Trop J Nat Prod Res, October 2020; 4(10):812-816 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Membrane Stabilization, Albumin Denaturation, Protease Inhibition, and Antioxidant Activity as Possible Mechanisms for the Anti-Inflammatory Effects of Flavonoid-Rich Extract of *Peltophorum pterocarpum* (DC.) K.Heyne (FRE*PP*) Stem Bark

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ARTICLE INFO

Article history:
Received 29 August 2020
Revised 10 October 2020
Accepted 25 October 2020
Published online 02 November 2020

ABSTRACT

The ever increasing discovery of large number medicinal plants and the screening of their bioactivity to provide data that will help physicians and patients make wise decision before using them. The study evaluated the membrane stabilization, albumin denaturation, protease inhibition and antioxidant activities as possible mechanisms for the anti-inflammatory effects of the stem-bark flavonoid-rich extract of *Peltophorum pterocarpum* (FREPP). The antioxidant activities were assessed using the hydrogen peroxide, nitric oxide (NO) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assays. The anti-inflammatory effect of FREPP was determined using the membrane stabilization model, albumin denaturation, protease inhibitor assay, while the total flavonoid was examined using the aluminium chloride colorimetric test. At 0.1, 0.4, 0.8 mg/mL, FREPP inhibited hypotonicity-induced haemolysis by 61.03, 76.84 and 83.84%, respectively. FREPP was effective in inhibiting albumin denaturation in a concentration-dependent manner with the highest inhibition of 69.89% at 50 µg/mL. Protease activity was significantly ($P < 0.05$) reduced at all concentrations compared to the control. The results showed that FREPP has antioxidant and anti-inflammatory activities.

Keywords: *Peltophorum pterocarpum,* Stem bark, Antioxidant, Anti-inflammatory, Flavonoid.

Introduction

Inflammation is considered as a protective mechanism employed to eliminate harmful stimuli such as pathogens, damaged cells or irritants thereby initiating the healing process in the body.[1] The early phase of inflammation is characterized by the production of reactive oxygen species (ROS) and the recruitment of inflammatory mediators to the site of injury. When the production of ROS outweighs the neutralization mechanism by antioxidants, oxidative stress is bound to occur.[2] Oxidative stress is implicated during inflammatory processes. The prolonged release of inflammatory mediators can provoke oxidative stress leading to chronic inflammatory diseases. Oxidative stress results in gene expression of pro-inflammatory mediators and oxidative inactivation of anti-proteinases. Previous studies have shown that medicinal plants having both antioxidant and anti-inflammatory potentials can mitigate against oxidative stress and improve immune function.[3] One of such plants with both antioxidative and anti-inflammatory properties is *Peltophorum pterocarpum* (DC.) Baker ex Heyne. It is a specie of Peltophorum in the family of Fabaceae. It is also called yellow-flamboyant. The bark has been reported to be used in the treatment of pains and management of sores.[4] The infusion of *P. pterocarpum* stem is used in preparing tooth powder, aqueous extracts of both bark and leaf of *P. pterocarpum* were found to exhibit antioxidant activity against DPPH radical.[6] The presence of flavonoids in these plant parts may be responsible for exhibiting this activity.[7] Due to the limitations posed by existing and mostly synthetic agents, the search for alternative therapies from nature has received much attention recently.[8] Interestingly, safer and more effective drugs have been developed from medicinal plants on the basis of their ethnomedicinal importance.[9]

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Citation: Enechi CO, Okeke SE, Nwankwo EN, Nweze JE, Obilor CP, Okoye CI, Awoh OE. Membrane Stabilization, Albumin Denaturation, Protease Inhibition, and Antioxidant Activity as Possible Mechanisms for the Anti-Inflammatory Effects of Flavonoid-Rich Extract of *Peltophorum pterocarpum* (DC.) K.Heyne (FRE*PP*) Stem Bark. Trop J Nat Prod Res. 2020; 4(10):812-816. doi.org/10.26538/tjnpr/v4i10.25

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

Materials and Methods

Plant materials

Freshly collected stem-bark of *Peltophorum pterocarpum* was gotten within the school premises of the University of Nigeria, Nsukka, Enugu State, Nigeria in March, 24, 20019 at the time this research was carried out. The stem-bark was authenticated by Mr. A. O. Ozioko of the International Centre for Ethnomedicine and Drug production (InterCEED), Nsukka Nigeria. Voucher specimen of the plant with No. INTERCEED/076 was deposited at the InterCEED Herbarium.

