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GC-MS screening and evaluation of the anti-inflammatory and antioxidant activities of ethanolic leaves and stem barks extracts from *Dialium guineense* Willd, *Parkia biglobosa* (Jacq.) R. Br. ex Benth. and *Tamarindus indica* L

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Abstract
Inflammation has been shown to be greatly involved in the degenerative processes of human skin such as photo aging and atopy. Reduction of low-grade inflammatory reactions by topical products may be necessary in case of skin aggression, to obtain an optimal wound healing and restore the physiological balance of human skin. Present work evaluated the antioxidant, anti-inflammatory and phototoxic properties of ethanolic extracts from leaves and stem barks of *Dialium guineense* Willd., *Parkia biglobosa* (Jacq.) *R. Br. Ex Benth. and Tamarindus indica* L. The antioxidant power of the extracts, measured *in vitro* by the KRL method, showed that each gram of D. guineense bark extract, *P. biglobosa* leaves extract and T. indica bark and leaves extracts has an antioxidant capacity equivalent to 1585, 2092, 5071 and 2246 mg of Trolox respectively. Simultaneously with their actions on cell viability, the anti-inflammatory activity of the extracts was monitored measuring their effects on NO production by mouse macrophages submitted to LPS from E. coli to determine the anti-inflammatory ratio of each extract. The bark and leaves of *D. guineense* Willd., the leaves of *P. biglobosa (Jacq.) R. Br. Ex Benth.* and the bark of *T. indica L.* have anti-inflammatory ratios from 161 to 458.2, whereas Dexamethasone (positive control) has a ratio of 37.87. The *in vitro* 3T3 NRU test was used on mouse fibroblasts to determine the phototoxicity of the six extracts. Only *D. guineense* Willd. stem bark was photo-toxic with a photo-irritation factor greater than 5 (PIF = 8.39). Our study report some molecules such as lupeol, amaryn, sitosterol for the first time in the extracts and shows that the use of these plants in traditional medicine is justified.

Keywords: anti-inflammatory, antioxidant, GC-MS, ethanolic extracts, lupeol, sitosterol, stigmasterol

Introduction
Living beings have two major common enemies: aging and diseases. Although a natural process, aging can be accelerated by oxidative stress which seems closely related to inflammatory processes [1, 2]. Inflammation is the set of defense mechanisms by which the body recognize, destroy and eliminate all foreign substances. Inflammatory reaction sometimes exceeds its objectives leading to deleterious effects. It then needs to be managed. On the other hand, oxidative stress (due to increase of oxygen reactive species) is linked to many pathologies [3, 2, 1]. As implication of oxidative stress and inflammation in occurrence of several pathologies (diabetes, cancer, cardiovascular diseases) was highlighted [3-5] search for molecules with antioxidant and anti-inflammatory power now goes beyond the anti-aging or cosmetic framework. Therefore, during two past decades, scientific research focused on them worldwide. In Africa and many low and middle income countries, traditional knowledge based on medicinal plants is used to fight against oxidative stress and inflammation [6]. Resort to this form of medicine have been linked to cultural and economic reasons [7, 8]. However, we note a worldwide increase of medicinal plants use [9, 10] because of their renewable character and association with no side effects. It is then important to investigate the effectiveness of those medicines. This is the purpose of this study focused on three plants recurrent in Benin traditional medicine: *Dialium guineense* Willd., *Parkia biglobosa* (Jacq.) *R. Br. Ex Benth., and Tamarindus indica* L.. Valorization and safeguarding of those threatened plants in Benin [11, 12] and their recurrence in our ethnobotanical survey justify our choice on them. They belong to the Fabaceae family which is well know for its medicinal purposes [13-16].

