



In vitro xanthine oxidase and albumin denaturation inhibition assay of *Barringtonia racemosa* L. and total phenolic content analysis for potential anti-inflammatory use in gouty arthritis

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ABSTRACT

Aim: This study was conducted to evaluate the *in vitro* anti-inflammatory activities and total phenolic content (TPC) of methanolic extracts of inflorescence axes, endosperms, leaves, and pericarps of *Barringtonia racemosa* L. **Methods:** The anti-inflammatory study was conducted by assessing the potential through xanthine oxidase (XO) and albumin denaturation inhibition assays. Meanwhile, the TPC in the extracts were assessed by Folin-Ciocalteu assay. **Results:** In the XO inhibition assay, the inflorescence axes extract was found to exert the highest inhibition capacity at 0.1% (w/v) with $59.54 \pm 0.001\%$ inhibition followed by leaves ($58.82 \pm 0.001\%$), pericarps ($57.99 \pm 0.003\%$), and endosperms ($57.20 \pm 0.003\%$) extracts. Similarly in the albumin denaturation inhibition assay, the inflorescence axes extract had shown the greatest inhibition capacity with $70.58 \pm 0.004\%$ inhibition followed by endosperms ($66.80 \pm 0.024\%$), leaves ($65.29 \pm 0.006\%$), and pericarps extracts ($43.33 \pm 0.002\%$). Meanwhile, for TPC analysis, leaves extract was found to have the highest phenolic content (53.94 ± 0.000 mg gallic acid equivalent [GAE]/g DW) followed by inflorescence axes (31.54 ± 0.001 mg GAE/g DW), endosperms (22.63 ± 0.001 mg GAE/g DW), and the least was found in pericarps (15.54 ± 0.001 mg GAE/g DW). **Conclusion:** The results indeed verified the *in vitro* anti-inflammatory activities of *B. racemosa* and supported its potential to be used in alleviating gouty arthritis and XO-related diseases.

KEY WORDS: Anti-inflammatory activity, *Barringtonia racemosa*, protein denaturation inhibition, total phenolic content, xanthine oxidase inhibition

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INTRODUCTION

According to the data from the World Health Organization, the value of worldwide annual market for herbal medicinal products approaches US\$60 billion [1]. Modern science has proven that the association of plants in medicinal approach is scientifically sound due to the bioactive compound constituents in plants which later paved the way for further discovery of drug developments from plants.

Barringtonia racemosa (L). which is also known as Putat, “fish poison” or “powder puff” is a type of plant species found to be widely distributed from Eastern Africa and Madagascar to Micronesian and Polynesian Island. Due to such diverse distribution, this species has been very well

associated ethnobotanically in various tribes around the world. Pharmacologically, this species had been scientifically proven to possess various medicinal benefits [2], among which are antibacterial [3-5], anti-mycobacterial [6], and anti-fungal [4]. Apart from being proven to have anti-infective activities, *B. racemosa* also successfully showed promising anti-tumor [7] and anti-arthritic activities [8] with excellent analgesic [5,9,10] and antioxidant properties [11-13].

One of the most common and essential properties of secondary metabolites is anti-inflammatory activity. Inflammation is caused by release of chemicals from tissues and migrating cells [14]. The occurrence of inflammation has been regarded to be associated in a number of disorders and prominently related to the painful condition. Gouty arthritis has been

identified to be the most common inflammatory arthritis in general practice and may significantly impair quality of life due to restricted mobility [15]. This study was carried out to evaluate the potential of *B. racemosa* to be used as a natural remedy for the treatment of inflammatory diseases. Particular focus on inflammatory gouty arthritis was emphasized since xanthine oxidase (XO) inhibitory activity assay was being scrutinized; wherein the XO has been identified as the culprit in gouty arthritis pathogenesis due to the formation of uric acid deposition in gouty patients.