Chemicals and reagents:

Chemicals utilized for this study were of the analytical grade and products of May and Baker England, British Drug House (BDH) England, Fluka Germany, Burgoyne, India, Harkin and Williams, England and Sigma Aldrich.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Preparation of plant material
The plant was collected, washed, cut into small sizes and shade-dried. The dried stem-bark was pulverized into the powdered form using a mechanical grinder. A weighed quantity (1000 g) was macerated in 3.5 L absolute ethanol using a maceration flask. It was allowed to stand for 72 h with frequent stirring, and then filtered into a flat-bottomed flask with the aid of a muslin cloth. Whatman No 1 filter paper was used for further filtration. A rotary evaporator was used to concentrate the filtrate at a temperature of 45°C to obtain the crude ethanol extract. The concentrated extract was stored at a temperature of 2-4 ^{0}C in a labeled sterile bottle in the refrigerator.

Preparation of flavonoid-rich extract
This was done using the method previously described by Chu *et al*.[10] Crude extract (3 g) was dissolved in 20 mL of 10% H_2SO_4 in a small flask. This was hydrolysed by heating on a water bath for 30 min at 100^{o}C. The mixture was placed on ice for 15 min, so as to allow the precipitation of the flavonoids aglycones. The cooled solution was filtered and the filtrate (flavonoid aglycone mixture) was dissolved in 50 mL of warm 95% ethanol (50^{o}C). The resulting solution was again filtered into 100 mL volumetric flask which was made up to the mark with 95% ethanol. The filtrate collected was concentrated to dryness using a rotary evaporator. The procedure was repeated several times to obtain enough quantity for the study.

Ethical clearance
Ethical clearance and approval for the conducive experimental conditions and humane use and handling of blood samples from human subject were given by the ethical committee of the Department of Biochemistry, University of Nigeria Nsukka (with approval numberUN/BCH/1872). Informed consent was obtained from all study subjects.

Effect of the flavonoid-rich extract of Peltophorum pterocarpum stem-bark on hypotonicity-induced hemolysis of human red blood cells (HRBC)
The method of Oyedepo and Femurewa,[11] with slight modification was used.

Preparation of Human Red Blood Cells Suspension
Blood sample (3 mL) obtained from a healthy volunteer was placed into an EDTA bottle, centrifuged at 3,000 rpm for 10 min and washed three times with an equal volume of normal saline. The blood volume was measured and reconstituted as a 40% (v/v) suspension with normal saline.

Procedure
The extract and indomethacin used were dissolved in distilled water which served as the hypotonic solution. An aliquot (1 mL) of varying concentrations of the extract (0.1, 0.2, 0.4, 0.6, 0.8 mg/mL) was put into each of a set of five test tubes. Another tube contained 1 mL of 0.6 mg/mL indomethacin. The contents of the respective tubes were made up to 4.9 mL with the distilled water. Two control tubes were used for this test. A control tube contained 4.9 mL of the vehicle while another contained 4.9 mL of normal saline (isotonic solution). HRBCs suspension (0.1 mL) was added to each tube, and after gentle mixing, the mixtures were incubated for 1 h at 37^{o}C. After incubation, the reaction mixture for each tube was centrifuged at 3,000 g for 10 min and the absorbance of the supernatant measured at 540 nm using a spectrophotometer. The tests were carried out in triplicates. The reaction media containing 1 mL varying concentrations of extract or indomethacin made up to 5.0 mL with normal saline, without HRBCs suspension were used as the respective blank for each test. The blank for the control tubes contained normal saline without HRBCs suspension. The percentage inhibition of haemolysis was calculated using the relation below:

% Inhibition of haemolysis = 100 $A = 100\left(1 - \frac{OD2-OD1}{OD3-OD1}\right)$

Where OD1 = Absorbance of control I (isotonic solution), OD2 = Absorbance of test sample, OD3 = Absorbance of control II (hypotonic solution)

Effect of the flavonoid-rich extract of Peltophorum pterocarpum stem bark on albumin denaturation
The method of Mizushin and Kobyashi[12] with minor modifications was used. The reaction mixture consisted of test extract and 1% aqueous solution of bovine albumin fraction; pH of the reaction mixture was adjusted using a small amount of HCl at 37^{o}C. The extract sample was incubated at 37^{o}C for 20 min and then at 51^{o}C for 20 min, after cooling the samples, the turbidity was read spectrophotometrically at 660 nm. The experiment was performed in triplicate. Percentage inhibition of protein denaturation was calculated as follows:

Percentage inhibition (%) = [{Abs control - Abs sample}/Abs control] x 100

Effect of flavonoid-rich extract of Peltophorum pterocarpum stem bark on protease activity
The test was performed according to the method described by 11.13 with minor modification. The reaction mixture (2 mL) was containing 0.06 mg trypsin, 20 Mm Tris HCl buffer (pH 7.4) and 1 mL test sample of different concentrations (0.1-0.5 µg/mL). The mixture was incubated for an additional 20 min. 2 mL of 70% perchloric acid was added to arrest the reaction. The cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210 nm against buffer as blank. The experiment was performed in triplicate. The percentage inhibition of protease inhibitor activity was calculated as:

Percentage inhibition (%) = [{Abs control - Abs sample}/ Abs control] x 100

Hydrogen peroxide scavenging activity of extract of P. pterocarpum stem bark
This activity of the plant extract was evaluated by the method of Ruch *et al*.[14] 80 µL of the aqueous plant extract was added to 150 µL of 4 mm hydrogen peroxide solution prepared in phosphate buffer (0.1 M, pH 7.4). This was incubated for 10 min, and the absorbance was read at 230 nm.

Nitric oxide (NO) scavenging activity of extract of P. pterocarpum stem bark
Nitric oxide scavenging activity was by the method of Garrat,[15] the sample was determined by adding 400 µL of 100 mM sodium nitroprusside, 100 µL of PBS (pH 7.4) and 100 µL of different concentrations of the plant extract. The reaction mixture was incubation at 25^{o}C for 150 min. To 0.5 mL of above solution, 0.5 mL of Griess reagent was added (0.1 mL of sulfanilic acid and 200 µL) naphthyl ethlenediamine dichloride (0.1% w/v)). This was incubated at room temperature for 30 min, and finally absorbance was read at 540 nm. All the reactions were performed in triplicates and their percentage inhibition was calculated by the formula:

Percentage inhibition (%) = [{Abs control - Abs sample}/ Abs control] x 100

Determination of DPPH radical scavenging activity of extract of P. pterocarpum stem bark
DPPH (1, 1- diphnyl-2- picrylhydrazyl) was used to determine the free radical scavenging activity of the extract by method of the Bloiss.[16] DPPH radical was prepared by dissolving 0.0024 g DPPH in 100 mL methanol (purple). To 1.0 mL of different concentrations from 0.1-0.6 µg/mL, 1.0 mL DPPH was added and left in the dark for 20 min, and then the absorbance was read at 517 nm. Ascorbic acid was used as a reference drug and the percentage inhibition of DPPH formation was calculated as:

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Percentage inhibition (%) = [{Abs control - Abs sample}/ Abs control] x 100

Statistical analysis

The data obtained were analysed using a one-way analysis of variance (ANOVA) in Statistical Product and Service Solution (SPSS) version 22.0 and presented as Mean ±SD. Mean values with $p < 0.05$ were accepted as significant.

