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Triterpenes and steroids from the stem bark of *Gambeya boiviniana* Pierre

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ABSTRACT
A chemical study was done on the stem bark of *Gambeya boiviniana* Pierre. This plant has been used in traditional medicine for treatment of different kinds of inflammation disorders. In the present study, anti-inflammatory activities of ethanolic, dichloromethane, ethyl acetate and butanolic extracts were assayed in mice using carrageenan-induced paw edema. Ethyl acetate extract was found to possess the most significant anti-inflammatory effect (67, 78%). These results are in accordance with the folk use of this plant. However, more research is needed for its use in clinical studies. The separations of the chemical compounds of ethyl acetate extract were carried out by different chromatographic technics and their structures were elucidated by spectroscopic method including nuclear magnetic, mass spectrometry, IR spectrometry, GC/MS. Nine compounds were identified during this investigation. There are lupeol acetate **3**, β amyrin acetate **4**, α amyrin acetate **6,** taraxasterol acetate **5**, fatty acid ester of lupeol **2** and fatty acid ester of β-amyrin **1**, chondrillasterol **7**, β-sitosterol **8**, β-sitosterol-3-O-glucoside **9**.

Keywords: *Gambeya boiviniana*, Sapotaceae, anti-inflammatory, Triterpenes, steroids, stem bark.

1. Introduction
Gambeya, syn *Chrysophyllum* Linn is a large genus of Sapotaceae about 80 species [1]. Ten species of *Chrysophyllum* have been represented in Madagascar [1, 2]. Of these *Gambeya boiviniana* Pierre, syn. *Chrysophyllum boivinianum* Baehni, *Gambeya madagascariensis* Lecomte known as vernacular name "famelona" with biggest leaves is distributed mainly in Comore and in the east of Madagascar. It is used for interior and exterior joinery, furniture, molding, paneling, flooring, light scales and in shipbuilding because of its elasticity [1]. In Madagascar, the leaves of *Gambeya boiviniana* provide one of the ingredients in herbal mixtures used to relieve the symptoms of malaria, fatigue, muscle pain, treat poisoning and heals the child subject to simple febrile seizure [1, 2].
The African species of *Gambeya* showed interesting pharmacological properties as anti-tumoral, antimicrobial, an anti-inflammatory [1, 2, 3, 4]. The chemical constituents of some *Gambeya* species of Africa have been studied such as steroids, steroids glycosides, pentacyclic triterpenoids, and fatty acid esters of triterpenoids have been identified [1, 2].
In this paper we study the acute anti-inflammatory activity of the extracts and the isolation and identification of steroids, triterpenoids and fatty acid esters of triterpenoids from the AcOEt extract of the stem bark of *Gambeya boiviniana*. Nine compounds were also identified as fatty acid ester of β-amyrin **1**, fatty acid ester of lupeol **2**, lupeol acetate **3**, β amyrin acetate **4**, α amyrin acetate **6,** taraxasterol acetate **5**, and chondrillasterol **7,** β-sitosterol **8**, β-sitosterol-3-O-glycoside **9**. This is the first chemical and biological study of the stem bark of this plant to the best of our knowledge.

2. Materials and Methods
2.1 General experimental procedures
1D (^{1}H, ^{13}C, DEPT) and 2D (^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC) NMR spectra were recorded on a Bruker Varian 300 NMR and 600 NMR operating at 300.15/100.6 MHz and 600MHz using $CDCl_3$, CD_3OD or DMSO-d_6 as solvent and TMS as an internal standard. Column chromatography (CC) was carried out on silica gel F_{254} (Merck) in glass blades. Thin

layer chromatography was performed on precoated TLC plates (Merck, silica 60F254) and visualized by UV light and by spraying with vanillin in H_2SO_4. Mass spectra were measured with Waters 2995/2996-Micromass Q-Tof micro spectrometer (ES^+-MS) and Agilent 5975 spectrometer (EI-MS).

2.2 Plant

Gambeya boiviniana Pierre (Sapotaceae), stem bark collected in mountain Analabe, rural district of Andapa, SAVA's Region Madagascar in Jun 2011, were compared with 11 herbarium Tan identified by Desiré Ravelonarivo *et al*. at the Parc National Botanic and Zoologique Tsimbazaza, Antananarivo, Madagascar.A voucher specimen has been deposited in the "Laboratoire de Chimie des Substances Naturelles et Chimie Organique Biologique".

