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D:B-friedo-olean-5-en-3 α -ol isolated from *Cladogelonium madagascariense* Leandri (Euphorbiaceae) extracts and its anti inflammatory activity in mice

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Abstract

This study was conducted to isolate the active components from *Cladogelonium madagascariense* Leandri and to investigate the anti inflammatory activity of the isolated compound. Ethanolic, hexanic, ethyl acetate, and butanolic extracts from *Cladogelonium madagascariense* Leandri were investigated for their *in vivo* acute anti-inflammatory activity (at doses of 250 mg/kg), using carrageenan injection in mice hind paw. The active component was isolated from the most active extract using different chromatographic technics and its structure was established by spectroscopic evidence (UV, 1D- and 2D-NMR, MS). The anti inflammatory activity of the isolated compound (at doses 12.5, 25 and 50 mg/kg) was studied in acute inflammation induced by carrageenan injection in mice hind paw, subchronic inflammation produced by subcutaneous implantation of cotton pellet and chronic inflammation from intra articular injection of complete Freund's adjuvant to induced arthritis. The results showed that the hexanic extract exhibited potent inhibitory activity against both bioassay model (70%). D:B-friedo-olean-5-en-3 α -ol was isolated from this extract. D:B-friedo-olean-5-en-3 α -ol inhibited the paw edema (77.40% at 50 mg/kg) induced by subplantar injection of carrageenan. D:B-friedo-olean-5-en-3 α -ol is active on subchronic inflammation (37.17% at 50 mg/kg) and on chronic inflammation (67.51% at 50 mg/kg). *Cladogelonium madagascariense* Leandri and its isolated compound D:B-friedo-olean-5-en-3 α -ol possessed anti inflammatory activity.

Keywords: *Cladogelonium madagascariense* Leandri, anti-inflammatory, Hexanic extract, D:B-friedo-olean-5-en-3 α -ol

1. Introduction

Medicinal plants in general are believed to be an important source of new chemical substances with potential therapeutic efficacy [1]. They still represent a large source of structurally novel compounds that might serve as lead for new drug development [2]. *Cladogelonium madagascariense* Leandri, vernacularly known as "tsontso" (figure 1) is a monotypic plant genus of the EUPHORBIACEAE family. It is an endemic plant growing in North Madagascar [3] with single genus and single species. A steam bath of the aerial parts are used in traditional medicine to treat fever and flu-like symptoms [4] which suggests its anti inflammatory activity. Inflammation is as a primary physiologic defense mechanism that helps the body to protect itself. An uncontrolled and persistent inflammation may act as an etiologic factor for many chronic diseases [5]. Many studies are being directed to find anti-inflammatory agents from natural sources [6]. The present work was undertaken to isolate a biological active compound (s) from the most active extracts of *Cladogelonium madagascariense* Leandri by using various chromatography techniques. The structure of D:B-friedo-olean-5-en-3 α -ol was elucidated by NMR spectroscopic methods. Anti-inflammatory activity was studied *in vivo* using experimental acute, chronic and subchronic inflammation models induced by phlogistic agents. This is the first chemical and biological studies carried out on this plant and its isolated compound.



Fig 1. *Cladogelonium madagascariense* Leandri (“tsontso”)

2. Materials and methods

2.1 Plant material

Cladogelonium madagascariense was collected from Sadjoavato, in the Region of “Diana” in the Northern part of Madagascar. This species was identified at the Department of Botany of Botanical and Zoological Park of Tsimbazaza (Antananarivo). A voucher specimen was deposited at the Sciences Faculty of Antananarivo University for future references.

