protection percentage (\%)} = 100-(X/Y)$$

X-Absorbance of the seedcoat extract,

Y-Absorbance of the control.

Protein denaturation method

The anti-inflammatory activity of *Borassus flabellifer* seedcoat extract was determined using the concept followed by Padmanabhan *et al.*,[5] and Dharmadeva *et al.*,[18] with slight modifications. Various concentrations of seedcoat extract were

taken in the test tubes and diluted to make 1 mL using phosphate buffer saline (pH 6.4) followed by 4 mL of egg albumin and the test tubes were incubated for 15 min at 37ºC. The process of denaturation was induced by placing the reaction mixture at 70ºC in a boiling water bath for 10 min. The reaction mixture was left to cool down at 28ºC for 15 min. The absorbance of the reaction mixture was analyzed at 660 nm. Diclofenac (1 mg/mL) was used as standard and control was prepared without seedcoat extract. The percent inhibition of protein denaturation was estimated on a percentage basis considering the control using the following formula,

Inhibition (%) =(Y-X /Y)x100

X-Absorbance of the seedcoat extract,

Y-Absorbance of the control.

Albumin denaturation method

The *in vitro* anti-inflammatory activity of *Borassus flabellifer* seedcoat extract was analyzed using the concept followed by Dharmadeva *et al.*,[19] and Leelaprakash *et al.*,[20] with minor modifications. Various concentrations of seedcoat extract were taken in the test tubes and diluted to make 1 mL using phosphate buffer saline (pH 6.4) followed by 0.5 mL of 5% aqueous solution of bovine albumin fraction. Using 0.1N HCl, the pH (6.4) of the reaction mixture was adjusted and incubated at 37ºC for 20 min. Then, the reaction mixture was maintained at 51ºC for about 20 min. The mixture was allowed to cool down at 28ºC for 15 min. The absorbance of the reaction mixture was noted at 660 nm. The standard used was Diclofenac (1 mg/mL) and the control was prepared without seedcoat extract.

The percentage inhibition of albumin denaturation was estimated by applying the following formula,

Inhibition (%)=(Y-X /Y)x100

X-Absorbance of the seedcoat extract,

Y-Absorbance of the control.

RESULTS

Evaluation of Antioxidant Property

The antioxidant potential of *Borassus flabellifer* seedcoat was assessed using four different methods namely DPPH assay, FRAP assay, H_2O_2 assay and Catalase assay.

DPPH assay

DPPH assay is a universally accepted method followed to assess the anti-oxidant activity in *in vitro* studies. In this present study, BHT [Butylated Hydroxy Toluene] was used as the standard to compare with the *Borassus flabellifer* seedcoat extract.

As shown in the graph (Figure 2), the seedcoat extract of *Borassus flabellifer* proved to be a better antioxidant agent than the standard used, BHT. At all the concentrations (100 mg-500 mg) of seedcoat extract, the percentage of inhibition is higher at concentrations ranging from 64.4%-92.1%, whereas the percentage of inhibition of the standard used [BHT] was 81.3% even at the maximum concentration (500 mg) used. As the concentration of the seedcoat extract increased, the antioxidant potential also increased much more significantly than the standard used.