2.3 Animals

Under a protocol approved by the Animal Care and Use Committee, mice swiss (25–30 g) of either sex were used for the pharmacological activities. All the animals were acclimatized to the laboratory conditions for a week before use. They were fed with standard animal feed and water *ad libitum*.

2.4 Extraction

The air-dried powder stem bark (400 g) of *Gambeya boiviniana* was extracted by maceration with 80% ethanol. Crude ethanol extract (15,85 g), after removal solvent under reduced pressure, was suspended in water and partitioned successively with hexane, dichloromethane, ethyl acetate and n-butanol to give respectively hexane extract (0,01 g), dichloromethane extract (0,28 g), ethyl acetate extract (1,78 g) and n-butanol extract (6 g). The ethyl acetate extract was submitted to silica gel column. Gradient elution was carried out using cyclohexane and increasing the polarity with dichloromethane and then with dichloromethane increasing with ethyl acetate and methanol.

2.5 Antiinflammatory activity

Carrageenan – induced paw edema

The method of Winter *et al*. 1962 was employed in this experiment [1]. The mice were divided into six groups of six animals each (4 tests groups, reference group and vehicle control group). The volume of the right hind paw of each mouse was measured with a plethysmometer (model 7140, Ugo Basile) [1]. The ethanolic, dichloromethane, ethyl acetate, aqueous extracts of *Gambeya boiviniana* Pierre and the standard drug (phenylbutazone) were suspended in mixture of Tween 80 - distilled water (10:90) as solvent and administered orally at the dose of 250 mg/kg for all extracts and 100 mg/kg for phenylbutazone. The control group received the vehicle only (10 ml/kg, p.o.).

Thirty minutes after the administration, acute paw edema was induced in the right hind paw by subplantar injection of 0.05 ml of 1% Carrageenan dissolved in physiological saline. The paw volume was measured just after carrageenan injection and at 30, 60 and 120 minutes [1].

The difference between the volume of the paw before and after carrageenan injection indicated the severity of edema. The percentage inhibition of the inflammatory reaction was determined for each animal by comparing with controls and calculated by the formula:

$$\% \text{ inhibition} = \frac{Vc - Vt}{Vc} \times 100$$

Where, Vt means increase in paw volume in rats treated with test compounds. Vc means increase in paw volume in control group of mice [1].

Statistical analysis

The experimental data were expressed as the mean ± standard error of the mean (SEM). The statistical analysis was carried out using unpaired Student's 't ' test. P values <0.05 were considered as significant.

2.6 Isolation procedure

The ethyl acetate extract (1,5 g) was subjected to column chromatography over silica gel (60 g silica gel 60, 80x2 cm). A total of 540 fractions were eluted with mixtures of cyclohexane/ methylene chloride (from 100:0 to 0:100), then with mixture methylene chloride/ethyl acetate (from 100:0 to 0:100) and finally with ethyl acetate/ methanol (from 100:0 to 0:100). The elutes were monitored using TLC and viewed under UV light (254 and 365 nm) and by spraying with 1% vanillin/5% H_2SO_4/EtOH reagent followed by heating at 100 °C. The fractions were combined on the basis TLC profiles and purified with MeOH. Further chromatography of the combined fractions [35-40] (10 mg) over silica gel 60 (20 mg) eluted with cyclohexane-CH_2Cl_2 (9:1) showed one spot containing esters of β-amyrin **1** with fatty acids (5 mg). Combined fractions [70-90] (25 mg) from MeOH exhibited one TLC spot containing esters of lupeol **2** with fatty acids (20 mg). The fraction 131 (12 mg) eluted with cyclohexane-CH_2Cl_2 (8:2) yielded one spot for a mixture (β-amyrin acetate **4**, lupeol acetate **3,** taraxasterol acetate **5**). α-amyrin **6** (5 mg) was obtained in fraction 132. The combined fractions [390-400] (20 mg) eluted with AcOEt/MeOH (1:9) furnished chondrillasterol **7** (8 mg). The combined fractions [540-549] (10 mg) eluted with AcOEt/MeOH (1:9) yielded β-sitosterol **8** (8 mg). Fractions [575-589] (40 mg) eluted with AcOEt/MeOH (2:8) furnished β-sitosterol glucoside **9** (38 mg).