Results and Discussion

In table 1, the FREPP stem-bark and indomethacin in a concentration-dependent manner significantly ($p < 0.05$) inhibited the lysis of the human erythrocyte membrane which was induced by hypotonic solution when compared to the control. The flavonoid-rich extract inhibited hypotonic solution induced lyses of HRBCs membrane. The preponderance of polyunsaturated fatty acids (PUFAs) within the RBCs membrane makes the cells enormously prone to oxidative harm[17] leading to hemolysis through which hemoglobin and different internal cellular components are released. Injurious agents such as hypotonic solution, heat, etc can lead to lyses of the RBC membrane. The FREPP stem-bark at various concentrations significantly ($p < 0.05$) prevented the lysis of the HRBC membrane. The results indicated the capacity of FREPP to inhibit haemolysis. The inhibition of RBC membrane lyses is a measure of the anti-inflammatory activity.[18,19] The ability of the FREPP to stabilize the erythrocyte membranes implies that it might stabilize lysosomal membranes as well. Stabilizing the lysosomal membrane is very crucial regulating inflammatory responses which help to prevent the release of lysosomal constituents of activated leukocytes (such as bactericidal enzymes and proteases) which upon extracellular release lead to further tissue inflammation and consequently leads to tissue damage.[20-22] The result thus provides evidence for membrane stabilization as a potential mechanism of the anti-inflammatory effect of flavonoid-rich extract of *P. pterocarpum* stem-bark.

Table 2 shows the effect of flavonoid-rich extract of *P. pterocarpum* stem bark on albumin denaturation. The FREPP stem-bark was effective in inhibiting albumin denaturation. Varying concentrations of the plant extract significantly ($P < 0.05$) inhibited the denaturation of albumin when compared to the control group. Diclofenac sodium showed a similar trend. The inhibition by the extract is concentration-dependent with 10 µg/mL having an inhibition of 6.95% and 50 µg/mL with the highest inhibition of 69.89%. Protein denaturation is a process in which proteins lose their structure by application of external stress or compounds and is seen as a marker for inflammatory and arthritic diseases.[23] The ability of flavonoid-rich extract of *P. pterocarpum* stem bark to inhibit protein denaturation also lend credence to its anti-inflammatory activity. The flavonoid-rich extract was effective in inhibiting albumin denaturation.

Table 3 shows the protease activity of flavonoid-rich extract of *Peltophorum pterocarpum* stem bark. The stem bark inhibited significantly ($P < 0.05$) protease activity at different concentrations when compared to the control. The standard drug showed similar results (Table 3). Protease inhibitors play important role for the better interpretation of basic principle of protein interaction. Proteolytic enzymes such as bromelain, papain, pancreatin, trypsin, chymotrypsin and rutin are essential regulators and modulators of inflammatory responses. Neutrophils are known to be a rich source of serine protease and are localized at lysosomes. It has been previously reported that leukocytes protease plays important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by protease inhibitors.[24] Different concentrations of extract significantly ($p < 0.05$) inhibited protease activity at different concentrations. This could be as a result of its high flavonoid content. This assay provides another evidence for its promising anti-inflammatory properties.

Table 4 showed that different concentrations of the plant extract (10, 20, 30, 40 and 60 µg/mL) significantly ($p < 0.05$) inhibited the oxidative stress resulting from hydrogen peroxide (H_2O_2) radicals when compared to the control. As the concentration of the extract (10, 20, 30, 40, 60 µg/mL) increases, the percentage inhibition increased having 23.8, 44.0, 57.8, 73.1, 86.2%, respectively, and ascorbic acid caused a percentage inhibition of 80.0%. H_2O_2 is known for its ability to penetrate into biological membranes. H_2O_2 itself is not very reactive, but its hydroxyl radicals are very toxic to cells. Scavenging of H_2O_2 by the extract may be attributed to the high concentration of flavonoids present in the extract which can donate electrons to H_2O_2, thus neutralizing H_2O_2 to water (H_2O). The results show that the FREPP had potent H_2O_2 scavenging activity which may be due to the flavonoid. The results show an excellent inhibition percentage in a concentration-dependent manner.