2-2 Extraction and isolation procedures

Dried stem leaves of *Cladogelonium madagascariense* Leandri were reduced to a fine powder with a mechanical grinder. The powdered plant material (200 g) was extracted by maceration with a mixture of Ethanol-water (80:20) (3500 ml) for 72 h, and concentrated to dryness under vacuum at a low pressure and low temperature 60 °C. The crude extract was dissolved in water and partitioned successively with hexan, ethyl acetate and n-butanol to give respectively hexanic, ethyl acetate and n-butanol extracts. The preliminary study has shown that the hexanic extract exhibited the highest biological activity. Therefore, our investigation has been focused on this extract. Sixty grams of silica gel (60 F254; 70-230 Mesh) were prepared in hexan and packed in column (length 100 cm, internal diameter 3.5 cm). One gram of the hexanic extract was subjected onto the column chromatography and eluted with a gradient cyclohexan - CH₂Cl₂ (100:0; 95:5; 90:10; 80:20; 70:30); 135 aliquots of 10 ml each were collected and analysed with thin layer chromatography (TLC), using precoated silica gel (60 F254) plates aluminium base (Merck) of 0.2 mm thickness. Five microliters of aliquots were deposited on TLC plates and eluted with a mobile phase cyclohexan - CH₂Cl₂ (70:30). The plates were dried and separated compounds were detected under UV lamp (254/365nm) or by spraying with freshly prepared vanillin spray reagent (0.1 g of vanillin dissolved in a mixture of: 28 ml methanol and 1 ml sulphuric acid)^[7], then heated at 110 °C for 1 min. The spots exhibiting the same R_f on TLC were combined^[8]. The aliquots 99 to 120 eluted with cyclohexan - CH₂Cl₂ (80:20) were combined and

precipitated with MeOH. The structure of isolated compound was elucidated by NMR spectral data (UV, 1D- and 2D-NMR, MS).

2-3 Animals

Male Swiss mice weighing 25 to 30 g were used for anti-inflammatory studies. The animals were acclimatized to the laboratory conditions for a week before use. They were fasted overnight before each experiment and had access to water *ad libitum*. They were used only once and in accordance with the ethical guidelines for the care of laboratory animals.

2-4 Antiinflammatory activity

Preliminary assay to determine the active fraction of *Cladogelonium madagascariense* Leandri Preliminary assay was established to determine the most active of *Cladogelonium madagascariense* Leandri extracts (ethanolic, hexanic, ethyl acetate and butanolic) using carrageenan induced paw edema. They were tested at the same dose of 250 mg/kg. The mice were divided into six groups of six animals each (4 tests groups, 1 standard group and 1 control group). The extracts of *Cladogelonium madagascariense* Leandri and standard drug were freshly prepared in a mixture of Tween 80-distilled water (10:90). Phenylbutazon (100 mg/kg) was used as standard drugs. All the products were administered orally to the mice. The extracts of *Cladogelonium madagascariense* Leandri (250 mg/kg) were given for the tests groups. Phenylbutazon was given for the standard groups. Vehicle (Tween 80-distilled water (10:90)) at 10 ml/kg was given for the control group. Thirty minutes after the administration, acute paw edema was induced by injection of 0.05 ml of 1% (w/v) freshly prepared carrageenan in a physiological saline (0.9%) into the subplantar tissues of the right hind paw of each mouse. The paw volume was measured at 1, 2, 3, 4, 5 and 6 hours after carrageenan injection^[9].

Acute inflammation activity of D:B-friedo-olean-5-en-3 α -ol

Paw edema induced by carrageenan injection was used as a model of acute inflammation according to Winter *et al.*, 1962^[10] and described previously^[11]. The volume of each mouse right hind paw was measured initially with a plethysmometer (Ugo Basile, Model 7140). All the products were administered orally to the mice. D:B-friedo-olean-5-en-3 α -ol at three doses (12.5 mg/kg, 25 mg/kg, 50 mg/kg) were given to the test groups. Phenylbutazon, Indomethacin were given for the standard groups. Vehicle (Tween 80-distilled water (10:90)) at 10 ml/kg was given to the control group. Thirty minutes after the administration, acute paw edema was induced by injection of 0.05 ml of 1% carrageenan into the subplantar tissues of the right hind paw of mice. The paw volume was measured at 1, 2, 3, 4, 5 and 6 hours after carrageenan injection^[9].

The percentage inhibition of paw edema was calculated as:

$$\text{Percentage inhibition} = [1 - (a / b)] \times 100$$

Where *a* is the increase of the right hind paw volume in test group, and *b* the increase of the right hind paw volume in control group^[12].

Activity of D:B-friedo-olean-5-en-3 α -ol on Subchronic inflammatory

The subchronic inflammatory model was evoked by granuloma induced by cotton pellet in mice according to the method of Meier *et al.*, (1950) [13]. The mice were anaesthetized with ether and the back skin was shaved. Cotton pellets (20 mg) was soaked in carrageenan 1% (w/v) dissolved in physiological saline (0.9%) and were implanted subcutaneously into both sides of the groin region of each animal [14, 15]. The products were administered orally for 7 consecutive days from the day of cotton pellet implantation. Indomethacin (10 mg/kg), or phenylbutazon (100 mg/kg) was administered to the standard group, D:B-friedo-olean-5-en-3 α -ol (12.5 mg/kg, 25 mg/kg and 50 mg/kg) for the test groups, and solvent (10 ml/kg) (Tween 80-distilled water (10:90)) to control group. On the eighth day, the mice were sacrificed and the pellets with the surrounding granulation tissues were collected. The granulomas were dried at 60 °C for 24 h and their weights were measured and compared to the control group. Increase in the dry weight of the pellets was taken as a measure of granuloma formation [14]. The anti-inflammatory effect was calculated by the following equation.