Acid hydrolysis of sterol glycoside

Compound **9** (9 mg) was heated at reflux with 10 ml of 2N HCl-MeOH (1:1) for 2 h. The MeOH was evaporated and the aqueous solution was extracted with CH_2Cl_2. The aglycone in the dichloromethane layer was analysed by TLC [CH_2Cl_2-MeOH 19:1]. The remaining aqueous phase was evaporated to dryness and monosaccharide glucose was identified by comparison with authentic samples on TLC.

2.7 Physical and spectroscopic data

Fatty acid esters of β-amyrin 1: white powders (MeOH) ^{1}H NMR ($CDCl_3$, 600 MHz) δ (ppm):5.13 (1H,t,H-12), 1.13 (3H,s,CH_3-27), 0.962 (3H,s,CH_3-26), 0.96 (3H,s,CH_3-25), 0.87 (6H,s, CH_3-29 and CH_3-30), 0.86 (3H,s,CH_3-24), 0.83 (3H,s,CH_3-23), 0.82 (3H,s,CH_3-28) Long chain: 2.3(2H, H-2'), 1.25($(CH_2)_n$), 0.90(3H, CH_3ter) ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm); 145.38 (C-13), 121.53 (C-12),80.64(C-3), 55.3(C-5), 47.6(C-9), 47.3(C-18), 46.89 (C-19), 41.8(C-14), 39.90 (C-8), 38.3 (C-1), 37.2 (C-4), 37.1 (C-22), 36.90 (C-10), 34.8 (C-21) , 33.3 (C-29), 32.6(C-7), 32.5(C-17), 31.2 (C-20), 28.4 (C-24),26.2 (C-16), 25.92(C-27), 26.2 (C-15), 23.40 (C-28), 22.27 (C-2), 23.5(C-11), 16.78(C-23), 18.71(C-6), 15.51(C-25).

Long chain 173.9 (C-1'), 34.75 (C-2'), 30.0 $(CH_2)_n$, 13.90 (CH_3 ter)

Fatty acid esters of Lupeol 2: white powders (MeOH) ^{1}H NMR ($CDCl_3$, 600 MHz) δ(ppm): 4.67(H-29β), 4.65(H-29α), 4.46(H-3),

2.36(H-19), 1.69(H-30), 1.03(H-26), 0.94(H-27), 0.85(H-25), 0.84(H-24), 0.83(H-23), 0.78(H-28)
Long chain: 2.28(H-3'), 1.61(H-4'), 1.25$(CH_2)_n$, O.87(CH_3 ter)
^{13}C NMR ($CDCl_3$, 600 MHz) δ (ppm); 151.02 (C-20), 109.36 (C-29), 80.1 (C-3), 55.5 (C-5), 50.34 (C-9), 48.11 (C-18), 48.00 (C-19), 43.19 (C-17), 42.96 (C-14), 40.85 (C-8), 40.00 (C-22), 38.44 (C-1), 38.05 (C-13), 37.90 (C-4), 37.09 (C-10), 34.87 (C-16), 34.22 (C-7), 29.9 (C-21), 27.87 (C-24), 27.44 (C-15), 25.18 (C-12), 23.75 (C-2), 20.95 (C-11), 19.04 (C-30), 18.22 (C-6), 18.01 (C-28), 16.04 (C-26), 16.00 (C-23), 15.98 (C-25), 14.45 (C-27)
Long chain: 174.01 (C-1'), 35.58 (C-2'), 25.10 (C-3'), 30.0 $(CH_2)_n$, 22.80 (CH_2- CH_3 ter), 14.03 (CH_3 ter)