MATERIALS AND METHODS

Sample Preparation and Extraction

The plant materials of *B. racemosa* were collected from Nasuha Herbal Farm, Johor, Malaysia. The determination of botanical term for plant parts used was made by referring to glossary of botanical terms [16] and Prance [17]. Four plant parts were studied which were inflorescence axis, leaf [Figure 1], endosperm, and pericarp part of fruits [Figure 2]. The samples were air dried



Figure 1: The (a) inflorescence axis and (b) leaf of *Barringtonia racemosa*

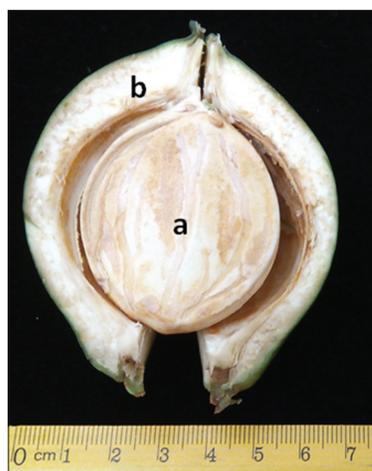


Figure 2: The (a) endosperm and (b) pericarp part of *Barringtonia racemosa* fruit

under the shade at ambient temperature ranging from 28°C to 32°C. The dried plant samples were further crushed into coarse powder using a domestic electric grinder (Philips, Netherlands). The extracts were prepared by soaking 5 g of samples in 200 ml of 70% methanol (Merck, Germany) diluted with 30% deionized water (Arium® pro Sartorius, Germany). The macerated samples were filtered using Whatman filter paper Number 1 (70 mm) (GE Healthcare, UK) placed on Buchner funnel whereby the filtration was assisted by a vacuum pump (GAST, USA). The resulting filtrates were then concentrated under vacuum using rotary evaporator system (Buchi, Switzerland) to yield concentrated extracts of samples. The resulting extracts were kept in glass vials and refrigerated at 4°C until further use.

Anti-inflammatory Activity Assays

XO inhibitory activity of *B. racemosa*

The assay method was carried out according to Azmi *et al.* [18] with some modifications in which the positive standard used in the current study was oxypurinol. The concentrations of samples and standards used in the study were expressed in the form of a percentage (%). The plant extracts were prepared at a concentration of 0.1% each (1.0 mg/ml). The assay mixtures were prepared by adding 300 µl of 50 mM potassium phosphate buffer (pH 7.5), 100 µl sample solutions, 100 µl of freshly prepared XO enzyme solution (0.2 U/ml in phosphate buffer), and 100 µl of deionized water. The mixtures were incubated at 37°C for 15 min. Afterward, 200 µl substrate solution (0.15 mM of xanthine) will be added into the assay mixture and further incubated at 37°C for 30 min. The reaction was stopped by the addition of 200 µl of 0.5 M hydrochloric acid. The XO inhibitory activities were assayed spectrophotometrically at 295 nm (indication of uric acid formation at 25°C) using UV/vis spectrophotometer (Jasco V-630 Spectrophotometer, Japan), and the data were processed by Spectra Manager system. Oxypurinol (001 %) was used as a positive control. The assay mixture without sample extract served as a negative control. All assays were done in triplicate; thus, inhibition percentages are the mean of three observations. The XO inhibitory activities were expressed as the percentage of inhibition of XO, calculated as follows:

$$\% \text{ XO inhibition} = (1 - B/A) \times 100\%$$

Where B is the absorbance reading of the test sample, and A is the absorbance reading without test sample (negative control).

Various concentrations of 0.025%, 0.050%, 0.075%, and 0.100% of oxypurinol and the most optimum extract were evaluated for XO inhibitory activity. The dose-response graph was utilized to generate a linear equation to estimate the concentration at which maximal inhibition (100%) is obtained.

Albumin denaturation inhibitory activity of *B. racemosa*

The assay was carried out by adopting the methods described by Kumari *et al.* [19] with some modifications in which the volume of each component in the reaction mixtures was reduced

by half. The plant extracts and positive standards (ibuprofen and diclofenac) were prepared at a concentration of 0.1% each (1.0 mg/ml). A reaction vessel for each mixture was prepared consisted of 200 μ l of egg albumin, 1400 μ l of phosphate buffered saline, and 1000 μ l of the test extract. Distilled water instead of extracts was used as a negative control. Afterward, the mixtures were incubated at 37°C for 15 min and then heated at 70°C for 5 min. After cooling, their absorbances were measured at 660 nm (Jasco V-630 Spectrophotometer, Japan) and the data were processed by Spectra Manager system. The inhibition percentage of protein denaturation was calculated using the following formula:

$$\% \text{ Denaturation inhibition} = (1 - D/C) \times 100\%$$

Where D is the absorbance reading of the test sample, and C is the absorbance reading without test sample (negative control).