Anti inflammatory activity (%) = $1 - (T/C) \times 100$.

Where, T represents the dry weight of the pellets in treated groups and C the dry weight in control groups.

Activity of D:B-friedo-olean-5-en-3 α -ol on Chronic inflammatory

Arthritis model induced by Freund's adjuvant was used to evaluate the activity of D:B-friedo-olean-5-en-3 α -ol on chronic inflammation on mice as described by Freund *et al.*, (1937) [16]. The paw volume of the right hind paw of mice was measured using plethysmometer (UgoBasile Italy). All products including D:B-friedo-olean-5-en-3 α -ol (12.5 mg/kg, 25 mg/kg and 50 mg/kg), Indomethacin (10 mg/kg), Phenylbutazon (100 mg/kg), and the vehicle (Tween 80-distilled water (10:90)) (10 ml/kg) were administered orally to the mice 30 min prior to CFA injection (0.05ml) into the metatarsal of the right hind paw of mice and continued daily for 28 consecutive days [17]. The paw volume was measured on 7th, 14th, 21st and 28th days after the injection of Freund's adjuvant. The differences between paw volume on day 28 after CFA injection and that on the day (V0) were calculated to estimate chronic inflammatory response. Percent inhibition of paw edema was calculated using formula:

% Inhibition = $(Vc - Vt) / Vc \times 100$

Where, Vc is the mean changes in paw volume of control group and Vt is the mean changes in paw volume of treated group.

2-5 Statistical analysis

The experimental data were expressed as the mean \pm SEM (standard error of the mean). Data were assessed by statistical analysis using unpaired Student's 't' test. P values < 0.05 was considered as significant.

3. Results and discussion

3.1 Results of extraction and structure of isolated compound

Fractionation of EtOH-H₂O extract (122 g) yielded 0.36 g (0.29 %) of hexanic extract, 7.44 g (6.09 %) of ethyl acetate extract and 12.27 g (10.05 %) of butanolic extract.

The aliquots of hexanic extract from 99 to 120 eluted with cyclohexane - CH₂Cl₂ (80:20) and combined with MeOH gave 20 mg of compound (Rf = 0.44). The structure elucidation of this compound based on spectral techniques indicated that this compound is a triterpene. The peaks assigned of D:B-friedo-olean-5-en-3 α -ol, the carbonyl group (213.15 ppm) were detected. The data 1D and 2D NMR of this compound is given in Table I. The structures of isolated compound was identified as D:B-friedo-olean-5-en-3 α -ol after comparing with a previous spectral data in the literature [18, 19] (Figure 2). Lupeol (5 mg) [20] and Friedelin (10 mg) [21] were also found in this hexanic extract and they were most extensively studied concerning pharmacological activity [22, 23]. Few reports are available on the biological activity of D:B-friedo-olean-5-en-3 α -ol.

D:B-friedo-olean-5-en-3 α -ol: White powder

m/z (rel%): 426 [M]⁺, ¹H NMR spectral data (400.15MHz, CDCl₃) 1.5 (H-1), 1.69(H-2 α), 1.86(H-2 β), 3.46(H-3), 5.64(H-6), 1.84(H-7 α), 1.96(H-7 β), 1.50(H-8), 2.00(H-10), 1.37(H-11 α), 1.55(H-11 β), 1.34(H-12 α), 1.69(H-12 β), 1.16(H-15 α), 1.47(H-15 β), 1.25(H-16 α), 1.37(H-16 β), 1.57 (H-18), 1.25(H-19 α), 1.37(H-19 β) 1.22(H-21 α), 1.47(H-21 β), 0.90(H-22 α), 1.53(H-22 β), 1.04 (H-23), 1.14(H-24), 0.85(H-25), 1.09(H-26), 1.00 (H-27), 1.16 (H-28), 0.99 (H-29), 0.95 (H-30)¹³C NMR spectral data (100.15MHz, CDCl₃) 141.5 (C-5), 121.8 (C-6), 75.6(C-3), 49.5(C-10), 47.3(C-8), 40.63(C-4), 39.1(C-14), 38.75 (C-22), 37.6(C-13), 35.7(C-16), 34.8(C-5), 34.8 (C-21), 34.6(C-18), 34.4 (C-11), 34.3(C-30), 32.9(C-19), 32.2(C-29), 31.9(C-15), 31.8(C-28), 30.1 (C-), 29.4(C-17), 29.4(C-20), 28.7(C-23), 25.2(C-24), 23.52(C-7), 27.6(C-2), 19.3(C-26), 18.26(C-27), 18.06(C-1), 16.05(C-25),

