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ORIGINAL ARTICLE

Comparative Studies of Selected *Calophyllum* Plants for their Anti-inflammatory Properties

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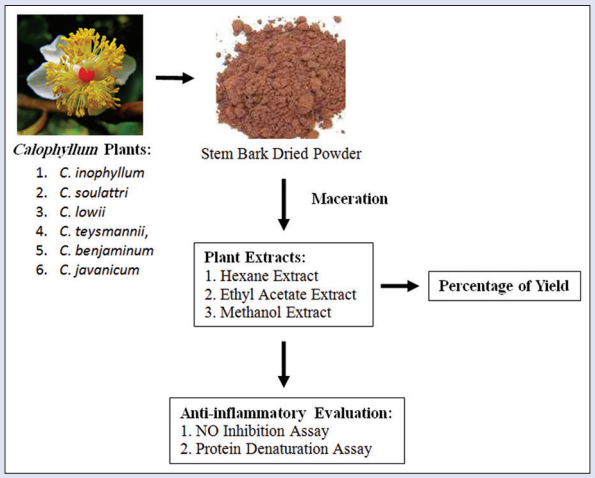
ABSTRACT

Background: *Calophyllum* (*Clusiaceae*) plants have been used as traditional medicine for rheumatism, vein problems, hemorrhoids, and gastric ulcers. The traditional uses of this genus prompted us to investigate six Malaysian *Calophyllum* species which are *Calophyllum inophyllum*, *Calophyllum soulattri*, *Calophyllum lowii*, *Calophyllum teysmannii*, *Calophyllum benjaminum* and *Calophyllum javanicum* for their anti-inflammatory properties. **Materials and Methods:** The stem bark of six plants was extracted with *n*-hexane (Hex), ethyl acetate (EA), and methanol (MeOH). The activities of the extracts were evaluated by determining the inhibition of nitric oxide (NO) production by lipopolysaccharide-induced RAW 264.7 cells, as well as protein denaturation. **Results and Discussion:** The *C. lowii* extracts showed the most significant activities against NO production with IC_{50} values of <40 μg/mL. For the protein denaturation test, *C. teysmannii* extracts showed the strongest effect with IC_{50} values of <100 μg/mL. The results indicated that *C. lowii* and *C. teysmannii* are effective in both assays. Besides, the Hex extracts of these *Calophyllum* plants possess stronger activities than the EA and MeOH extracts. The anti-inflammatory properties are contributed by the secondary metabolites present in the crude extracts, specifically flavonoid, triterpenes, and xanthones. **Conclusion:** These results confirm the potential of *Calophyllum* plants to serve as lead agents in preventing inflammation.

Key words: *Calophyllum,* inflammation, nitric oxide inhibition, phytochemical, protein denaturation

SUMMARY

- The hexane extract of *Calophyllum lowii* and *Calophyllum teysmannii* exhibited good anti-inflammatory effects
- The plant extract of *Calophyllum lowii* showed the strongest activities against nitric oxide production of RAW 264.7 cells
- The plant extract of *Calophyllum teysmanii* exhibited the strongest inhibitory effect against protein denaturation in egg albumin.



Abbreviations used: NO: Nitric oxide; spp.: Species; Hex: Hexane; EA: Ethyl acetate; MeOH: Methanol.

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INTRODUCTION

Inflammation is defined as a localized protection of tissues from infection or destruction of tissues or irritation which are characterized by pain, swelling, and redness. This is usually accompanied by a disturbance in physiological functions such as enhancement of protein denaturation and alterations in the membrane.[1-3] Inflammation is associated with the nonfunctioning of endothelial cells[4] and carcinogenesis.[5] In addition, inflammation can lead to a wide range of diseases, such as cardiovascular,[6] bowel,[7] and autoimmune diseases.[8] Nonsteroidal anti-inflammatory drugs (NSAIDs) are usually administered in the treatment of inflammatory conditions. These drugs usually have severe side effects such as stomach ulcers.[1] Therefore, investigations leading to the discovery of natural active therapeutic principles from our rich bioresources must be promoted. These lead compounds could well be used in the place of the NSAIDs.