Their aerial organs are widely used in the treatment of various infectious diseases in Benin. Because inflammatory reaction may occur in response to infections, natural compounds having both activities would be very interesting.
D. guineense is a dialioideae exclusively present in regions of tropical Africa and exceptionally in Sao Tome et Principe. It grows mainly in the wild as a shrub on land left fallow and in dry or dense forests, as well as forest galleries, from Senegal to southern Nigeria. In Benin, it is present from the coast to the southern region. Antibiotic properties are attributed to its roots, leaves and barks used alone or in combination with other plants in the treatment of malaria, coughs, bronchitis, diarrhea, palpitations, dysmenorrhea, ulcer, anemia, hemorrhoids [17, 18]. Concerning *P. biglobosa*, it is a Mimosoideae [19] present in tropical Africa between 3 ° and 15 ° North. It grows in tropical regions with high rainfall but also in arid environments. In Benin, it is an almost ubiquitous tree occuping the fifth position among the most used plants in traditional medicine [20]. Finaly, *T. indica* is a detarioideae up to 25 meters tall [21] growing very well in semi-humid or arid regions [22]. With well kwon laxative and carminative effects, its organs are used in the treatment of a wide range of pathologies such as toothaches [23], bacterial infections, malaria [24].

Material and Methods

Leaves and bark of the plants were collected in three localities of Benin (Abomey-Calavi, Cotonou and Parakou). Samples were formally identified at the Benin National Herbarium under the voucher numbers AA 6727 / HNB, AA 6728 / HNB and AA 6729/ HNB for *Dialium guineense Willd*., *Parkia biglobosa* (Jacq.) *R. Br. Ex Benth*., and *Tamarindus indica L.* respectively.
RAW 264.7 macrophage assay has been used to monitor the inhibitory effects of the extracts on the low-grade inflammatory cascade (RAW 264.7, Sigma-Aldrich, N° P6110401, Lot. 09I006). Phototoxicity was assessed using Balb /c 3T3 mouse fibroblasts (3T3-L1) (ATCC, United States, ATCC® CL-173™, N° P6110401, Lot. 09I006). DMEM (Dulbecco's Minimum Essential Medium) with stable L-glutamine supplemented with Penicillin 100 IU/ml, streptomycin 100 µg/ml, and 10% of inactivated calf serum served as culture medium. It was freshly prepared with a pH of 7.2. Standardized horse blood was used for KRL test. All test materials were diluted in dimethyl sulfoxide (DMSO, Sigma-Aldrich).

Anti-inflammatory test and cell viability

This *in vitro* anti-inflammatory assay is based on the ability of macrophages to generate a strong inflammatory response when stimulated with antigens. Mouse immortalized macrophages (RAW 264.7 cell line) were stimulated by *E. coli* LPS and exposed to the extracts for 24 hours. At the end of the incubation period, NO production was evaluated indirectly by measuring the accumulation of nitrite/nitrate in the culture medium using a spectrophotometric method based on the Griess reaction. Negative and positive controls were DMSO and Dexamethasone (1, 5, 10, 50 and 100 µM) respectively. Cell mitochondrial respiration measurement allowed to assess cell viability. Inhibition of NO release and inhibition of cell viability were expressed as percentages as compared to the negative controls:

Percentage of NO release (%) = 100 x ($OD_{\text{test well}} - OD_{\text{blank}}$) / $OD_{\text{control}} - OD_{\text{blank}}$

Percentage of Cell viability (%) = 100 x ($OD_{\text{test well}} - OD_{\text{blank}}$) / $OD_{\text{control}} - OD_{\text{blank}}$

The concentrations of the test material causing respectively a 50% decrease of NO release ($IC_{50\text{-NO release}}$) and a 50% decrease of cell viability ($IC_{50\text{-cell viability}}$) were calculated using the software Table curve Version 2.0. The anti-inflammatory ratio corresponded to the ratio between the anti-inflammatory activity and the toxicity. It was expressed as follows:
Anti-inflammatory ratio = $IC_{50\text{-cell viability}} / IC_{50\text{-NO release}}$

Phototoxicity and cytotoxicity measurement

The *in vitro* 3T3 NRU phototoxicity test was used to evaluate phototoxicity of the six ethanolic extracts measuring relative reduction in viability of cells exposed to them in presence of light versus absence of light. The Photo-Irritation-Factor (PIF) was calculated with concentrations (obtain by the software Phototox Version 2.0.) of the test material causing a 50% release of the preloaded Neutral Red without irradiation (IC_{50} -Irr) and with irradiation (IC_{50} +Irr) as compared to the control culture using the following formula:

$PIF = IC_{50}$ (-Irr) / IC_{50} (+Irr)

