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Lignans from of the seeds of *Brochoneura acuminate* (Myristicaceae) and the antioxidant and wound healing properties of the methanol extract

Marie Claire Raharisoa, Claudine Aimée Rasolohery, Nantenaina Tombozara, David Ramanitrahasimbola, Solofoniaina Andriantiaray Razafimahefa, Bernard Bodo and Marcelle Rakotovao

Abstract
The seeds of *Brochoneura acuminata* (Lam.) Warb. (MYRISTICACEAE), endemic to Madagascar, are used in Malagasy traditional medicine for their wound healing virtue in the South-Eastern part of the island. Two lignans named Otobanone (1) and 1-hydroxyotobain (2) were isolated for the first time from the methanol extract. Their chemical structures were established using MS and NMR technics. The free radical DPPH scavenging assay was used to determine the antioxidant capacity of the methanol extract. The wound healing effect of the methanol extract was assessed on the rat dorsal created experimental wound. The methanol extract showed a potent scavenging capacity with an IC50 value of 105.31 ± 0.24 µg / mL. Topical application of both 2% (w/w) methanol extract of the seeds of *B. acuminata* and OFM® ointments in excision wound model significantly increased ($p < 0.001$) wound contraction rate at the 3rd, the 6th and the 9th day and shortened the epithelization time compared with control treatment. These results contribute to the chemotaxonomy of the Myristicaceae family and support the traditional uses of *B. acuminata*.

Keywords: *Brochoneura acuminata*, Myristicaceae, antioxidant, healing activity, lignans, otobain

1. Introduction

Plants remain the first source of new drugs and discovery of new molecules. The development of new drugs is taking place in various scientific and medical research settings (Chominot, 2000) [1]. Since years, plants have been used in traditional medicine to treat a large range of human diseases (Sofowora *et al.*, 2013; Dawid- Pać, 2013) [2, 3]. Leaves and barks are generally prepared in infusion for internal use and oil and/or extract from the fruits or seeds is rather used topically (Dzoyem *et al.*, 2014) [4]. Madagascan flora biodiversity is well- known by its high rate of endemicity estimated at 85% from 12,000 to 14,000 species (Rasoanaivo *et al.*, 1992) [5]. The majority of Malagasy people still rely on traditional medicine to take care of their health due to the unaffordable price of conventional drugs.

The Myristicaceae family includes at about 300 species. Among the 16 genera of this family, the genus *Brochoneura* is endemic to the littoral rainforests along the eastern coast of Madagascar consisting on three species including *Brochoneura acuminata* (Lam.) Warb., *B. vouri* (Baill.) Warb. and *B. madagascariensis* Warb. (Schatz, 2001; Cadotte at *al.*, 2002; Figueiredo et Smith, 2020) [6, 7, 8]. *B. acuminata* is a tree (10 – 15 m tall) with elliptical and obovate evergreen leaves. The inflorescence is entirely strewn with a very short and ferruginous purulence where the flowers are grouped. The fruits are overglobulous, irregularly obovate- compressed, 5 cm long and wide, apiculate. The seed is enveloped in a ceraceous and crustacean aril with a very sweet smell (De la Bâthie, 1952; Sauquet, 2004) [9, 10]. *B. accuminata* is known as Madagascar nutmeg and by other local names such as 'Rara', 'Hafotra', 'Vory'or 'Rarahoraka'. The seeds of this specie are used by the local population as the principal ingredient in the preparation of the local cosmetics and for the treatment of wound and sore (De la Bâthie, 1952; Doyle *et al.*; 2004; Edmond. *et al.*, 2005) [9, 11, 12]. The leaves are used to treat injury, scabies and abcess (Razafindraibe *et al.*, 2013) [13]. The latex from the bark of *B. acuminata* is used to treat fungal infection and stomachaches (Sattle r and Razafindravao, 2017) [14]. According to our best knowledges, neither pharmacological nor chemical studies have been undertaken on this specie. Moreover, several Madagascan and other Myristicaceae species were reported for their uses in the treatment of infectious diseases, scabies and scarring (Gottlieb, 1979; Walker and Walker, 1981; Sauquet, 2003 and 2004;