Table 5 shows that the plant extract at different concentrations (10, 20, 30, 40, 60 µg/mL) in a concentration-dependent manner inhibited significantly ($p < 0.05$) the oxidative stress caused by DPPH radicals when compared to the control. The free-radical scavenging activity by DPPH is a widely used method to evaluate the free radical scavenging ability in various plant species in short period.[25] The extract showed an excellent scavenging activity compared to ascorbic acid. The results are comparable to other free radical scavenging methods.[26] The flavonoid-rich extract and ascorbic acid neutralized the free radical character of DPPH by transferring either electrons or hydrogen atoms to DPPH,[27] thereby changing the colour from purple to the yellow colored stable diamagnetic molecule diphenylpicrylhydrazine.

Table 1: Inhibition of hypotonicity-induced hemolysis of human RBC cells by flavonoid-rich extract of *P. pterocarpum* stem-bark on hypotonicity-induced hemolysis of HRBC

		OD_{540nm}		Difference in OD values		
Treatment	**Concentration (mg/mL)**	**Hypertonic solution (OD_3)**	**Hypotonic (OD_2)**	**Isotonic (OD_1)**	**(OD_2-OD_1)**	**% inhibition of Haemolysis**
Control	-	0.429 ± 0.002	-	-	-	-
Control	-	-	-	0.100 ± 0.001	-	-
Extract	0.1	-	0.228 ± 0.001^{g}	-	0.128	61.03
	0.2	-	0.197 ± 0.002^{e}	-	0.09	70.52
	0.4	-	0.176 ± 0.001^{d}	-	0.076	76.84
	0.6	-	0.167 ± 0.001^{c}	-	0.067	79.64
	0.8	-	0.153 ± 0.003^{b}	-	0.053	83.84
Indomethacin	0.4	-	0.218 ± 0.001^{f}	-	0.118	64.11

Values are Mean ± Standard Deviation, n = 3, values with superscripts b, c, d, e, f, g are considered significant ($p < 0.05$).

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

The degree of discolouration indicated the scavenging potential of the flavonoid-rich extract of *P. pterocarpum* stem bark in terms of hydrogen donating ability.[28]

Table 6 showed that the plant extract at different concentrations (10, 20, 30, 40, 60 µg/mL) in a concentration-dependent manner inhibited significantly ($p < 0.05$) oxidative stress caused by Nitric oxide (NO) radicals when compared to the control. The percentage inhibition is 20.4, 29.9, 36.2, 48.8, 49.4%, respectively while ascorbic acid caused a percentage inhibition of 50.9%. The flavonoid-rich extract of *P. pterocarpum* exhibited NO scavenging activity leading to the reduction of the nitrite concentration in the assay medium. In the study, the NO scavenging capacity was concentration-dependent with the highest concentration with the most scavenging activity. The ethanol flavonoid-rich extract of *P. pterocarpum* in sodium nitropruside (SNP) solution significantly inhibited ($p < 0.05$) the accumulation of nitrite, a stable oxidation product of NO liberated from SNP in the reaction medium with time compared to ascorbic acid. The toxicity of NO increases when it reacts with superoxide to form the peroxynitrite anion, which is a potential strong oxidant that can decompose to produce OH and NO_2.[29] The flavonoid-rich extract also exerted significant H_2O_2 radical scavenging activity.

Table 2: Effect of flavonoid-rich extract of *P. pterocarpum* stem bark on albumin denaturation

Treatment	Concentration (µg/mL)	O.D (660 nm)	% Inhibition
Control	-	0.518 ± 0.006^{g}	-
Extract	10	0.482 ± 0.001^{f}	6.95
	20	0.359 ± 0.001^{e}	30.70
	30	0.195 ± 0.002^{c}	62.36
	40	0.165 ± 0.002^{b}	68.15
	50	0.156 ± 0.001^{a}	69.89
(Diclofenac Sodium)	100	0.276 ± 0.004^{d}	46.72

Values are Mean ± Standard Deviation, n = 3, values with superscripts a, b,c, d, e, f, g are considered significant ($p < 0.05$).