Total Phenolic Content (TPC) Analysis of *B. racemosa*

The TPC analysis using Folin-Ciocalteu method was carried out according to Almey *et al.* [20] with minor modifications in the duration of incubation before absorbance measurement being recorded which was altered to be 1 h (60 min). Gallic acid was used as a positive reference standard. The plant extracts as well as the positive standard were prepared as stock solutions at a concentration of 0.1% each (1.0 mg/ml). Working standards of between 0.01 and 0.05% were prepared for TPC analysis. The reaction vessels were prepared by placing 100 μ L plant extract added with 750 μ L Folin-Ciocalteu reagent in each vessel. The mixture was incubated at room temperature (25°C) for 5 min. Afterward, 750 μ L of 6.0% (w/v) sodium carbonate was added into each reaction vessel and mixed thoroughly. The mixtures were incubated at room temperature (25°C) for 60 min before the absorbance of each sample was read at 765 nm by spectrophotometer (Thermo GENESYS™ 20 Visible Spectrophotometer, USA). The assays were done in triplicate. The standard calibration curve of gallic acid was plotted and used for the determination of TPC in the test samples. A linear equation was generated from the curve to identify related gallic acid concentration by substituting the corresponding absorbance value as “y” values in the equation to find the resulting concentration of gallic acid (mg/ml) as the “x” values. Afterward, the TPC of each plant sample was calculated according to the following formula. The results were expressed as mg GAE/g DW (mg gallic acid equivalent/g dry weight of extract):

$$\text{TPC} = (C) V/M$$

Where, TPC = Total phenolic content (mg GAE/g DW)

C = Concentration of GA from calibration curve linear equation (mg/ml)

V = Volume of the extract solution (ml)

M = Weight of extract used (g).

Statistical Analyses of Results

The results were expressed as mean \pm standard deviation of triplicate readings and statistically analyzed by IBM SPSS

Statistics version 17.0. The data were subjected to one-way analysis of variance. Tukey's honest significant difference (HSD) test ($P < 0.01$) was performed to determine the significance of the difference between means of different plant part extracts.

RESULTS

Anti-inflammatory Activity Assays

XO inhibitory activity of *B. racemosa*

All extracts had shown more than 50% inhibition against XO activities [Table 1]. This signified that the extracts effectively inhibited XO from catalyzing the action of converting xanthine to uric acid at a considerably low concentration of 0.1%. The inflorescence axes extract was found to exert the highest inhibition capacity at 0.1% (v/v) with $59.54 \pm 0.001\%$ inhibitory activity followed by leaves ($58.82 \pm 0.001\%$), pericarps ($57.99 \pm 0.003\%$), and endosperms ($57.20 \pm 0.003\%$) extracts. Meanwhile, the positive control, oxypurinol recorded 68.36% inhibition of XO activities at a relatively similar concentration.

Albumin denaturation inhibitory activity of *B. racemosa*

All extracts had given more than 50% inhibition [Table 1]. The inflorescence axes extract had shown the greatest inhibition capacity with $70.58 \pm 0.004\%$ followed by endosperms ($66.80 \pm 0.024\%$), leaves ($65.29 \pm 0.006\%$), and pericarps extracts ($43.33 \pm 0.002\%$). Meanwhile, the non-steroidal anti-inflammatory drugs (NSAIDs) drugs used which were ibuprofen and diclofenac sodium exhibited relatively higher inhibition capacity of $83.53 \pm 0.003\%$ and $94.90 \pm 0.004\%$, respectively.

TPC Analysis of *B. racemosa*

A calibration curve had been plotted using the absorbance data of gallic acid over serial concentrations [Figure 3]. An equation of $y = 6.2500x + 0.0429$ was obtained for its linear equation with the correlation value of $R^2 = 0.9944$.