Sauquet *et al*, 2003) [10, 15, 16, 17, 18], but chemical and pharmacological studies remain insufficiently exploited.
Recently, there is a growing interest in natural antioxidants due to their ability to delay the oxidation of other molecules and inhibit the initiation or propagation of oxidizing chain reactions by free radicals, protecting the human body and reducing oxidative damages that may significantly cause the development of many chronic diseases, as diabetes, cancer and cardiovascular diseases (Donno *et al.*, 2018, Shiekh *et al.*, 2020) [19, 20]. They are also used in the enhancement of wound healing by accelerating the tissue remodeling (Xian *et al.*, 2020) [21].
In this work, we reported the isolation of two lignans, the major compounds, from the seeds of *B. accuminata* as well as the antioxidant capacity of the seed methanol extract compared to gallic acid and the wound healing effect of the seed methanol extract compared to the reference product OFM® (Madécassol).

2. Methods

2.1 Plant materials

The seeds of B. acuminata were harvested in December 2016, from the dense humid forest of the Ranomafana National Park, located at 450 Km in the south-east of Antananarivo during the fruiting period. A voucher specimen was identified by Dr Hanta Vololona RAZAFINDRAIBE and deposited at fauna and flora department of the Tsimbazaza Zoological Park (RHKV-025/2016), Antananarivo, Madagascar.

2.2 Extraction and phytochemical screening

Plant materials were dried in a cool and airy place, away of sunlight, before to be ground into fine powder. 100 g of raw materials was extracted by the soxhlet for 4 hours with methanol. The resulting solution was then evaporated under reduced pressure, at 40 °C using a rotary evaporator (Buchi R114). The phytochemical screening was carried out according to the protocols described by Tombozara *et al.* (2017) [22].

2.3 Isolation and structure elucidation

Vacuum liquid chromatography was carried out for the fractionation of the methanolic extract. 1 g of the methanol extract was deposited on a column of 60 Merck silica gel with a particle size of 40-60 Å and eluted with a mixture of dichloromethane and methanol with an increasing polarity starting 100 – 0 to 0 – 100 (v/v) giving 178 fractions which were pooled on the basis of their TLC profile affording six fractions (F1 - F6). Repeated column chromatography of the fraction F2 with a mixture of dichloromethane, ethyl acetate and methanol (5/3/1; v/v/v) (isocratic elution) led to the isolation of compound 1 (the major compound from F2, 17 mg) which was purified using preparative TLC eluted with a mixture of dichloromethane, ethyl acetate and methanol (5/3/1; v/v/v). Repeated column chromatography of the fraction F3 with a mixture of dichloromethane, ethyl acetate and methanol (5/3/2; v/v/v) (isocratic elution) led to the isolation of compound 2 (the major compound from F3, 19 mg) which was purified using preparative TLC eluted with a mixture of dichloromethane, ethyl acetate and methanol (5/3/2; v/v/v).
The spots were visualized under UV light (254nm and 365nm) then revealed by the methanolic solution of sulfuric vanillin in order to verify the purity of the compounds. Compound 1 and 2 structures were established using a mass spectrometer (Bruker Compass Maxi II EDT) in ESI positive mode and NMR spectrometer (Bruker 500, Germany) for the 1H and 13C NMR registration in CDCl3 and confirmed by the literature reviews.

2.4 Antioxidant activity

2.4.1 Autobiography assay

The autobiography assay is based on the protocol described by Dieng *et al*. (2015) slightly modified. The methanol extract or gallic acid (reference antioxidant) were dissolved in methanol and placed on a plate of silica gel. Silica plate was eluted a mixture of dichloromethane, ethyl and methanol (2/5/3, v/v/v) acidified with 2 drops of acetic acid. At the end of the development, DPPH methanolic solution (2mg/mL) was sprayed on the dried plate. The presence of antioxidant compounds was revealed by light yellow areas on a purple background after an optimal time of 30 minutes.