Moreover, natural products derived from medicinal plants have been verified to be a major resource of biologically active compounds, many of which have become new lead chemicals to be developed as pharmaceuticals.[1,9] Another merit is the possibility to discover new drugs with reduced adverse effect as there are many cancers or pathogens which are resistant toward existing drugs or develop resistance during prolonged chemotherapy.[9] Surprisingly, only small amount of plant species have been studied scientifically albeit there are plenty of plants worldwide.

Calophyllum spp. (*Clusiaceae*) are known as Bintagor or Tamanu by local communities and are widely distributed in tropical countries.[10] These plants have been used as traditional medicines to treat rheumatism, vein

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problems, diarrhea, hemorrhoids, and gastric ulcers.[11-13] Previous studies on *Calopyllum* spp. have shown them to possess potential pharmaceutical development which include anti-HIV,[14,15] cytotoxic,[16] antioxidant,[16,17] antimicrobial,[18] anti-*Helicobacter pylori*,[19] anti-proliferative,[20] antitumor,[20] antifungal,[21] and anti-inflammatory[22] activities. Since *Calophyllum* exhibits a wide range of biological activities, we performed a comparative study for anti-inflammatory properties by the nitric oxide (NO) and protein denaturation assays on eighteen crude extracts of six *Calophyllum* species, which are *Calophyllum inophyllum* Linn., *Calophyllum soulattri* Burm. exF. Mull., *Calophyllum lowii* Planch et Trian, *Calophyllum teysmannii* Miq, *Calophyllum benjaminum,* and *Calophyllum javanicum* Miq. The anti-inflammatory activities of five plant bark extracts have not been reported previously. It was found that *C. lowii* and *C. teysmannii bark* extracts could provide new leads for the development of anti-inflammatory drugs.

MATERIALS AND METHODS

Plant materials

The stem bark of the six *Calophyllum* plant species was collected in December 2010 from the Sri Aman district, Sarawak, Malaysia. The plant materials were examined and identified by the botanist Dr. Rusea Go from the Department of Biology, Faculty of Science, Universiti Putra Malaysia. All the plant specimens were deposited in the herbarium located in the Department of Biology, Faculty of Science, Universiti Putra Malaysia with voucher specimen codes of RG205, RG202, RG321, RG208, RG105, and RG201 for *C. inophyllum*, *C. soulattri*, *C. lowii*, *C. teysmannii*, *C. benjaminum*, and *C. javanicum*, respectively.

The air-dried and milled materials of *Calophyllum* species stem bark were macerated consecutively with *n*-hexane (Hex), ethyl acetate (EA), and methanol (MeOH). The macerates were evaporated until dryness to yield Hex, EA, and MeOH extracts, respectively which are nonpolar, semipolar, and polar extracts.

Evaluation of *in vitro* anti-inflammatory activity

Nitric oxide assay

The anti-inflammatory properties of plant samples were evaluated by NO assay as reported previously by Ee *et al.*[22] In brief, the RAW 264.7 cells were seeded in a 96-well plate until confluency of the cells observed, followed by treatment with the plant sample and then induced with 10 µg/mL of lipopolysaccharide (LPS). After incubation for 24 h, 50 µL of Griess reagent was used to react with the same volume of cell-free culture supernatant, followed by 10 min incubation at ambient temperature. The absorbance of the mixture was measured at 550 nm. The experiment was carried out in triplicate for accuracy. A standard curve of sodium nitrite with the concentration of 0–100 µM was plotted for the detection of the amount of nitrite in the plant samples. Diclofenac sodium was used as a standard drug in this assay. The average absorbance of plant samples was calculated and used to determine the percentage of NO inhibition using the following formula:

Percentage of NO inhibition = ([A − B] − [C − B])/(A − B) × 100

Where, A = average of absorbance of positive control,

B = average of absorbance of blank,

C = average of absorbance of the sample.