On the other hand, the degree of membrane damage (i.e. the increase of released Neutral Red) was measured by a fluorescence-luminescence reader Infinite M200 Pro (TECAN). The Optical Density (OD) of each well was read at 540 nm. The results obtained for wells treated with the test material were compared to those of untreated control wells (HBSS, 100% viability) and converted to percentage values. Neutral Red desorbed solution serves as blank.
The percentages of cell viability were calculated as:

$$\text{Viability (\%)} = \frac{\text{Mean OD of test wells} - \text{mean OD of blanks}}{\text{Mean OD of negative control} - \text{mean OD of blanks}}$$

Kit Radicaux Libres (KRL) test

Evaluation of the overall resistance of standardized horse blood submitted to oxygen reactive species attack was made in absence versus presence of our extracts. The overall resistance of the control blood to the radical attack in the presence or absence of our products was expressed by the time at which 50% of the blood cells were lysed (T1 / 2 in minutes). The antiradical efficacy of the products was then expressed as a percentage of overall potential for antiradical defense of the control blood (% T1 / 2 of the control blood). The results were standardized in Trolox equivalents (water soluble analogue of vitamin E) and in equivalents Gallic acid (phenolic acid).

Gas chromatography and mass spectrometry analysis

100µl of each filtered extract were dried with nitrogen, then 100µl of BSTFA were added and the whole is incubated at 70 °C for 20 min. 2µl were injected for GC-MS analysis. The analyses were carried with a GC-MS apparatus consisting of a chromatograph Shiwazu QP, a mass spectrometer GCMS-QP2010S equipped with an electronic impact ion source and a quadrupole analyzer type, a GC Solution acquisition software as well as banks (NIST, WILEY). The compounds were separated using a DB-1MS capillary column (30 m x 0.25 x mm internal diameter x 0.25 µm film thickness from JW Scientific) which temperature limits were between -60 °C and 350 °C. The mobile phase was helium. Pressure in the column

was 3psi and the flow rate was 1.59 mL /min. The injector temperature (Inlet) was 280 °C, interface temperature was 280 °C, the source and the quadrupole at 150 °C. Temperature gradient was programmed as follows: 1 min at 60 °C, then increasing from 100 to 260 °C at 4 ° / min, then 30 min at 260 °C. The total elution time was 82 min. We focus on compounds with high retention time (>60min) in this work.

Results and Discussion

Results of anti-inflammatory activities and phototoxicity are reported in Tables 1&2 below.

Table 1: Anti-inflammatory, cytotoxic activity and anti-inflammatory ratio of the extracts

	NO release IC_{50} (µM or µg/mL)	Cell viability IC_{50} (µM or µg/mL)	Anti-inflammatory ratio
Dialium guineense barks	0.15 ± 0.01	68.74	458.2
Dialium guineense leaves	0.22 ± 0.05	43.17 ± 8.2	196.2
Parkia biglobosa barks	77.78 ± 9.4	>100	>1.3
Parkia biglobosa leaves	0.62 ± 0.08	>100	>161
Tamarindus indica barks	0.28 ± 0.02	48.91 ± 10.1	174.6
Tamarindus indica leaves	>100	>100	-
Dexamethasone	4.31 ± 1.45 µM	163.22 ± 24.96 µM	37.87

Extracts of *Dialium guineense* (both leave and stem), those of leaves of *Parkia biglobosa* and barks of *Tamarindus indica* exert higher anti-inflammatory activity than Dexamethasone, the positive control (Fig. 1). Barks of *Dialium guineense* extract showed the best anti-inflammatory activity (Table 1). Sitosterol (0.77%) and lupeol acetate (0.55%) are triterpenoids we found and reported for the first time in this extract. Those two molecules have been reported to have interesting anti-inflammatory activity [25, 26]. In association with phenolic compounds contained in this extract [27-29] they could explain our result and support the use of *Dialium guineense* by traditional healers. This extract has a high photo-irritation factor (Table 2). Therefore, it appears suitable to investigate the three others as anti-inflammatory drugs for cutaneous use. The leaves of this plant for example have half of the anti-inflammatory activity compared to the barks but is not phototoxic. It has higher tenor of sitosterol (3.5%) and lupeol acetate (1.7'%) which seems to play a key role in phototoxicity as they are also more abundant in leaves of *P. biglobosa* (2.02% and 4.41%) and barks of *T. indica* (0.80% and 1.53%) (Tables 7&8).