2.4.2 DPPH free radical scavenging assay

The methanol extract free radical removal capacity was evaluated using the radical DPPH assay, described by Tombozara *et al*. (2020) slightly modified. Briefly, 25 µL of the methanol extract with a concentration ranging from 5 to 125 µg/mL was added to 175 µL methanol DPPH solution (0.25 mmol/L) in a 96-well microplate and incubated at room temperature for 30 min. Methanol was used as blank and a methanol solution of DPPH was used as negative control. Gallic acid with a concentration ranging from 1.25 to 40 µg/mL was used as antioxidant reference compound. Results were expressed as means of inhibiting concentration (IC), calculated using the following equation: IC (%) = 100 x (A0 – A1) / A0 where A0 and A1 are the values for the absorbance of the negative control and sample at 517 nm. The IC50 (inhibition concentration at 50%) values of extract and gallic acid were calculated by linear regression (concentration of tested sample or gallic acid vs average percent of scavenging capacity by three replicates).

2.5 Wound healing effect of the methanol extract

The *in vivo* wound healing effect of the methanol of the seeds of B. acuminate consist on the creation of an experimental wound on previously anesthetized rat and the treatment of this wound with the ointment preparations to be tested according to the protocol described by Sagliyan *et al*. (2010) [24].

2.5.1 Animals

Wistar adult male rats (weighing 200 ± 20 g and aged between 2 and 3 months), kept under controlled conditions (12 h dark and 12 h light cycle, 25 ± 2°C temperature and 50 ± 10% humidity) at the Institut Malgache de Recherches Appliquées animal house, were used. The animals received a standard food pellet (1420, Livestock Feed Ltd.) and remained fasting for one night before the experiment. The experiment was approved by the local ethic committee.

2.5.2 Realization and treatment of surgical wounds

Adult rats were divided into three groups of 9 animals and subjected daily to a standard diet (25 g/Kg) and tap water throughout the study. A 20 mm diameter circular incision wound (around 300 mm2 circular area and 2 mm depth) was surgically excised on the skin of the dorso-lumbar region of the anesthetized rats with 10 mg/kg ketamine after shaving with an electric clipper. The excisional wounds were disinfected with 90°C ethanol after the surgery. Group I received a daily treatment with an ointment consisting of vaseline (100%) and served as a control, however, group II

and III were treated immediately with a 0.40 g of ointment consisting of the methanol extract – vaseline (2%; w/w) and OFM® (Madécassol) respectively. Animals are then placed in individual cages. The wound dressing was done daily due to once a day with an amount of ointment about 0.40 g for each group until the total wound recovering.

2.5.3 Assessment of the scar process

The dimensions of the excisional wounds were measured after the excision process and each 3 days until complete healing using a digital caliper. Percent shrinkage or progression of wound contraction was calculated using the following formula given by Lodhi *et al*. (2006) and epithelization period was registered:

$$Pr.D_n = \frac{M.D_1 - M.D_n}{M.D_1} \times 100$$

$Pr.\ Dn$: Percentage of the wound reduction at the nth day
$M.\ D1$: Wound size on the 1st day
$M.\ Dn$: Wound size on the nth day

2.6 Statistical analysis

All the experiments were carried out at least in triplicate and the results expressed as mean ± standard deviation (S.D.) or standard error of the mean (S.E.M.). Data were statistically analyzed using Student's *t*-test and one- way analysis of variance (ANOVA) followed by Tukey Post Hock test using an IBM SPSS Statistics 20 software.