Protein denaturation assay

Protein denaturation assay was carried out by referring to the methods reported by Mizushima and Kobayashi[23] with minor modification. The reaction mixture (5 mL) was made up of 200 µL of egg albumin, 2.8 mL of phosphate buffered saline, and 2 mL of plant sample. The mixture was incubated at 37°C for 15 min and followed by 70°C for 5 min. The absorbance was then measured at 660 nm. Diclofenac sodium was used as reference drug. The percentage inhibition of protein denaturation was calculated using the following formula:

Percentage of inhibition = ([A − B] − [C − B])/(A − B) × 100

Where, A = average of absorbance of positive control,

B = average of absorbance of negative control,

C = average of absorbance of the sample.

RESULTS

The stem bark of six *Calophyllum* spp. was extracted and the yields of the crude extracts are presented in Table 1. The anti-inflammatory activities of *Calophyllum* plant extracts were evaluated against NO and protein denaturation, and the results are summarized in Tables 2 and 3. The concentration of nitrite present in the extracts was determined from the standard curve obtained from the serial concentration of sodium nitrite. It was observed that the plant extracts of *C. lowii* showed the most significant activity against the NO production of RAW 264.7 cells with IC_{50} values of 24.45, 38.02, and 24.48 µg/mL for the Hex, EA, and MeOH extracts, respectively. This was followed by the extracts of *C. javanicum* and *C. teysmannii*, where the IC_{50} values of the MeOH extracts of both

Table 1: Weights of the crude extracts obtained from *Calophyllum* spp.

Plant species	Dry sample weight (kg)	Dry crude extracts (g)		
		Hex	EA	MeOH
Calophyllum inophyllum	3.0	80.7	40.1	60.9
Calophyllum soulattri	1.0	101.2	32.4	97.3
Calophyllum lowii	1.5	50.8	47.6	40.5
Calophyllum teysmannii	1.5	52.4	68.1	30.0
Calophyllum benjaminum	3.0	22.3	11.0	221.3
Calophyllum javanicum	2.5	26.0	15.6	260.0

Hex: Hexane; EA: Ethyl acetate; MeOH: Methanol

Table 2: IC_{50} values and percentage of inhibition of plant extracts (100 µg/mL) against nitric oxide by RAW 264.7 cells

Plant extracts	IC_{50} (µg/mL)	NO (µg/mL)	Percentage of inhibition
Calophyllum inophyllum			
Hex	57.31±2.38	7.92±0.38	85.62±1.95
EA	66.52±1.06	8.99±0.68	74.93±2.07
MeOH	>100	12.51±0.13	44.31±1.12
Calophyllum soulattri			
Hex	64.69±0.95	9.69±0.38	78.48±2.23
EA	26.42±3.01	5.04±0.85	99.27±4.17
MeOH	83.21±2.05	10.90±0.32	60.00±2.09
Calophyllum lowii			
Hex	24.45±0.71	6.04±1.21	98.61±1.18
EA	38.02±2.82	6.92±1.18	93.97±2.21
MeOH	24.48±0.49	6.40±0.89	96.76±1.73
Calophyllum teysmannii			
Hex	32.62±1.93	6.40±0.18	95.48±0.89
EA	35.37±2.60	8.70±0.68	87.23±2.46
MeOH	61.33±4.61	13.64±0.90	67.83±2.46
Calophyllum benjaminum			
Hex	21.07±0.22	6.01±0.19	95.47±1.37
EA	65.27±1.37	9.11±0.91	78.40±1.76
MeOH	82.05±6.99	13.41±1.67	54.44±1.65
Calophyllum javanicum			
Hex	23.66±0.32	5.83±0.41	96.58±1.86
EA	37.92±1.45	6.45±0.23	93.17±1.97
MeOH	54.96±2.06	10.26±0.97	73.13±1.80
Diclofenac sodium	5.02±0.38	10.07±1.36	70.11±2.12

Hex: Hexane; EA: Ethyl acetate; MeOH: Methanol; NO: Nitric oxide

Table 3: IC_{50} values and percentage of inhibition of crude extracts (250 µg/ml) against protein denaturation

