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Spasmolytic Effects Of Kauranoid And Ester Of Linear Alkaloid From The Aerial Part Of Croton Borarium (Euphorbiaceae) Endemic In South Part Of Madagascar

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Abstract – The aerial part of the plant known under the vernacular name Somorombohitse (Malagasy name) and scientifically named *Croton Borarium (Euphorbiaceae)* endemic in the South part of Madagascar, is used by the local communities to treat asthma and hypertension. The biologic test result riddling of the isolated trachea of guinea pig stimulated by the acetylcholine in 210^{-2}M showed antiasthmatic activity with the CE50= 0.85±0.001µg/ml (n=6). Effet concentration of carbachol from 10^{-9}M to 10^{-3}M have been realized in absence and in presence of the hydro-ethanolic extract of *Cr. Borarium* to 0.1mg/ml, verapamil to 10µM and atropine to 10nM. The relaxing activity of hydro-ethanolic extract from 0.1 to 3 mg/ml has been executed on the isolated aorta and jejunum of rabbit stimulated with the KCl to 50nM. White the using of the extract technic by partitioned liquid-liquid to permitted localizing the active fraction (Hex/DCM: 3/1) with CI50= 0.275±0.003µg/ml (n=6).
The bioassay-guided fractionation of the active fraction using repeated silica gel column chromatography resulted in the isolation of two pure compounds active as evidenced by analytical TLC and HPLC analysis. The elucidation of pharmacologic mechanism of these active principles has showed that, to first and active principle bring into play the calcic movement through the cellular membrane of vascular and polished muscle and the second is the by way of NO intermediate. Their chemical structures of two pure compounds were determined by 1D and 2D NMR spectroscopy and spectroscopy High-resolution MS.

Keyword – C. borarium, Euphorbiaceae, Spasmolytic effects, kauranoid, alkaloid.

INTRODUCTION

Asthma is a lung disease characterized by respiratory problems [Godard P, 2001; Godard P and al, 2000; Global Initiative for Asthma, 2021] and symptoms such as shortness of breath, wheezing, chest contraction and coughing [Plojoux J, and al, 2011; Jeffery PK, 2001; Bousquet J and al, 2000; Barnes PJ, 2000 and 1996]. According to "Global Burden of Astma Report", asthma symptoms vary depending on age and its severity differs from one individual to another [Henry M, 2021; Gergen and al, 2001; Djukanovic R and al, 1990; Chung KF et al 1999; Elias JA and al, 1999] and this disease affects around 300 million people worldwide and around 100 million new cases will be added in 2025[Neukirch and al, 1995].

Over several decades, the treatment of asthma has been considerably improved in developed countries due to the availability of several classes of drugs with different mechanisms of action [Drazen and al, 2005; Li JT, 2006; Christiaens T, and al, 2020] but

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in poor countries, particularly the African continent, asthma is one of the leading causes of mortality after infectious and parasitic diseases [National Institute of Health, 1997].

According to Rivière et al, 2005, asthma still represents a major proportion of mortality in all age groups in Madagascar and in particular the region of this large island because respiratory infections affect 12.63% of people consulted in health centers basic health.

Madagascar is the poorest country in the world because their economic growth is very low, the insufficiencies of medical infrastructure, the lack of access to care hinder the effective management of asthma because of very low purchasing power and those which leads to a marked increase in the frequency of this disease [Randrianarivo T.R and al, 1987; Michel T.H and al, 2017; Rakotomaro J and al2023]. This is why Malagasy patients turn to traditional medicine to treat them [Fatiany PR and al, 2013]. The practice of traditional medicine holds an important place in Malagasy society [Fatiany PR, 2015] to heal and keep human beings in good health because of their custom, their very low purchasing power and the inadequacies of the infrastructure of the modern medical. However, in our time, plant extracts and similar preparations can no longer be considered as true “medicines” because of the evolution and improvement of modern drug studies for which rigorous identification and very great precision are required purity according to pharmaceutical laws and the order of modern medicine. This is why the World Health Organization (WHO) and the African Union (AU) have developed national policies for the evaluation and regulation of traditional medicine practices.

In addition, Madagascar has been designated by the international scientific and conservation community as a national research heritage because of its richness in terms of biodiversity and its endemicity [Fatiany PR et al 2014]. Its flora is unique and diverse [Schwartz, 200], it has 12,000 listed species [Humbert, 1952] and more than a thousand unlisted species [Pierre de la Bâthie H, 1921; Pernet R, 1951]. In addition, there appears to be a huge problem in the management of plant resources in Madagascar due to deforestation, bush fires, illegal exploitation and natural damage which leads to the disappearance of plant species [Parmard et al, 2003]. The threats that present to Malagasy fauna and flora, the entire population must also face significant health problems that affect them. Indeed, despite the fact that diseases linked to lifestyles are in decline, the prevalence of cardiovascular and viral pathologies [American Lung Association Asthma Clinical Research Centers, 2007], respiratory and bacterial diseases remain high and constitute the main causes of mortality [Dandouau D et al 1913]. Faced with these facts, we have focused our research on the study of plants used in traditional medicine with the aim of scientifically proving the therapeutic virtues of these plants, of isolating their active ingredients, of elucidating the mechanism of activity of the plant and their active ingredients and in order to determine their chemical structures.

2. MATERIAL AND METHODE

2.1. Plant of selection

Several methods have been described in the literature to make a selection of plants to study, but for our part we used ethnobotanical surveys with the local population close to nature, healers (fig.1) and traditional practitioners for us. Inform the traditional use of plants and collect information given by traditional therapists which could help us choose one or more plants to study.

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Figure.1: A healer from southern Madagascar

2.2. Ethnobotanical Survey

The therapeutic virtues of certain plants are known by the Malagasy people. For a long time, they used these plants to heal and treat illnesses. Based solely on experiences and passed down from generation to generation. So this survey, described as exhaustive, concerns all individuals in a population close to nature: traditional practitioners, healers and well-practiced people, to know the name, dose and quantity of plants to use. The investigations take place as follows: before talking about medicinal plants, we give a clear and detailed presentation in the local language on the or/and the diseases that will be targeted at the end of the presentation. We ask questions regarding the plants used by the local population or by the traditional practitioner and healer to combat these diseases and then the parts taken to carry out the treatments, the doses and the duration of treatments. Finally, we try to ask for the different vernacular names according to the dialects in order to facilitate the collections and understand the instructions for use and guide the biological screening tests and we ask traditional practitioners to accompany us during the plant collections (fig. 2).

2.3. Collecting the plant

Ethnobotanical surveys made it possible to select the plant known by the vernacular name Somorombohitse (dialect name Mahafaly, one of a tribe in Madagascar). The stem bark of this plant was collected in the Mahafaly forest in Ankiliabo Ampanihy District South-West of Madagascar in March 2019.

Figure.2: Photo plant collection

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It was identified in the Botanical department at the Tsimbazaza National Botanical and Zoological Park in Antananarivo. The herbarium was deposited at the Applied Chemistry Laboratory of the University of Toliara Rue Layflaylle Toliara 601 under number AR-01.

2.4. Preparation of crude.

The stem bark of the collected plant matrix was dried and then made into powder it was dry. The extraction of this powder is done by cold maceration in the hydro-ethanol mixture (20/80), for seventy-two hours with permanent stirring at ambient laboratory temperature. After each filtration, the solutions obtained were evaporated to dryness using a rotavapor under reduced pressure, at 40°C of the bath and we have a crude extract noted AR-01.

This extract was taken up in demineralized water, the mixture obtained was washed three times successively with the mixture of hexane/dichloromethane (3/1), then with ethyl acetate and finally with n-butanol. Each of the organic phases obtained is collected then evaporated to dryness and we have the different crude extracts of increasing polarities. Chemical screening was done according to a well-known protocol as previously reported [5-8, 14, 15].The results of the extractions were recorded in Table-I.

2.5. Pharmacological testing protocol

2.5.1. Antiasthmatic activity test

a. Relaxing effect on isolated guinea pig trachea.

The trachea of the sacrificed guinea pigs is removed, cleaned in Krebs-Henseleit solution (survival solution) and cut about 3 mm long.