Table 2: Phototoxicity results

	Toxicity in non-irradiated cells IC_{50}(-irr) (µM)	Toxicity in irradiated cells IC_{50}(+irr) (µM)	PIF	Phototoxicity
Dialium guineense barks	75.31 ± 8.54	8.97 ± 1.54	8.39	phototoxic
Dialium guineense leaves	47.56 ± 9.64	36.87 ± 4.21	1.28	Not phototoxic
Parkia biglobosa barks	130.85 ± 9.21	202.78 ± 9.47	0.64	Not phototoxic
Parkia biglobosa leaves	123.61 ± 2.21	66.33 ± 1.34	1.86	Not phototoxic
Tamarindus indica barks	53.64 ± 8.88	49.94 ± 9.54	1.07	Not phototoxic
Tamarindus indica leaves	8.76 ± 9.47	>100	-	Not phototoxic
Chlorpromazine	46.33 ± 2.96	1.04 ± 0.25	44.54	phototoxic

-irr: not irradiated, +irr: irradiated

It clearly appears on Figure 1 that, despite its great anti-inflammatory activity, only *Dialium guineense* barks extract exerted phototoxicity in our study. Leaves extract of this plant have lower anti-inflammatory activity than barks but are not phototoxic. This can be due to the difference of abundance in terpenoids like sitosterol (0.78% vs trace) and lupenone (0.42% vs trace). *Parkia biglobosa* leaves and *Tamarindus indica* barks extracts also aren't phototoxic and have higher amount of sitosterol, lupeol or lupenone than Dialium guineense barks extract.

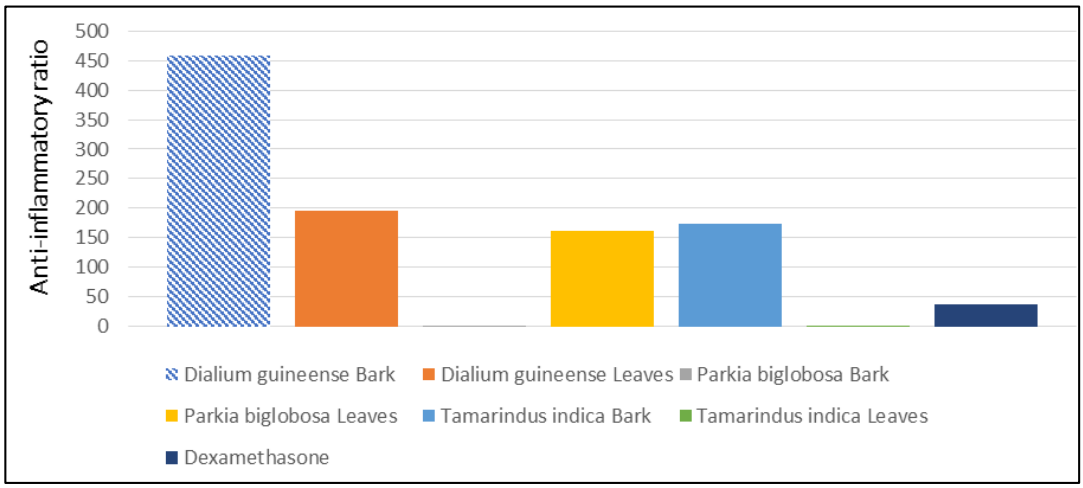

Fig 1: Anti-inflammatory activity and phototoxicity of ethanolic extracts

Table 3: antioxidant activity of the extracts

Concentrations (mg/L) *	% increase of half-hemolysis time ($T_{1/2}$) of control blood					
	Dialium guineense		*Parkia biglobosa*		*Tamarindus indica*	
	Barks	leaves	Barks	leaves	Barks	leaves
0	0.00	0.00	0.00	0.00	0.00	0.00
1	5.64	3.64	6.94	7.30	21.87	10.93
2	15.85	5.39	9.83	14.08	38.19	20.34
5	36.70	13.63	19.65	30.63	73.14	45.10
10	61.33	20.98	30.72	59.86	118.05	75.95
20	82.93	36.80	47.83	109.48	265.36	117.55
50	149.18	73.47	106.94	266.33	451.18	224.66
100	438.64	85.30	277.53	181.70	342.87	338.91