Different plant parts had given different TPC values in *B. racemosa* [Table 1] with the leading phenolic content

Table 1: The anti-inflammatory activities (in xanthine oxidase inhibitory and albumin denaturation assay) and TPC of different plant parts of *B. racemosa*

Plant part	Assay		TPC (mg GAE/g DW \pm SD)
	Xanthine oxidase inhibition	Albumin denaturation inhibition	
Inflorescence axis	59.54 ± 0.001^a	70.58 ± 0.004^a	31.54 ± 0.001^a
Endosperm	57.20 ± 0.003^b	66.80 ± 0.024^a	22.63 ± 0.001^b
Leaf	58.82 ± 0.001^c	65.29 ± 0.006^a	53.94 ± 0.000^c
Pericarp	57.99 ± 0.003^d	43.33 ± 0.002^b	15.54 ± 0.001^d

Means in each assay followed by the same letter are not significantly different from each other according to Tukey HSD test at $P < 0.01$.

SD: Standard deviation, *B. racemosa*: *Barringtonia racemosa*,

TPC: Total phenolic content

was found in leaves extract (53.94 ± 0.000 mg GAE/g DW). This was followed by inflorescence axes (31.54 ± 0.001 mg GAE/g DW), endosperms (22.63 ± 0.001 mg GAE/g DW) and the least was found in the pericarp part of fruits extracts (15.54 ± 0.001 mg GAE/g DW).

DISCUSSION

In Vitro Anti-inflammatory Activities

XO inhibitory activity of *B. racemosa*

According to a review by Borges *et al.* [21], XO is a type of enzyme which is ubiquitously found among species and tissues of mammals and it catalyzes the oxidative hydroxylation of purine substrates and subsequent reduction of O_2 with generation of reactive oxygen species, either superoxide anion radical or hydrogen peroxide. In rheumatological diseases point of view, XO is an enzyme responsible for catalyzing the oxidation of hypoxanthine to xanthine and further lead to the formation of uric acid [22] in which the elevated beyond normal levels will be the underlying reason of gouty arthritis attack [23]. Even though XO is the factor which leads to the occurrence of gouty arthritis, there is an overwhelming acceptance that XO serum levels are significantly increased in various pathological states such as hepatitis, inflammation, ischemia-reperfusion, carcinogenesis, and aging [21].

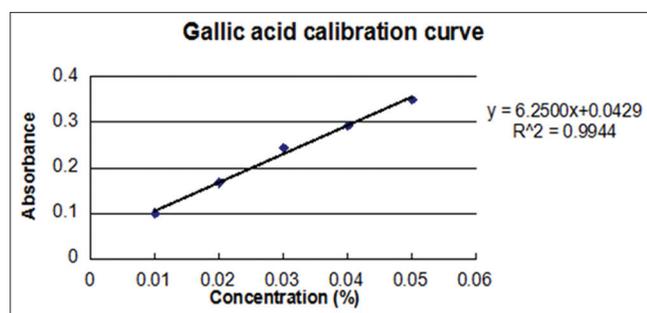


Figure 3: Gallic acid calibration curve for Folin-Ciocalteu total phenolic content assay of *Barringtonia racemosa*

The action of XO inhibitor is therefore required in the prevention and treatment of gout whereby it inhibits the biosynthesis of uric acid from purine [Figure 4] and allopurinol is the widely used synthetic XO inhibitor used in modern medicine in the treatment of gout [24]. Both allopurinol and its active metabolite, oxypurinol (isosteres of hypoxanthine and xanthine, respectively), inhibit XO. Their competitive inhibitions thereby limiting the biosynthesis of uric acid hence promoting renal clearance of hypoxanthine and xanthine [21,25].

Nevertheless, allopurinol may exert certain side effects due to allergy and may generate rashes [18]. In serious cases, allopurinol may also lead to fatality due to adverse drug reactions attributed to allopurinol hypersensitivity [18,26]. Due to such reason, the search for natural sources of plant-based medicines with profound effects of XO inhibition activities is seen to be worthwhile.

Considering the present study, it had been proven that *B. racemosa* effectively inhibits XO activity which resulted in great inhibition percentage in the most optimum extract from the inflorescence axes part ($59.54 \pm 0.001\%$) at a concentration of as low as 0.10%. Oxypurinol was used as a positive standard for XO inhibition in this study whereby it is known as an active metabolite of allopurinol; hence functions as an inhibitor of XO. Oxypurinol had shown greater XO inhibition by exerting $68.36 \pm 0.003\%$ inhibitory activity at a concentration of 0.1%. The linear equations generated from the dose-response plot of inflorescence axes extract and oxypurinol were utilized to estimate the effective concentration for maximal inhibition in both samples. On value substitution, maximal inhibition (100%) in inflorescence axes extract was estimated to be at a concentration of 1.07% (10.7 mg/ml) while oxypurinol exhibited 0.41% (4.10 mg/ml) as its estimated effective concentration for maximal XO inhibition. According to *post-hoc* analysis of Tukey HSD, there were significant differences ($P < 0.01$) between means of each sample in the XO inhibition activity.