The organ rings are mounted in the tanks (20 ml) with isolated organs, containing survival solution, aerated with carbogen (5% CO_2 and 95% O_2), and maintained at 37 °C in a thermostatic bath. The preparation is subjected to an isometric tension of 2 g. The equilibration period is 90 min, during this time, the organ is washed every 30 min.

At the end of this equilibration, the organ is stimulated with acetylcholine (10^{-4} M) for 15 min, and then rinses are carried out 30 min after the last one, it is contracted again with acetylcholine (2 10^{-5} M).

At the contraction plateau, cumulative and increasing concentrations of the tested extracts are injected in order to highlight the relaxant activity. The relaxations obtained are evaluated in % in order to calculate the EC50. The test result carried out on C. borarium is recorded in Table II.

b. Inhibitory effect of active extracts on the contractile activity of carbachol on isolated organs.

The protocols are the same during the equilibration period, but after, the contracting agent used is carbachol. The result is summarized in Table III

- **Isolated rabbit aorta**

The rabbit thoracic aorta is removed and cleaned in a petri dish containing Krebs-Henseleit solution. Then it is cut into 3 mm rings. The aorta pieces are then mounted in the organ tanks containing 20 ml of the survival solution aerated with carbogen and maintained at 37 °C.

The preparations are subjected to an isometric tension of 2 g. During the 2-hour equilibration period, the organs are washed every 30 minutes. At the end of this period, they are sensitized with 50 mM KCl for 15 minutes. Then, the washes are carried out 4 times. 30 minutes after the last wash, the aorta rings are stimulated again with 50 mM KCl. At the contraction plateau, the active extract is injected into the tanks. The relaxations obtained are evaluated in % in order to construct the effect-concentration curves and calculate the EC50 values. The result is recorded in Table IX.

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- **Isolated rabbit jejunum**

The rabbit jejunum is collected. Freed from adipose and connective tissues, it is cut to a length of 2 cm and mounted in organ tanks containing tyrode's solution for intestine, aerated with carbogen and maintained at 37 ° C. During an equilibration period of 1 hour, the organs are washed every 15 minutes. At the end of this period, they are stimulated with 50 mM KCL for 10 minutes, then the preparation is washed at least 3 times. 15 minutes after the last wash, the pieces of jejunum are stimulated again with KCL. At the contraction plateau, cumulative and increasing concentrations of the test products are injected into the tanks. The results obtained (Table IX) are expressed in % in order to determine the EC50 values.

c. Relaxing effect on isolated rat aorta

The thoracic aorta of rat (Wistar breed) removed, then cleaned in the survival solution and cut into rings 3 to 4 mm long, is mounted in the isolated organ tanks containing Krebs-Henseleit solution aerated with carbogen (5% CO_2 and 95% O_2), maintained at 37 °C in the thermostated bath. The equilibration period is done in 2 hours, and during this period, the preparation is washed every 30 min. At the end of this time, the aorta is sensitized with noradrenaline (10^{-5}M), at the contraction plateau, acetylcholine (10^{-6}M) was injected into the tanks, to check the activation of functional endothelium, then the organ is washed 3 times every 10 min, after 10 min of this last rinse, the rings are contracted again with noradrenaline (10^{-6}M).

At the contraction plateau, the tested product is injected at the increasing cumulative concentration in order to construct the concentration-effect curve, and to calculate the EC50 value of relaxation of the extract. The result is summarized in Table IV.

d. Elucidation of the mechanisms of pharmacological activities of active molecules on vascular effects

The mechanisms of pharmacological activities of active molecules were elucidated thanks to the different tests of the inhibitory effects of active molecules with respect to the contractile activities caused by antagonists and specific agonists.

e. Inhibitory effect of active molecules with respect to the contractile activity caused.

The test protocol is identical as in paragraph 1.3.1., but after the equilibrium period the contracting agent was varied according to the case. Thus:

- L-NAME: was used to verify the involvement of NO in the vascular activity of pure active molecules. The 10^{-4}M L-NAME solution was used as an endothelial NO synthetase inhibitor.

- Propranolol: aims to show the involvement of cAMP (Cyclic Adenosine Monophosphate) and the β_2 pathway in the vascular activity of pure molecules. The 10^{-5}M propranolol solution was used as a competitive antagonist of β_2 adrenergic receptor.

- Indomethacin: it was used as a type-1 cyclooxygenase inhibitor in order to know that the Prostacyclin pathway was involved in the activity of pure active molecules.

- Inhibitory effect of pure active molecules on the contractile activity of $CaCl_2$ on the isolated rat aorta in depolarizing medium without calcium:

30 min after the last wash, the preparation is washed with the calcium-free survival solution enriched with KCl, using EDTA to eliminate calcium from the extracellular medium. Note that EDTA is no longer used during the last rinse. Then, $CaCl_2$ concentration effects were performed, and then the same washing concentration is carried out as before, 30 min after the last wash, different concentrations of pure molecules are put in contact with the organ for 30 min, then the calcium concentration effect is performed again.

The inhibition caused by the active molecules is evaluated as a percentage in order to calculate the IC50 (product concentration that inhibits 50% of the maximum concentration induced by the $CaCl_2$ contracting agent).

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2.6. Isolation of anti-asthmatic molecules from Croton borarium

The application of bio-guided fractionation methods, namely the combination of different series of chromatographic analyses (TLC, CLBP, HPLC and Preparative TLC) coupled with targeted biological tests on the active fractions made it possible to purify and isolate the molecules responsible for the activity that were targeted, the isolated pure products were then tested on the target to verify their activity. Finally, these products were tested with the different agonists and antagonists of the isolated organ models to elucidate the mechanisms of pharmacological activities of these pure active ingredients.

3. RESULTS

3.1. Survey results

The ethnobotanical surveys conducted in the southern and southwestern parts of Madagascar have made it possible to identify about twenty different species of plants used by healers, traditional practitioners and local populations to combat various common diseases. Among these species, the plant known by the vernacular name Somorombohitse (fig.3). After botanical identification, this plant belongs to the Euphorbiaceae family, genus of Croton and species of Borarium and scientifically called Croton borarium.

Figure.3: Photo of Somorombohitse (Scientific Name: *Croton borarium*)

Ethnobotanical surveys carried out among the local population of Fonkotany Ankiliabo district Ampanihy, have made it possible to know that the leaves of this plant are used by the population in case of breathing problems, by practicing the inhalation of steam and in addition the traditional practitioners named Mahazonta and Lahivao indicate that the stem bark of this plant is used to treat hypertension, by practicing the decoction method.

3.2. Result of extraction and coarse separation

The extraction of the powder of this plant is carried out by cold maceration using organic solvent of increasing polarity. The extraction results have been recorded in Table I.

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Table I: - Extraction results of Croton borarium stem bark

Species	*Croton borarium*
Studied part	Stem bark
Powder mass (g)	750
H2O/EtOH extract (g)	28.18
Hex/DCM extract (g)	11.16
ACoET extract (g)	7.45
But extract (g)	2.56
H2O extract (g)	3.92

3.3. Isolation of pure active principle

5g of the hexane-dichloromethane extract (3/1) was subjected to operation using silica gel chromatography. This column was eluted using a mixture of petro-chloroform ether-methanol (1/8/1) in isocratic mode. At the end of the column, followed by grouping and evaporation to dryness, three fractions are obtained (F-I: 2.43g), (F-II: 846.5mg and (f-III 1.20g). The three fractions were subjected to biological tests to locate the active fraction(s). The in vitro test shows that the F-I fraction has a better activity. 2g of FII was fractionated using silica gel column chromatography, eluted by a mixture of hexane-ethyl acetate (8/2) in acetone gradient. In order to obtain the column, four fractions FI-1 (64mg), F I-2 (743mg), F I-3 (1.02g) and F I-4 (146.2mg) are obtained. Among these fractions, it is the F I-1 fraction which presents the best activity according to the result of the in vitro test. .There is also the fraction F II-2 but their activity is very low compared to F I-1. Our isolation is taken from these two active fractions. 135mg of F II-1 was subjected to the cold crystallization technique using hexane for 4 hours. After crystallization followed by washing with cyclohexane, we obtain a pure product noted AR-011 (25mg).