Lupeol acetate 3: white powder^{1}H NMR ($CDCl_3$, 600 MHz) δ(ppm): 4.68(H-29β), 4.56(H-29α), 4.48(H-3), 2.37(H-19), 2.03(CH_3-COO), 1.69(H-30), 1.03(H-26), 0.94(H-27), 0.85(H-25), 0.84(H-24), 0.83(H-23), 0.78(H-28) ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm); 151.19 (C-20), 109.78 (C-29), 81.22 (C-3), 55.40 (C-5), 50.49 (C-9), 48.43 (C-19), 48.00 (C-18), 43.17 (C-17), 42.97 (C-14), 40.96 (C-8), 40.20 (C-22), 38.50 (C-1), 38.10 (C-13), 37.86 (C-4), 37.23 (C-10), 35.72 (C-16), 34.26 (C-7), 29.86 (C-21), 28.09 (C-24), 27.58 (C-15), 25.01 (C-12), 23.86 (C-2), 21.07 (C-11), 19.47 (C-30), 18.39 (C-6), 18.05 (C-28), 16.04 (C-26), 16.55 (C-23), 16.33 (C-25), 14.50 (C-27)
Acetate 171.02 (C-1'), 21.42 (CH_3)

α-amyrine acetate 6: ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm); 139.5 (C-13), 124.2 (C-12), 80.8 (C-3), 59.00 (C-18),55.40 (C-5), 47.67 (C-9), 39.6 (C-19), 38.07 (C-18), 33.7 (C-17), 42.0 (C-14), 39.6 (C-8), 41.5 (C-22), 38.40 (C-1), 37.94 (C-4), 36.6 (C-10), 32.89 (C-7), 31.2 (C-21), 28.09 (C-24), 27.58 (C-15), 26.72 (C-16), 25.01 (C-12), 23.86 (C-2), 21.07 (C-11), 19.47 (C-30), 19.32 (C-27), 18.39 (C-6), 18.05 (C-28), 16.04 (C-26), 14.53 (C-23), 16.33(C-25).
Acetate 171.02 (C-1'), 21.42 (CH_3)

Chondrillasterol 7: ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm):5.08 (1H,t, H-7), 5.05 (1H,t, H-22), 4.96 (1H,t, H-23), 3.59 (1H,t, H-3), 1.02 (3H,d,H-21), 0.85 (6H,d,H-26, H-27), 0.79 (3H,d,H-19), 0.74 (3H,d,H-29), 0.54 (3H,d,H-18), 13C NMR ($CDCl_3$, 100 MHz) δ (ppm); 139.5 (C-8), 138.0 (C-22), 129.5 (C-23), 117.32 (C-7), 70.8 (C-3), 55.94(C-14), 54.83(C-17), 50.83 (C-24), 49.18 (C-9), 42.96 (C-13), 40.53(C-20), 39.90 (C-5), 39.24 (C-12), 37.48 (C-1), 33.78 (C-10), 31.55(C-25), 31.20 (C-2), 29.90 (C-6), 29.80 (C-16), 25.16 (C-28), 23.90 (C-11), 23.80 (C-15), 21.08 (C-21),20.76 (C-26), 20.76 (C-27), 18.75 (C-19), 12.33 (C-29), 11.84 (C-18).

β-sitosterol 8: ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm); 5.33 (1H, H-6),1.01(3H, s, H-19), 0.92 (3H, s, H-21), 0.84 (3H, d, H-26), 0.82 (3H, d, H-27), 0.80 (3H, d, H-29), 0 .68 (3H, s, H-18) ^{13}C NMR (C_5D_5N, 100 MHz) δ (ppm); 140.0 (C-5), 121.4(C-6), 71.62 (C-3), 56.5(C-14), 55.89 (C-17), 49.94(C-9), 45.65(C-24), 42.3 (C-4), 42.10(C-13), 39.20 (C-12), 37.4(C-1), 36.5(C-20), 35.82(C-10), 34.00(C-22), 32.00(C-7), 31.93(C-8), 31.72(C-2), 29.00(C-25), 28.3(C-16), 26.02 (C-23), 24.6 (C-15), 23.06 (C-28), 21.12 (C-11), 19.34(C-19), 19.34(C-21), 19.02(C-26), 18.75(C-27), 11.72 (C-29), 11.63(C-18).