Plant extracts	IC_{50} (µg/mL)	Percentage of inhibition
Calophyllum inophyllum		
Hex	106.38±2.16	100.00±0.21
EA	>250	-
MeOH	33.22±0.37	100.00±1.18
Calophyllum soulattri		
Hex	204.95±1.74	63.04±1.67
EA	>250	18.22±4.77
MeOH	>250	44.16±1.45
Calophyllum lowii		
Hex	81.26±6.46	97.56±0.95
EA	96.33±3.52	97.06±1.06
MeOH	80.80±1.78	97.09±0.41
Calophyllum teysmannii		
Hex	31.10±0.64	100.00±1.43
EA	62.44±2.04	97.22±3.42
MeOH	88.61±2.90	100.00±2.66
Calophyllum benjaminum		
Hex	102.21±1.39	97.75±1.90
EA	147.90±1.18	85.90±0.94
MeOH	>250	-
Calophyllum javanicum		
Hex	>250	-
EA	>250	-
MeOH	>250	-
Diclofenac sodium	304.72±9.08	42.74±1.26

Hex: Hexane; EA: Ethyl acetate; MeOH: Methanol; NO: Nitric oxide

plants are 54.96 and 61.33 µg/mL. The plant extracts of *C. benjaminum* and *C. soulattri* showed moderate NO inhibition while *C. inophyllum* exhibited the weakest activity among the *Calophyllum* species, with the EA exhibiting the highest IC_{50} values of 66.52 µg/mL and the MeOH extract more than 100 µg/mL.

The percentage inhibition of the plant extracts against protein denaturation and their IC_{50} values are presented in Table 3. Almost all the plant extracts showed significant inhibition effects at a concentration of 250 µg/mL, except for the crude extracts of *C. javanicum* and *C. soulattri* and the EA extract of *C. inophyllum* and the MeOH extract of *C. benjaminum*. The crude extracts of *C. teysmannii* exhibited the strongest inhibitory effect against protein denaturation with IC_{50} values of 31.10, 62.44, and 88.61 µg/mL for the Hex, EA, and MeOH extracts, respectively. In addition, the MeOH extract of *C. inophyllum* gave significant inhibition effect with an IC_{50} value of 33.22 µg/mL. All the crude extracts of *C. lowii* gave similar moderate activities with IC_{50} values in the range of 80.80–96.33 µg/mL.

DISCUSSION

Macrophages, a type of phagocytic white blood cells involved in the immune defense system will be activated during inflammation leading to the production of inflammatory mediators and cytokines.[24] The production of these cytokines and mediators, particularly NO is induced by a macrophage activator such as the bacterial LPSs. Inducible NO synthase, one of the isoforms of NO, is activated by LPS in macrophages and the activated NO produced leads to a number of biological processes, for instance, inflammation, and immunoregulation.[25] Therefore, inhibition of NO production is known to have potential anti-inflammatory therapeutic value. Besides, protein denaturation is one of the many factors that will lead to various types of inflammatory and arthritic diseases at which one of the previous studies has reported that denaturation of tissue protein could lead to the production of auto-antigen in several arthritic diseases.[3,26]

Denaturation of proteins is a well-documented and parallel physiopathological phenomenon with inflammation process. The mechanism of protein denaturation is due to overproduction of ROS and RNS by neutrophils and macrophages during inflammation and attacking body tissues, which are susceptible to undergo denaturation. Nowadays, protection against protein denaturation is the main consideration in developing conventional drugs to treat both free radical damage and inflammation. In the present study, the anti-inflammatory properties of *Calophyllum* plants were evaluated by NO inhibition and protein denaturation assays.