700mg of F II-2 subjected to chromatography on a sephadex gel column LH-2O eluted by the chloroform-methanol mixture V / V in isocratic mode and in order to obtain four fractions F II-1 (145mg), F II-2 (308.2mg), F II-3 (45mg) and F II-4 (151.42mg). Among them, it is the fraction F II-3 which presents an interesting activity. 40mg of F II-3 undergoes a preparative layer chromatography with two developments, the first using a mixture of chloroform-methanol (V/V) by adding 2µl of ammonia (NH+ OH-) and in the second step Hexane-dichloromethane-methanol (2/6/2) and we obtain two pure products noted AR-012 (2.8mg) and AR- 013 (1.2mg). The three isolated pure products were tested and the results show that the product AR-011 and AR-012 are active but AR-013 is inactive.

3.4. Pharmacological studies

- **Relaxing effect of various Croton borarium extracts on isolated guinea pig trachea pre-contracted with acetylcholine**

The results of the test carried out on the various Croton Borarium extracts are shown in figure.4 and the various IC50 values are recorded in table.2.

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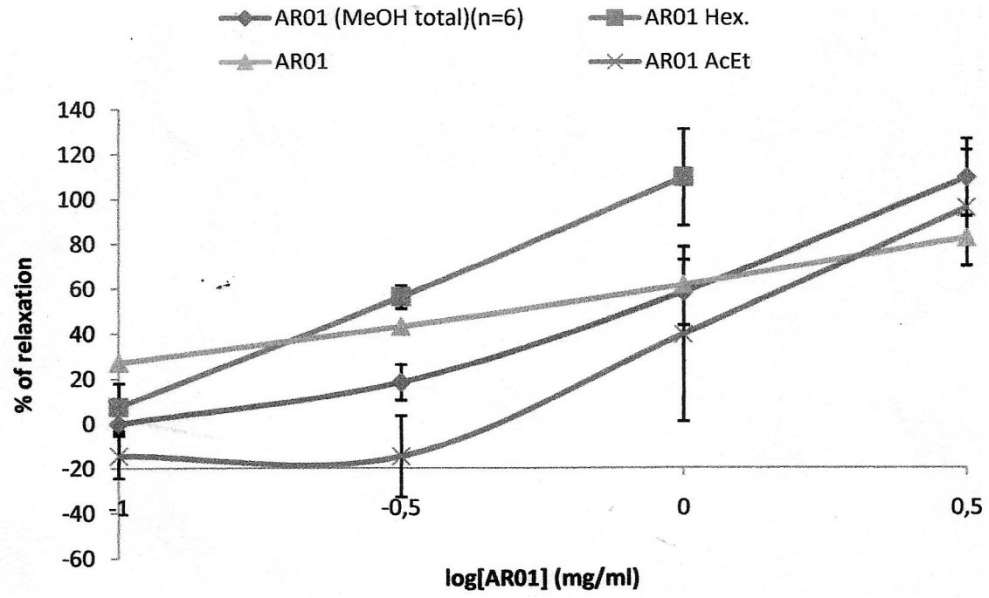

Figure.4: Relaxing effect of various croton borarium extracts

Table.2: The different IC50 values for relaxation of the 4 Croton borarium extracts

Extracts tested	Crude extract (MeOH)	**Hex-DCM (ARO1 Hex.)**	N butanol (AR01)	Ex. AcEt (AR01 AcEt)
CI50	0.850±0.041mg/ml	**0.275±0.021mg/ml**	2.46±0.341mg/ml	1.41±0.106mg/ml

This figure.2 and the IC50 values show that the Hex-DCM extract has a very interesting activity.

- **Inhibitory effect of Hex-DCM extract, verapamil and atropine on carbachol contractile activity in isolated guinea pig trachea**

The aim of this study is to highlight the activity of Hex-DCM extract compared with that induced by Verapamil and atropine.

The inhibitory effect of Hex-DCM extract on carbachol-induced contractility in isolated guinea pig trachea is shown in figure.5.

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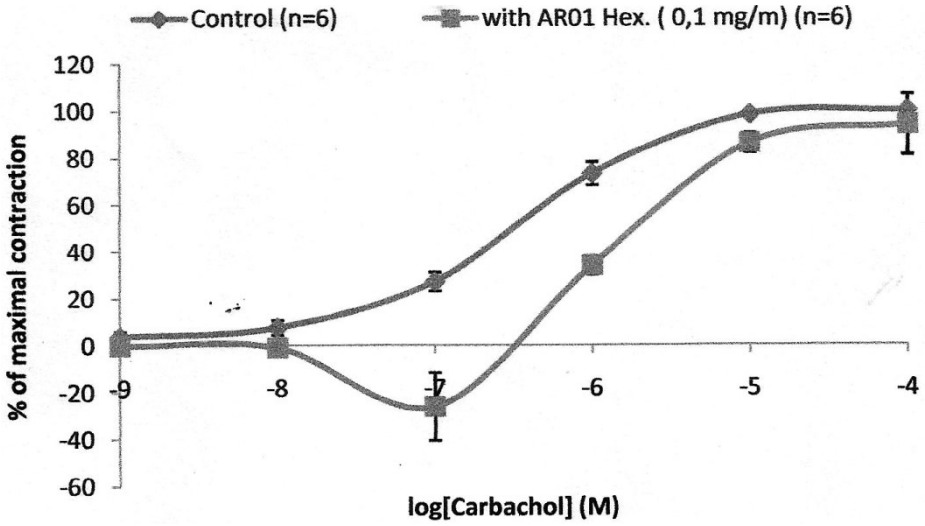

Figure.5: Inhibitory effect of Hex-DCM extract (AR01hex) on carbachol-induced contraction in isolated guinea pig trachea

The results reported in the figure above (fig.5) show that Hex-DCM extract inhibits carbachol-induced contraction in isolated guinea-pig trachea in the same way as verapamil (fig.6) and atropine (fig.7) inhibit contraction induced by the same contracting agent.

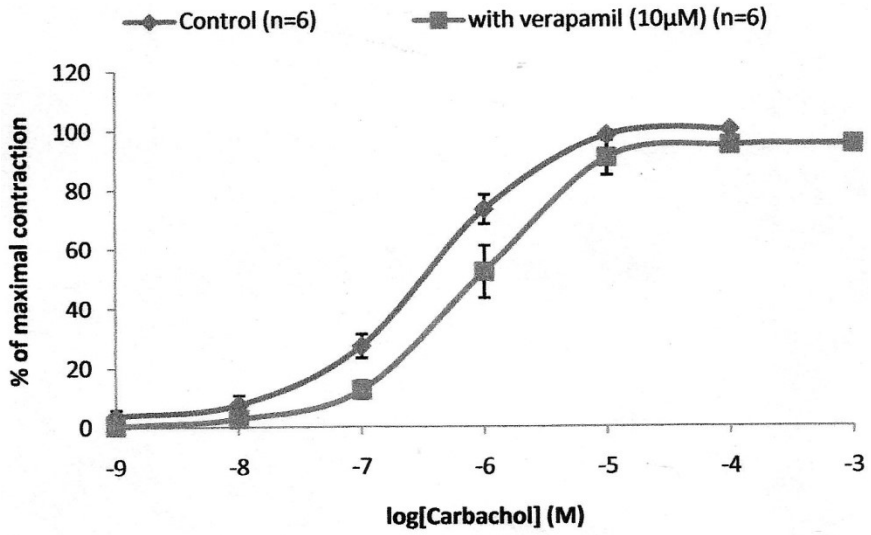

Figure.6: Inhibitory effect of Verapamil (10µM) on carbachol induced contraction in isolated guinea pig trachea

Table.3: EC50 value for verapamil (10µM) versus carbachol-induced contraction

Test	Control	With verapamil (10µM)
CE50(carbachol) (x10^{-7}M)	5.48±0.82	35.75±3.27