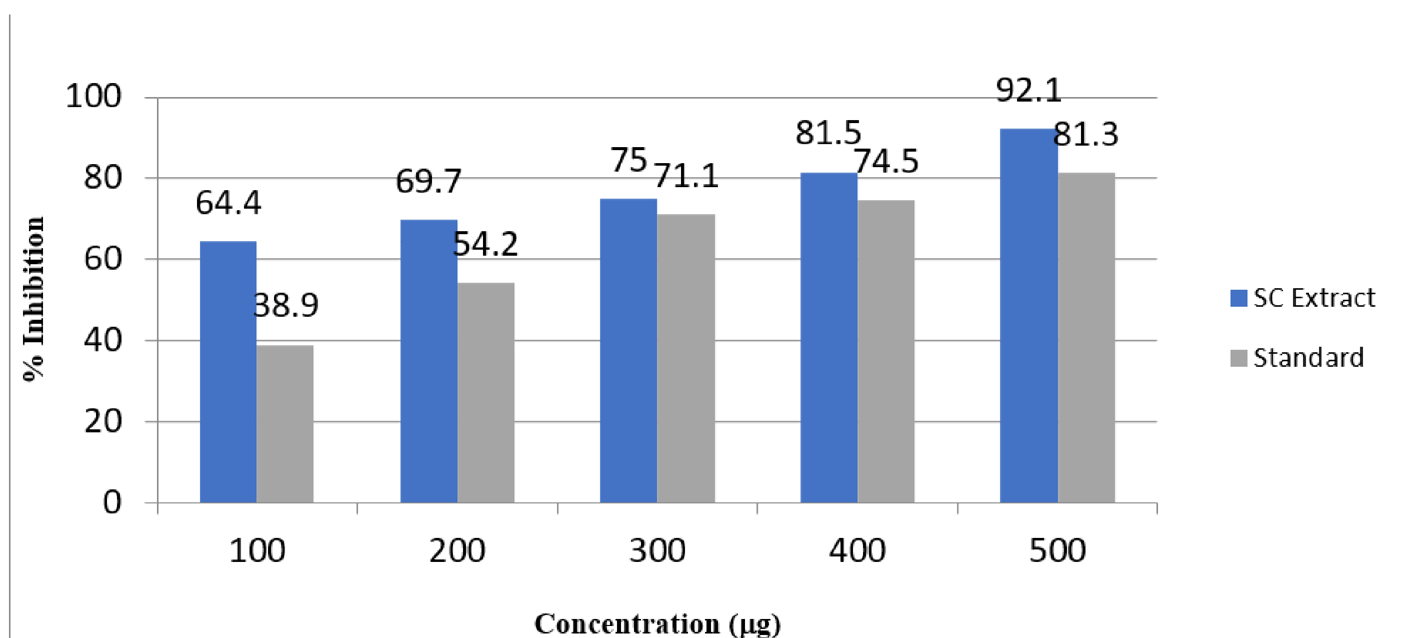

Figure 2: Antioxidant activity of *Borassus flabellifer* seedcoat extract using DPPH assay.