* Concentration in mg of extract per liter of reaction medium

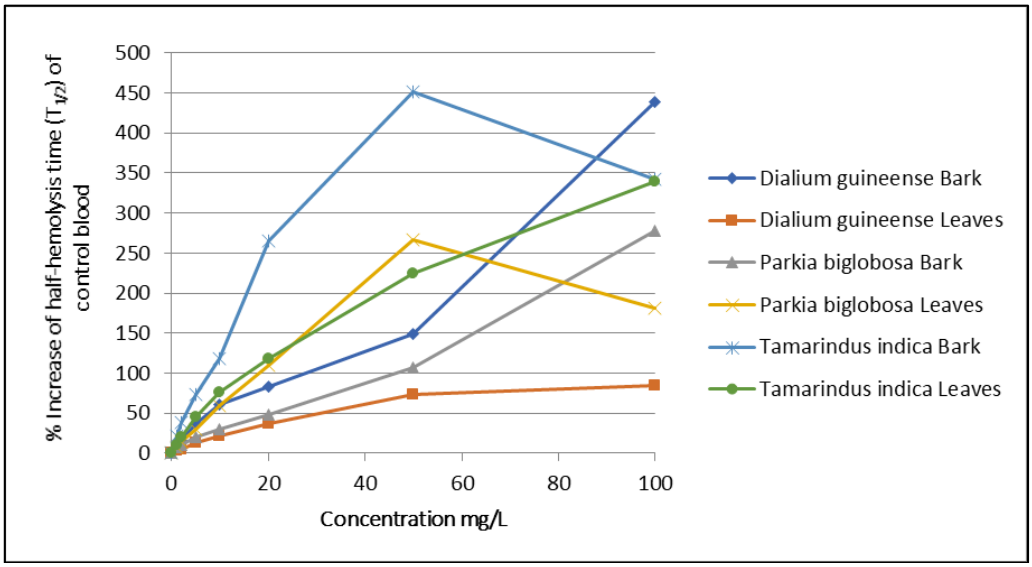

Fig 2: Antioxidant activity of plant extracts

KRL test results indicate that all the tested plant extracts have a dose-dependent antiradical capacity (Fig. 2) and this antioxidant power is strongly increasing in a concentration range of 0 to 20 mg/L (Table 3). At 20 mg/L, leaves of *Parkia biglobosa*, leaves and barks of *Tamarindus indica* extracts have remarkable results. They increased the resistance of the control blood to a radical attack up to 109.48, 265.36 and 117.55% respectively (Table 3). Also, those extracts were at least as effective as Trolox and gallic acid.

Table 4: Anti-radical power of extracts vs Trolox (Equivalent Trolox mg / g product)

Concentrations (mg/L) *	Antioxidant activity of plant extracts of Benin: Equivalents mg of Trolox/g of extract					
	Dialium guineense		*Parkia biglobosa*		*Tamarindus indica*	
	Barks	leaves	Barks	leaves	Barks	leaves
0	0.00	0.00	0.00	0.00	0.00	0.00
1	2157,55	1391,50	2654,27	2789,66	8360,74	4177,09
2	3028,74	1029,43	1879,17	2689,96	7297,49	3887,81
5	2805,47	1041,60	1502,12	2341,37	5591,19	3447,83
10	2344,19	802,04	1174,09	2287,81	4512,03	2902,86
20	1584,90	703,19	914,09	2092,13	5071,12	2246,38
50	1140,39	561,58	817,49	2035,83	3448,87	1717,37
100	1676,51	326,03	1060,73	694,48	1310,49	1295,33

Table 5: Anti-radical power of extracts vs Gallic acid (Equivalent Trolox mg / g product)