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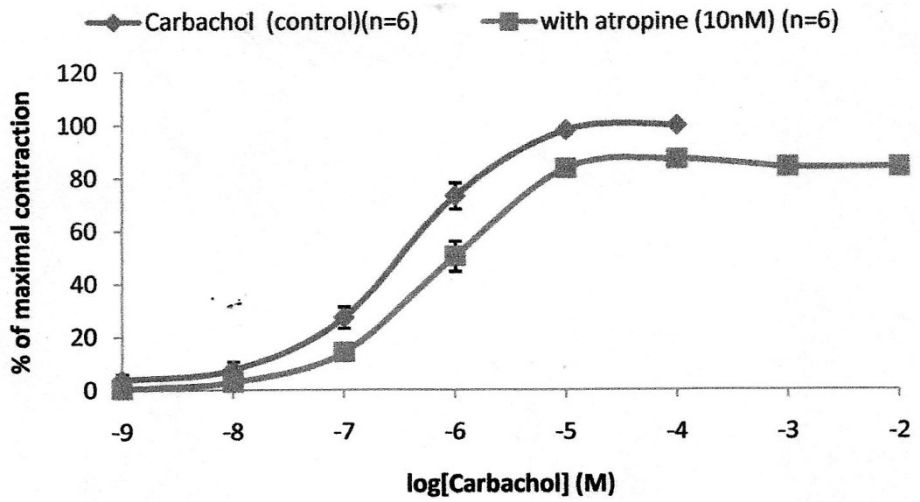

Figure.7: inhibitory effet of atropine (10nM) on carbachol-induced contraction in isolated guinea pig trachea.

Table.4: EC50 value for atropine (10nM) versus carbachol-induced contraction

Test	Control	With atropine (10nM)
CE_{50} (carbachol) (x 10^{-7}M)	5.48 ± 0.82	45± 4.59

❖ **Relaxing effect of the extract Hex-DCM on rabbit isolated aorta.**

The aim of this test is to evaluate the vasorelaxant activity of Hex-DCM extract on isolated rabbit aorta. The results obtained are shown in figure 8.

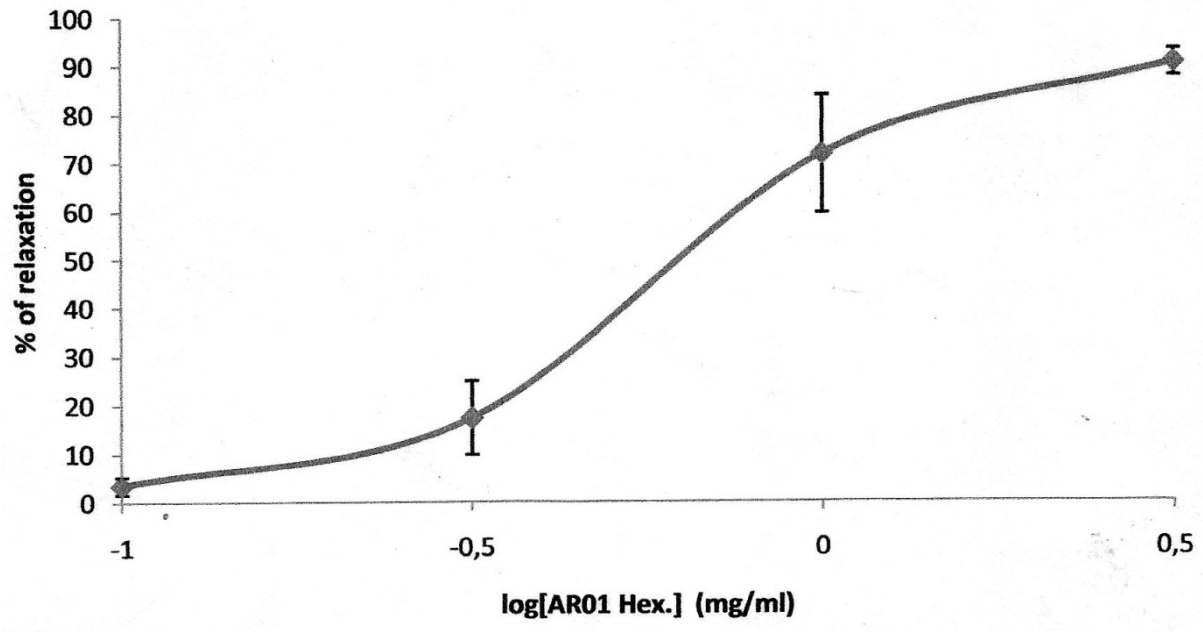

Figure.8: Relaxant effect of AR01 Hex-DCM on rabbit isolated aorta stimulated with 50mM of KCI (n=6).

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The result presented in figure.8 above shows that Hex-DCM extract is able to release the isolated aorta rabbit aorta pre-contracted with mM KCl whose relaxation EC50 value is equal to 0.70±0.14mgml^{-1}.

❖ **Spasmolytic effect of extract Hex-DCM on the jejunum isolated of rabbit**.

This manipulation goal is to up to the fore the relaxing activity of extract Hex-DCM in the isolated rabbit jejunum stimulated with the KC1 (50mM).

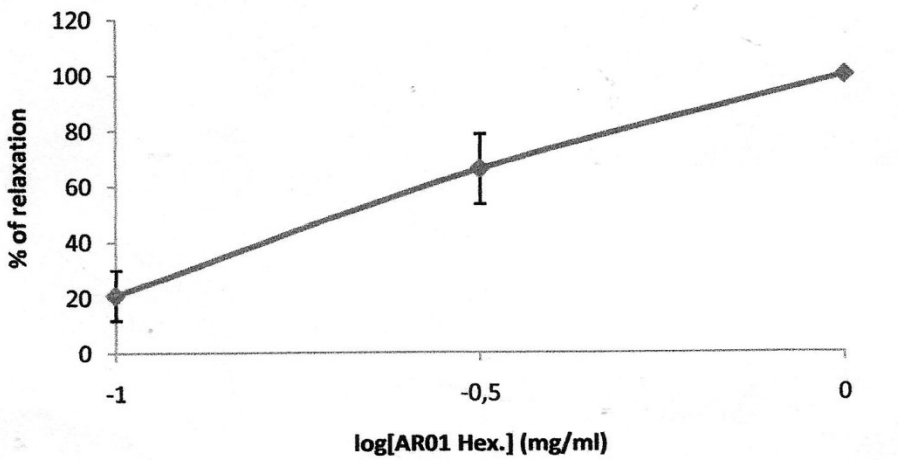

Figure.9: Relaxant effect of AR01 Hex-DCM on KCI (50mM) induced-contraction in isolated rabbit jejunum. (n=6).

The results presented on the figure-9 show the extract Hex-DCM of AR-01 relaxed the jejunum isolated of rabbit pre contracted with the KCL.

This relaxation is concentration-dependent with one value of CE_{50} equal 0.24 ≠ 0.056mg/ml.

e. Isolated aorta rat.

❖ **Relaxing effect of extract Hex-DCM and the pure product on aorta provided of**

The relaxing effect of extract Hex-DCM and this of compound pure are compared on different conditions (aorta with endothelium, aorta without endothelium). The results obtained are reported on the following figures.

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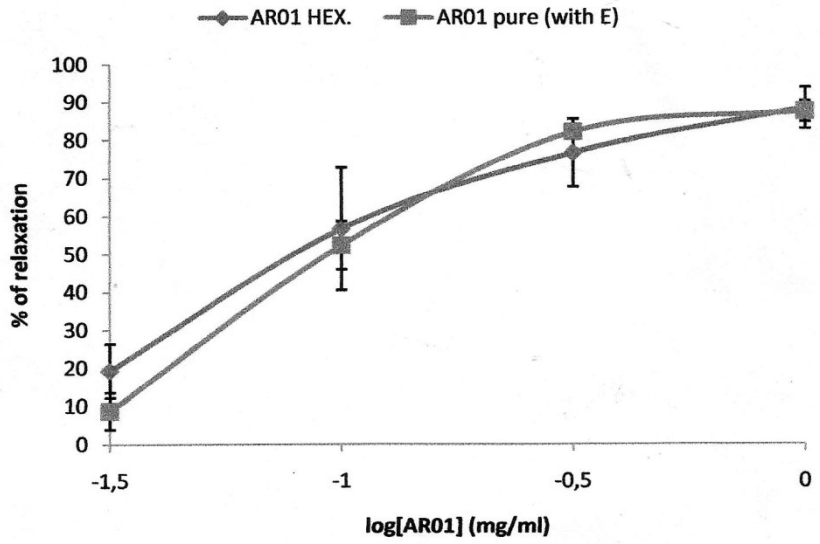

Figure.10: The activity of extract Hex-DCM and of the compound pure (with endothelium) isolated of rat pre-contracted with the noradrenalin in 10^{-6}M.