3. Results and discussion

3.1 Extraction and phytochemical screening

The extraction on 100 g of raw materials led to 25.6 g of brown pasty methanol extract (25.6% yield). The phytochemical screening of the methanolic extract of *B. acuminata* seeds showed the presence of alkaloids, flavonoids, steroids, terpenoids, phenolic compounds, anthraquinones and polysaccharides.

3.2 Isolation and structural analysis

The isolation procedures on the seed methanolic extract of *B. acuminata* afforded to two compounds (figure 1). Their chemical structures were identified according to their 1H and 13C NMR and ESI-MS (+) spectra. The HR-ESI-MS of compound **1** showed a peak at m/z 339.1223 (*339.1232 calculated mass*) corresponding the pseudo molecular ion [M+H]+ leading to the formula C20H1905+. On the basis of this information, the formula of compound **1** is C20H1805 with twelve degrees of insaturation. The 1H NMR spectrum revealed the presence of two secondary methyl groups at 0.98 (3H-10, *d*, J 6.5 Hz) and 1.19 (3H-9, *d*, J 6.7 Hz) ppm, two methylenedioxyphenyl groups at 5.67 (2H-11, *d,* J 1.4 Hz) and 5.91 (2H-7', *d*, J 1.4 Hz) ppm, two methine groups at 2.36 (H-2, *dq*, J 11.8 Hz; 6.7 Hz) and 1.93 (H-3, *ddq,* J 11.8 Hz; 9.8 Hz; 6.5 Hz) ppm, one oxymethine group at 3.69 (H-4, *d*, J 9.8 Hz) and five aromatic protons suggesting the presence of two aromatic rings binding with two heterocyclic rings. The two last insaturation were attributed to a hexacyclic ring and a carbonyl group confirmed by the 13C NMR spectrum at 198.6 ppm (C1). The peak data of compound **1** were as follow:

Compound 1: White needle crystal. ESI-MS (+): m/z 699 [2M+Na]+, 361 [M+Na]+, 339 [M+H]+. 1H NMR (600 MHz, CDCl3): 2.36 (H-2, *dq*, J 11.8 Hz; 6.7 Hz), 1.93 (H-3, *ddq*, J 11.8 Hz; 9.8 Hz; 6.5 Hz), 3.69 (H-4, *d*, J 9.8 Hz), 6.77 (H-7, *dd*, J 8.3 Hz; 0.7 Hz), 7.63 (H-8, *d*, J 8.3 Hz), 6.53 (H-2', *d*, J 1.7 Hz), 6.69 (H-5', *d*, J 7.9 Hz), 6.59 (H-6', *dd*, J 7.9 Hz; 1.7 Hz), 1.19 (3H-9, *d*, J 6.7 Hz), 0.98 (3H-10, *d*, J 6.5 Hz), 5.67 (2H-11, *d,* J 1.4 Hz), 5.91 (2H-7', *d*, J 1.4 Hz). 13C NMR (150 MHz, CDCl3): 198.6 (C1), 47.5 (C2), 43.7 (C3), 49.1 (C4), 126.8 (C4a), 145.1 (C5), 151.8 (C6), 107.5 (C7), 122.2 (C8), 127.8 (C8a), 137.6 (C1'), 108.7 (C2'), 147.5 (C3'), 146.0 (C4'), 107.8 (C5'), 122.0 (C6'), 12.5 (C9), 17.4 (C10), 101.6 (C11), 100.9 (C7'). The structure was established as Otobanone or 1-oxo-otobain after comparison with the literature (Kuo *et al.*, 2001) [26]. Otobanone was already isolated from the heartwood of *Chamaecyparis obtusa var. formosana* (Kuo *et al.*, 2001) [26].