Table I: NMR spectral data of D:B-friedo-olean-5-en-3 α -ol (CDCl₃, ¹H 600.15 MHz, ¹³C 100MHz).

Position	Position	$\delta^{13}\text{C}$ (ppm)	$\delta^1\text{H}$ (ppm)	Correlation HMBC			COSY
				² J _{CH}	³ J _{CH}	⁴ J _{CH}	
1	1 α	18.06	1.5	10	9		
	1 β						
2	2 α	27.6	1.69				3.2 β
	2 β		1.86	3			3.2 α
3		75.6	3.46	4	23, 24		2 β , 2 α

4		40.63	-			
5		141.5	-	4, 10, 6	23	2 α , 6 β
6	6 α 6 β	121.8	5.64			5, 6 β 5, 6 α
7	7 α 7 β	23.52	1.84 1.96			
8		47.3	1.50			
9		34.6	-	11, 8, 10	1	
10		49.5	2.00			
11	11 α 11 β	34.4	1.37 1.55		10 9	1, 7 10
12	12 α 12 β	30.1	1.34 1.69			
13		37.6	-			
14		39.1	-			
15	15 α 15 β	31.9	1.16 1.47	16	17	
16	16 α 16 β	35.7	1.38 1.55	15,17		
17		29.4	-			
18		34.8	1.57	19	16	19
19		32.9	1.25 1.37	20,18,21	29, 30	21 α , 21 β , 18
20		29.4	-			
21	21 α 21 β	34.8	1.22 1.47		20	21 β 19,21 α ,22 α
22	22 α 22 β	38.75	0.90 1.53			21 β , 22 β 22 α
23		28.7	1.04	4	3, 5, 24	
24		25.2	1.14	4	3, 5, 23	
25		16.05	0.85	8	5, 1, 9	
26		19.3	1.09	10	9, 14, 7	
27		18.26	1.00	14	10, 13, 15	
28		31.8	1.16	17	18, 22, 16	
29		32.2	0.99	20	30, 19	
30		34.3	0.95	19, 30		

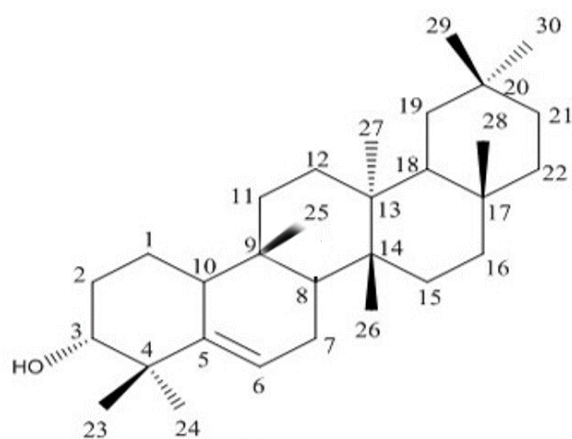


Fig 2: D:B-friedo-olean-5-en-3 α -ol isolated from hexanic extract of *Cladogelonium madagascariense* Leandri

3-2 Anti-inflammatory activity

Determination of the active fraction of *Cladogelonium madagascariense* Leandri

From the preliminary assay, all the fractions of *Cladogelonium madagascariense* Leandri at the dose of 250 mg/kg reduce the paw edema induced by carrageenan injection, and the hexanic extract has the highest anti-inflammatory activity. The

ethanolic and hexanic extracts reduce 51.61% the paw edema for ethanolic extract, 70% for hexanic extract, 19.35% for ethyl acetate and 25% for butanolic extracts ($P < 0.05$). Phenylbutazon a commonly used anti-inflammatory drug produced a significant inhibition of 87.22. The present results indicate the efficacy of hexanic extract of *Cladogelonium madagascariense* Leandri in acute anti-inflammatory conditions (Figure 3).