The results represented on the figure above show an extract Hex-DCM and the compound pure relax the isolated aorta stimulated with the noradrenalin in 10^{-6}M in way concentration dependent which the respective values of CE_{50} ARE 0.15± 0.011 mg/ml and 0.13±0.026 mg/ml.

Even in its pure form, AR-01's vasorelaxant activity is not enhanced. This could be explained by the fact that the activity of this plant is due to the combination of one or more molecules.

- **Relaxing effect of the pure compound on the aorta with on without endothelium**.

The made tests consist of studying the mechanism of the vasorelaxing activity of the pure compound.

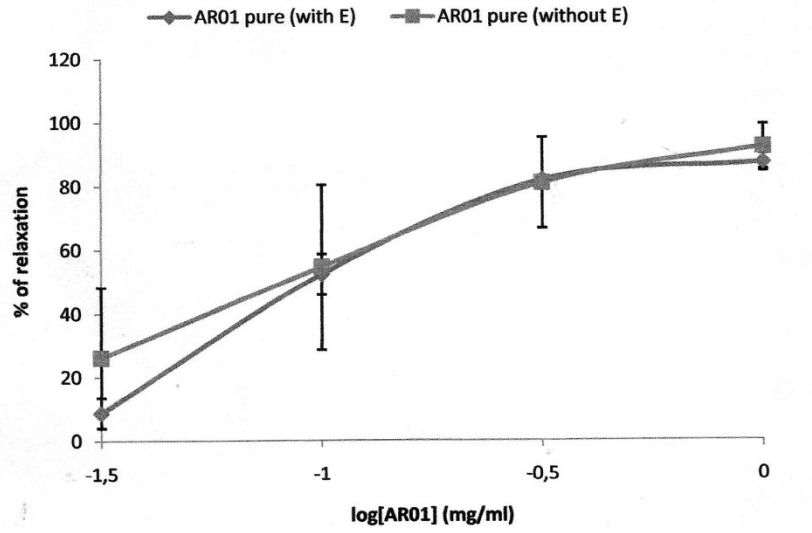

Figure 11: Relaxing activity of the pure compound on the aorta of stimulated rat with the noradrenalin in 10^{-6}M.

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The results above show that pure compound relax the aorta with and without endothelium without modifying significally the value of CE_{50}: 0.13±0.026 mg/ml whit aorta provided of endothelium and 0.18 ± 0.05 mg/ml whit aorta devoid endothelium. The presence or not of endothelium doesn't influence vasorelaxing activity of pure compound.

- **Inhibitory effect of pure compound on the contraction induced by the $CaC1_2$ on the isolated aorta of rat in depolarizing spot without calcium.**

This study consists to put to the fore implication of calcic and membranous canals voltage dependent or VOC on the pharmacologic activity of pure compound.

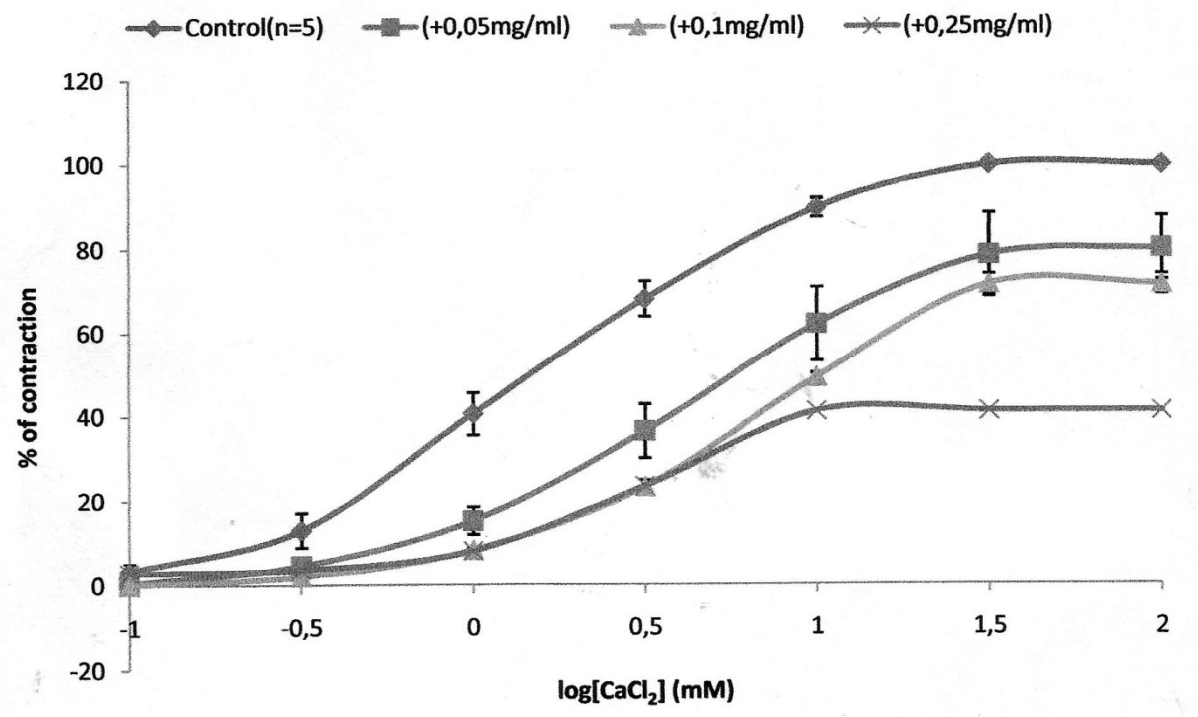

Figure 12: Effect concentration of calcium on the isolated aorta in the depolarizing spot without calcium in absence and in presence of different pure compound.

These results show that pure compound inhibit the maximum contraction produced by the $CaC1_2$ on the isolated aorta of rat so as to concentration dependent. The value de CI_{50} calculated is 0.2 ± 0.015 mg/ml.

- **Sharp toxicity**

Any modification of behavior has been observed just after administration till the third day.

During these 03 days of observation, there is not mortality.

3.5. STRUCTURAL ANALYSES

a. Determination of the chemical structure of compound AR-011

The results of the TOF-MS-EIS 6.79 eV mode mass spectrometry analysis in methanol allowed to determine the molecular ion at m/z = 302.24510 $[M+H]^+$, attributed to the molecular mass of the product AR-011 and corresponding to the gross formula $C_{20}H_{30}O_2$.

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Nuclear Magnetic Resonance (NMR) Analysis

The use of proton NMR, carbon- and 2D H-C NMR (HSQC) spectra of the product AR-011 allows us to detect the presence of the acid carbonyl carbon at δc 185.5 (C-18), two alkene carbons (C=CH2) at δc 155.70 (C-16), δc 103.5 (C-20), three quaternary carbons at δc 39.9 (C-1), δc 43.8 (C-9) and δc 44.4 (C-3), three methynyl (CH) groups at δc 44.2 (C-5), δc 55.2 (C-2) and δc 57.7 (C-8), nine methylene groups (-CH2-) at δc 18.6 (C-7), δc 19.8 (C-11), δc 22.5 (C-14), δc 33.3 (C-6), δc 38.6 (C-10), δc 39.9 (C-4), δc 40.2 (C-12), δc 40.7 (C-7) and δc 48.5 (C-15) and two methyl groups (-CH3) at δc 15.6 (C-17) and δc 29.3 (C-19).

The interpretations of the HMBC spectrum of the product AR-011 allowed to have the carbon proton assignments and the definitive structure of this product. Indeed for the first fragmentation of the molecule was identified from two methyl protons which come out respectively at 1.04ppm and 1.33ppm.