Looking at the polarities of the crude extracts and the anti-inflammatory activities against RAW 264.7 cells results in Table 2, there is indication that the most nonpolar extract appears to be the most active in the assays except for the *C. soulattri* Hex extract. Among the Hex extracts, *C. benjaminum* showed the highest activity with an IC_{50} value of 21.07 µg/mL followed by *C. javanicum, C. lowii*, and *C. teysmannii* with IC_{50} values of 23.66, 24.45, and 32.62 µg/mL, respectively. The Hex extracts of *C. inophyllum* and *C. soulattri* showed the lowest activities among all the Hex extracts tested but the NO inhibition effects fell on the moderate zone with IC_{50} values of 57.31 and 64.69 µg/mL. Besides, most of the *Calophyllum* plants showed correlations between the anti-inflammatory activities against RAW 264.7 cells and the polarities of the plant extracts. These species are *C. inophyllum, C. teysmannii, C. benjaminum*, and *C. javanicum* as seen in Table 2.

Similarly, the results of protein denaturation inhibition effects of *Calophyllum* spp. also showed a correlation between the inhibition activities and polarities of the crude extracts. This can be seen for the plant extracts of *C. soulattri*, *C. teysmannii*, and *C. benjaminum* [Table 3]. The nonpolar extracts exhibited the highest activities in the protein denaturation inhibition assay except for *C. inophyllum* and *C. javanicum*. Among the crude extracts, the Hex extract of *C. teysmannii* exhibited the strongest activity with an IC_{50} value of 31.10 µg/mL. This was followed by the Hex extracts of *C. lowii*, *C. benjaminum*, and *C. inophyllum* with IC_{50} values of 81.26, 102.21, and 106.38 µg/mL, respectively.

Calophyllum spp. are found to be rich in the biologically active secondary metabolites such as coumarins,[27,28] flavonoids,[29] triterpenes,[27,30] and xanthones.[21,28] Among these constituents, flavonoids are well studied for their potential anti-inflammatory properties. Examples are kaempferol, quercetin, and genistein which have been studied widely both *in vitro* and *in vivo*.[31-34] Kaempferol and quercetin were found in the plant extracts of *C. inophyllum* and *C. brasiliense,* and this was reported previously.[35,36] Lupeol and stigmasterol are triterpenoids which exhibited good anti-inflammatory activities.[37] The study of the mechanism of action of lupeol reveals that it is a multi-target agent with immense anti-inflammatory potential targeting key molecular pathways.[38,39] These terpenoid compounds have been isolated from the plant extracts of *C. benjaminum* and *C. nodusum* previously.[40,41] A variety of xanthones, such as inophinnin and jacareubin, has also been reported to give good anti-inflammatory properties based on previous studies.[22,42] Most of these phytochemical constituents have been isolated from the plant extracts of *Calophyllum* spp.,[22,43-45] particularly the Hex extracts. Thus, it can be deduced that the xanthone and triterpenoid constituents mentioned above which are present in the *Calophyllum* spp studied here contribute to the positive test results against NO and protein denaturation.

Overall, a similar pattern of the inhibitory effects between RAW 264.7 cells and protein denaturation was observed for the crude extracts of *C. inophyllum*, *C. soulattri*, *C. lowii*, *C. teysmannii*, and *C. benjaminum* except for *C. javanicum*. Our current preliminary screening results revealed that the plant extracts of *C. lowii* and *C. teysmannii* are effective against both the RAW 264.7 cells and protein denaturation. Moreover,

the nonpolar extracts of *Calophyllum* spp. exhibited the highest activity as compared to the EA and MeOH extracts. Thus, further study on the detailed mechanism of anti-inflammatory effects by these plant extracts is highly recommended.

CONCLUSION

From the anti-inflammatory test results of the *Calophyllum* spp., we can conclude that *C. lowii* and *C. teysmannii*, particularly their Hex extracts, demonstrated significant NO production inhibition effect against LPS-induced RAW 264.7 cells, as well as heat-induced denaturation of egg albumin protein. A future study on the identification of the corresponding bioactive constituents will be conducted together with the detail mechanisms of anti-inflammatory activity, such as vascular permeability, nociceptive, cyclooxygenase (COX-1), and COX-2.

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Conflicts of interest

There are no conflicts of interest.

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