The HR-ESI-MS of compound **2** revealed a peak at m/z 323.1273 (*323.1283 calculated mass*) corresponding the pseudo molecular ion [M–H2O+H]+ leading to the formula C20H1904+. On the basis of this information, the formula of compound **2** is C20H2005 with eleven degrees of insaturation. The 1H NMR spectrum is very similar to compound **1**. The differences between the two spectra are the presence of the oxymethine group at 4.347 (H-1, *d,* J 10.0 Hz) and the singlet signal at 7.240 ppm corresponding to a hydroxyl group. These differences are confirmed by the 13C NMR spectrum with the shifted signal of the carbonyl from compound **1** at 198.6 ppm to 74.6 ppm for the compound **2**. The peak data of compound **2** were as follow:

Compound 2: White crystal. ESI-MS (+): m/z 323 [M–H2O+H]+. 1H NMR (600 MHz, CDCl3): 7.24 (OH, *s*), 4.34 (H-1, *d*, J 10.0 Hz), 1.38 (H-2, *ddq*, J 11.0 Hz; 10.0 Hz; 6.4 Hz), 1.52 (H-3, *ddq,* J 11.0 Hz; 9.9 Hz; 6.5 Hz), 3.48 (H-4, *d*, J 9.9 Hz), 6.72 (H-7, *dd*, J 8.2 Hz; 0.4 Hz), 7.14 (H-8, *d*, J 8.2 Hz), 6.51 (H-2', *d*, J 1.7 Hz), 6.67 (H-5', *d*, J 7.8 Hz), 6.58 (H-6', *dd*, J 7.8 Hz; 1.7 Hz), 1.16 (3H-9, *d*, J 6.4 Hz), 0.95 (3H-10, *d*, J 6.5 Hz), 5.89 (2H-11, *d,* J 1.4 Hz), 5.64 (2H-7', *d*, J 1.4 Hz). 13C NMR (150 MHz, CDCl3): 74.6 (C1), 44.0 (C2), 42.7 (C3), 49.6 (C4), 122.7 (C4a), 144.0 (C5), 145.6 (C6), 106.9 (C7), 118.4 (C8), 134.9 (C8a), 139.4 (C1'), 108.8 (C2'), 146.5 (C3'), 145.0 (C4'), 107.6 (C5'), 122.0 (C6'), 15.5 (C9), 16.8 (C10), 100.7 (C11), 100.7 (C7'). The structure of compound 2 was established as 1-hydroxyotobain after a deduction from the structure of compound 1.

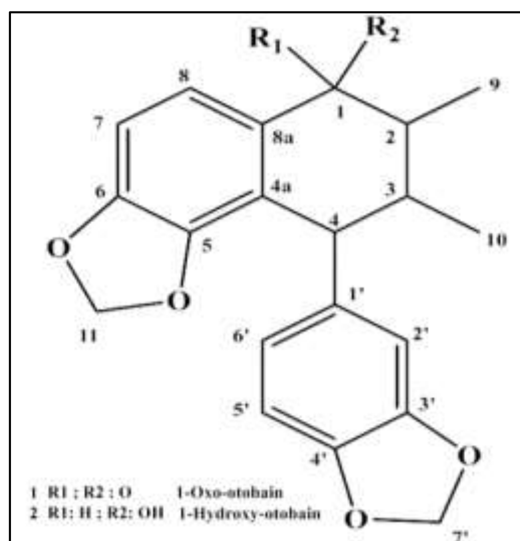

Fig 1: Chemical structure of isolated compounds

Otobain and analogues have been isolated from several species of the Myristicaceae family since the 1960s including the seeds of *Myristica otoba* and *Virola cuspidata* in occurrence the hydroxyotobain however the position of the hydroxyl group is not the same as in compound **2** (Wallace *et al.*, 1963; Blair *et al.*, 1969) [27, 28], *Virola carinata* (Gottlieb *et al.*, 1976) [29], *Virola sebifera* (Lopes *et al.*, 1982) [30], *Virola calophylloidea* (Martinez V *et al.*, 1985) [31] and *Virola elongata* (Kato *et al.*, 1990) [32] suggesting that otobain and analogues could become a marker compounds of Myristicaceae family. The isolation of Otobanone and 1-hydroxyotobain from the seeds of *B. acuminata* contribute to the chemotaxonomy of this plant family. Besides, several reports indicate the use of *B. acuminata* in several herbal preparation (Bascopea et Sternera, 2006; Yankep E. *et al.*, 1999) [33, 34] and, recently, Lamblin *et al.,* (2008) [35] have demonstrated the use of lignans in the prevention and treatment of cancers.