The proton at δH 1.33 carried by the carbon at δc 29.3 correlates with carbons that exit respectively at 57.7ppm (CH), at 43.8ppm a quaternary and with carbon at 38.6ppm is a CH2. It also correlates with the carbon exiting at 185.5ppm, is a carbonyl group attributed to an acid function.

The of a methyl exits at 1.04ppm carried by the carbon exiting at δc 15.6 correlates with the carbons that exit respectively at δc 39.9, δc 54.3, δc 57.7 and δc 40.2.

According to the interpretations of the COSY spectrum, the protons that have different chemical environments carried by the carbon at δc 40.2 coupled with the two protons carried by the carbon δc 19.8 (C-11), then these two protons carried by the carbon C-11 are coupled with the two protons carried by the outgoing carbon δc 38.6 (C-10). Now the protons of the methyl carried by the carbon at δc 29.3 correlate with the carbon C-10. This gives the first fragmentation of the molecule AR-011 (fig.13)

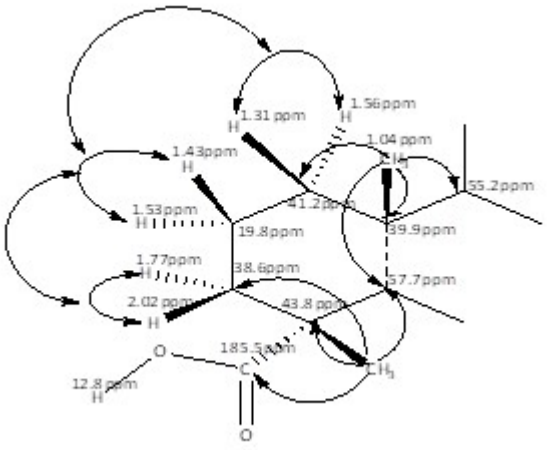

Figure.13: The first sequence of the AR-011 molecule

The second sequence of the AR-011 molecule was identified from two methyne protons carried by the carbons at δc 55.2 (C-2) and δc 57.7 (C-8). The proton exits at δH 1.39 carried by the carbon exits at δc 55.2 (C-2) show correlations with the quaternary carbons which exit at δc 39.8 and δc 44.4 respectively. It also correlates with the carbon exits at δc 18.6. The proton exits at 1.76 ppm carried by the C-8 carbon exits at δc 57.7 in the α position relative to C-1, exhibit strong correlations with the carbons exiting at δc 43.8, δc 39.9, δc 22.50 and weak correlations with carbons exiting at δc 29.3, δc 41.5 and δc 185.5 respectively.

According to the first sequence of the AR-011 molecule, the carbons exiting at δc 29.3 and δc 185.5 respectively are in the β position relative to the C-8 carbon, while the carbon at δc 41.50 is clearly the β position relative to the C-8 carbon. Now the protons which exit respectively at δH 1.31 and δH 1.56 carried by the carbon at δc 41.50 correlate strongly with the carbons

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exiting respectively at δc 22.50 and δc 44.40 and then these are coupled with the protons exiting at δH 1.28 and δH 1.53 carried by the carbon at δH 22.50, whereas the protons mentioned above are in vicinal positions, because they are carried by two different carbons. Hence the second sequence of the AR-011 molecule (fig.14).

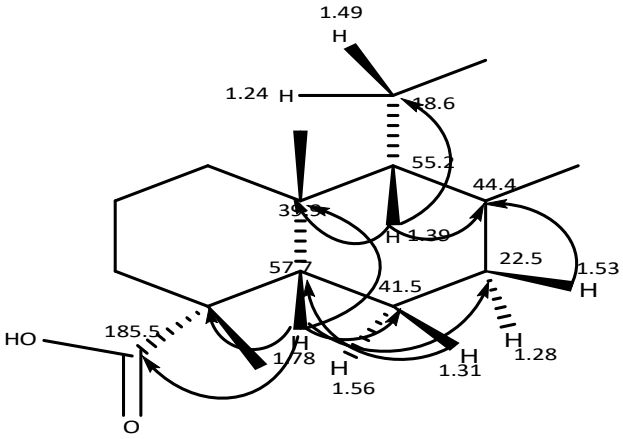

Figure- 14: Second sequence of the AR-011 molecule

Then the third sequence of the AR-011 molecule was identified from the protons that exit respectively at δH 1.28 and δH 1.53 carried by the carbon exiting at δc 18.6. The COSY spectrum of this molecule allowed us to note that both are coupled with the protons carried by the carbon at δc 33.30 and these protons exit respectively at δH 1.31 and δH 1.56, while they are in vicinal positions.

The interpretations of the HMBC spectrum of this molecule show that the protons carried by the carbon exiting at δc 33.30 correlate with the carbons that exit respectively at δc 18.60 and δc 44.21. They show weak correlations with the outgoing quaternary alkene carbon at δc 155.7 and the methylene carbon at δc 39.95, in addition COSY mentioned that the protons carried by each carbons that exit at δc 39.95 and δc 44.21 respectively were correlated.

So the protons exit at δH 1.24 and δH 1.49 respectively carried by the carbon exiting at δc 39.95 show strong correlations with carbons exiting at δc 44.21 and δc 44.40. Combining this information, we have the third sequence of the AR-011 molecule (fig.15).

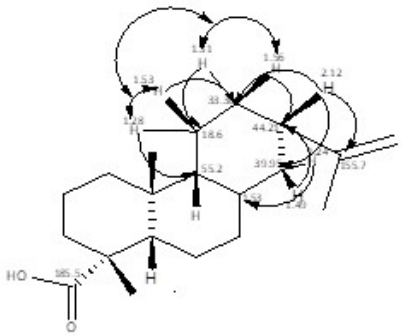

Figure .15: Third sequence of the AR-011 molecule

The last sequence of the AR-011 molecule, which was identified from two linear alkene protons carried by the carbon leaving at δc 103.52. These two protons leaving respectively at 4.83ppm and 5.03ppm, correlate with carbons leaving at δc 22.50 a quaternary alkene, at δc 44.21 (CH) a methynyl and at δc 49.23 (-CH2-) a methylene. Then, the two vicinal protons which exit

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respectively at 1.76ppm (H-15a) and at 2.00ppm (H-15b) carried by the carbon at δc 49.23, present strong correlations with carbons which exit at δc 44.40 and at δc 155.7.

All the information raised made it possible to determine the last sequence of the AR-011 molecule (fig.16).

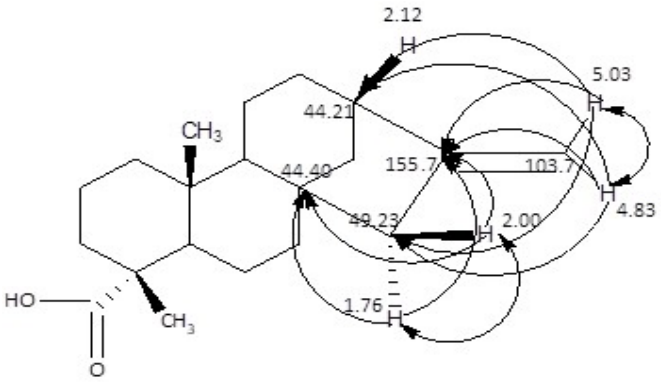
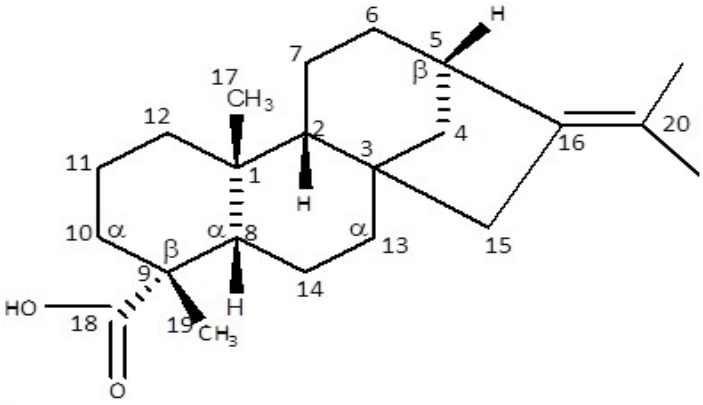


Figure .16: The fourth sequence of the AR-011 molecule

This is the last sequence, which allowed us to determine the complete chemical structure and configuration of the AR-011 molecule. It is attributed to a diterpenoid derived from a kauranoid called: (5β, 8α, 9β, 10α, 13α)-Kau-16en-18oic acid (fig.17). All the results from the NMR analyses (1D and 2D) are recorded in table-VII.