The previous studies done on *B. racemosa* showed its protective effects against adjuvant-induced arthritis in animal models [8], and therefore, corroborated the potential use of this species to

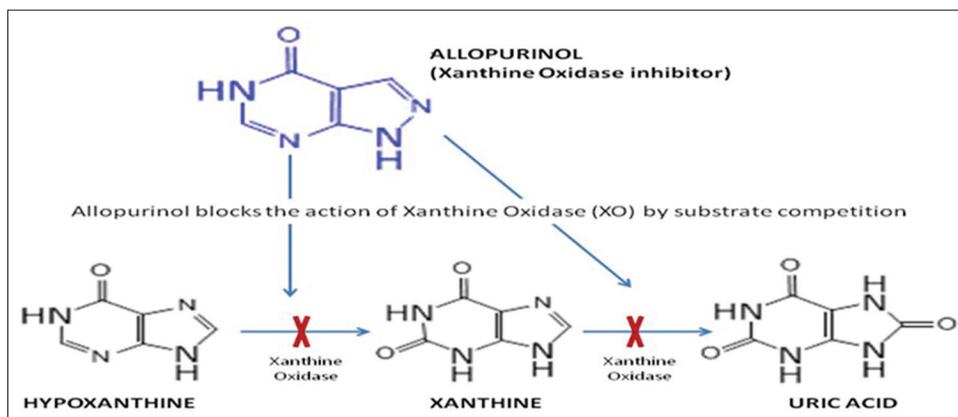


Figure 4: The reaction cascade of allopurinol in xanthine oxidase inhibition mechanism and uric acid biosynthesis in gouty arthritis pathogenesis

alleviate inflammatory symptoms in rheumatic-related diseases. In the study carried out by Patil *et al.* [8], the consumption of *B. racemosa*-derived bartogenic acid resulted in protection against primary and secondary arthritic lesions, body weight changes, and hematological perturbations. In addition, the serum markers of inflammation and arthritis in the arthritic rats were also reduced. On radiological analysis and pain score, the effectiveness of the species was found to be promising and protective against arthritis. Considering its effectiveness in rheumatological disorders, therefore in the current study the potential use of *B. racemosa* in alleviating gouty arthritis was being assessed since to date, there were neither *in vivo* nor *in vitro* studies had ever been recorded to evaluate its potential to be used as gouty remedies. In addition, according to toxicological studies done on *B. racemosa*, it had been verified that the species is devoid of toxicity [9,10] and may serve as a potential candidate to be further developed into a herbal-based formulation for gouty arthritis treatment.

Albumin denaturation inhibitory activity of B. racemosa

Inflammation is the reaction process of living tissues to stimuli evoked by inflammatory agents such as physical injuries, heat, microbial infections, and noxious chemical irritations. The response of cells toward inflammation will lead to certain pathological manifestations characterized by redness, heat, swelling, and pain with even impaired physiological functions. Inflammation has been implicated in the pathogenesis of many diseases including arthritis, stroke, and cancer [27]. Protein denaturation has been well correlated with the occurrence of the inflammatory response and leads to various inflammatory diseases including arthritis [28]. According to Opie [29], tissue injury during life might be referable to denaturation of the protein constituents of cells or of intercellular substance. Hence, the ability of a substance to inhibit the denaturation of protein signifies apparent potential for anti-inflammatory activity.

The capacity of different plant parts of *B. racemosa* to inhibit protein denaturation of albumin which was ranging from $43.33 \pm 0.002\%$ to $70.58 \pm 0.004\%$ inhibition in this assay had therefore provided another evidence for its promising anti-inflammatory properties. In the current study, ibuprofen and diclofenac sodium, the two routinely used NSAIDs for arthritis had been used as the reference compound anticipated to exert optimally positive inhibition percentage. A statistical analysis had shown that there were no significant differences ($P > 0.01$) between means of different plant parts except in pericarp, whereby the extract exhibited significant differences in multiple comparison analysis of *post-hoc* Tukey HSD test.