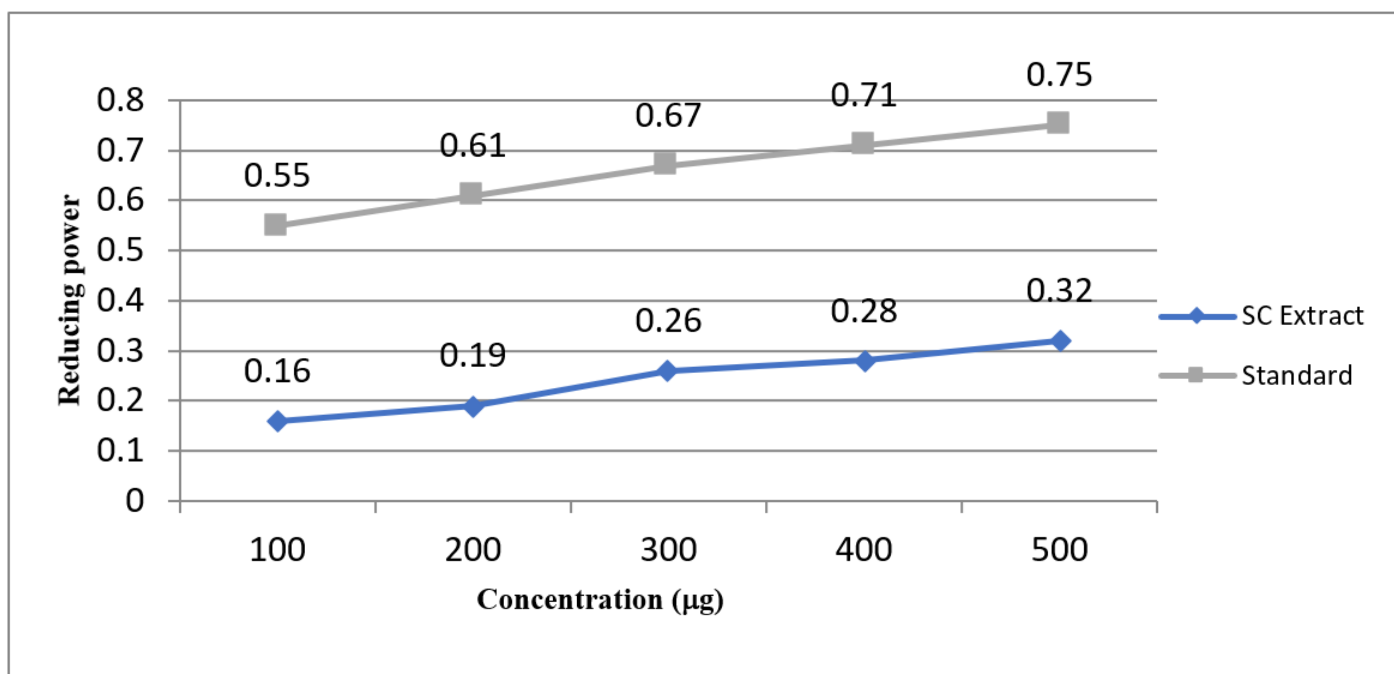

Figure 3: Antioxidant activity of *Borassus flabellifer* seedcoat extract using FRAP assay.

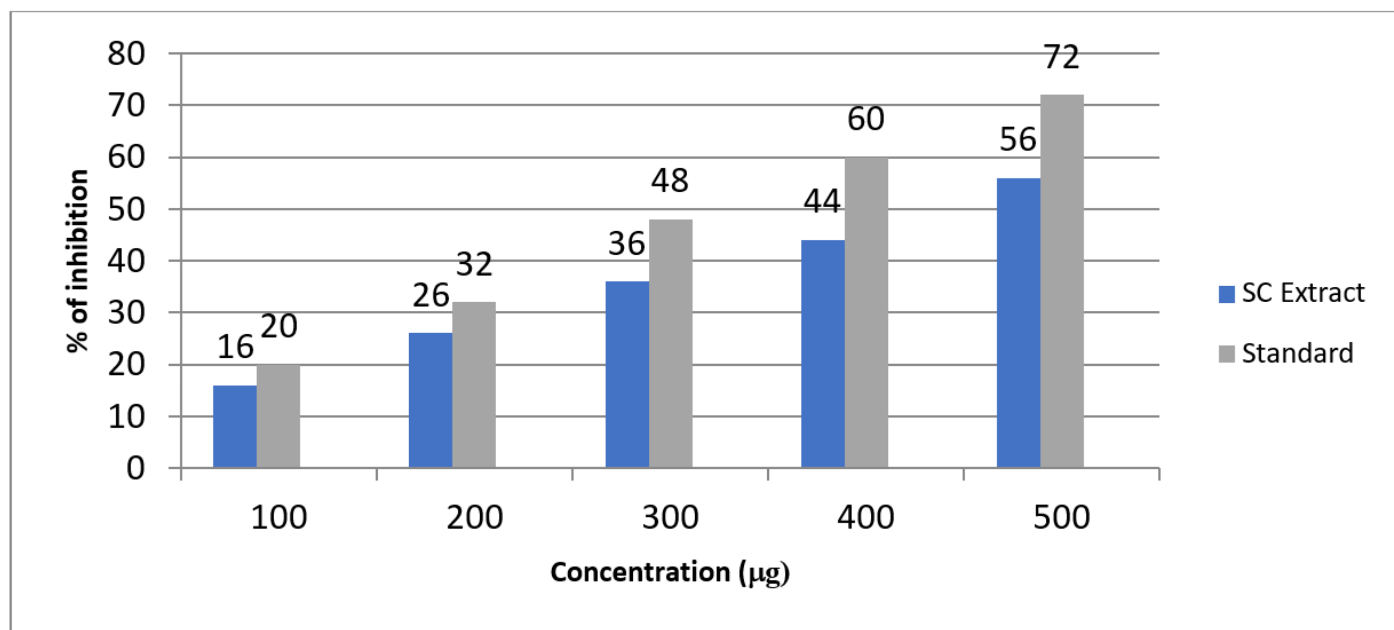

Figure 4: Antioxidant activity of *Borassus flabellifer* seedcoat extract using H_2O_2 assay.