Molecular formula: C20H30O2

Molecular mass: m/z = 302.24510 [M+H]+

Scientific name: (5β, 8α, 9β, 10α, 13α)-Kau-16en-18oic acid

Figure.17: Complete chemical structure of active molecule AR-011

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Table. VII: ^{1}H and ^{13}C chemical shift and important HMBC correlation

Atom N°	δ H multiplicity	δ ^{13}C shift	COSY	HMBC
1		39.9 s		
2	1.39 dd	55.2 d	H-7	C1, C3 and C7
3		44.4 s		
4a	1.24 m	39.9 t	H-5	C3 and C5
4b	1.49 m			
5	2.12 q	44.2 d	H-4 and H-6	C4 ; C6 and C16
6a	1.31 m	33.3 t	H-5 and H-7	C4 ; C5 and C7
6b	1.56 m			
7a	1.28 m	18.6 t	H-2 and H-6	C2 ; C6
7b	1.53 m			
8	1.78 t	57.7 d	H-14	C1 ; C9 and C14
9		43.8 s		
10a	2.02 dd	38.6 t	H-11	C9 and C11
10b	1.77 td			
11a	1.43 m	19.8 t	H-10 and H-12	C10 ; C12
11b	1.53 m			
12a	1.56 dt	41.1 t	H-11	C9 and C11
12b	1.31 dt			
13a	1.56 dt	41.5 t	H-14	C3 and C14
13b	1.31 t			
14a	1.28 m	22.5 t	H-8 and H-13	C8 and C13
14b	1.53 m			
15a	2.00 d	49.2 t	itself	C3 and C16
15b	1.76 d			
16		115.7 s		
17	1.04 s	15.6 q		C1 ; C2 ; C8 and C12
18	12.08 s	185.0 s		
19	1.33 s	29.3 q		C8 ; C9 ; C10 and C18
20a	5.03 d	103.5 t	It self	C5 ; C15 and C16
20b	4.83 d			

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(s): singlet; (d): doublet; (t): triplet; (q): quadruplet; (m): multiplet; (dd): doublet of doublet; (td): triplet-doublet; δ: shift in ppm.

b. Determination of the chemical structure of compound AR-012

The chemical structure of pure product AR-012 was elucidated by mass spectrometry (MS) and one- and two-dimensional nuclear magnetic resonance spectroscopy (1D and 2D NMR).

The results of mass spectrometry analysis in TOF-MS-EIS mode 6.79 eV in methanol allowed to determine the molecular ion at m/z = 192.1245 [M+H]+ attributed to the molecular mass of product AR-012 and corresponds to the gross formula C8H17NO4. The bibliographic research carried out concerning the molecular mass of this molecule allowed to conclude that it is a molecule not yet described.

The UV spectrum of the molecule recorded by HPLC-UV-DAD presents an absorption maximum at 210 nm, which corresponds to the chemical family of an amino acid. The analysis of its IR spectrum presents a band at 3300 Cm-1 which indicates the presence of hydroxyl functions.

The exploitation of the spectra of the 1D proton and carbon NMR 2D NMR (HSQC) of the product AR-012, allowed to identify the presence of the carbonyl function at δc 170.80, a carbon of type C-O at δc 61.89, two symmetrical secondary alcohol groups (-CH_2OH). These carbons come out at δc 59.65 and the hydroxyl proton (OH) comes out at δH 8.72 and the two non-hydroxyl protons carried by the same carbon. We observe the presence of a heteroatom included in the halide family attributed to a tri-substituted nitrogen, three methylene groups (-CH_2-) at δc 55.18 and the two are symmetrical and linked with the alcohol group, and a methyl group at δc 20.90. The displacements of the identified protons, COSY and the HMBC correlations are recorded in Table-VIII. All of this information above made it possible to determine the complete chemical structure of the product AR-012 (fig.18). This attributed to an alkaloid ester. It is a new molecule called Boraïne and scientifically named 2-(bis (2hydroxylethyl) amino) ethyl acetate.

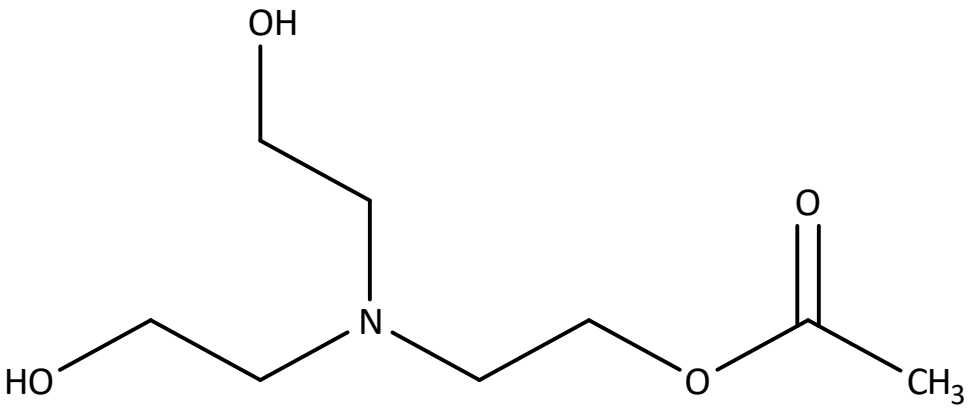

Figure.18: Complete chemical structure of compound-2

Table-2: ^{1}H and ^{13}C chemical shift and important HMBC correlation of compound-2

Atom N°	δ^{1}H, order of multiplicity	δ ^{13}C order of multiplicity	COSY	HMBC
1	2.21 s	20.90 q		
2		170.80 s		
3	4.35 t	61.89 t	H_4	C_4

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4	2.97 t	55.18 t	H_3	C_3
5	2.53 t	56.79 t	H_6	C_6
6	3.45 t	59.65 t	H_5	C_5
6-OH	4.16 s			
7	2.53 t	56.79 t	H_8	C_8
8	3.45 t	59.65 t	H_7	C_7
8-OH	4.16 s			

(s) : singulet ; (t) : triplet ; δ : déplacement en ppm.

4. DISCUSSION

The decoction of the aerial part of Croton borarium has been used by the local population in the southwestern part of Madagascar in case of breathing difficulty and to stabilize blood pressure. The physiological phenomenon of this breathing difficulty derives either from bronchial hypersecretion or from the reduction in the caliber of the airways. The results of the tests carried out on the isolated guinea pig trachea show that the crude extract of Croton borarium relaxes the isolated guinea pig trachea stimulated with acetylcholine at 2.10-5M. This result justified the traditional use of this plant.

The application of the extraction technique by liquid-liquid partition between two immiscible solvents water and organic solvent of increasing polarity provides three (03) extracts: hexane-dichloromethane (3/1), ethyl acetate and n-butanol. With the same pharmacological test, the Hex-DCM extract (3/1) shows significant relaxing activity. According to this result, the molecule(s) responsible for the activity in this plant belongs to the chemical family of moderately polar or apolar molecule because all the products soluble in Hex/DCM solvent are moderately polar or apolar molecules. In addition, the drug commonly used in case of asthma attack is theophylline and its active ingredient is an apolar molecule and it acts inside the tracheobronchial muscle cells by inhibiting PDE, while it was easy to cross the cytoplasmic membrane of these smooth muscle cells is therefore an asset for an apolar molecule to act on this mechanism. The application of bioguided fractionation methods on Hex-DCM extract (3/1) of the aerial part of Croton borarium, using 60 mesh silica gel chromatographic column (0.063 to 0.200mm diameter), Sephadex gel LH-20 column, thin layer chromatography analysis, HLPC analysis allowed to isolate two pure products. The chemical structures of the isolated products were elucidated using NMR spectral methods (1D and 2D) and TOF.MS-ESI 6.97eV mass spectrometry. The pure product AR-011 is attributed to a diterpenic kauranoid: (5β, 8α, 9β, 10α, 13α)-Kaur-16en-18oic acid of crude formula $C_{20}H_{30}O_2$ and molecular mass 302.4510 [M+H]+ after calculation. After the literature, this molecule was isolated the first time in coffee [Wahlberg et al, 1957] and in Eupatorium album [Herz et al, 1976] but it is also the first time that its isolation was on the genus of Croton endemic to Madagascar. Chemically, the basic structure of this product comes from the hypothetical carbocation formed from the active labdadienyl which cyclizes in the following way: - attack of C-13 by the exocyclic double bond, migration of double bond13 (14), stabilization of the cation by elimination of a proton at C-7 or C-14 gives the tetracyclic chain (fig.19).