β- Sitosterol-3- β_-D-glucoside 9: ^{1}H NMR (C_5D_5N, 400 MHz) δ (ppm); 5.33 (H-6,), 5.03 (Glc H-1', d, J = 7.6 Hz), 0.97 (3H, d, J = 6.8 Hz, H-21), 0.92 (3H, s, H-19), 0.85 (3H, t, J = 7.6 Hz, H-26), 0.83 (3H, d, J = 6.8 Hz, H-27), 0.84 (3H, t, J = 6.8 Hz, H-29), 0.65 (3H, s, H-18) and ^{13}C NMR (C_5D_5N, 100 MHz) δ (ppm); 141.80 (C-5), 122.80 (C-6), 103.47 (C-1'), 79.50 (CH sucre), 79.39 (2C, CH sucre, C-3), 76.2 (CH sucre), 72.5 (CH sucre), 63.7 (C-6'), 57.74 (C-14), 57.15 (C-17), 51.25 (C-9), 46.96(C-24), 43.39 (C-13), 40.86 (C-4), 40.24 (C-12), 38.3 (C-1), 37.3 (C-20), 35.1 (C-22), 33.09 (C-7), 32.96 (C-8), 31.16 (C-2), 30.03 (C-25), 27.27 (C-16), 25.4 (C-23), 24.3 (C-28), 22.2 (C-11), 21.1 1 (C-26), 21.1 (C-27), 20.9 (C-19), 19.9 (C-21), 13.08 (C-29), 12.89 (C-18); Q-tof premier UPCL/MS [M+Na]:599

3. Results and Discussion

The results of anti-inflammatory activity studies of *Gambeya boiviniana* Pierre show that the ethanolic and ethyl acetate extracts at doses of 250 mg/kg reduced significantly the paw edema (52.82% and 67.78%; $p<0.05$) 2h after carrageenan injection when compared with control but it was not as strong as phenylbutazone (87,22%; $p<0.05$). Dichloromethane and butanolic extracts didn't show a significant effect (Table I).

Table 1: Oral anti-inflammatory activity of extracts of the bark of *Gambeya boiviniana* Pierre on acute model of inflammation by carrageenan-induced edema in left hind paw of mice (mean ± SEM; n=6. *Statistically significant from control P<0,05).

Treatments	Doses (mg/kg)	Volume in ml of the left hind paw of mice before and after carrageenan injection± SEM					
			0		2 h		(% of inhibition)
Control			0,27±0,006		0,58±0,003		
ethanolic extract	250		0,28±0,016		0,41±0,005		52,82*
CH_2Cl_2 extract	250		0,21±0,02		0,52±0,02		17,90
ethyl acetate extract	250		0,29±0,01		0,35±0,008		67,78*
butanolic extract	250		0,25±0,01		0,55±0,01		10,55
Phenylbutazone	100		0,23±0,07		0,27±0,01		87,22*

It is well known that carrageenan induced paw edema is characterized by biphasic event with involvement of different inflammatory mediators. In the first phase (during the first 2 h after carrageenan injection), chemical mediators such as histamine and serotonin play role, while in
Second phase (3–4 h after carrageenan injection) kinin and prostaglandins are involved [2]. Our results revealed that administration of ethanolic and ethyl acetate extract inhibited the

edema starting from the second hours of inflammation, which is probably may be due to the inhibition of different aspects and chemical mediators of inflammation.

The ethyl acetate extract of the stem bark of *Gambeya boiviniana* Pierre yielded triterpenoids and steroids by silica gel chromatography. Assignments of the ^{1}H and ^{13}C-NMR of these compounds were accomplished from ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC and HMBC experiments. These compounds were identified by comparison of their spectral data with those of β-amyrin fatty acid ester **1** [2, 3], α-amyrin acetate **6,** β-amyrin acetate **4** [2], lupeol fatty acid ester **2** [2], lupeol acetate **3** [2], taraxasterol acetate **5**, chondrillasterol **7** [2], β-sitosterol **8** [2], and β-sitosterol glucoside **9** [2] (figure 1) reported in the literature.

Otherwise the nature of the sugar contents in compound 8 was identified by TLC comparison with authentic sample produced through acid hydrolysis reaction.

These compounds isolated from ethyl acetate extract of *Gambeya boiviniana* Pierre found in many species have never been isolated before from species *Gambeya* other chondrillasterol [11, 12].