FRAP assay

FRAP assay is based on the compound's reducing power. This assay is unique in that it detects antioxidants directly, as opposed to assessing how well free radicals are inhibited. Ferrous sulfate [$FeSO_4$] was the standard used to compare with the seedcoat extract of *Borassus flabellifer*.

The antioxidant activities were expressed as the concentrations of antioxidants having a ferric-reducing ability equivalent to that of 1 mM of $FeSO_4$. Linearity of the FRAP and the dose–response line is exhibited for both the seedcoat extract and standard ($FeSO_4$).

As depicted in the graph (Figure 3), the reducing power of the *Borassus flabellifer* seedcoat extract increased as the concentration (100 mg-500 mg) increased but was not significant to the standard [$FeSO_4$] used.

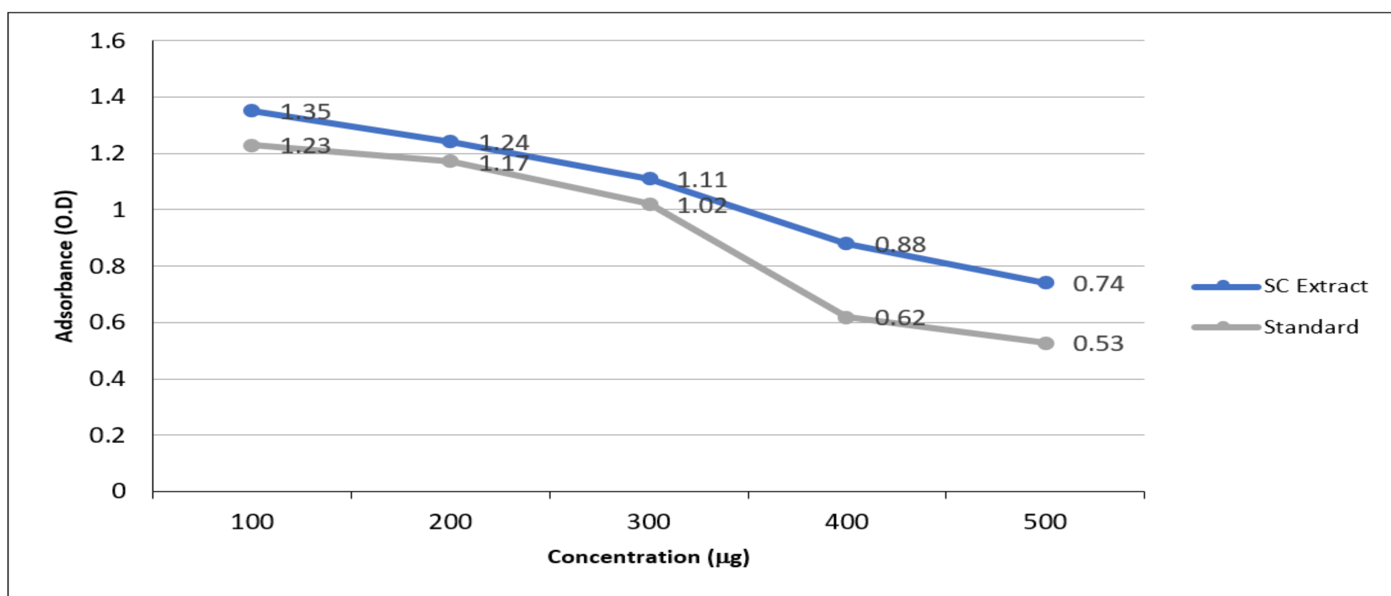

Figure 5: Antioxidant activity of *Borassus flabellifer* seedcoat extract using Catalase assay.

Hydrogen peroxide [H_2O_2] assay

As shown in the graph (Figure 4), the seedcoat extract of *Borassus flabellifer* served as a good scavenger of H_2O_2. As the concentration (100 mg-500 mg) of seedcoat extract increased, the antioxidant potential increased but not more than the standard used, Ascorbic acid. The percentage of inhibition of seedcoat extract was 56% even at the maximum concentration of 500 mg whereas there was 72% inhibition was exhibited by the standard used which was higher than the seedcoat extract.