3.3 Antioxidant activity
DPPH is a stable free radical with a purple color in methanol solution. DPPH solution turns into yellow in the presence of scavengers following the chemical reaction DPPH• + H-A → DPPH-H + A• where H-A is an antioxidant compound; during this reaction, the absorbance decreases (Soares *et al*., 1997) [36]. The sample potential scavenging is indicated by the degree of DPPH solution discoloration when the sample contains one or more compounds able to share hydrogens acting in single or synergistic action.

3.3.1 Autobiography revelation
The qualitative free radical DPPH scavenging capacity of the methanol extract and gallic acid was done by the autobiography revelation assay. The sprayed TLC with methanolic DPPH solution is highlighted in the figure 2. The TLC showed several yellow spots at different Rf with the gallic acid at the Rf 0.71. These several yellow spots from the seed extract confirm the presence of free radical DPPH scavenger compounds and showed the probable potentiality of its antioxidant activity.

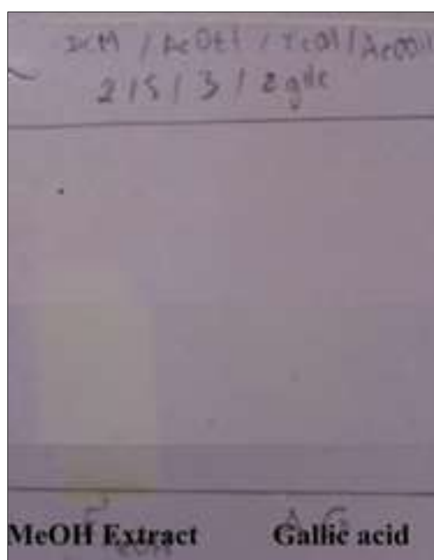

Fig 2: Autobiography of methanol extract of *B. acuminate vs* gallic acid revealed with DPPH solution (2mg/mL)

3.3.2 Quantitative free radical DPPH scavenging capacity
The IC50 of the methanol extract and the gallic acid were reported in the table 1 and illustrated on the figure 3. The antioxidant capacity of the methanol extract (105.31 ± 0.24 µg/mL) is five times lower than the gallic acid antioxidant capacity (20.40 ± 1.01 µg/mL), it does not signify that the methanol extract is not active, because compared to several extract of the endemic plants from Madagascar such as the methanol extract of the aerial parts of *Vaccinium secundiflorum* Hook. (76.07 ± 1.08 µg/mL), the methanol extract of leaf and bark of *Uapaca bojeri* Bail. (47.36 ± 3.00 and 33.32 ± 0.69 µg/mL respectively) or the methanol extract or the decoction of the aerial parts of *Lygodium lanceolathum* Devs. (24.66 ± 2.86 and 107.05 ± 3.41 µg/mL respectively), using the same protocol, the difference is not really significant (Tombozara *et al.*, 2020; Razafindrakoto *et al.*, 2020, Razafin-drabazo *et al.*, 2020) [37, 38, 39]. Moreover, phenolic compounds and flavonoids detected in the methanol extract were previously reported as great antioxidant active phytochemicals (Aksoy *et al.*, 2013; Montefusco-Pereira *et al.*, 2013; Donno *et al.*, 2016) [40, 41, 42], they could be the origin of this property.