Although bioassays were not conducted on the isolated compounds from active ethyl acetate extract, there were previous studies reported on their biological activities. Acetylated alpha- and beta-amyrin presents sedative, anxiolytic, analgesic and anticonvulsant properties [2]. β-Sitosterol shows antiinflammatory, antiprostatic, anti-pyretic, antiarthritic, anti-ulcer, insulin releasing and oestrogenic effects and inhibition of spermatogenesis. It reduces risk of cancer and prevention of oxidative damage through its antioxidant activity [2].

Long-chain fatty acid esters of lupeol extracted from an African plant, *Holarrhena floribunda* (Apocynaceae), were shown to have strong antimalarial activity [2]. Lupeol acetate exhibited antinociceptive and anti-inflammatory activity [2].

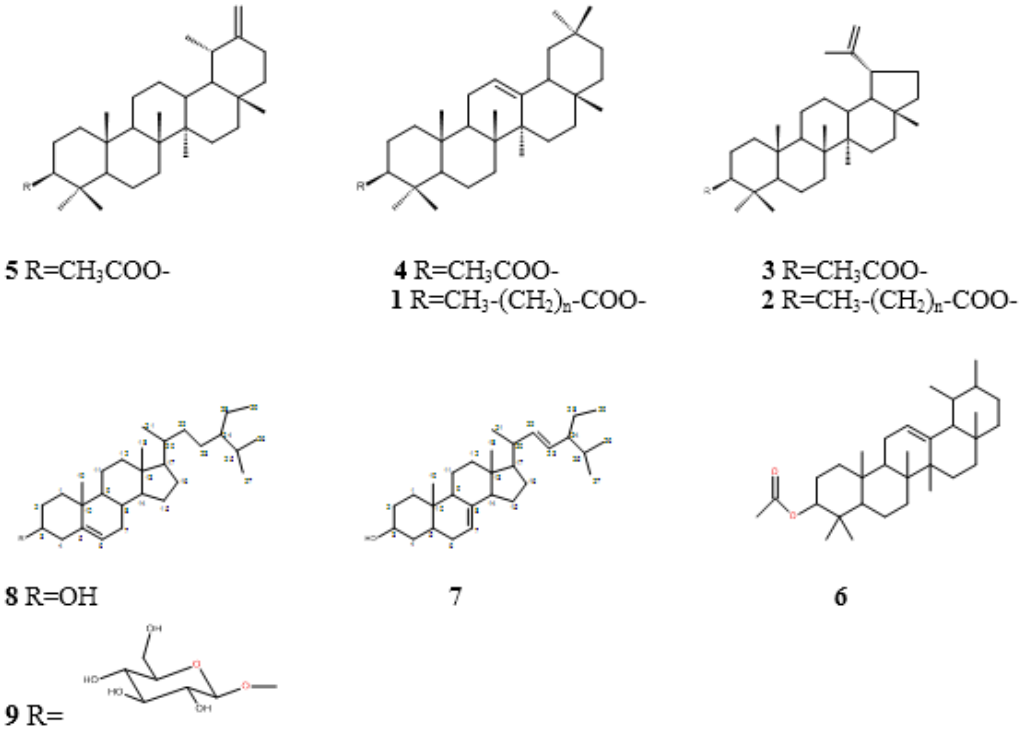

Fig 1: Structure of triterpenes and steroids from the stem bark of *Gambeya boiviniana* Pierre.

Presence of triterpenoids and steroids as the major compounds in ethyl acetate extract can approximately explain antiinflammatory activity of this extract to have strong effect. Triterpenoids have been linked with analgesic, antiinflammatory and antipyretic activities, not surprising therefore to observe activities in ethyl acetate extract since it contained triterpenoids as a major constituent [2]. The relative reduction in the activities observed with the ethanolic extract as compared with ethyl acetate extract is might be due to interaction between the other components.

More studies are needed to show the mechanisms of the anti-inflammatory effects of these agents found in Gambeya *boiviniana* Pierre.

4. Conclusion

The results of the present study showed that *Gambeya boiviniana* Pierre has anti-inflammatory properties and it justifies the traditional use of this plant in the treatment of various types of pains and inflammation.

5. Acknowledgments

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