Catalase assay

Catalase, an antioxidant enzyme, acts as a catalyst for the conversion of hydrogen peroxide [H_2O_2] to oxygen and water. This qualitative assay measures the decrease in absorbance of H_2O_2 at a specific wavelength (240 nm), which is directly related to the catalase activity in the sample. At all the concentrations of seedcoat extract ranging from 100 mg-500 mg, the absorbance was found to be decreasing gradually. This signifies the fact that higher catalase activity was noted as more H_2O_2 decomposed which led to a gradual decrease in absorbance. Linearity is exhibited for both the Seedcoat Extract and standard [BSA] used.

As depicted in the graph (Figure 5), catalase activity is noted and found to be increasing as the concentration of the seedcoat extract increases. Decrease in the absorbance of H_2O_2 corresponds to an increase in catalase activity. As Catalase is an antioxidant enzyme, it's evident that the seedcoat extract of *Borassus flabellifer* possesses antioxidant potential.

Evaluation of Anti-Inflammatory Activity

The *in vitro* anti-inflammatory ability of the seedcoat extract of *Borassus flabellifer* was found using the Membrane stabilization method, Protein denaturation method and Albumin denaturation method. The standard anti-inflammatory used was Diclofenac (1 mg/mL) in all these methods.

Membrane stabilisation method

As shown in the graph (Figure 6), the seedcoat extract exhibited anti-inflammatory activity from the lowest concentration taken but less than the standard, Diclofenac used. The inhibition percentage of seedcoat extract was noted to be in the range of 15.8% -34.9% at the concentrations ranging from 100 mg-500 mg respectively. On the contrary, the standard [Diclofenac] used was found to exhibit a maximum of 65.3% at the concentration of 500 mg whereas seedcoat extract exhibited 34.9% only even at the maximum concentration of 500 mg. As the concentration increased, the anti-inflammatory ability of the *Borassus flabellifer* seedcoat extract also increased.

Protein denaturation method

Standard [Diclofenac] used was found to exhibit a maximum of 84.6% at the concentration of 500 mg whereas seedcoat extract exhibited 38.4% only even at the maximum concentration of 500 mg.

As pictured above (Figure 7), the seedcoat extract of *Borassus flabellifer* did not exhibit any anti-inflammatory activity at the lowest concentrations (100 mg, 200 mg) used but started exhibiting activity as the concentration increased though the activity was lesser than the standard Diclofenac used.

Albumin denaturation method

As depicted in the bar diagram (Figure 8), the seedcoat extract of *Borassus flabellifer* exhibited some anti-inflammatory activity (12.5%, 25%) only at the highest concentrations (400 mg, 500 mg) respectively but much less than the standard Diclofenac used.