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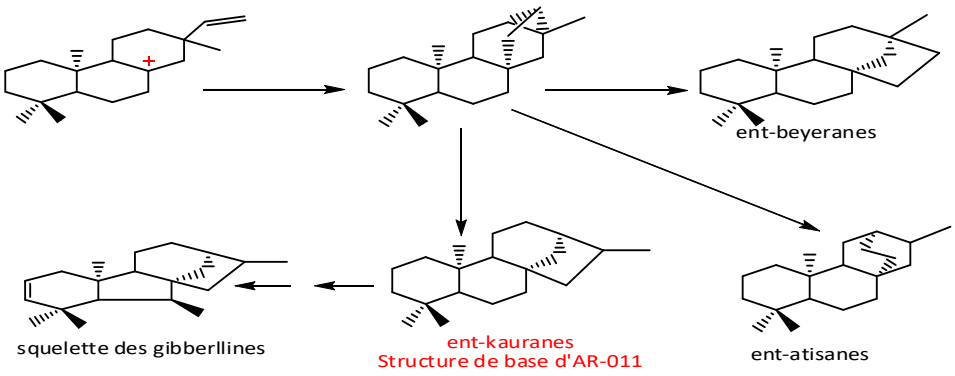

Figure.19 : Formation structure de base AR-011

Regarding the biological activities of this molecule, Harson et al 1974 and Ellames et al, 1976 described that kauranoid derivatives exhibit antibacterial activity if the unsaturated group becomes saturated; moreover the OH of the carboxylic acid was substituted by a glucose, thus our hypotheses were confirmed by the literature data. The long-range correlations of the AR-011 molecule allowed to determine the exact configuration of this molecule.

These results of the ROESY correlation studies (fig.20) and the configuration of AR-011 were justified by the stereochemistry of carbons C-5, C-8 and C-9 and the position of C-10 and C-13 [Herz .W et al 1974]

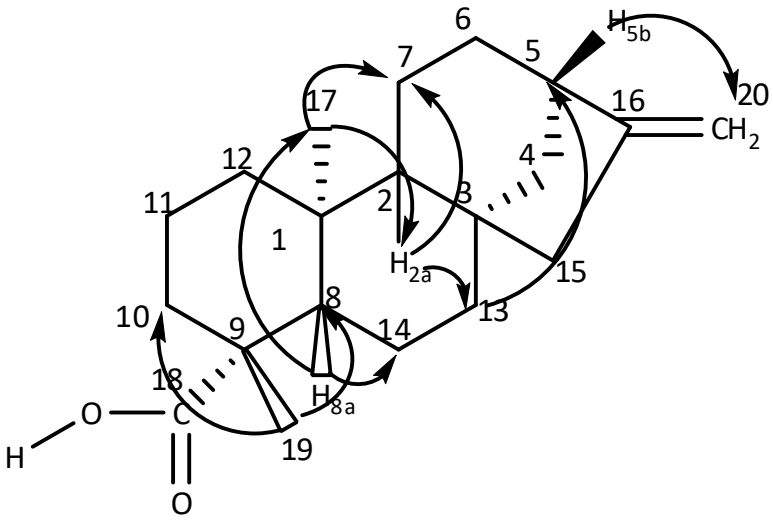

Figure.20: ROESY correlation of the AR-011 molecule

The pure product AR-012 is an alkaloid ester of the crude formula C8H1704N and molecular mass m/z= 192.1827, it is attributed to 2bis (2hudroxyl-ethyl) amine ethyl acetate. This is the first time isolated on Croton borarium and is a new molecule identified is named ‘’ Boraïne ’’.

The results of the tests obtained on the isolated rat aorta show that the Hex-DCM extract and the two pure products are endowed with vasorelaxant activity on both the aorta with and without endothelium. However, at the levels of the endothelial cells reside the production of EDRF (Endothelium Derived Relaxing Factor) which, in turn, will act on the smooth muscle cells in the form of NO. By activation of guanylate cyclase, the cytoplasmic concentration of cyclic GMP increases to result in muscle relaxation. The relaxant activity of this molecule does not use the relaxation mechanism via NO. As calcium channel blockers are classified as antihypertensive drugs, tests of the effect of AR-011 on calcium flux were undertaken. The opening of membrane calcium channels was induced by depolarization of the membrane of vascular smooth muscle cells by increasing the concentration of

extracellular K+ ion (depolarizing medium). AR-011 inhibits the maximum concentration of the aorta induced by CaCl2 in a concentration-dependent manner. This molecule therefore involves the movement of calcium across the cell membrane of vascular smooth muscle by inhibiting entry. The relaxation obtained with the Hex-DCM extract on the aorta and jejunum of rabbit stimulated with 50mM KCl confirms the involvement of V.O.C. calcium channels. The result of the pharmacological activity test of the product AR-012 shows that it exerts a very interesting Vasorelaxant effect and their mechanism of pharmacological activity goes through the β2 adrenergic receptor. The toxicity studies carried out on the crude extract of this plant did not show any sign of significant toxicity. These results justify the traditional uses of Croton borarium to treat asthma.

CONCLUSION

Ethnobotanical surveys carried out in the south and south-west of Madagascar have identified a plant known by the vernacular name Somorombohitse (Mahafaly tribe name). This plant has been used by the local population in the region to treat high blood pressure and breathing difficulties.

The application of the cold maceration extraction technique using a hydro-ethanol (20/80) organic solvent mixture to the powdered aerial part of the plant, followed by filtration and dry evaporation, yielded a dry crude extract. After biological testing, the total hydro-ethanolic extract showed relaxing activity on isolated guinea-pig trachea. The use of a liquid-liquid partition extraction method on the crude extract yielded three extracts, of which Hex-DCM(3/1) was the most active after testing.

The application of bioguided fractionation techniques to Hex-DCM extract, using column, thin layer and preparative layer chromatography, enabled us to isolate two pure, active principles.

The chemical structures of the two pure products and their molecular masses were elucidated using NMR spectroscopic and mass spectrometric analysis methods.

The compound AR-011 has the molecular formula C20H30O2, molecular mass m/z= 302.4510 [M+H]+ attributed to (5β, 8α, 9β, 10α, 13α)-Kau-16en-18oic acid is a diterpene Kauranoid. It produces a vasorelaxant effect of EC50= 0.21±0.06µg/ml at n=6, on isolated rat aorta (with and without endothelium), stimulated with noradrenaline. This relaxant activity could result from the blockade of calcium influx through vascular smooth muscle cells, as the contraction produced by CaCl2 on aorta in depolarizing medium without calcium was inhibited by AR-011 in a concentration-dependent manner. The relaxation induced by Hex/DCM extract (3/1) in rabbit aorta and jejunum stimulated with 50mM KCl supports this mechanism of anticalcium activity at membrane level.

Compound AR-012, molecular formula C8H17O4N, molecular mass m/z = 191.3865 [M+H]+ attributed to 2bis(2hydroxyethyl)-acetate ethylamine is a new molecule named Boraïne. It exerts a vasorelaxant effect with EC50= 13.21±2.65µg/ml at n=6 and pharmacological mechanism via the β2-adrenergic receptor. Preliminary acute and chronic toxicity studies carried out on the hydro-ethanolic crude extract of this plant showed no significant toxic signs.

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