Concentrations (mg/L) *	Antioxidant activity of plant extracts of Benin: Equivalents mg of Gallic acid/g of extract					
	Dialium guineense		*Parkia biglobosa*		*Tamarindus indica*	
	Barks	leaves	Barks	leaves	Barks	leaves
0	0.00	0.00	0.00	0.00	0.00	0.00
1	1027,26	662,53	1263,76	1328,22	3980,74	1988,81
2	1442,05	490,14	894,72	1280,75	3474,50	1851,08
5	1335,75	495,93	715,19	1114,78	2662,09	1641,59
10	1116,12	381,87	559,01	1089,28	2148,28	1382,12
20	754,61	334,80	435,22	996,11	2414,48	1069,55
50	542,96	267,38	389,23	969,31	1642,08	817,68
100	798,22	155,23	505,04	330,66	623,96	616,74

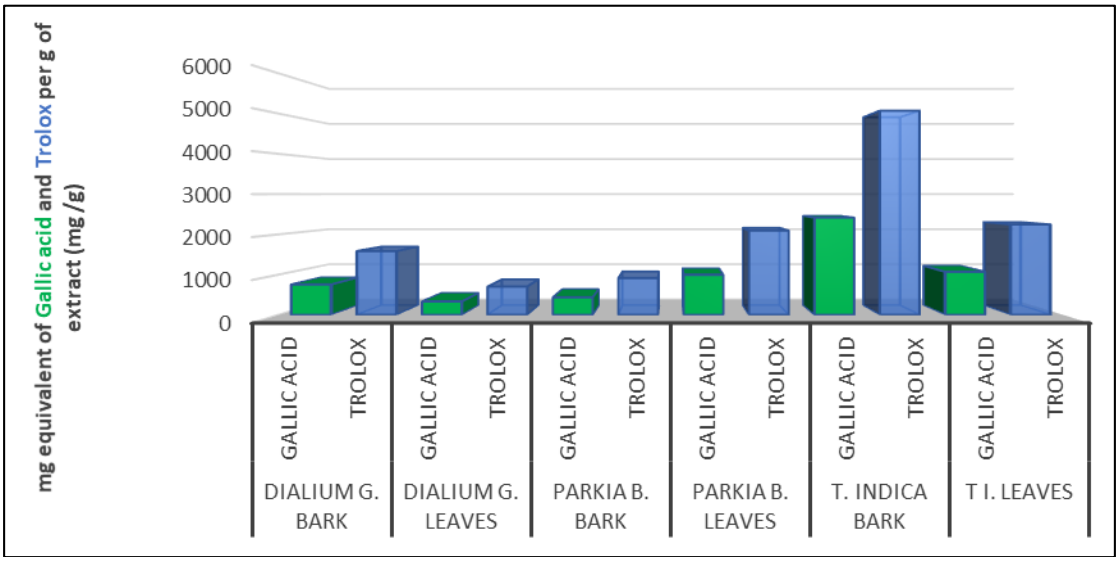

Fig 3: Comparison of the antioxidant power of the extracts with Gallic acid and Trolox at a concentration of 20 mg/L

As shown in Fig. 3, *Tamarindus indica*' s barks ethanolic extract is particularly interesting: it is five times more active than Trolox (Table4) and 2.5 times more active than gallic acid (Table 5). Lupeol (0.83%), amyrin (0.65%) and lupeol acetate (1.53%) were detected in this extract by GC-MS analysis (Table 8). Those molecules were reported in the plant [30] and their free radical scavenging property is well known [31-33]. Leaves extract of this plant also showed high free radical scavenging activity [34, 35].

Chemical compounds identified by GC-MS in the extracts are summarized below. Identification was based on direct comparison and at least 80% matching of the retention times and mass spectral data with those in the National Institute of Standards and Technology (NIST) and WILLEY libraries. DPPH free radical scavenging test of the methanolic extract of *Dialium guineense* leaves showed a concentration-dependent antioxidant activity supposed to be due to the phenolics compounds [36]. Leaves ethanolic extracts of *Parkia biglobosa* showed an antioxidant activity of 109.48% according to the KRL test. It contains lupenone (2.59%), β-Amyrin (1.05%), Sitosterol (2.02%) and lupeol acetate (4.41) all known for biologic activities. Other studies reported similar antiradical activity of the barks according to Diphenyl Picryl Phenyl Hydrazine (DPPH) test: 87.68% for aqueous extract [37].