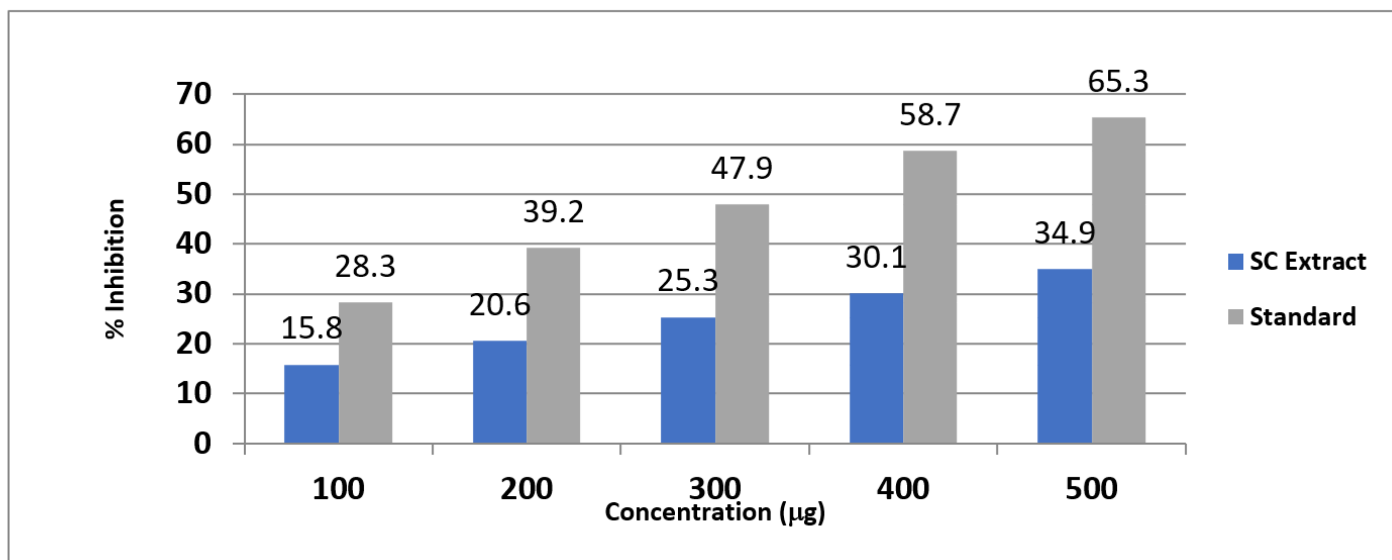

Figure 6: Anti-inflammatory activity of *Borassus flabellifer* seedcoat extract using Membrane stabilization method.

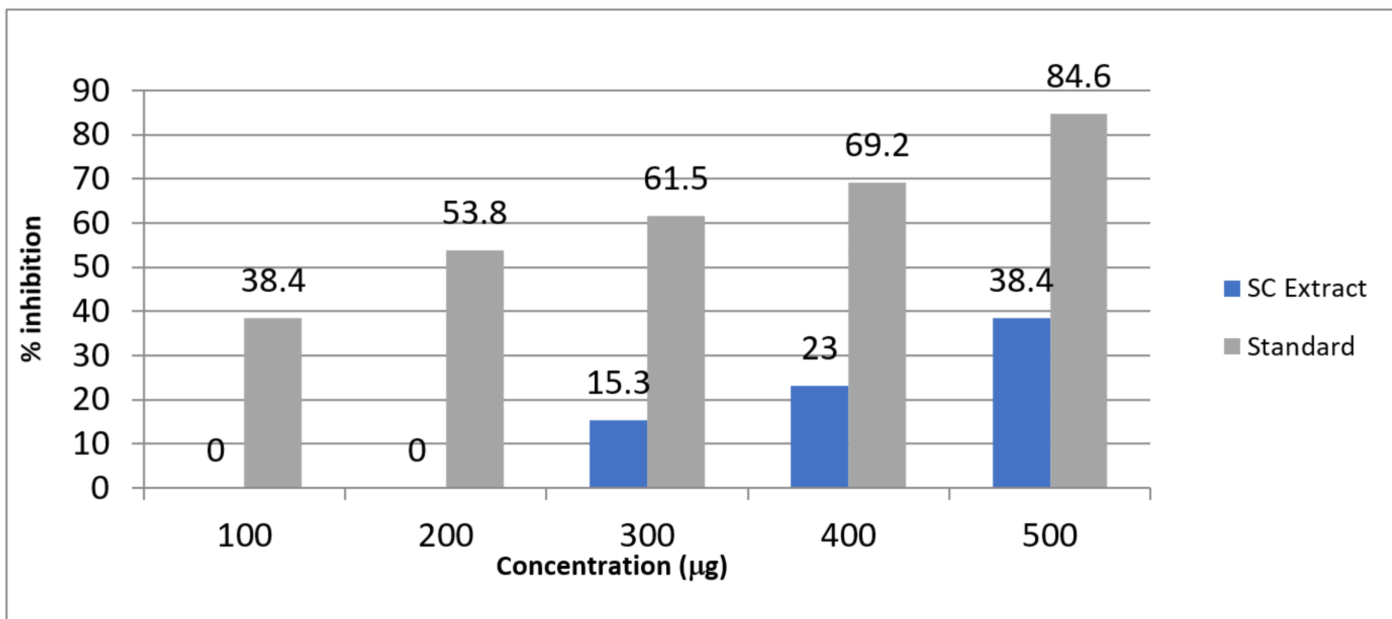

Figure 7: Anti-inflammatory activity of *Borassus flabellifer* seedcoat extract using Protein denaturation method.