Table 1: IC50 of methanol extract of *B. acuminata*

Sample	Equation	R2	IC50 (µg/mL)
Methanol extract	y = 0,4526x + 2,3352	0,9963	105.31 ± 0.24*
Gallic acid	y = 2,5618x - 2,2724	1	20.40 ± 1.01

Values are represented as mean ± S.D.
**p* < 0.001 *vs* gallic acid

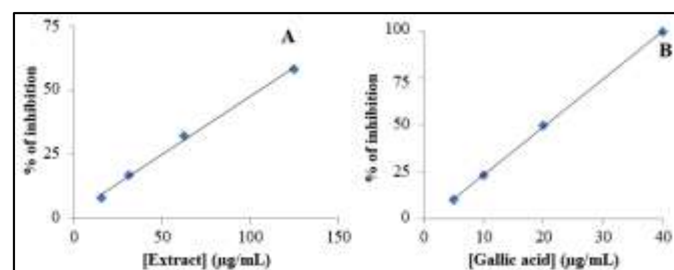

Fig 3: Free radical scavenging effect of the methanol extract of the seeds of *B. acuminata* (A) and Gallic Acid (B)

3.3 Wound healing effect of the methanol extract
The wound healing process proceeds in three overlapping phases: a gradual disappearance of inflammation (0 – 3 days), a contraction phase (3 – 12 days) and a remodeling phase (3 – 12 months). Topical application of both 2% (w/w) methanol extract of the seeds of *B. acuminata* and OFM® ointments in excision wound model significantly increased ($p < 0.001$) wound contraction rate at the 3rd, the 6th and the 9th day and shortened the epithelization time (Table 3) compared with control treatment. The wound closure achieved by the extract on the 6th post wounding day (about 60%) was almost the same to the contraction value of reference drug OFM® (about 60%) but that of negative control group was only about 22%. The 2% ointment of the methanol extract showed slightly lower rate of wound contraction (93.63 ± 1.58 %) at the 9th day than of that the OFM® (99.74 ± 0.15 %) but they are not statistically different. On the other hand, both of the methanol extract and OFM® wound contractions showed significant differences compared with the control (51.83 ± 1.63 %) at the 9th day. These results indicate the methanol seed extract of *B. acuminata* potentiality in healing wounds. Moreover, Jian et Bari (2010) [43] reported the wound healing properties of flavonoids and terpenoids are known to promote wound-healing process, mainly due to their astringent and antimicrobial activities which seem to be responsible for wound contraction and

increased rate of epithelization (Scortichini et Pia Rossi, 1991) [44].
These phyto-constituents could act synergistically affording to the effectiveness of the use of this treatment with the seed methanol extract of *B. acuminata*

Table 3: Effect of methanol extract of the seeds of *B. acuminata* ointment (2%, w/w) and OFM® (daily treatment with 0.4 g) on wound contraction and epithelization period

Day of treatment	Wound contraction (%)		Epithelization Period	
	3rd day	6th day	9th day	(Days)
Control	7.27 ± 0.32	21.54 ± 1.26	51.83 ± 1.63	16.78 ± 0.28
Methanol extract (2%)	39.81 ± 2.79*	59.46 ± 1.63*	93.63 ± 1.58*	10.11 ± 0.20*
OFM®	36.12 ± 1.10*	60.34 ± 0.87*	99.74 ± 0.15*	9.22 ± 0.22*

Values are expressed as mean ± S.E.M.
* $p < 0.001$ *vs* control
OFM®: Ody Fery Meva or Madécassol

4. Conclusion
Two lignans including Otobanone and 1-hydroxyotobain have been isolated from the first time in the seed of *acuminata* and identified using spectrometric methods. Phenolic compounds and flavonoids detected in the methanol extract could be the responsible for the antioxidant activity. The wound healing activity could be due to the synergistic action of flavonoids and terpenoids in the methanol extract. These findings contribute to the chemotaxonomy of the Myristicaceae family and confirm the claim of the traditional uses of the seeds of *B. acuminata* as wound healing.

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