Table 6: Triterpenoids identified in *Dialium guineense* by GC-MS

		Leaves	Stem-barks
RT	**Compounds**	5.92 %	1.48 %
61.774	Free Sitosterol	0.78	trace
62.337-62.442	Stigmasterol TMS	0.26	0.2
63.086	Lupenone	0.42	-
63.794-63.873	Lupeol	trace	trace
64.603-64.838	Sitosterol TMS	2.72	0.77
66.108-66.433	Lupeol acetate	1.74	0.51

RT: Retention time

Sitosterol and lupeol acetate are reported here for the first time in those extracts. Despite their low amount, we suppose they contribute to the anti-inflammatory role and the antioxidant activities of the extracts as reported by several papers [38, 25, 33].

Table 7: Triterpenoids identified in *Parkia Biglobosa* by GC-MS

		Leaves	Stem-barks
RT	**Compounds**	**10.44%**	**7.31%**
60.665	Taraxerone	-	**0.66**
62.372-62.442	Stigmasterol TMS	0.37	0.72
62.883-63.086	Lupenone	2.59	1.14
63.840-63.873	Lupeol	trace	trace
64.457	beta-Amyrin TMS	1.05	-
64.636	beta Sitosterol	-	1.18
64.838	Sitosterol TMS	2.02	-
66.196-66.433	Lupeol acetate	4.41	3.61

Beta-Amyrin is present in *Parkia Biglobosa* leaves extract which is as active as gallic acid and two times more active than Trolox (Fig. 3). It is well kwon for its antioxidative and anti-inflammatory activities [39, 40].

Table 8: Triterpenoids identified in Tamarindus indica by GC-MS

		Leaves	Stem-barks
RT	**Compounds**	**4.04%**	**3.81%**
62.25	**Alpha-Amyrin TMS**	-	**0.23**
62.316 & 62.385	Stigmasterol TMS	trace	trace
62.869 & 63.023	Lupenone	1.75	trace
63.817	Lupeol	-	0.83
64.345	beta-Amyrin TMS	-	0.42
64.612	beta-Sitosterol	-	0.80
64.724	Sitosterol TMS	1.07	-
66.138 & 66.219	Lupeol acetate	1.22	1.53

Lupeol, beta amyrin and beta sitosterol could to be involved in the good antioxidant and anti-inflammatory activity of Tamarindus indica barks extract. This extract is five times more active than Trolox and two times more active than gallic acid (Fig 3).

Conclusion

In the present study, ethanolic extracts of leaves and barks of *Dialium guineense*, *Parkia biglobosa* and *Tamarindus indica*, showed interesting antioxidant and anti-inflammatory properties. Lupeol acetate and Sitosterol were reported for the first time in *Dialium guineense* barks. Lupenone, amyrin, stigmasterol and taraxenone were also identified in the extracts and could explain their biological activity. The amounts of those compounds and their synergy also seem to play a key role in the biological activities. Except barks

extracts of *Dialium guineense* which is phototoxic, the other extracts can be investigate as cosmetic agents.

Abbreviations
DMSO: Dimethyl sulfoxide
DPPH: Diphenyl Picryl Phenyl Hydrazine
KRL: Kit Radicaux Libres
LPS: Lipopolysaccharide
PIF: Photo-Irritation-Factor

Declarations

- **Ethics approval and consent to participate:** Not applicable.
- **Consent to publish:** Not applicable
- **Availability of data and materials:** All data generated or analyzed during this study are included in this published article (However, raw data are available from the corresponding author on reasonable request).
- **Competing interests:** "The authors declare that they have no competing interests."
- **Funding:** France and Benin governments
- Authors' Contributions

S.M.G. is corresponding author, PhD student working on the subject. He carried out most of the test and wrote this manuscript. S. A. preformed KRL tests, figures and tables. L. A. make GC-MS analyzes. C.D.G. performed anti-inflammatory tests. M.R. make extractions and assisted L.A for GC-MS analyzes. E.D.A. is PhD supervisor and direct and correct this manuscript. P.P. is co- Supervisor of thesis. He also direct and correct the manuscript. D.C.S is member of thesis committee and he correct the manuscript.

All authors read and approved the final manuscript for submission. They have no conflict of interest to declare.

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