DISCUSSION

Despite having a broad spectrum of modern synthetic medicines, researchers are dedicated to the progress of safe and biologically potential plant-derived compounds for the development of novel drugs since the synthetic compounds mostly come with the price of toxicity even though the benefits might outweigh the risks. Pharmacologists are targeting drug development from plant-derived compounds to get the most benefit of it without the risk of their dangerous adverse effects like hepatotoxicity and nephrotoxicity. Mother Nature has solutions for all human ailments but we are yet to explore many.

Antioxidants work through a variety of mechanisms, which explains the diversity in antioxidant activity for the seedcoat extract across various assays. DPPH assay exhibits strong antioxidant activity amongst the other performed assays. FRAP assay evaluates the reducing power of antioxidants, which reflects their electron-donating capacity. The relatively lower activity in this assay might be due to reduced capacity of the seedcoat extract to donate electrons or participate in redox reactions. H_2O_2 assay possesses a comparable amount of antioxidant activity as the standard used. Catalase assay confirms the presence of antioxidant activity and it increases as the concentration increases.

The overall results of this current study suggested that *Borassus flabellifer* seedcoat extract possesses good antioxidant potential using DPPH assay, Hydrogen peroxide assay, Catalase assay and also expressed limited anti-inflammatory activity using

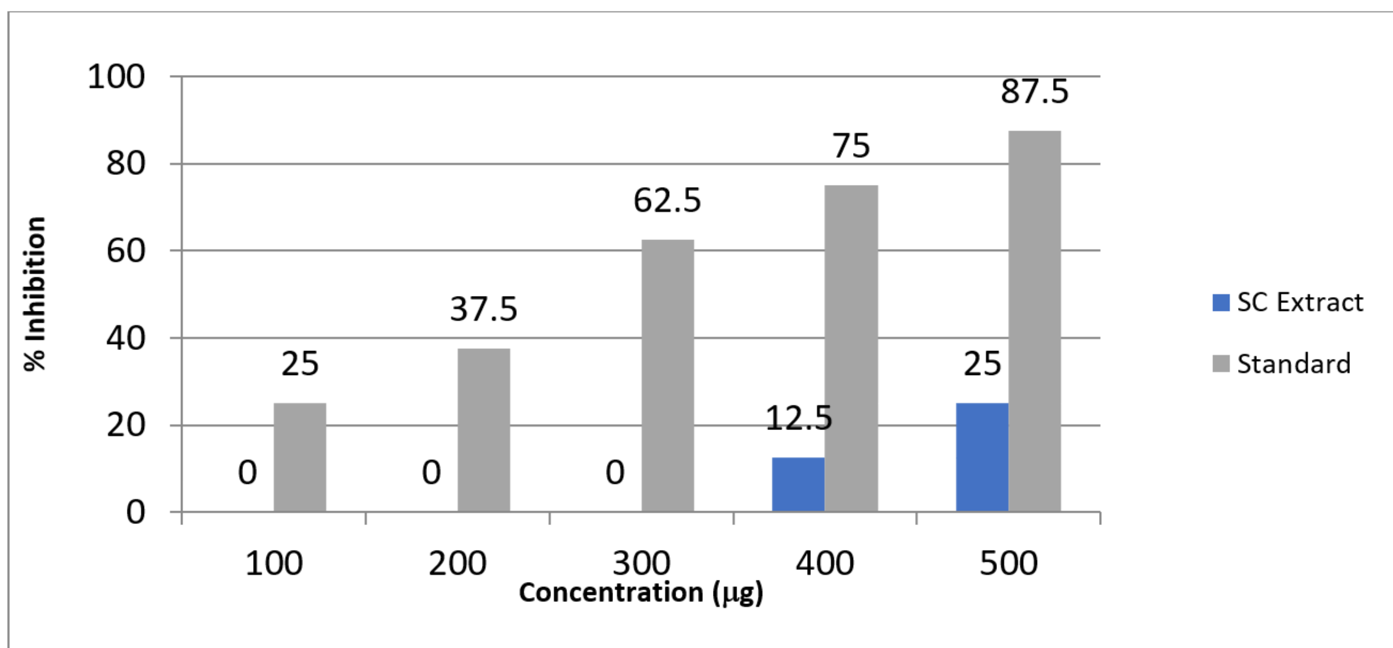

Figure 8: Anti-inflammatory activity of *Borassus flabellifer* seedcoat extract using Albumin denaturation method.