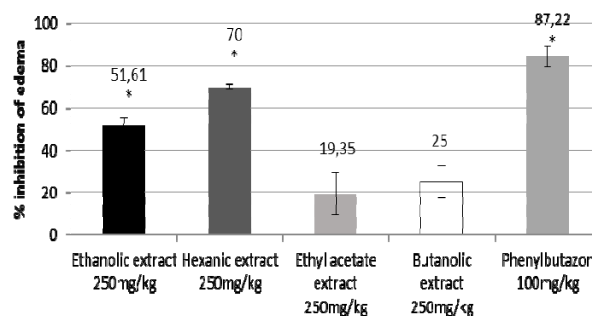


Fig 3: Anti-inflammatory activity of *Cladogelonium madagascariense* Leandri extracts and phenylbutazon administered per os on acute model of inflammation by carrageenan-induced paw edema in mice, two hours after carrageenan injection (mean \pm SEM; n=6. *Statistically significant from control $P < 0.05$)

Effect of D:B-friedo-olean-5-en-3 α -ol on acute inflammation

D:B-friedo-olean-5-en-3 α -ol isolated from hexanic fraction, at different doses inhibited the paw swelling induced by carrageenan injection, compared to the control groups. The maximum inhibition was observed 3 h after the injection of carrageenan with a percentage inhibition of inflammation 37.55 and 77.40 respectively at the doses 12.5 mg/kg and 50

mg/kg. Compared with indomethacin (10 mg/kg), the effect of D:B-friedo-olean-5-en-3 α -ol at the same dosage (12.5 mg/kg) was less; 69.94% for indomethacin and 37.55% for D:B-friedo-olean-5-en-3 α -ol. The effect of D:B-friedo-olean-5-en-3 α -ol was higher than phenylbutazon, because used at the dose of 100 mg/kg, phenylbutazon showed 87.22% inhibition and 77.40% with D:B-friedo-olean-5-en-3 α -ol at 50 mg/kg ($P < 0,05$) (Figure 4).

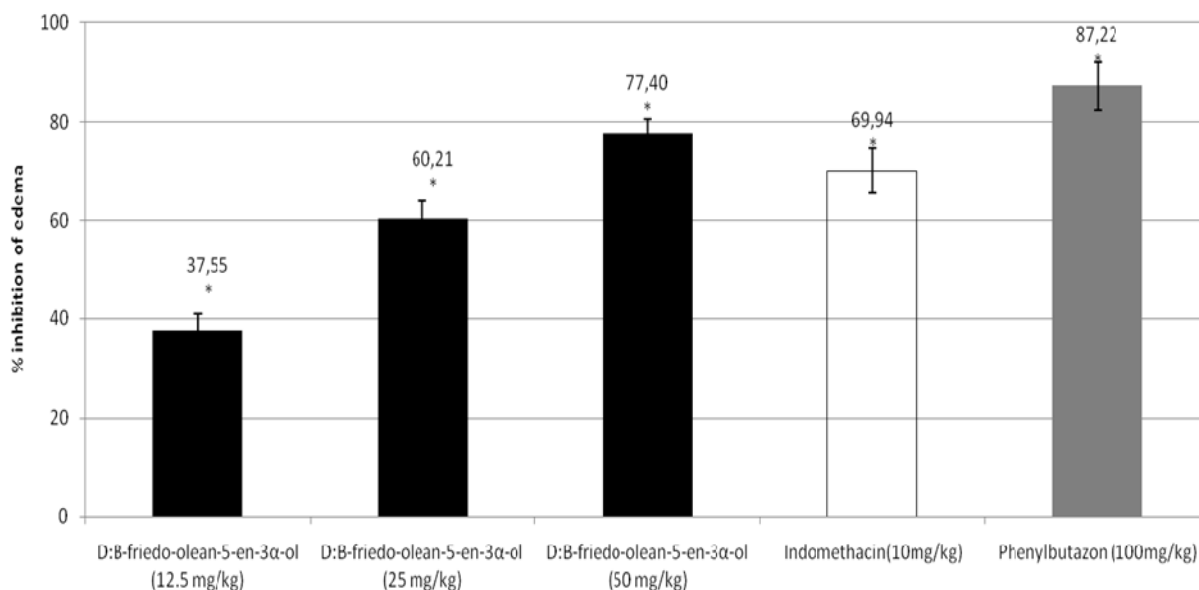


Fig 4: Acute anti-inflammatory activity of D:B-friedo-olean-5-en-3 α -ol, indomethacin and phenylbutazon administered per os in subplantar injection of carrageenan (1%) in the right hind paw of the mice, three hours after carrageenan injection (mean \pm SEM; n=6; * $P < 0,05$)