Table 3: Effect of flavonoid-rich extract of *P. pterocarpum* stem bark on protease activity

Treatment	Concentration (µg/mL)	Absorbance $(O.D)_{210nm}$	% Inhibition
Control	-	0.300 ± 0.001^{g}	-
Extract	10	0.923 ± 0.001^{f}	29.00
	20	0.876 ± 0.001^{e}	32.61
	30	0.777 ± 0.002^{c}	40.15
	40	0.750 ± 0.002^{b}	42.30
	50	0.735 ± 0.003^{a}	43.46
	100	0.808 ± 0.005^{d}	37.85

Values are Mean ± Standard Deviation, n = 3, values with superscripts b, c, d, e, f, g are considered significant ($p < 0.05$).

Conclusion

The results of the investigations show that the plant extract exhibited significant antioxidant and potent anti-inflammatory effects. The rich flavonoid content of the extract may be responsible for these outcomes. The study provides a scientific basis for the ethnomedicinal claims for the use of this plant in curing inflammatory ailments.

Table 4: H_2O_2 scavenging activity of flavonoid-rich extract of *P. pterocarpum*

Treatment	Concentration (µg/mL)	O.D (230 nm)	% Inhibition
Control	-	1.804 ± 0.002^{g}	
Extract 1	10	1.374 ± 0.005^{f}	23.8
Extract 2	20	1.010 ± 0.003^{e}	44.0
Extract 3	30	0.764 ± 0.005^{d}	57.6
Extract 4	40	0.486 ± 0.003^{c}	73.1
Extract 5	60	0.249 ± 0.002^{a}	86.2
(Ascorbic Acid)	100	0.361 ± 0.002^{b}	80.0

Values are Mean ± Standard Deviation, n=3, values with superscripts b, c, d, e, f g are considered significant ($p < 0.05$).

Table 5: DPPH Scavenging activity of flavonoid-rich extract of *P. pterocarpum*

Treatment	Concentration (µg/mL)	O.D (517nm)	% Inhibition
Control	-	0.218 ± 0.003^{e}	
Extract 1	10	0.164 ± 0.004^{d}	24.8
Extract 2	20	0.118 ± 0.002^{c}	45.9
Extract 3	30	0.110 ± 0.002^{b}	49.5
Extract 4	40	0.094 ± 0.002^{a}	56.9
Extract 5	60	0.089 ± 0.003^{a}	59.2
(Ascorbic Acid)	100	0.092 ± 0.005^{a}	57.8

Values are Mean ± Standard Deviation, n=3,
Superscripts b, c, d, considered significant ($p < 0.05$)

Table 6: NO scavenging activity of flavonoid-rich extract of *P. pterocarpum*

Treatment	Concentration (µg/mL)	O.D (540 nm)	% Inhibition
Control	-	1.123 ± 0.004^{g}	
Extract 1	10	0.894 ± 0.003^{f}	20.4
Extract 2	20	0.787 ± 0.002^{e}	29.9
Extract 3	30	0.717 ± 0.003^{d}	36.2
Extract 4	40	0.575 ± 0.003^{c}	48.8
Extract 5	60	0.568 ± 0.003^{b}	49.4
(Gallic Acid)	100	0.551 ± 0.002^{a}	50.9

Values are Mean ± Standard Deviation, n = 3, values with superscripts b, c, d, e, f g are considered significant ($p < 0.05$).

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgements

We the authors wish to extend our gratitude to the academic staff and technologist of the Natural Science Unit, School of General Studies, and those of Biochemistry Department University of Nigeria, Nsukka for their technical assistance during the study.

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