Membrane stabilization method, Protein denaturation method and Albumin denaturation method. Hence, instead of using synthetic antioxidant supplements, as an alternate, antioxidant supplement can be formulated using *Borassus flabellifer* seedcoat and it can also be used as an adjuvant in treating several inflammatory diseases.

A study done by Sahni *et al*., (2014) reported that the *Borassus flabellifer* roots inculcate high antioxidant properties and can be considered as a valuable ingredient in nutraceutical supplements for the promotion of health.[21] Jamkhande *et al*., (2016) revealed that *Borassus flabellifer* methanolic leaf extract possesses a strong scavenging ability which was evaluated using DPPH and Hydrogen peroxide assay. Ascorbic acid was used as standard. They concluded that it can be utilized in agro-food and nutraceutical industries as they are good sources of natural antioxidants.[22]

Kavatagimath *et al*., (2016) reported that the ethanolic flower extracts of *Borassus flabellifer* were observed to have concentration-dependent (10 mg-1000 mg) antioxidant activity using DPPH assay. BHT was used as a standard in this current study. In this present study, the ethanolic seedcoat extract of *Borassus flabellifer* exhibited 81.3% of inhibition but ethanolic flower extract of the same exhibited only 54.2% of inhibition.[23]

A study done by Rani *et al*., (2018) performed research on the aqueous fruit extract of *Borassus flabellifer*,[24] which revealed 67.72% of scavenging activity using DPPH assay at the maximum concentration performed whereas this present study on ethanolic seedcoat extract of *Borassus flabellifer* had 92.1% antioxidant activity using DPPH assay.

Banu *et al*., (2021) reported that the aqueous seed powder extract of *Borassus flabellifer* had significant antioxidant activity using DPPH assay, H_2O_2 assay, NO assay and some anti-inflammatory activity found using albumin denaturation assay.[25]

A study done by Tunit *et al*., (2022) reported that the *Borassus flabellifer* male flower ethanolic extract[26] exhibited superior antioxidant activity than ascorbyl glucoside assessed by DPPH assay and limited anti-inflammatory potential using Inhibition of protein denaturation method where positive control used was Diclofenac as we described in this present study.

CONCLUSION

Our present research on the *Borassus flabellifer* seedcoat ethanolic extract demonstrated significant antioxidant and limited anti-inflammatory activity. Plants have been widely used for healing diverse diseases, since ancient times. The drugs developed from plant sources are relatively safe and possess less serious side effects.

Hence, the seedcoat of *Borassus flabellifer* can be used to develop safe and effective novel antioxidant and anti-inflammatory drugs against several chronic diseases. In the future, further *in vivo* and clinical studies have to be conducted to build a therapeutic strategy to fortify the long-term safe treatment against several human ailments.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

COPD: Chronic Obstructive Pulmonary Disease; **NSAID:** Non-steroidal anti-inflammatory drug; **SLE:** Systemic Lupus Erythematosus; **DPPH:** 2,2′-diphenyl-1-picrylhydrazyl; **BHT:** Butylated Hydroxy Toluene; **FRAP:** Ferric Reducing Antioxidant Power; **TPTZ:** Tri Pyridyl Triazine; **$FeSO_4$:** Ferrous Sulfate; **H_2O_2:** Hydrogen Peroxide; **BSA:** Bovine Serum Albumin; **FOX:** Ferrous Oxidation-Xylenol Orange; **HRBC:** Human Red Blood Cell; **NaCl:** Sodium Chloride.

SUMMARY

Borassus flabellifer, commonly known as Ice apple, Nungu (Tamil) and Tale Hannu (Kannada) is abundantly present in South Asia. The seeds of the fruit are usually eaten leaving the seedcoat behind, wasted. In this study, the seedcoat of *Borassus flabellifer* has been proven to possess a strong anti-oxidant and limited anti-inflammatory effect. As numerous adverse effects are being faced in daily life due to the misuse/overuse of medicines, novel formulations of herbal products would be very valuable to the medical world for treating several chronic ailments.

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