Edema developed after carrageenan injection is a biphasic event. The first phase (0–2 hours) is attributed to the release of histamine and serotonin, followed by kinin and finally through bradykinin. The second phase (2–6 hours) is to be correlated with the enhanced production of NO, PGs, TNF- α , IL-1 β , and IL-6 [24, 25]. It has been reported that the second phase of edema is sensitive to drugs like hydrocortisone, phenylbutazon and indomethacin [23]. Our results showed that oral administration of tested doses of D:B-friedo-olean-5-en-3 α -ol reduced the paw edema 3 h after carrageenan injection, which means, D:B-friedo-olean-5-en-3 α -ol possess an anti-inflammatory activity and it is more effective in the second phase of inflammation than in the first phase.

Granuloma induced by cotton pellet

The subcutaneous implantation of cotton pellet into the mice induced a formation of granuloma at the site of implantation. D:B-friedo-olean-5-en-3 α -ol inhibited the granuloma formation by 21.98% and 37.17% at the doses of 25 mg/kg and 50 mg/kg as compared to control group. Indomethacin (10 mg/kg) and phenylbutazon (100 mg/kg) decreased the granuloma formation by 32.83% and 18.97% (Table II). Indomethacin is more effective than phenylbutazon to inhibit the granuloma formation, as reduces the influx of mononuclear cells and the formation of giant cells [25].

Table 2. Effect of oral administration of D:B-friedo-olean-5-en-3 α -ol, indomethacin and phenylbutazon on granuloma induced by subcutaneous implantation of cotton pellet in mice (n=6). Each point represents the mean \pm S.E.M. * $P < 0.05$ vs. control groups

Groups	Doses (mg/kg)	Weight of dry cotton Pellet granuloma (mg \pm sem)	Percentage of inhibition
Control	-	57,3 \pm 0,23	-
D:B-friedo-olean-5-en-3 α -ol	12,5	50,36 \pm 1,97	12,11%
D:B-friedo-olean-5-en-3 α -ol	25	44,7 \pm 1,85*	21,98%*
D:B-friedo-olean-5-en-3 α -ol	50	36 \pm 2,3*	37,17%*
Indomethacin	10	38,83 \pm 1,06*	32,23*
Phenylbutazon	100	46,43 \pm 1,08*	18,97*

The cotton pellet granuloma method has been employed to assess the various components of subacute inflammation such as transudative, exudative, and proliferative phases [26]. The implantation of cotton pellet soaked in carrageenan induced an immune response [27]. The resident mononuclear cells such as

macrophages resident in the site of lesions are unable to kill the pathogen agent, in which case they release various cytokines such as TNF- α , IL-1 β and IL-6 [28] contributing to the recruitment, differentiation and activation of immune cells in the blood vessels that migrate to the site of lesion.

The recruitment of immune cells is initiated when the levels of cytokines pro inflammatory increase. This induce differentiation and activation of inflammatory cells subsequently migrate also to the site of lesions. This migration and accumulation of macrophages, T cells and other immune cells around the site of lesions and their subsequent interactions induce the formation of granuloma. An uncontrolled liberation of cytokines pro inflammatory increase the formation of granuloma containing large numbers of infected cells and widespread caseation. The granuloma growth could be reduced if the levels of pro inflammatory cytokines are reduced, or their function is blocked [27, 29].

Therefore the decrease in granuloma weight in the present study indicates inhibition of the proliferative phases by D:B-friedo-olean-5-en-3 α -ol. This effect may be due to the inhibition of cytokines pro-inflammatory levels.