In clinical setting, major pharmacological agents used for the anti-inflammatory and pain-relief management are NSAIDs due to their capacity in inhibiting protein denaturation [30]. However, this type of drugs is associated with adverse effects on gastrointestinal tract leading to the formation of gastric ulcers and may result in cardiovascular complications as well [31-33]. Interestingly, *B. racemosa* had been documented to be used as a natural remedy for gastric ulcer as quoted by Hussin *et al.* [4] according to Deraniyagala *et al.* [9]. Indeed, this provides

another added value for the species to be considered as a potential candidate for anti-inflammatory agent, deliberating the expectation that the risk of developing gastric ulcers could be minimized considering its ethnopharmacological use.

TPC Analysis of *B. racemosa*

Phenolics are broadly distributed in the plant kingdom and are the most abundant secondary metabolites of plants [34]. Due to such reason, high intake of fruits and vegetables in daily diet are being recommended. Apart from being frequently associated with anti-oxidative properties [35], the presence of phenolic compounds in plants has been attributed to a number of significant pharmacological activities for instance as a cancer cell growth and development inhibitor [36-38], as natural antiulcer due to their gastroprotective nature [39] and providing pain relief for arthritis-related diseases [40].

In this study, the highest TPC was found in the extract of leaves followed in descending order by inflorescence axes, endosperms, and pericarps. The *ex vitro* leaves extract of *B. racemosa* had demonstrated the greatest content of phenolic compounds (53.94 ± 0.000 mg GAE/g DW) and the least was found in the pericarp part of fruits (15.54 ± 0.001 mg GAE/g DW). The differences between means of plant parts in this TPC study were statistically significant ($P < 0.01$) according to *post-hoc* Tukey HSD test.

It has been observed that the values of TPC from the samples in the current research recorded greater phenolic content from those documented in the previous researches. Nurul-Mariam *et al.* [12] reported that the highest TPC was obtained from the methanolic extract of a stick of *B. racemosa* which recorded the value of 29.9 ± 0.02 mg GAE/g freeze dried weight. Meanwhile, Zawawi *et al.* [41] were investigated the value of TPC of *B. racemosa* in methanolic extract of young leaves, and the TPC value of the most optimum samples was found to be relatively low (0.34 mg GAE/g DW) as compared to that of TPC obtained from the current research. The differences in the findings could be due to many factors and may be affected by genotype, plant age, and developmental stages as well as sample preparation procedures.

Another point to be highlighted in the present study is the superiority of inflorescence axis in its anti-inflammatory activities among other plant parts in both anti-inflammatory assays. It has been noted that even though the inflorescence axis part was having the greatest anti-inflammatory activities; nevertheless in terms of plant phenolic content, its superiority was lower than leaves extract. Therefore, it could be suggested that the anti-inflammatory activities shown by the inflorescence axes extract were possibly not solely due to the phenolic content of the species. The activities were, therefore, could be anticipated to be influenced by any other factors such as fatty acids compositions. The findings were almost similarly portrayed in *Jatropha curcas* L. in which the roots sample of *J. curcas* showed the highest anti-inflammatory activity but contained lower phenolic compounds than the leaves extract [42].

Nevertheless, more further studies are underway to investigate the distinctive presence of phenolic compounds in *B. racemosa* since it has been reported by Hussin *et al.* [4] that there was significant detection of phenolic compounds in the species (leaf, stick and bark parts) by using high performance liquid chromatography analysis. Therefore, this indeed requires further analyses to elucidate the compounds responsible for *B. racemosa*'s anti-inflammatory activities.

CONCLUSION

This study showed promising properties of *B. racemosa* to be potentially used as a plant-derived anti-gouty arthritis remedy. The superior activities of inflorescence axis had shown its potential to be optimally harnessed as a candidate in the mitigation of inflammatory diseases. Due to its XO inhibitory activity, this species would be useful in preventing the progress of other XO-related diseases as well. It is suggested that intense *in vivo* studies to be conducted to determine the amount recommended for consumption. Owing to its pharmacological importances, further studies and investigations are therefore required for this species to be optimally developed as pharmaceutical preparations in alleviating inflammation.

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