Complete Freund's adjuvant (CFA) induced arthritis

Inflammation can also occur in articular joints. Arthritis is a chronic inflammation resulting in progressive destruction of articular and periarticular structure [28]. Injection of CFA in right hind paw of mice provoked inflammatory reactions: swelling, redness and an increase in the paw volume. The repeated administration of different doses of D:B-friedo-olean-5-en-3 α -ol or the standard drugs (indomethacin, phenylbutazon) for 28 days inhibited the development of joint swelling induced by complete Freund's adjuvant as compared to control group. In the present study, D:B-friedo-olean-5-en-3 α -ol reduces the chronic inflammation in the joint of the hind paw of mice, 20.80% at 25 mg/kg and 67.51% at 50 mg/kg. This effect at the same dosage (12.5 mg/kg) was less than indomethacin (61.8%) at 10 mg/kg. At the higher dosage (50 mg/kg), D:B-friedo-olean-5-en-3 α -ol was more effective than phenylbutazon (57.57%) at 100 mg/kg (Figure 5).

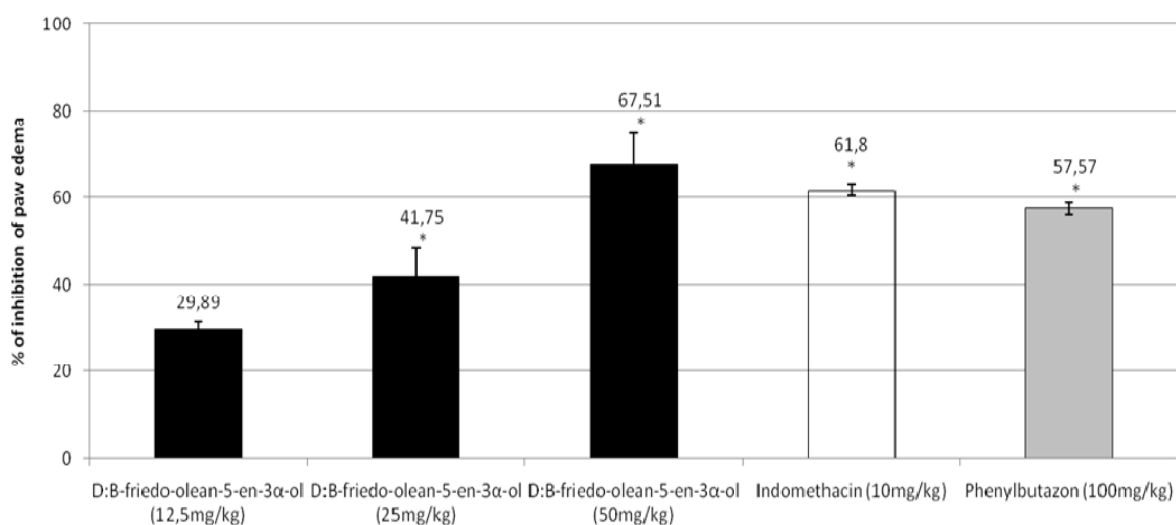


Fig 5: Anti-arthritic activity of D:B-friedo-olean-5-en-3 α -ol, Indomethacin and Phenylbutazon in Complete Freund's adjuvant (CFA) injected in metatarsal of the right hind paw of mice at 28 day after CFA injection (mean \pm SEM; n=6; *P<0,05)

Complete Freund's adjuvant induced arthritis are characterized by chronic proliferative and inflammatory reactions in synovial membranes, producing pain, disability and eventually destruction of joints [17]. Synovial membrane contains activated B and T cells, plasma cells, mast cells, and particularly activated macrophages. The progression of arthritis is characterized by increase of the paw footpad and tibiotarsal joint diameters which can be attributed to the release of number of mediators like proinflammatory cytokine such as TNF- α , IL-1 and IL-6. They play a fundamental role in the processes that cause inflammation, articular destruction, and the comorbidities associated with arthritis and they are the major proinflammatory cytokines with high concentrations in the inflamed joint synovium [30, 31, 32]. In this study, D:B-friedo-olean-5-en-3 α -ol inhibits the joint inflammation. The mechanism of D:B-friedo-olean-5-en-3 α -ol may be due to depletion of pro-inflammatory cytokines that occurred in the joint homogenate of arthritic mice, leading to moderate control on progression of arthritis, suggesting that a balance between proinflammatory and anti-inflammatory cytokines is necessary for controlling the progression of arthritis.

Conclusion

This work has shown that hexanic extract of *Cladogelonium madagascariense* Leandri possess the highest anti-inflammatory activity. This extract is rich in triterpene and D:B-friedo-olean-5-en-3 α -ol is active orally on acute, subchronic and chronic models of inflammation. Further studies are in progress to elucidate the anti-inflammatory mechanism of D:B-friedo-olean-5-en-3 α -ol.

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